# *Gomphrena claussenii*, a Zn and Cd hyperbioindicator species

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# *Gomphrena claussenii*, a Zn and Cd hyperbioindicator species

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Dedicated to my beloved family, Álvaro and João

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# CHAPTER 1

## General introduction

## Metal tolerance in plants

Plant species have been occupying suboptimal environments due to their adaptation and acclimation mechanisms evolved for stress tolerance (Mickelbart *et al.*, 2015). Among the species adapted to abiotic stresses, a unique group called metallophytes, have been identified as being extremely tolerant to high concentrations of metal and metalloid trace elements (Baker, 1987). While most plant species are sensitive to these toxic conditions, metal tolerant species have evolved the ability to survive and reproduce in such environments (Baker & Brooks, 1989; Ernst, 2006).

Plant species are classified as metal sensitive or resistant according to their response to toxic metals (Baker, 1987). While sensitive species present toxicity symptoms or even die during long metal exposure, other species can resist this stress by avoiding or tolerating high concentrations of metals. Depending on the mechanism used to cope with high metal levels and the capacity to accumulate them, resistant species can be classified as metal excluder or hyperaccumulator (Lin & Aarts, 2012; van der Ent *et al.*, 2013). Despite being highly metal tolerant both the hypertolerant and hyperaccumulators species have a distinct metal distribution within the plant. While hypertolerant species accumulate most metals inside the roots and avoid accumulation in shoots, hyperaccumulators present a low accumulation in roots and an extraordinary leaf metal accumulation (Rascio & Navari-Izzo, 2010).

Considerable variation of transition metal concentrations in soil has induced plant species to develop a precise mechanism to efficiently acquire and distribute micronutrients (Ernst, 2006; Puig & Penarrubia, 2009; Merchant, 2010). This mechanism is known as metal homeostasis and it allows plants to grow, and adapt to different

environment conditions. Substantial information regarding metal and metalloid homeostasis in plants has been collected in recent decades wherein important progress has been made by studying natural metal hypertolerant species (Baker, 1987; Reeves, 1988; Schat, 1999; Krämer *et al.*, 2007; Hanikenne *et al.*, 2008; Sinclair & Krämer, 2012).

Metal hyperaccumulator research has also been motivated by the possibility to use these plants to remediate metal polluted areas through phytoremediation techniques (Chaney *et al.*, 2007; Marques *et al.*, 2009). Ideally, hypertolerant species are suitable for stabilizing the soil (phytostabilization) at highly contaminated areas while hyperaccumulators would stabilize and also extract metals (phytoextraction). The ideal plant species to efficiently achieve remediation techniques should comprise high metal tolerance and high metal accumulation along with high biomass production (Chaney *et al.*, 2007). Unfortunately, metal hyperaccumulators species are often small plants, producing relatively little biomass and consequently have a low capacity for metal removal. The two major strategies considered to overcome this limitation are to breed or to genetically engineer hyperaccumulator species to increase their biomass; or to genetically engineer high-biomass species aiming to increase their metal accumulation and tolerance capacity. These are long term strategies and until now the goals to convert high biomass plants into efficient crops or to sufficiently increase hyperaccumulator biomass for phytoremediation purposes have not been reached.

## Zn and Cd highly tolerant species

Plants growing naturally in metal-enriched soils are in general metal tolerant, which makes the native vegetation at contaminated areas an important source of metal-adapted species (Baker & Brooks, 1989). A large number of metalliferous soils are

ultramafic serpentine sites, presenting high concentrations of nickel (Ni) which justifies the relative abundance of Ni hypertolerant/hyperaccumulators species identified, compared to Zn or Cd hyperaccumulators (Baker *et al.*, 2010). There are very few natural Zn-enriched sites, limiting the number of evolved Zn tolerant species (Krämer, 2010). Most toxic Zn-contaminated sites result from human activities such as metal mining and smelting, which often generate a concomitant contamination with other metals, such as cadmium (Cd), lead (Pb) and copper (Cu). Very often Cd-contaminated areas are also enriched with high levels of Zn (Kabata-Pendias & Mukherjee, 2007b) hence, Cd hyperaccumulator species are in general also highly Zn tolerant (van der Ent *et al.*, 2013).

Metal hyperaccumulation has been described in approximately 500 plant taxa (~0.2% of all angiosperms). A widely known definition made by Reeves (1992) for hyperaccumulators having nickel as example is: " a plant in which a Ni concentration of at least 1000 μg $g^{-1}$ has been recorded in the dry matter of any above-ground tissue in at least one specimen growing in its natural habitat". These species are described to accumulate high concentrations of metals in the shoot tissues even when grown in soils with low metal concentrations. While zinc (Zn) is required by most plant species in a typical shoot concentration of 15-20 μg $g^{-1}$, Zn hyperaccumulators accumulate more than 3000 μg $g^{-1}$ dry weight of Zn (van der Ent *et al.*, 2013). Cd normally becomes toxic already when accumulated at very low concentrations (1-5 μg $g^{-1}$) while hyperaccumulators are able to growth with more than 100 μg $g^{-1}$ of Cd in their shoots. In addition to high metal accumulation, a high bioconcentration factor (i.e. the shoot to soil metal concentration ratio) higher than 1, but often higher than 50 and a translocation ratio (i.e. the shoot to root metal concentration ratio) higher than 1 are also criteria used to define a species as metal hyperaccumulator (van der Ent *et al.*, 2013; van der Ent *et al.*, 2015).

Despite the energy costs required to avoid potential toxic effects, metal hyperaccumulators have a selective advantage over other species when growing at metal-enriched sites (Maestri *et al.*, 2010). Several hypotheses have been proposed to explain the ecological benefit of metal hyperaccumulation, such as drought resistance, interference with neighbouring plants (allelopathy), and defence against natural enemies (Pollard & Baker, 1997; Rascio & Navari-Izzo, 2010). So far, the latter hypothesis, defence against natural enemies, has gained most experimental support, with several descriptions of cases where hyperaccumulators loaded with metals are more tolerant to herbivores and fungi than plants with normal metal concentrations (Pollard & Baker, 1997; Huitson & Macnair, 2003; Jhee *et al.*, 2005; Jiang *et al.*, 2005; Quinn *et al.*, 2010; Hörger *et al.*, 2013; Kazemi-Dinan *et al.*, 2014).

Until now, *Arabidopsis halleri* (Küpper *et al.*, 2000), *Noccaea caerulescens* (Assunção *et al.*, 2003b), *Noccaea praecox* (Pongrac *et al.*, 2009), *Sedum alfredii* (Yang *et al.*, 2004) (recently renamed as *S. plumbizincicola* (Wu *et al.*, 2013)), and *Viola baoshanensis* (Wu, C *et al.*, 2010) are identified as Zn/Cd hyperaccumulator species. Found growing at old mine sites across Europe, but also widely distributed on non-metallicolous soils, *N. caerulescens* and *A. halleri* are the two most investigated hyperaccumulator species, whereas *S. plumbizincicola* and *V. baoshanensis*, identified in Zn mining areas in China, are increasingly better studied.

## Physiology of Zn and Cd homeostasis and tolerance

Zinc as a micronutrient is essential for plant growth and reproduction. As an indispensable element for plants it ensures the catalytic function of enzymes and is directly involved in transcription regulation through specific binding sites (Broadley *et al.*,

2007). For most species the right Zn concentration, on average 20 mg $kg^{-1}$, guarantees optimal plant performance (Marschner & Marschner, 2012). Zn deficiency is known as a common crop problem and its toxicity restricts plant growth at contaminated sites. Usually plants present phytotoxic symptoms when the leaf Zn concentration is higher than 300 mg $kg^{-1}$ dry weight although some species are affected even with 100 mg $kg^{-1}$ of Zn. Under excess Zn conditions, most plants try to maintain an optimum Zn concentration through regulation of metal homeostasis processes such as metal uptake and distribution (Sinclair & Krämer, 2012).

Potentially highly toxic to plants Cd is a non-essential element which interferes with Zn, iron (Fe), manganese (Mn) and calcium (Ca) homeostasis (Clemens, 2006; Gallego *et al.*, 2012).When exposed to high levels of Cd, development and biomass production of non-tolerant plants are affected. In addition they show toxicity symptoms such as leaf chlorosis, water unbalance, and reduction of photosynthesis rate (Clemens, 2006). At the cellular level, Cd toxicity induces an oxidative stress due to reactive oxygen species (ROS) production (Cuypers *et al.*, 2010; Jozefczak *et al.*, 2015).

Metal tolerant plant species have evolved physiological strategies to minimize internal metal toxicity by allocating their metal surplus in metabolically less active tissues and removing the toxic ions from sensitive subcellular parts, such as the cytosol and organelles (Clemens, 2001). Therefore, hyperaccumulator species often compartmentalize metals in the trichomes, in the epidermis or in the apoplastic space. For instance, the hyperaccumulators *N. caerulescens* (Küpper *et al.*, 2004; Cosio *et al.*, 2005) and *N. praecox* (Vogel-Mikuš *et al.*, 2008a; Vogel-Mikuš *et al.*, 2008b) accumulate metals in the leaf epidermis cells, *A. halleri* preferentially accumulates Zn and Cd in the cells forming the base of trichomes (Hokura *et al.*, 2006; Fukuda *et al.*, 2008) and *S. alfredii* (*S. plumbizincicola*) stores Zn mainly in the epidermis of stems and leaves and Cd in the pith

of stems (Tian *et al.*, 2011). Intracellular Zn and Cd is mainly stored in the vacuoles in these hyperaccumulator species (Cosio *et al.*, 2005; Sarret *et al.*, 2009; Tian *et al.*, 2011).

As an important step during metal sequestration, metal chelation is important for reduction of toxic metal bioavailability and to avoid a possible reaction with other compounds (Mari & Lebrun, 2006; Lin & Aarts, 2012). Organic acids, amino acids and oligopeptides are the main metal chelators in plants. Especially the anions of malate, citrate and oxalate are relevant metal ligands and are found to be associated with metal hyperaccumulation and tolerance (Broadley *et al.*, 2007; Haydon & Cobbett, 2007). The acidic pH in the vacuoles favours the metal-organic acid complex formation, an important role for metal detoxification. For instance the citrate and malate concentrations found in *A. halleri, N. caerulescens* and *S. alfredii* correlate positively with Zn accumulation (Yang *et al.*, 2006; Sarret *et al.*, 2009; Monsant *et al.*, 2011).

Nicotianamine (NA), a non-proteinaceous amino acid, is another metal major Zn chelator (Clemens *et al.*, 2013). NA acts to reduce free reactive ions in the cytosol by making stable complexes with metals. These complexes contribute to Zn xylem loading thereby enhancing metal accumulation in shoot tissues (Deinlein *et al.*, 2012; Cornu *et al.*, 2015). The involvement of NA in Zn hyperaccumulation in *A. halleri* has been recently shown by Deinlein *et al.* (2012), who found that suppression of Nicotianamine synthase (NAS) expression strongly decreases Zn accumulation in leaf. The restriction of Cd accumulation in *NAS* knockdown plants also indicates an involvement of NA in Cd chelation for *A. halleri.* Histidine has also been described as is an important metal chelator, as it has a high affinity to bind metals. Especially the Ni hyperaccumulating *N. caerulescens* needs Ni chelation by histidine (Richau *et al.*, 2009), but histidine is also essential for *N. caerulescens* to chelate Zn at high pH such as in the cytosol (Küpper *et al.*, 2004).

## Molecular mechanism of metal tolerance

Our knowledge of molecular processes in metal tolerance and accumulation has been greatly enhanced in the past decade. Particularly, significant information has been obtained from extensive investigation of metal tolerant model species *A. halleri* and *N. caerulescens* (Verbruggen et al., 2009). Being close relatives of *Arabidopsis thaliana* has facilitated the molecular investigations of these two species. However, the lack of reference genomes has restricted molecular studies with these high metal tolerant and accumulator species. To overcome this limitation, an attractive approach used by most studies was to compare the transcriptome of metal hyperaccumulators, *A. halleri* and *N. caerulescens*, to related non-accumulator and metal-sensitive species, such as *A. thaliana* or *Thlaspi arvense*, to identify the genes involved in metal tolerance processes (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006; Weber *et al.*, 2006; van de Mortel *et al.*, 2008).

Until recently, the molecular knowledge about metal hyperaccumulation was restricted to Brassicaceae species. The progress in sequencing technologies enabled a deeper and more accurate investigation of the molecular biology in a wider number of metal hypertolerant and hyperaccumulator species. Approaches using these new techniques have already enabled investigations of non-model species such as *Psychotria gabriellae*, tolerant to nickel (Ni) (Merlot *et al.*, 2014); *Sedum alfredii*, tolerant to Zn and Cd (Gao *et al.*, 2013); and *Silene vulgaris*, tolerant to copper (Cu) (Baloun *et al.*, 2014).

These studies revealed that metal tolerance and accumulation in plants are related with different expressions of a similar set of genes (Halimaa *et al.*, 2014). A common evolutionary mechanism described for several genes involved in metal tolerance is copy number expansion in hyperaccumulator species, to increase their expression level compared to related non-accumulators (Krämer, 2010). For instance, while one copy of

the *HMA4* Zn transporter involved in root to shoot translocation was identified in *A. thaliana*, there is a triplication of this gene in *A. halleri* (Hanikenne *et al.*, 2008) and a quadruplication in *N caerulescens* (Lochlainn *et al.*, 2011).

Metal homeostasis is often found to be different in metal hypertolerant and hyperaccumulator species compared to non-accumulator species, with differences in metal uptake, transport and sequestration. Therefore, genes involved in those processes are expected to be main players in controlling metal tolerance and hyperaccumulation. When exposed to high levels of Zn or Cd, most non-tolerant plant species will regulate root plasma membrane transporters as a first step trying to reduce metal uptake. Members of the ZIP family (**Z**inc-regulated transporter, **I**ron-regulated transporter **P**rotein) such as Zn and Fe transporters have a major role in Zn uptake (Krämer *et al.*, 2007). Many ZIP members, including *AtZIP4*, *AtZIP6*, *AtZIP9* and *AtIRT3* are highly expressed in *A. halleri* and *N. caerulescens* compared with non-tolerant relatives. Characterization of *ZIP* genes in hyperaccumulator species such as *NcZNT1, NcZNT5* and *NcZNT6*, orthologues of *AtZIP4, AtZIP5* and *AtZIP6*, demonstrated their important role for Zn accumulation in plants (Wu *et al.*, 2009; Hassan, 2013; Lin, 2014).

Metal sequestration in the vacuoles is an important step for tolerance and accumulation and transporters from different gene families are involved in this process. AtMTP1, a member of **C**ation **D**iffusion **F**acilitator (CDF) family of **M**etal **T**ransport **P**roteins (MTPs), has a major role in Zn storage inside the vacuole. Higher Zn tolerance and accumulation in roots due to *MTP1* overexpression in *A. thaliana* was reported by van der Zaal *et al.* (1999). Higher expression of *AtMTP1* homologues in root and shoot tissues of *A. halleri* and *N. caerulescens* supports the importance of this transporter to enhance Zn vacuolar accumulation (Assunção *et al.*, 2001; Drager *et al.*, 2004). Cd accumulation in hyperaccumulator species has been associated with higher expression of

*HMA3*. This is a member of the P-type **H**eavy **M**etal transporting **A**TPases (HMA) gene family, HMA3 is a tonoplast transporter which contributes to Cd sequestration inside the vacuole (Ueno *et al*., 2011; Chao *et al*., 2012). For metal sensitive species, such as *A. thaliana* and rice, HMA3 expression in roots contributes to root Cd sequestration and Cd tolerance (Ueno *et al*., 2010; Chao *et al*., 2012). HMA4, another P-type ATPase, has a different function. It is a plasma membrane transporter which plays a major role in xylem loading of Zn and Cd and is thus responsible for Zn and Cd translocation from root to shoots in plants. Especially metal hyperaccumulators contain several copies of this gene, corresponding to increased expression compared to regular species (Mills *et al*., 2005; Hanikenne *et al*., 2008; Lochlainn *et al*., 2011; Iqbal *et al*., 2013).

Different from non-accumulator species, metal hyperaccumulators remobilize metals from vacuoles stored in the root. Proteins from the **N**atural **R**esistance **A**ssociated **M**acrophage **P**rotein family (NRAMP) play a role in metal uptake and remobilization with NRAMP1, involved in Cd accumulation (Milner *et al*., 2014), NRAMP3 and NRAMP4 (Oomen *et al*., 2009; Mary *et al*., 2015) involved in Zn and Fe transport, and NRAMP6 (Cailliatte *et al*., 2009) described as a Cd transporter. NRAMP1, a Mn transporter, has been recently described as also responsible to translocate Cd (Milner *et al*., 2014). Recent transcriptomic analysis of *N. caerulescens* supports a role of NRAMP1 in Cd accumulation (Halimaa *et al*., 2014). As a crucial step for high accumulation, hyperaccumulators translocate metals rapidly and efficiently to the shoot.

Most of these transporters can also transport other elements. As a result, changes in Zn homeostasis and Cd tolerance processes affect other micronutrient concentrations such as Fe and Mn. Fe deficiency is therefore often one of the symptoms of Zn and Cd toxicity in non-tolerant species. *IRT1*, *IRT3*, *FRD3* and *NRAMP3* have been suggested to take part in the cross-talk between Zn and Fe homeostasis (Oomen *et al*., 2009;

Shanmugam *et al.*, 2011; Pineau *et al.*, 2012). A specific regulation of these genes allows hyperaccumulators species to keep a balance between Zn tolerance and Fe homeostasis (Shanmugam *et al.*, 2013).

## *Gomphrena claussenii*

Shortly before the start of my PhD research, the first Zn/Cd hypertolerant species from South America was identified (Borin, 2010). The metallophyte *Gomphrena claussenii* Moq. was found growing at a Zn mining site near Vazante in the state of Minas Gerais (MG), Brazil, the state where almost all of the Zn mining of Brazil takes place (Filho & Viana, 2011). So far *G. claussenii* has been reported to grow in 'cerrado' and 'caatinga' ecosystems at few sites in the Minas Gerais and Bahia states of Brazil (Ferreira & Dias, 2004; Forzza *et al.*, 2010). *G. claussenii* is a perennial bushy species belonging to the Amaranthaceae family, with a high Zn/Cd accumulation, relatively large biomass production for a metal highly accumulator species and an easy regrowth capacity.

In contrast to most other Zn/Cd hyperaccumulators, *G. claussenii* produces a reasonable biomass in the field, which made it an attractive species to investigate further as it could be a promising candidate species to be used in Zn/Cd phytoextraction. Such studies could explain how this plant accumulates and tolerates heavy metals, providing further information on its physiology and the molecular genetic mechanisms supporting this. Since most of our knowledge of metal hypertolerant/hyperaccumulators is from temperate environments, the study of *G. claussenii* will be especially useful for future studies of (sub)tropical Cd and Zn accumulator species. Understanding the genomics and transcriptomics of *G. claussenii* will also contribute substantially to the genomic knowledge of Amaranthaceae species especially on the regulation of micronutrient levels

in Amaranthaceae crops such as spinach (*Spinacia oleracea*), beet (*Beta vulgaris*), quinoa (*Chenopodium quinoa*) and Amaranth species. In addition this species research will provide useful information for comparison of the evolution of Zn and Cd hypertolerance between *G. claussenii* (Amaranthaceae) and Brassicaceae species.

Despite being an attractive species for scientific research, there are some disadvantages associated with *G. claussenii* as the object of study. The scarcity of information about *G. claussenii* implicates in experimental difficulties when studying this species. For instance, with no genome information available for *G. claussenii* or any species in this genus, molecular genetic investigations are challenging. The recent developed technique of deep-coverage sequencing of cDNA or RNA-seq may be an efficient option to have a detailed transcriptome analysis of *G. claussenii* even without the availability of a reference genome. Another challenge is the lack of regeneration and genetic transformation protocols for this species, which limits the options to experimentally verify gene functions. The development of an optimum stable transformation protocol is not straightforward and can take a long time. This leaves only the alternative, to study interesting *G. claussenii* genes upon ectopic expression in well-known model species such as tobacco and *A. thaliana*. Fortunately, *G. claussenii* is part of the *Gomphrena* genus, for which *Gomphrena elegans* is identified as a very closely related species, which is sensitive to high Zn and Cd conditions. Thus, physiology and molecular genetics of both species can be compared to identify candidate genes or alleles that distinguish their contrasting metal-response characteristics. This species comparative analysis have been successfully used to study other metal tolerant species such as *N. caerulescens* and *A. thaliana* (van de Mortel *et al.*, 2006; van de Mortel *et al.*, 2008); *N. caerulescens* and *N. arvense* (Hammond *et al.*, 2006); *A. halleri* and *A. thaliana* (Talke *et al.*, 2006; Weber *et al.*, 2006).

## Outline of the thesis

In this PhD research, fundamental aspects of the Zn/Cd hypertolerant and -accumulating species *G. claussenii* were investigated in order to identify molecular mechanisms, including the genes involved, responsible for its metal tolerance and accumulation properties. The Zn and Cd tolerance of *G. claussenii* is assessed in chapter 2, and compared with a closely related non-tolerant species, *Gomphrena elegans*. The physiological effects of high Zn or Cd exposure on *G. claussenii* and *G. elegans* were evaluated through soil and hydroponics experiments. Metal accumulation capacity of shoots and roots for both species were also examined, to assess their phytoremediation potential. In chapter 3, the Zn and Cd cellular localization and distribution analyses of *G. claussenii* stems and leaves are present. This includes a metabolite profile analysis of *G. claussenii* roots and shoots comparing plants exposed to high Cd or high Zn with non-exposed plants, to identify possible metal chelators. In chapter 4 the attention moves from physiological to molecular genetic aspects of *G. claussenii* hypertolerance. A comparative transcriptomics analysis of *G. claussenii* and *G. elegans* was used to investigate molecular mechanisms and identify genes potentially responsible for the adaptation of *G. claussenii* to high Zn/Cd exposure. Transcript sequences were annotated, and expression level differences upon Zn and Cd exposure were analysed in *G. claussenii* and its metal-sensitive congener to distinguish differentially regulated processes. The relevance of the knowledge obtained in this thesis research for our understanding of plant metal hypertolerance and for application in phytoremediation, as well as the potential for future research of *G. claussenii*, is discussed in chapter 5.

# CHAPTER 2

## *Gomphrena claussenii*, the first South-American metallophyte species with indicator like Zn and Cd accumulation and extreme metal tolerance

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## ABSTRACT

Plant species with the capacity to tolerate heavy metals are potentially useful for phytoremediation since they have adapted to survive and reproduce under toxic conditions and to accumulate high metal concentrations. *Gomphrena claussenii* Moq., a South-American species belonging to the Amaranthaceae, is found at a zinc (Zn) mining area in the state of Minas Gerais, Brazil. Through soil and hydroponic experiments, the metal tolerance and accumulation capacities of *G. claussenii* were assessed and the effects on physiological characteristics were compared with a closely-related non-tolerant species, *Gomphrena elegans* Mart. *Gomphrena. claussenii* plants grown in soil sampled at the Zn smelting area accumulated up to 5318 μg $g^{-1}$ of Zn and 287 μg $g^{-1}$ of Cd in shoot dry biomass after 30 days of exposure. Plants were grown in hydroponics containing up to 3000 μM of Zn and 100 μM of Cd for *G. claussenii* and 100 μM of Zn and 5 μM of Cd for *G. elegans*. *Gomphrena claussenii* proved to be an extremely tolerant species to both Zn and Cd, showing only slight metal toxicity symptoms at the highest treatment levels, without significant decrease in biomass and no effects on root growth, whereas the non-tolerant species *G. elegans* showed significant toxicity effects at the highest exposure levels. Both species accumulated more Zn and Cd in roots than in shoots. In *G. elegans* over 90% of the Cd remained in the roots, but *G. claussenii* showed a root:shoot concentration ratio of around 2, with shoots reaching 0.93 % Zn and 0.13 % Cd on dry matter base. In *G. claussenii* shoots, the concentrations of other minerals, such as Fe and Mn, were only affected by the highest Zn treatment while in *G. elegans* the Fe and Mn concentrations in shoots decreased drastically at both Zn and Cd treatments. Taking together, these results indicate that *G. claussenii* is a novel metallophyte, extremely

tolerant of high Zn and Cd exposure and an interesting species for further phytoremediation studies.

## INTRODUCTION

In many parts of the world, soils have become polluted with high levels of heavy metals mainly due to industrial activities (Ernst, 2006). Most plant species are sensitive to these contaminated conditions whilst certain species have evolved the ability to survive and reproduce in such toxic environments. Such ability can be attained by plants mainly through two strategies: avoidance and tolerance. While species belonging to the first group invest in external mechanisms to keep metals chelated outside, metal tolerant plants developed a physiological machinery adapted to accumulate these high metal concentrations inside the root and/or shoot, dealing with the enhanced stress this will cause (Baker, 1987).

Exposure to high levels of metals is likely to cause alterations in plant physiology. Stunted growth, leaves chlorosis, iron (Fe) deficiency, water unbalance, and reduction of photosynthesis rate are symptoms usually displayed by non-tolerant species when exposed to high levels of zinc (Zn) and cadmium (Cd) (Clemens, 2006; Broadley *et al.*, 2007; Gallego *et al.*, 2012).While sensitive species present phytotoxic symptoms with concentrations of Zn from 100–400 μg $g^{-1}$ and of Cd from 5–30 μg $g^{-1}$ in shoots, hypertolerant plants can complete their life cycle accumulating more than 3000 μg $g^{-1}$ of Zn and/or 100 μg $g^{-1}$ of Cd (Kabata-Pendias & Mukherjee, 2007a; van der Ent *et al.*, 2013).

To overcome this stress condition, hypertolerant plants have selected physiological strategies to remove the toxic ions from the most sensitive subcellular parts, such as the cytosol and various organelles (Clemens, 2001). Metal surplus chelation and sequestration

into the vacuole or excretion to the apoplast, are mechanisms widely used by hypertolerant species to reduce internal metal bioavailability (Clemens, 2006; Ernst, 2006). Whereas some hypertolerant species accumulate most of the heavy metals inside the root, a particular group, defined as metal hyperaccumulators, have evolved the strategy to translocate and store the metals preferably in the shoot (Brown *et al.*, 1995).

In the last few decades hyperaccumulator species have received substantial attention because of their interesting metal homeostasis physiology and potential application in phytoremediation, a technology based on the ability of plants to extract or stabilize pollutants in the environment and thus contribute to functional restoration of contaminated areas (Marques *et al.*, 2009).

Phytoextraction theoretically is the ideal remediation technique, capable to reduce soil metal concentrations, at a low cost, to non-toxic levels (Dickinson *et al.*, 2009). To achieve such in an economically viable way, it is crucial to combine traits like high biomass and high metal tolerance and accumulation in the phytoextraction plants (Chaney *et al.*, 2007). At moderately contaminated sites phytoextraction has proved to be feasible using hyperaccumulator species (Rascio & Navari-Izzo, 2010; Hanikenne & Nouet, 2011), however, because such species usually have low biomass production, phytostabilization may be the appropriate technique for severely contaminated soils (Zhao & McGrath, 2009). In such a case, plants are used to prevent leaching of pollutants from the soil and provide cover vegetation to improve the soil quality and reduce wind contamination, to further minimize the risk of erosion and leaching leading to contamination of ground and surface waters (Dickinson *et al.*, 2009; Zhao & McGrath, 2009).

Hypertolerance has likely evolved independently within different angiosperm families (Ernst, 2006) and often this is a trait present only in one genus or even one species. Some families, such as the Brassicaceae, show a higher occurrence of Zn, Cd or Ni hyperaccumulators species, like the hyperaccumulator models *Noccaea caerulescens* and *Arabidopsis halleri* (Broadley *et al.*, 2001; Assunção *et al.*, 2003b; Hanikenne & Nouet, 2011).

Researches with hypertolerant and hyperaccumulator species from tropical environments falls far short of what is known about temperate taxa (Baker *et al.*, 2010). Latin America is the least studied continent, with few metallophyte (metal tolerant and/or hyperaccumulator plants) species reported: only 172 species among which 89% are related to nickel (Ni). So far no Zn or Cd hyperaccumulator species have been described (Ginocchio & Baker, 2004). Nevertheless, there is no clear geographic reason that Latin America is so poorly represented, as it has a uniquely diverse flora (8 of the 25 biodiversity hotspots in the world are in Latin America) but also due to the presence of countless sites rich in metal ores as well as metal smelter areas (Ginocchio & Baker, 2004; Reeves *et al.*, 2007; Baker *et al.*, 2010).

Plants naturally growing in metal-enriched soils are in general metal tolerant, which makes the vegetation native to contaminated areas an important potential source of metal tolerant and accumulator species (Baker & Brooks, 1989). One example is the Zn mining site near Vazante in the state of Minas Gerais, Brazil, where almost all of the zinc extraction in Brazil takes place (Filho & Viana, 2011). One species at this site, *Gomphrena claussenii* Moq. (Fig. 1) has the ability to grow and thrive at the locally high Zn and Cd levels, making it a potentially interesting species for phytoremediation. *Gomphrena claussenii* is a perennial species, belonging to the Amaranthaceae family, and native to Brazil (Marchioretto *et al.*, 2010). *Gomphrena elegans* Mart. is a related species, which is

widespread in South America, but not reported to be tolerant to excess metal exposure (Mussury *et al.*, 2006). It is used as a metal sensitive species for this study. The work presented here aims to evaluate the physiological effects of high Zn and Cd on *G. claussenii* when compared with *G. elegans*. Consequently, we assess the metal tolerance capacity of *G. claussenii* to toxic metals and evaluate its potential for use in phytoremediation.

Figure 1 - *Gomphrena claussenii* Moq. plants growing at a Zn mining site at Vazante, Minas Gerais, Brazil.

## MATERIALS AND METHODS

### Plant material and growth conditions

*Gomphrena claussenii* Moq. plants were collected from a zinc mine area at Vazante, in the state of Minas Gerais (MG), Brazil. *Gomphrena elegans* seeds were collected in the field at Antônio João, in the state of Mato Grosso do Sul, Brazil, and provided by Dr. Rosilda Mara Mussury from the Federal University of Grande Dourados,

Dourados, Brazil. Seeds could not be used as starting material for *G. claussenii* since field access was limited and seeds are only mature at restricted periods during the year. Instead, seven individual plants were collected at the site and vegetatively propagated. After a pilot experiment and confirmation of the high Zn and Cd tolerance of all plants, one line was brought into *in vitro* tissue culture and taken to The Netherlands for further experiments. *Gomphrena elegans* seeds were sterilized and germinated as start material.

Both species were vegetatively propagated through tissue culture using half-strength Murashige and Skoog (MS) medium containing, 2% sucrose and 0.8% agar at pH 5.8. Plant material were cultured in a growth room (24 °C, 250 μMoles $m^{-2}$ $s^{-1}$ light at plant level and a 16-h light / 8-h dark cycle). Two-week-old tissue-culture grown cuttings were used as starting material for soil and hydroponics experiments.

## Zn and Cd tolerance

**Soil assay** - Zinc ore extracted at Vazante is processed in the metal smelter at Três Marias, MG, Brazil. Soil samples were collected at four different sites around the zinc smelter, three contaminated sites and one non-contaminated control site at some distance from the smelter (Control: 18°12'16"S / 45°14'02"W; Site1: 18°11'25"S / 45°14'10"W; Site2 18°11'08"S / 45°14'07"W; Site3 18°11'06"S / 45°14'24"W). Pre-cultured *G. claussenii* plants were planted in the four different soils in 250-ml pots. The experiment was performed with three repetitions, each represented by one pot with one plant, during the winter season in a glass green house, at Lavras Federal University, Lavras, MG, Brazil,. After 30 days, shoots were harvested and washed with demi-water for biomass and mineral concentration measurements.

**Hydroponic assay** - *Gomphrena claussenii* and *G. elegans in vitro* cuttings were transferred to 600-ml polyethylene pots (one plant per pot and three replicate pots per

treatment) containing a modified Clark's full strength nutrient solution (Clark, 1975): 1.3 mM $KNO_3$, 2.53 mM $Ca(NO_3)_2$, 0.9 mM $NH_4NO_3$, 0.6 mM $MgSO_4$, 0.5 mM KCl, 34.5 µM $Ca(H_2PO_4)_2$, 19 µM $H_3BO_3$, 2 µM $ZnSO_4$, 7 µM $MnCl_2$, 0.5 µM $CuSO_4$, 0.086 µM $(NH_4)_6Mo_7O_{24}$, and 38 µM Fe(Na)EDTA. The pH buffer MES was added at 2 mM and the pH was set at 5.5 using KOH. After three weeks growing on quarter-strength Clark's solution, plants were exposed to half-strength solution with normal Zn (2 µM) or excess Zn/Cd: 100, 1000 and 3000 µM of $ZnSO_4$ or 10, 50 and 100 µM of $CdSO_4$ (at 2 µM $ZnSO_4$) for *G. claussenii* and 100 µM of $ZnSO_4$ or 5 µM of $CdSO_4$ (at 2 µM $ZnSO_4$) for *G. elegans*. The applied Zn and Cd concentrations were chosen to be in the range of bioavailable concentrations at the site of collection and based on pilot experiments. The solutions were replaced once a week and plant culture was performed in a climate chamber (20/15 °C day/night; 250 µMoles $m^{-2}$ $s^{-1}$ light at plant level; 12h day length; 70% RH). After three weeks of metal exposure or control treatment, the plants were harvested. Roots were first desorbed with ice-cold 5 mM $PbNO_3$ for 1h. Solubility of minerals was calculated using the solution speciation software Visual MINTEQ 3.0 (Gustafsson, 2000).

## Root elongation

The ability of *G. claussenii* and *G. elegans* to tolerate excess metal exposure was tested through root elongation measurements (Schat & Ten Bookum, 1992). Plants were grown in the same hydroponic conditions as described above, at normal (2 µM of Zn) or the highest metal exposure (3000 µM $ZnSO_4$ or 100 µM $CdSO_4$/2 µM $ZnSO_4$ for *G. claussenii* and 100 µM $ZnSO_4$ or 5 µM µM $CdSO_4$/2 µM $ZnSO_4$ for *G. elegans*). Before metal exposures, roots were stained with active coal powder to allow the measurement of the longest unstained root (Schat & Ten Bookum, 1992). Roots were measured after three and six days of exposure.

## Assessment of mineral concentrations

Shoot samples from *G. claussenii* plants were collected for mineral analyses from plants sampled at six different locations at the Zn mining site (Vazante). Plant materials were digested in a CEM® Mars-5 microwave oven system (CEM Corporation, Matthews, NC, USA), following the USEPA 3051 method (USEPA, 1995). For the soil experiment, both plant and soil materials were digested as mentioned before. Metal bioavailability (water-soluble fraction) was estimated from soil solution extracts obtained by the saturated-paste technique (Raij *et al.*, 2001). Filtrates were passed through 0.22-µm cellulose membranes to determine the total dissolved metals. The concentrations of Zn and Cd in all extracts were determined by using either flame or graphite-furnace atomic absorption spectrophotometry (Perkin-Elmer®AAnalyst™800). NIST standard reference materials (SRM 1573a Tomato Leaves, SRM 2710 Montana Soil, SRM 1640 Trace Elements in Natural Water) were used to check the accuracy of elemental determinations, which was found satisfactory, i.e., metal recoveries ranged from 78 to 122%. For the analysis of total metal concentrations in plant samples of the hydroponic experiments, 50-90 mg of each sample was wet-ashed in 2 ml of a 4 : 1 mixture of $HNO_3$ (65%) and HCl (37%), in Teflon bombs for 7 h at 140°C and thereafter had their volume adjusted to 5 ml with demineralised water. Metal concentrations (Zn, Cd, Fe and Mn) were determined using flame atomic absorption spectrophotometry (Perkin Elmer AAnalist 100; Perkin Elmer Nederland, Nieuwerkerk a/d IJssel, The Netherlands).

## Statistics analyses

Data was statistically evaluated through ANOVA tests following the Tukey test used to compare means.

# RESULTS

## Mineral analyses from the field

As expected for metal mining areas there is considerable variation in the soil metal levels at the site and consequently also the plants collected at different locations at the site showed variation for Zn and Cd concentrations in shoots. Plants contained between 230 to 10434 μg $g^{-1}$ of Zn and 6 to 96 μg $g^{-1}$ of Cd in shoot dry weight samples. Concentrations were correlated with soil levels meaning that higher levels were found in plants growing in more contaminated sites whereas the lowest levels were found in plants collected in an area close to the mine but were mining was not conducted.

## Soil and plant Zn and Cd analysis

Soil samples were taken from the zinc smelter at four different points. Total metal concentrations varied when comparing the different sampling sites and they showed extremely high Zn and Cd levels compared to the control sample (Table 1), as was expected for soil sampled at a zinc smelting area. Although slightly higher than metal concentrations normally found in non-contaminated soil, the metal concentrations of the control sample are within the range for non-contaminated soil, even though the sample was taken not far from the industrial area. The highest levels of both metals are found in sample 3, which is taken at the Zn smelter ore waste deposit site, while the other two samples are taken slightly more distant from this site. Comparing to the total levels, the water soluble (available) metal concentrations were always much lower, except for site 3, at which they were above what is considered to be within the normal range for plants (Kabata-Pendias & Mukherjee, 2007a) (Table 1). *Gomphrena claussenii* plants taken from the site grew well in a greenhouse in pots containing this soil and accumulated up to 5318

μg $g^{-1}$ of Zn and 287 μg $g^{-1}$ of Cd in their shoots after 30 days of exposure (Table 1). Since air contamination was excluded once plants were grown in a greenhouse far away from the Zn smelter, the high metal concentrations can only be caused by high uptake and root to shoot translocation of metals from the soil.

Table 1- Zinc (Zn) and cadmium (Cd) concentrations (µg g-1) in soil samples from the Zn smelting area at Três Marias, MG, Brazil, and in shoots of *Gomphrena claussenii* plants after growing for 30 days in control and metal contaminated soil collected at four sites around the Zn smelter. Mean total and water-soluble (available) soil and shoot metal concentrations are shown, with standard errors between brackets. n=3. DW - dry weight.

| Sample site | Soil (µg $g^{-1}$) | | | | Shoot (µg $g^{-1}$ DW) | |
|---|---|---|---|---|---|---|
| | Total concentration | | Available concentration | | | |
| | Zn | Cd | Zn | Cd | Zn | Cd |
| Control | 117.7 (2.1) | 3.6 (0.4) | 0.2 (0.1) | 0 | 22 (1.2) | 1 (0.1) |
| Site 1 | 3830.1 (62.9) | 73 (2.2) | 0.6 (0.01) | 0.06 (0) | 237.9 (1.4) | 7.5 (0.1) |
| Site 2 | 960.2 (104.3) | 12 (1.7) | 4.9 (0.1) | 0.08 (0) | 241.2 (1.1) | 7.3 (0.03) |
| Site 3 | 15212.9 (580.3) | 147.4 (4.4) | 168.2 (18.4) | 4.63 (0.1) | 5318.36 (10.33) | 287 (8) |

## Zn and Cd tolerance in hydroponic solution

Exposing plants to hydroponic solutions with high concentrations of metals can be misleading if metals precipitate upon preparing the solution and thus become unavailable to plants. Therefore we calculated solubility of Zn and Cd in the half-strength Clark's solution we used as growing medium. Even at the highest Zn and Cd concentrations, both metals were completely soluble and available for uptake (Table 2). The main ion forms for those elements in solution are $Zn^{2+}$ and $Cd^{2+}$. These forms are expected to be readily available for plant uptake.

Table 2 - Zinc (Zn) and cadmium (Cd) speciation in half strength Clark's solution containing the highest Zn or Cd concentrations which were used in exposure experiments (3000 µM Zn or 100 µM Cd), as calculated according to Visual MINTEQ 3.0. Speciations with less than 1% were not included. aq – aqueous.

| Component | % of total concentration | Speciation |
|---|---|---|
| $Cd^{2+}$ | 93.1 | $Cd^{2+}$ |
| | 1.9 | $CdCl^{+}$ |
| | 3.7 | $CdSO_{4\,(aq)}$ |
| $Zn^{2+}$ | 82.4 | $Zn^{2+}$ |
| | 16.6 | $ZnSO_{4\,(aq)}$ |

Zn and Cd tolerance was evaluated in *G. claussenii* and *G. elegans* plants based on three parameters: toxicity symptoms, growth rate (dry weight) and root elongation. The *G. claussenii* plants only exhibited slight metal toxicity symptoms, and exclusively at the highest treatment levels (3000 µM Zn or 100 µM Cd) (Fig. 2A and B), confirming their extreme tolerance to both Zn and Cd treatments. *Gomphrena elegans* plants already developed visual toxicity symptoms when exposed to 100 µM of Zn and 5 µM of Cd (Fig. 2C and D). They also showed a reduced growth rate and strong leaves chlorosis, starting in the first week of metal exposure. The toxicity symptoms became more severe with increasing exposure time. In the third week the oldest leaves started to fall off.

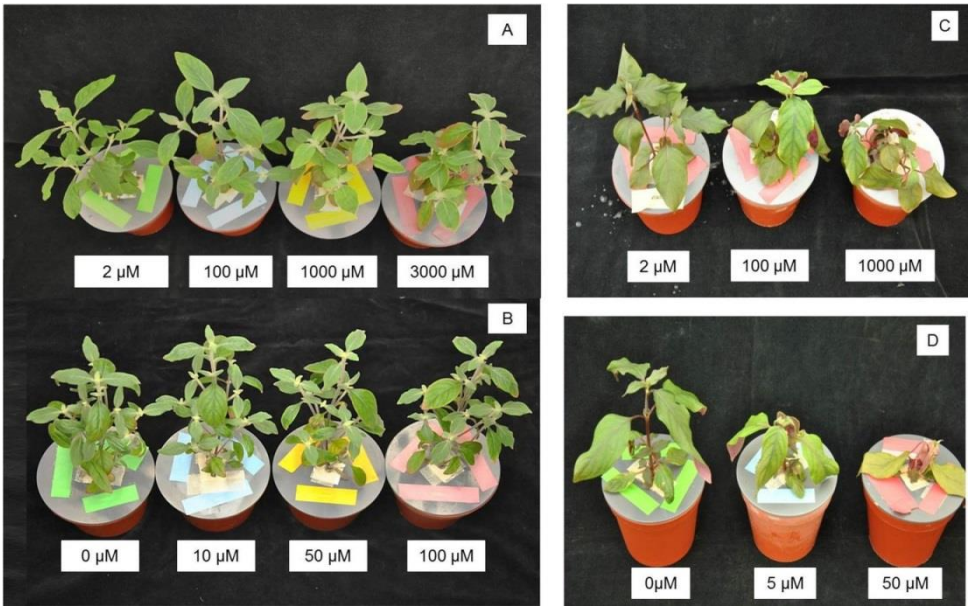


Figure 2 – *G. claussenii* (A,B) and *G. elegans* (C,D) plants after three weeks of exposure to different concentrations of zinc (Zn) (A,C) and cadmium (Cd) (B,D). Plants are grown hydroponically in half strength Clark's solution, containing 2 µM Zn, and supplemented to 100, 1000 or 3000 µM Zn, respectively 10, 50 or 100 µM Cd for *G. claussenii* and 100 or 1000 µM of Zn, respectively 5 or 50 µM of Cd for *G. elegans*.

Growth responses to high Zn and Cd concentrations were different for *G. claussenii* and *G elegans*. As expected for a non-metal-tolerant species, *G. elegans* biomass production decreased notably and significantly ($P < 0.05$; n=3) for shoot and roots when comparing plants grown at high metal exposures with the ones grown in control conditions. Such was not the case for *G. claussenii*, for which an increase in Zn or Cd concentration did not reduce root or shoot dry weight, not even at the highest metals concentrations (Fig. 3). In fact, the concentration of 2 µM Zn, which is considered to be sufficient for plants in general, may be suboptimal for *G. claussenii* plants, which produce

a higher, shoot biomass at elevated Zn concentrations, although the difference was not statistically significant with the low number of plants we tested ($P > 0.05$; n=3).

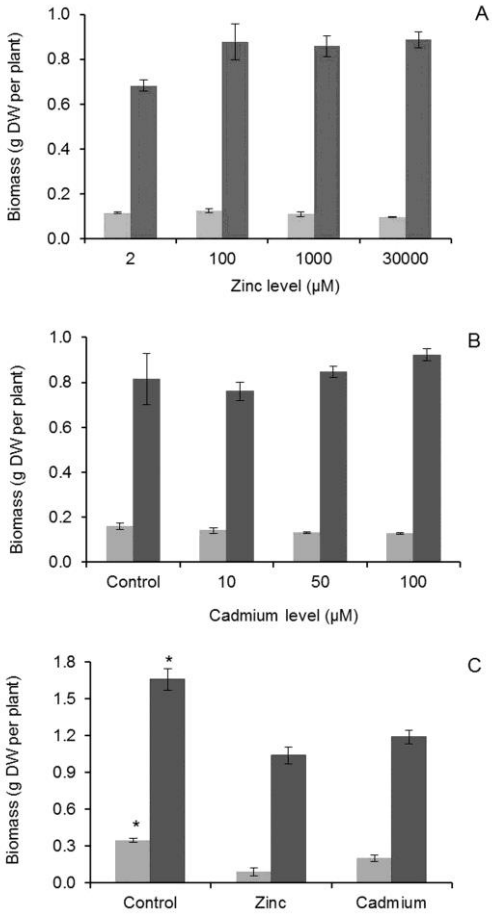


Figure 3 – Root (light grey bars) and shoot (dark grey bars) biomass of *G. claussenii* (A and B) and *G. elegans* (C). *G. claussenii* plants were exposed to different zinc (Zn; 2–3000 µM) and cadmium (Cd; 0-100 µM) concentrations and *G. elegans* plants were exposed to normal Zn (2 µM), high Zn (100 µM) and Cd (5 µM), for 3 weeks. Data points and error bars represent means (n = 3) and standard errors, respectively. DW - dry weight. Asterisks denote significant differences of control (2 µM Zn) from treatments as found upon Tukey's testing ($P < 0.05$).

Root elongation measurements confirmed the strong metal tolerance properties of *G. claussenii*, especially when compared to *G. elegans*. Upon Zn (3000 µM) and Cd (100 µM) treatments *G. claussenii* plants showed no significant effects in root growth ($P < 0.001$; n=6) compared with plants grown under control conditions (Fig. 4). Instead, roots seemed to grow even longer under high Zn and high Cd. *Gomphrena elegans* plants presented a drastic reduction in root growth, already after three days of exposure to 100 µM of Zn or 5 µM of Cd (Fig. 4B). With increased exposure time *G. claussenii* showed no reduction in growth while *G. elegans* plants after 6 days of exposure displayed an even higher reduction in root growth.

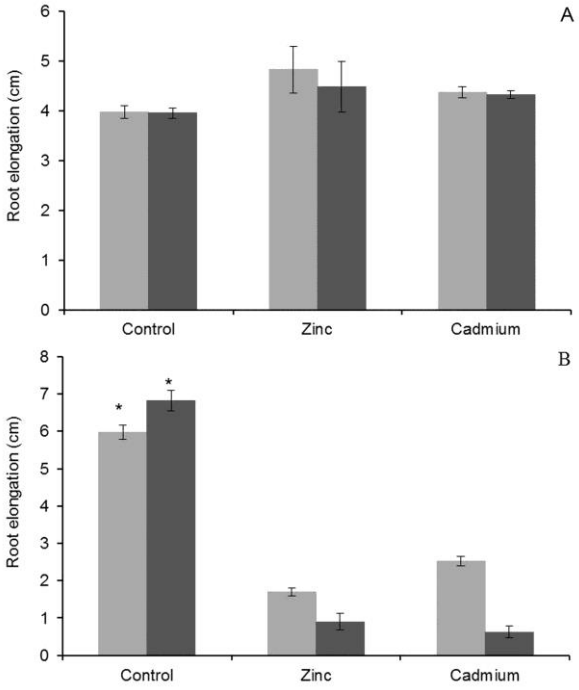


Figure 4 - Increase in root length of *G. claussenii* plants after 3 days (light grey bars) and 6 days (dark grey bars) of exposure to 2 µM zinc (Zn; Control), 3000 µM Zn (Zinc) and 100 µM Cd (Cadmium) (A); and of *G. elegans* plants after exposure to 2 µM Zn (Control), 100 µM Zn (Zinc) and 5 µM Cd (Cadmium) (B). Means values and standard errors are shown (n = 6). Asterisks denote significant differences of control (2 µM Zn) from treatments as found upon Tukey's testing ($P < 0.05$).

## Mineral concentrations

Mineral concentrations were measured from plants growing in hydroponic conditions after three weeks of exposure to elevated Zn/Cd conditions. Zn and Cd concentrations increased significantly for both studied species in roots and shoots with increased Zn or Cd exposure levels (Fig. 5). *Gomphrena claussenii* and *G. elegans* showed higher Zn and Cd concentrations in roots than shoots at all treatment levels. For *G.*

*claussenii* the metal concentrations increased nearly proportional with increasing exposure. In contrast to the non- metal-tolerant species, *G. claussenii* was able to store extremely high concentrations of these elements in shoots, eventually reaching 9.3 g Zn $kg^{-1}$ dry weight (Fig. 5A) and 1.3 g Cd $kg^{-1}$ dry weight at the highest exposure levels (Fig. 5B). The root : shoot accumulation ratios for Zn and Cd in *G. claussenii* averaged around two (Fig. 5). *Gomphrena elegans* was clearly not tolerant to Cd, not even at the modest exposure level of 5 µM. Although the plants managed to keep Cd out of the shoot, the root concentrations at this exposure level already exceeded those found in *G. claussenii* at 10 µM Cd exposure (Fig. 6D). At this concentration, more than 90% of the Cd was found in the roots of *G. elegans*. Although the shoot Cd concentrations were high, they remained significantly lower than those in *G. claussenii* shoots at 10 µM Cd exposure ($P < 0.01$; n=3). At exposure to 100 µM Zn, the Zn concentrations in *G. elegans* roots and shoots were about 2, respectively 2.5 times lower than in *G. claussenii* (Fig. 6A and B).

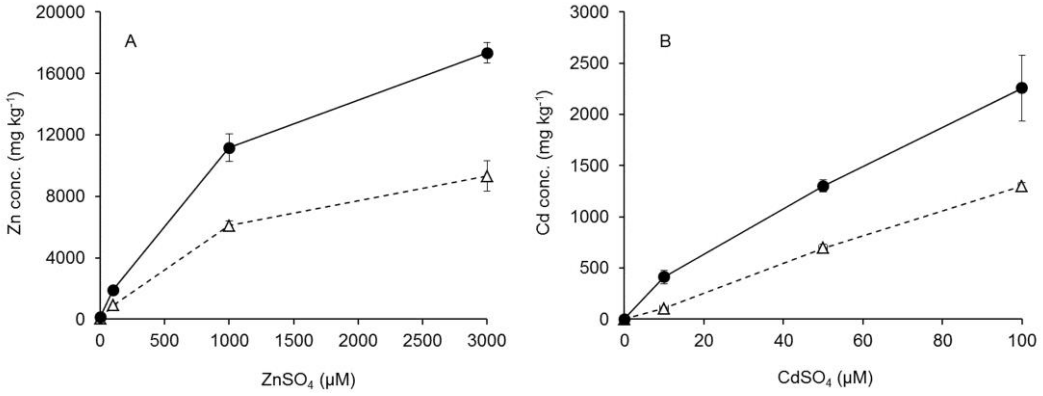


Figure 5 - Zinc (Zn) (A) and cadmium (Cd) concentrations (B) (in mg $kg^{-1}$ dry weight; mean ± SE) of *G. claussenii* shoots (Δ) and roots (●) upon growth in hydroponic nutrient solutions. Plants were grown for 3 weeks in a hydroponic solution containing 2 µM $ZnSO_4$ before exposure to elevated $ZnSO_4$ (100 µM; 1000 µM and 3000 µM) or $CdSO_4$ concentrations (0; 10 µM; 50 µM and 100 µM).

The exposure to elevated Zn and Cd concentrations was also expected to affect the concentrations of other minerals, such as iron (Fe) and manganese (Mn), for which homeostasis mechanisms often interact with those for Zn. *Gomphrena claussenii* and *G. elegans* Fe concentrations in roots increased with an increase of Zn and Cd supply (Fig. 7 BI and BII). Fe concentrations in *G. claussenii* shoots were statistically similar ($P > 0.05$; n=3) at all Zn and Cd exposure levels, even though at the highest Zn exposure, the Fe concentration appeared to be lower (Fig. 7AI and AII). In *G. elegans* the shoot Fe concentrations decreased significantly ($P < 0.01$; n=3) at both Zn and Cd treatments (Fig. 7AIII). Mn concentrations were also affected by the Zn and Cd treatments (Fig. 7C). Although the Mn concentration in roots of Zn-exposed *G. claussenii* plants seemed to decrease at the highest exposure level, this was statistically not significant ($P > 0.05$; n=3). The Mn concentration in shoots decreased significantly ($P < 0.05$) with increasing Zn exposure levels (Fig. 7CI). When exposed to Cd, Mn decreased drastically in roots but

stayed similar in shoots (Fig. 7CII). There was no difference between metals treatment in *G. elegans*, both Zn and Cd treatments significantly reduced the Mn concentrations in roots and shoots ($P < 0.01$; n=3) (Fig. 7CIII).

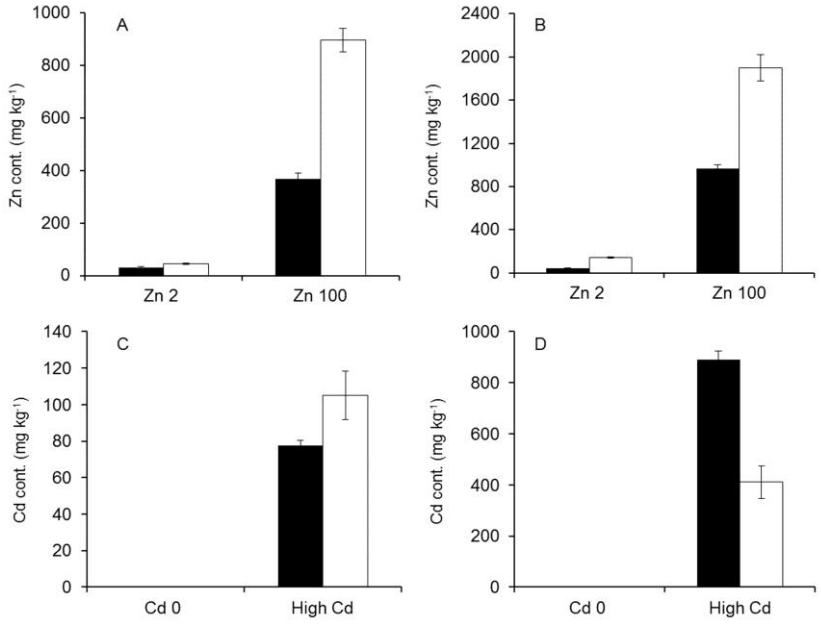


Figure 6 - Comparison between *G. elegans* (black bars) and *G claussenii* (white bars) zinc (Zn) and cadmium (Cd) concentrations (mg $kg^{-1}$; mean ± SE) in shoots (A, C) and roots (B,D) of plants exposed to comparable Zn (A, B) or Cd treatment levels (C, D). Zn treatment concentrations were 2 and 100 µM of $ZnSO_4$ (Zn2, Zn100). Cd treatment concentrations were either no exposure (Cd0) or 5 and 10 µM of $CdSO_4$ to *G. elegans* and *G. claussenii* respectively (High Cd). Plants were grown for 3 weeks on control solution (2 µM Zn) before 3 weeks of treatments exposure.

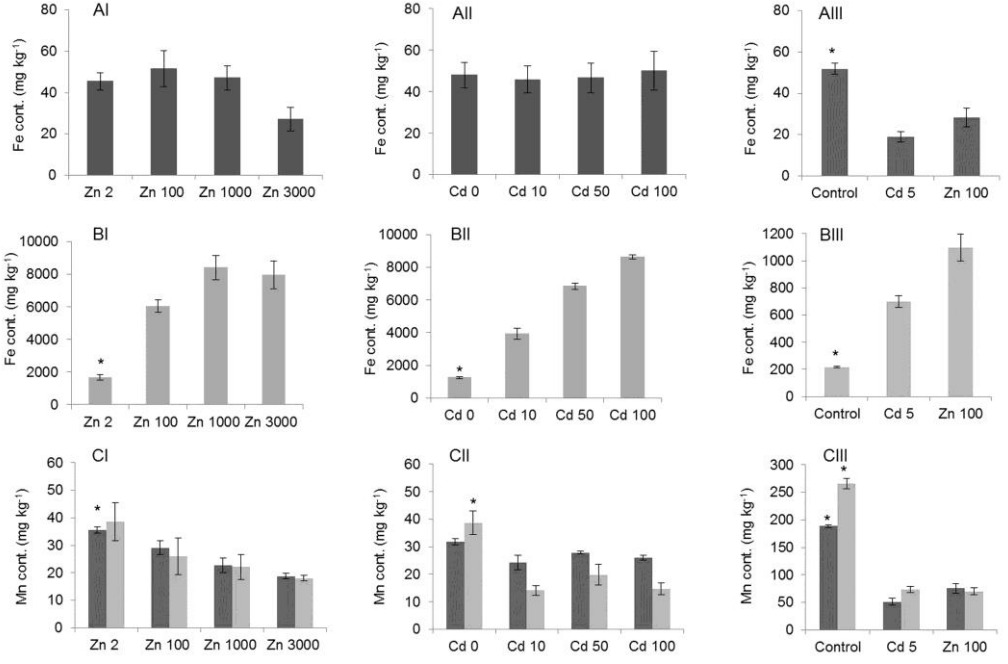


Figure 7 – Iron (Fe; A and B) and manganese (Mn; C) concentrations (mg $kg^{-1}$; mean ± SE) in *G. claussenii* (I and II) and *G. elegans* (III) shoot (dark grey bars) and root (light grey bars). Plants were grown for 3 weeks on control solution (Zn - 2μM) before exposure to Zn (2, 100, 1000 and 3000 μM) and Cd (0, 10, 50 and 100 μM) treatments for *G. claussenii* and to Zn (2 μM and 100 μM) and Cd (5 μM) treatments for *G. elegans*. Asterisks denote significant differences of control (Zn 2 μM) from treatments by Tukey's test ($P < 0.05$).

## DISCUSSION

The results we present here demonstrate that *G. claussenii* is indeed a novel metallophyte, extremely tolerant to high Zn and Cd exposure. This is the first report of a species of this kind from South America. Field results, together with pot experiments using soil from contaminated sites, present clues about the high Zn and Cd tolerance. These results also give an indication of the potential of this species for phytoremediation purposes under field-like conditions (Chaney *et al.*, 2007), where mixed contaminations are more rule than exception (Kabata-Pendias & Mukherjee, 2007b). While the high metal tolerance is not unexpected, given the abundance of the species at the Zn mining site, the high levels of accumulation are surprising. The reported shoot Zn and Cd concentrations of *G. claussenii* plants grown on contaminated soil collected at the site (max. 5300 and 280 μg $g^{-1}$ for Zn and Cd respectively; Table 1) clearly exceed the recently proposed threshold levels to classify species as metal hyperaccumulators, which are 3000 μg Zn $g^{-1}$ dry weight and 100 μg Cd $g^{-1}$ dry weight (van der Ent *et al.*, 2013). This means *G. claussenii* is not only a metallophyte, but also a Zn/Cd hyperaccumulator species, again the first one known from the South American continent.

The hydroponic metal exposure experiments we performed subsequently provided an excellent way to evaluate the maximum levels of Zn/Cd tolerance and accumulation. A crucial point to consider when using extremely high levels of metals in hydroponic solutions is the metal availability (Baker & Whiting, 2002). We preferred to use half strength Clark's nutrient solution (Clark, 1975) for hydroponics, which is different from the more often used half Hoagland's solution (Hoagland & Arnon, 1940), mainly because it allowed us to expose plants to higher Zn concentrations without precipitation of metals (Table 2).

To evaluate metal tolerance and accumulation of *G. claussenii*, we used the closely related species *G. elegans* as comparison, which is common in many South American countries (Mussury *et al.*, 2006) and not known to be adapted to heavy metal exposure. Although both species are taxonomically close, they are clearly separate species, with different plant morphologies. Also our attempts to cross both species have not been successful. For the evaluation, we considered the effect of metal exposure on both roots and shoots, which was possible when using hydroponic conditions. Upon metal exposure, root growth is more rapidly affected than that of other plants parts, therefore root elongation has previously been suggested to be an efficient parameter to evaluate metal tolerance (Macnair *et al.*, 1993). The treatment effects on root elongation easily distinguished both species in the highly Zn and Cd tolerant *G. claussenii* and the non-tolerant *G. elegans* (Fig. 4).

*Gomphrena claussenii* not only adapted to high metal exposure by evolving metal tolerance traits, but it also evolved the capacity to store substantial amounts of Zn and Cd within the plant. The concentrations of Zn and Cd accumulated in root and shoot tissues (Fig. 5) were approximately ten times higher than what is reported to be toxic for most plant species (Kabata-Pendias & Mukherjee, 2007a). The differences in Zn accumulation between *G. claussenii* and *G. elegans*, when exposed to the same Zn level, were not as prominent as would be expected based on comparisons of other hypertolerant species with their closest non-tolerant relatives (Lasat *et al.*, 1996; Ni *et al.*, 2004). This may be a consequence of the natural high Zn concentration which is found in shoots of Amaranthaceae species. From 48 studied families, Amaranthaceae species have in average the second highest Zn concentration in shoots, 108 mg Zn $kg^{-1}$ dry weight, while the average over all families was 77 mg Zn $kg^{-1}$ dry weight (Broadley *et al.*, 2007). Thus this plant lineage may be more prone to evolve Zn/Cd tolerance than other families.

Throughout the metal exposure treatments, *G. claussenii* showed hardly any signs of metal toxicity, not only in roots but also not in shoots, confirming its exceptional Zn and Cd tolerance. Only few other Zn and Cd hypertolerant species have been reported so far, such as *Arabidopsis halleri* (Küpper et al., 2000) , *Noccaea* (*Thlaspi*) *caerulescens* (Assunção *et al.*, 2003a), *Noccea praecox* (Pongrac *et al.*, 2009), *Sedum alfredii* (Yang *et al.*, 2004) and *Viola baoshanensis* (Wu, C *et al.*, 2010). However, different from these species that accumulate high levels of metals in shoots when exposed to low concentrations, *G. claussenii* presents an almost constant ratio between exposed and accumulated metal concentrations, typical of a metal bioindicator species (van der Ent *et al.*, 2013). Thus, *G. claussenii* appears to have evolved another tolerance mechanism. The adaptive mechanism which evolved in classical hyperaccumulators is focussed on preferentially accumulating metals in leaves to deter herbivores (Boyd, 2007; Fones *et al.*, 2010). Instead, *G. claussenii* accumulates metals at approximately twice the concentrations in roots than in shoots (Fig. 4), which indicates the ability to use shoots to store metals if storage capacity in roots is not adequate. This adaptation would probably require less modifications to the metal homeostasis mechanism than the evolution of metal hyperaccumulation. Releasing the barrier to prevent Zn and Cd translocation to the shoots, allowing the metals to follow the concentration gradient, would be sufficient. This is likely to involve genes of the HMA-like P-type ATPase metal transporters, which are involved in loading metals into the xylem (Wong & Cobbett, 2009). Of course an increased metal flux from roots to shoots should be dealt with by providing sufficient apoplastic and vacuolar metal storage capacity in shoots, otherwise plants will accumulate metals in shoots, but not tolerate them and succumb to the toxic consequences. The mechanism for this can be similar for root or shoot tissues, and does not require the tight tissue-specific regulation of metal transporter gene expressions as found in

hyperaccumulators, where roots appear to be actively involved in transporting metals to the vascular system and up into the shoots to keep root concentrations relatively low and shoot levels high, against the concentration gradient (Verbruggen *et al.*, 2009). Such could simply be achieved by increasing expression of transporters exporting metals from the cytoplasm, either to the apoplast or to the vacuoles.

The effects of Zn or Cd exposure on Fe and Mn homeostasis were clearly higher in *G. elegans* than in *G. claussenii*. The decrease of Fe concentration in *G. elegans* shoots is a common effect of Zn and Cd toxicity in metal sensitive plants. In contrast, tolerant species like *G. claussenii* are able to keep shoot Fe concentrations unaffected (Shanmugam *et al.*, 2011), avoiding the drastic symptoms that disturbance of Fe homeostasis will cause on photosynthesis. Root Fe concentrations in both species increased with increasing Zn or Cd exposure. For *G. elegans*, the increase of Fe in roots can be explained as a consequence of Fe deficiency in shoots, due to competition for uptake of Fe with Zn or Cd. The effect is much more pronounced for *G. claussenii* than for *G. elegans*. Metal hyperaccumulator species like *S. alfredii* and *N. caerulescens* also show an increase of root Fe concentration in response to high Zn or Cd exposure (Zhou & Qiu, 2005; van de Mortel *et al.*, 2006). However, even though the solution speciation analysis showed that more than 90% of the Zn and Cd are available as free ions, the possibility that the high Fe concentration in roots of *G. claussenii* is a consequence of apoplastic Fe precipitation, rather than symplastic uptake, cannot be discarded (Chaney *et al.*, 2007).

The combination of high metal tolerance and high metal accumulation along with high biomass production makes plants suitable for phytoextraction. Two major strategies have been considered to achieve these properties: to breed or genetically engineer hyperaccumulator species to increase their biomass or to genetically engineer high-biomass species to increase their metal accumulation and tolerance capacity (Chaney *et*

*al.*, 2007). These are not trivial challenges, but the main reason for this is the scarcity of natural metal hypertolerant and metal accumulating species that are high-biomass producing. *G. claussenii* is a Zn/Cd accumulating species, which produces considerable biomass in the field, and we believe that domestication of this species can be a promising approach to consider for non-GMO-based phytoextraction.

## ACKNOWLEDGEMENTS

We thank Dr. Rosilda Mara Mussury for providing the seeds of *Gomphrena elegans* and Dr. Henk Schat for his help with the mineral analyses and for critically reading the manuscript. The Brazilian governmental organizations CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FAPEMIG (Fundação de Amparo a Pesquisa do Estado de Minas Gerais) are acknowledged for their financial support.

# CHAPTER 3

## *Gomphrena claussenii*, a novel metal hypertolerant bioindicator species sequesters cadmium, but not zinc, in vacuolar oxalate crystals

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## SUMMARY


- *Gomphrena claussenii* is a recently described zinc (Zn) and cadmium (Cd) hypertolerant Amaranthaceae species displaying a metal bioindicator Zn/Cd accumulation response. We investigated the Zn and Cd distribution in stem and leaf tissues of *G. claussenii* at the cellular level, and determined metabolite profiles to investigate metabolite involvement in Zn and Cd sequestration.
- *G. claussenii* plants exposed to high Zn and Cd supply were analysed by scanning electron microscopy with energy dispersive X-ray (SEM-EDX) and micro-proton induced X-ray emission (micro-PIXE). In addition, GC-TOF-MS was used to determine metabolite profiles upon high Zn and Cd exposure.
- Stem and leaf tissues of *G. claussenii* plants exposed to control and high Cd conditions showed the abundant presence of calcium oxalate (CaOx) crystals, but upon high Zn exposure, their abundance was strongly reduced. Ca and Cd were co-localizing to the CaOx crystals in the Cd exposed plants. Citrate, malate and oxalate levels were all higher in shoot tissues of metal exposed plants, with oxalate levels induced 2.6 fold upon Zn exposure and 6.4 fold upon Cd exposure.
- Sequestration of Cd in vacuolar CaOx crystals of *G. claussenii* is found to be a novel mechanism to deal with Cd accumulation and tolerance.

# INTRODUCTION

Knowledge on metal and metalloid homeostasis in plants has increased considerably in the last decades (Merchant, 2010), however a better understanding of the role of these elements in plant metabolism is required to improve the use of plants in phytoremediation technologies (to clean up metal contaminated soil) and in biofortification (to improve nutritional quality) of the human diet (Zhao & McGrath, 2009). Important progress in understanding the physiological and molecular aspects of metal homeostasis in plants was made by studying natural metal hypertolerant species (Hanikenne & Nouet, 2011). These plants have the ability to grow at extremely high, toxic, metal conditions and often accumulate high levels of metals or metalloids, such as nickel (Ni), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb) (Krämer, 2010).

Besides identification of the genes involved in metal homeostasis (Verbruggen *et al.*, 2009), it is also important to know the cellular localization of metals and their chemical forms during uptake, transport and storage processes (Hall, 2002; Clemens, 2006). Once metals are taken up by plants, metal hypertolerance can only be achieved by minimizing internal metal toxicity. Two, often combined, strategies are reported as fundamental to accomplish this; metal sequestration, which is to physically compartmentalize metals to sites with low metabolic activity; and metal chelation, to reduce reaction of (sequestered) metals with other compounds (Mari & Lebrun, 2006; Lin & Aarts, 2012).

Especially in shoots, metals sequestration to sites of low metabolic activity is an important way to avoid interference with plant photosynthesis, potentially highly sensitive to disturbances contributing to increased formation of reactive oxygen species (Alaoui-Sossé *et al.*, 2004; Rascio & Navari-Izzo, 2010). Metal sequestration in leaves of

hypertolerant species therefore occurs mainly in the trichomes, in the epidermis or intercellularly, in the apoplastic space. Intracellularly, metals are mostly sequestered in vacuoles (Cosio *et al.*, 2005; Sarret *et al.*, 2009; Tian *et al.*, 2011). Proper metal sequestration ensures better metal detoxification and the potential for higher metal accumulation, which has the additional advantage that plants containing high concentrations of toxic metals may render the plant much less attractive to herbivores, compared to their non-accumulating neighbours (Leitenmaier & Küpper, 2013).

Organic acids, amino acids and oligopeptides are generally good metal chelators, both in metal exposure adapted and non-adapted species (Sharma & Dietz, 2006; Haydon & Cobbett, 2007). For instance, studies with model plant species like (non-adapted) *Arabidopsis thaliana*, (Ni-adapted) *Alyssum lesbiacum* and (Zn/Cd/Ni-adapted) *Noccaea caerulescens* provided extensive knowledge on Ni chelation by histidine and the contribution of this chelator to high Ni tolerance (Krämer *et al.*, 1996; Richau *et al.*, 2009). The anions of organic acids, such as malate and citrate (Broadley *et al.*, 2007; Haydon & Cobbett, 2007), as well as nicotianamine (Clemens *et al.*, 2013), are important chelators implicated in Zn tolerance.

Recently a new metallophyte, *Gomphrena claussenii*, has been described as the first Zn and Cd hypertolerant species belonging to Amaranthaceae family (Villafort Carvalho *et al.*, 2013). In addition to its capacity to grow at metal contaminated sites and to accumulate high levels of Cd and Zn in its shoots (from 0.1 to 1% of its dry matter), the substantial shoot biomass produced by *G. claussenii* makes it also a potentially useful species for phytoremediation applications. It differs from the well-studied metal hyperaccumulators of the Brassicaceae family, like *Arabidopsis halleri* or *N. caerulescens*, in that it is not preferentially taking up metals at low exposure conditions. Instead, it acts like a bioindicator (van der Ent *et al.*, 2013) , accumulating Zn and Cd in a more linear

relation with metal exposure (Villafort Carvalho *et al.*, 2013). This uncommon response prompted us to hypothesize that *G. claussenii* may use a novel mechanism of metal tolerance, different from what has been described for other hypertolerant species. Both from an evolutionary perspective and a mechanistic perspective it will be very interesting to determine how this novel tolerance mechanism differs from what is known about the well-studied metal-adapted Brassicaceae species (Krämer, 2010). Understanding metal tolerance and accumulation in *G. claussenii* will also contribute to its application in soil remediation programs, and it may provide valuable information on the regulation of micronutrient levels in Amaranthaceae crops like spinach (*Spinacia oleracea*), beet (*Beta vulgaris*), quinoa (*Chenopodium quinoa*) and Amaranth species. The objectives of this study were to investigate Cd and Zn distribution and sequestration in leaves and stems of *G. claussenii* using scanning electron microscopy with energy dispersive X-ray (SEM-EDX) and micro-proton induced X-ray emission (micro-PIXE) technologies. The results of these analyses prompted us to also analyse the changes in primary metabolites in roots and shoots during high Zn and Cd exposure, to uncover possible ligands for these metals in *G. claussenii*.

## MATERIALS AND METHODS

### Plant culture

Clones of one genotype of *Gomphrena claussenii* Moq., originally collected from a Zn mine at Vazante, Minas Gerais, Brazil, were cultivated hydroponically as described by (Villafort Carvalho *et al.*, 2013). In short, *G. claussenii* cuttings were transferred to 600-ml pots (one plant per pot), filled with modified Clark's nutrient solution (1.3 mM $KNO_3$, 2.53 mM $Ca(NO_3)_2$, 0.9 mM $NH_4NO_3$, 0.6 mM $MgSO_4$, 0.5 mM KCl, 34.5 µM $Ca(H_2PO_4)_2$,

19 µM $H_3BO_3$, 2 µM $ZnSO_4$, 7 µM $MnCl_2$, 0.5 µM $CuSO_4$, 0.086 µM $(NH_4)_6Mo_7O_{24}$, and 38 µM Fe(Na)EDTA, set at pH 5.5). After three weeks of pre-culture in a quarter strength solution and high humidity, plants were moved to half strength solution supplemented with 2 µM $ZnSO_4$ (control conditions), or exposed to high Zn (1000 µM $ZnSO_4$, no Cd) or high Cd (2 µM $ZnSO_4$, 100 µM $CdSO_4$). Nutrient solutions were refreshed every week. Each treatment consisted of three replicates. Plants were harvested 21 days after the start of the metal exposure. Three experiments were performed according to these conditions, one for mineral analysis, the second one for microscopic analyses and the third one to obtain metabolite data.

## Analysis of zinc and cadmium concentration

Zinc and Cd concentrations were determined for leaf, stem and root samples by using atomic absorption spectroscopy as described by (Villafort Carvalho *et al.*, 2013).

## Microscopic analysis

Hand-cut sections of leaves and stems from control and metal-treated plants were initially examined using light microscopy (Nikon Eclipse 80i; http://www.nikoninstruments.comwith differential interference contrast (DIC)). Both cryo and dry samples were prepared for scanning electron microscopy analyses. For cryo samples, fresh leaf and stem material was glued onto an aluminium Leica sample holder by carbon glue (Leit-C, Neubauer Chemicalien, Germany), directly frozen in liquid nitrogen and subsequently fitted in the cryo-sample loading system (VCT 100). The Leica sample holder was transferred to a non-dedicated cryo-preparation system (MED 020/VCT 100, Leica, Vienna, Austria) onto a sample stage at -93 °C. In this system samples were freeze fractured and subsequently freeze dried for 3 min at -93 °C at 1.3 x $10^{-6}$ mbar to remove water from the fractured sample surface. Samples were sputter-coated

with a layer of 10 nm tungsten (W) at the same temperature and directly transferred in high vacuum into the field emission scanning microscope (Magellan 400, FEI, Eindhoven, the Netherlands) using the sample stage set at -122 °C and 4 x $10^{-7}$ mbar. The morphological analysis was performed with secondary electrons at 2 kV, 13 pA. For dry sample preparation, leaf and stem parts were sampled and put directly into 100% methanol. After one hour the methanol was substituted to pure acetone in a series of a methanol : acetone mixtures (2:1, 1:1, 1:2, 15 min per step). Finally the samples were critical-point-dried with carbon dioxide (CPD 030, BalTec, Liechtenstein). The dry samples were glued by double sticky carbon adhesive tabs (Spectro-grade EMS, Washington, USA) on aluminium stubs and coated with about 20 nm carbon (K950X, Quorum Technologies, UK).

## Energy dispersive X-ray analyses

Samples were qualitatively and quantitatively analysed by energy-dispersive X-ray (EDX) in the same scanning electron microscope (SEM) using an AzTec X-ray analyser with X-Max Silicon Drift Detector (80 $mm^2$, Oxford Instruments Analytical, High Wycombe, England), at an acceleration voltage of 15 kV, 200 pA, working distance = 4 mm. CGraphs calculation and EDX images were performed using the AZtec software package from Oxford instruments. For the evaluation of Cd concentration, potassium (K) was always taken into account since the Cd L lines overlap with the Ka line of K. It meant that for each Cd measurement we took care not to let the K levels interfere with the Cd measurements. Thus we managed to avoid an overestimation of Cd.

## Micro-PIXE analyses

Small (3 × 3 mm) pieces of fresh plant leaves were excised from an area encompassing the main vein in the lower part of the leaf. They were embedded in tissue

freezing medium (Jung, Leica) and frozen in liquid propane, cooled with liquid nitrogen(Vogel-Mikuš *et al.*, 2009). Cryotome sectioning was performed at -25 °C at 60 µm thickness. The sections were placed in custom-made pre-cooled aluminium containers and covered with fitting stainless steel covers. Using a cryo-transfer-assembly, cooled with liquid nitrogen (Vogel-Mikuš *et al.*, 2009), the containers were transferred to an Alpha 2–4 Christ freeze-dryer where sections were freeze-dried at -30 °C at 0.3 mbar for three days. Freeze-dried sections were mounted between two layers of Pioloform foil stretched over aluminium holders. Micro-proton-induced X-ray emission (micro-PIXE) analysis of the samples was performed at the Jožef Stefan Institute (JSI), Slovenia, as previously described (Vogel-Mikuš *et al.*, 2008a; Lyubenova *et al.*, 2013). Two upgrades were recently made to the nuclear microprobe. Firstly, the previous SiLi detector was replaced by a new SDD detector (e2v SiriusSD® High Wycombe, Buckinghamshire, UK) for detection of X-rays of the lower Z-elements. Secondly, lateral resolution was improved using a new high brightness multicusp ion source (Pelicon *et al.*, 2014). The size of the beam and the lateral resolution of the maps was 0.8 µm. The GeoPIXE II software package (Ryan 2000) was used to analyse the micro-PIXE spectra (analysis of Cd was based on Cd K-lines) and to generate numerical matrices. These numerical matrices were used to create spatial distribution maps with PyMCA software (Solé *et al.*, 2007) and to calculate the concentrations of Ca and Cd using ImageJ software (Abràmoff *et al.*, 2004). In ImageJ the Intensity Correlation Analysis plug-in was used to calculate Pearson's correlation coefficients (as in Pongrac *et al.*, 2013) and the Co-Localisation Highlighter plug-in to generate the co-localisation maps with white co-localisation points, red Ca distribution and green Cd distribution.

## Metabolites analyses

Relative metabolite content was determined by extracting polar metabolites (Lisec *et al.*, 2006; Carreno-Quintero *et al.*, 2012). Briefly, polar metabolite fractions were extracted from ~200 mg fresh weight shoots (i.e. aboveground parts) and roots. After extraction, aliquots of the polar phase (100 µl) were dried by vacuum centrifugation for 12-16 hours. The dried samples were derivatized online as described by Lisec et al. (2006) using a Combi PAL autosampler (CTC Analytics AG; www.ctc.ch). First, 12.5 µl O-methylhydroxylamine hydrochloride (20 mg $ml^{-1}$ pyridine) was added to the samples and incubated for 30 min at 40 °C with agitation. Then the samples were derivatized with 17.5 µl MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) for 60 min. An alkane mixture (C10-C30) was added to each sample to determine retention indices of metabolites. The derivatized samples were analysed by a gas chromatography, time-of-flight, mass spectrometry (GC-TOF-MS) system consisting of an Optic 3 high performance injector (ATAS GL Int., Eindhoven, the Netherlands) and an Agilent 6890 gas chromatograph (Agilent Technologies, http://www.agilent.com) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments, http://www.leco.com). 2 µl of each sample was introduced into the injector at 70 °C using a split flow of 19 ml $min^{-1}$. The injector was rapidly heated with 6 °$Cs^{-1}$ to 240 °C. The chromatographic separation was performed using a VF-5ms capillary column (Varian; , http://www.varian.com; 30 m x 0.25 mm (internal diameter) x 0.25 µm (film thickness)) including a 10-m guardian column with helium as carrier gas at a constant column flow rate of 1 ml $min^{-1}$. The GC oven temperature was isothermal for 2 min at 70 °C, followed by a 10 °C per minute ramp to 310 °C, and was held at this temperature for 5 min. The transfer line temperature was set at 270 °C. The column effluent was ionised by electron impact at 70eV. Mass spectra were

acquired at 20 scans $sec^{-1}$ within a mass range of m/z 50 – 600, at a source temperature of 200 °C. A solvent delay of 295 s was set. The detector voltage was set to 1400 V.

## GC-TOF-MS data processing methods

Raw data were processed by ChromaTOF software 2.0 (Leco instruments) and MassLynx software (Waters Inc., http://www.waters.com) and further analysis was performed using MetAlign software (Lommen, 2009) to extract and align the mass signals (s/n≥ 3). Mass signals that were present in less than two samples were discarded. Signal redundancy per metabolite was removed by means of clustering and mass spectra were reconstructed (Tikunov *et al.*, 2012). Metabolites were identified by matching mass spectra to those of authentic reference standards, available mass spectra databases (NIST08, National Institute of Standards and Technology, Gaithersburg, MD, USA, http://www.nist.gov/srd/; Golm DB, http://gmd.mpimp-golm.mpg.de/) and by comparison of retention indices (RIs) with known RIs. RIs were calculated using a series of alkanes that were fitted with a third order polynomial function (Strehmel *et al.*, 2008). Metabolite data were statistically analysed using the SPSS package (version 21; http://www.ibm.com/software/analytics/spss). The mean and standard error (SE) of each treatment as well as the fold changed compared to control conditions were calculated. Analysis of variance (ANOVA) was performed on the data followed by a post hoc Tukey test using a significance threshold of $P< 0.05$.

## Oxalic acid quantification

Oxalic acid concentrations were quantified using an authentic reference standard. The linear calibration curve was calculated by injecting samples at 0.1, 0.2, 0.5 and 1.0 mg $ml^{-1}$ along with extracted plant samples. Raw data were baseline-corrected and aligned

using MetAlign software (Lommen, 2009). Quantification was based on the extracted quantifier mass for oxalic acid (m/z 147).

## RESULTS

### *Gomphrena claussenii* hyperaccumulates Zn and Cd only at high metal exposure

*G. claussenii* was originally collected from a Zn mine site, highly contaminated with Zn and Cd. Previously we showed those plants to grow well in the field, and that plants collected from site grew well in the metal contaminated soil collected at the site. We also showed this species to be similarly tolerant to high Zn and Cd conditions when grown hydroponically, showing no reduction in biomass when exposed to up to 3000 μM Zn or up to 100 μM Cd (Villafort Carvalho *et al.*, 2013). For the experiments described here, new plants were grown hydroponically. The concentrations of Zn and Cd in these plants were measured after three weeks of exposure to control (2 μM Zn), high Zn (1000 μM) or Cd (100 μM) levels. In plants exposed to high Zn and Cd, these metals were hyperaccumulated, with Zn and Cd concentrations in the stems more than two times higher compared to leaves (Fig. 1a,b). This is atypical when compared to other metal hyperaccumulators, which generally accumulate metals at higher concentrations in shoots than in roots and can do this already at low metal exposure concentrations. It is why we previously classified *G. claussenii* as a metal bioindicator, rather than a metal hyperaccumulator species (Villafort Carvalho *et al.*, 2013). At high Cd exposure, Cd competes with Zn uptake as Zn concentrations did not exceed the levels found at normal Zn supply (Fig. 1a). The partitioning of Zn and Cd over the three examined plant parts was more or less equal under control conditions, but at high metal exposure, the

partitioning in roots was reduced compared to that in leaves and stems (Fig. 1c,d). At those exposures, plants contained more than 80% of the stored Zn or Cd in shoots.

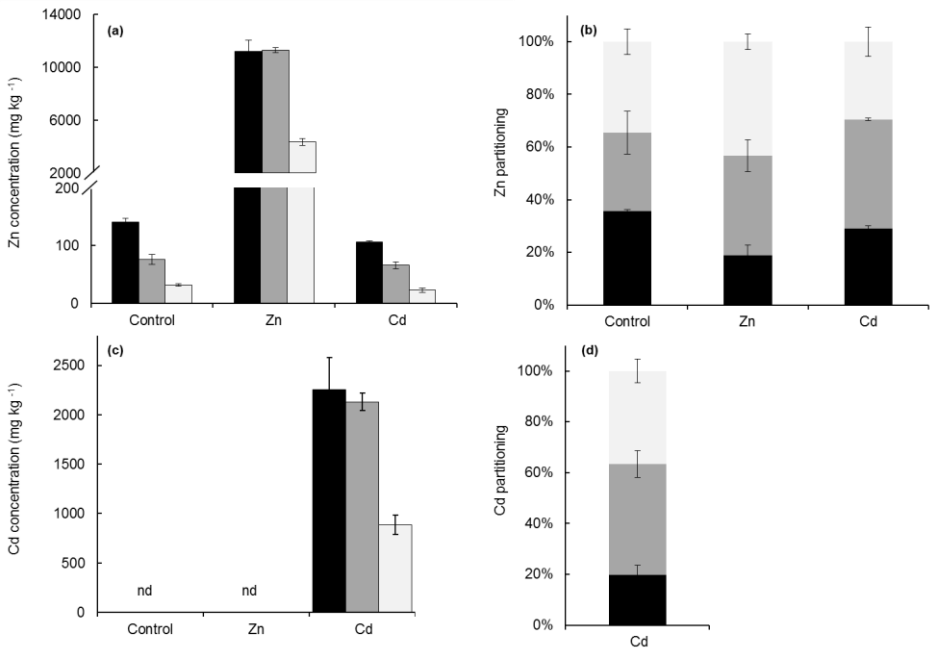



Figure 1 - Zinc (Zn) (a) and cadmium (Cd) (b) concentrations (in mg $kg^{-1}$ dry weight; mean ± SE) and Zn (c) and Cd (d) partitioning (%) of *Gomphrena claussenii* roots (black bars), stems (dark grey bars) and leaves (light grey bars). Plants were grown for three weeks in hydroponic solution containing 2 µM $ZnSO_4$ and thereafter continued to grow in these control conditions or exposed to high Zn (1000 µM $ZnSO_4$) or high Cd concentrations (100 µM $CdSO_4$, 2 µM $ZnSO_4$) for another three weeks.

## Detection of Cd-containing Ca-oxalate crystals in Cd exposed plants

Leaf sections were made of these plants for light microscope analysis. The high Zn or Cd treatments did not induce any obvious histological changes when compared to control conditions (data not shown). Large crystals were observed inside the vacuoles of pith cells in stems of plants growing at control conditions (Fig. 2a). Those crystals were morphological classified as calcium oxalate ($CaC_2O_4$; CaOx) druse crystals and were easily identified by light microscope using polarized light (Fig. 2a,b). Crystals were only found

within the vacuoles of the cells and not outside the cells. In stems of plants exposed to high Zn, no crystals could be found (Table 1), while the high Cd exposure did not affect the number and appearance of crystals (Table 1 and Fig. 2d). Crystals could still be seen in stems of plants exposed to 100 µM Zn, but less than that under control conditions (4.25 crystals per stem segment).

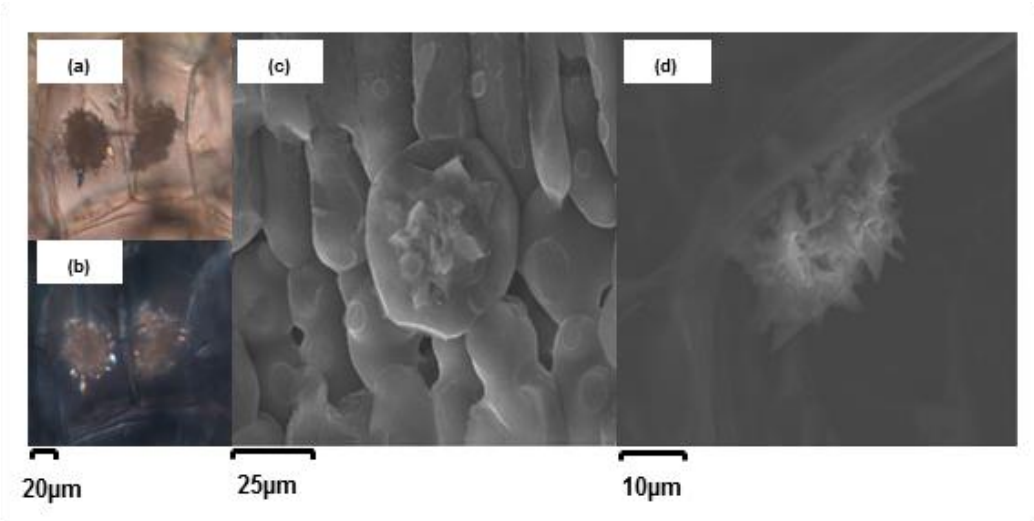



Figure 2 -Visualization of calcium oxalate (CaOx) crystals inside the vacuoles of stem pith cells of *Gomphrena claussenii* plants growing in control conditions (2 µM $ZnSO_4$) as observed with a light microscope under normal light (a) and polarized light (b). CaOx crystals were also observed through scanning electron microscopy in plants exposed to high cadmium (100 µM $CdSO_4$, 2 µM $ZnSO_4$) in leaf (c) and stem (d) cells. Leaf samples were prepared using flash-frozen material (cryo technique) (c); stem samples were dried and carbon-coated (d).

Table 1- Effects of high zinc (Zn) and high cadmium (Cd) exposure on the average number (± SE) and elemental composition (in weight %) of calcium oxalate crystals observed in stem sections of *Gomphrena claussenii*

| | Control (2 µM Zn) | | 1000 µM Zn | 100 µM Cd (2 µM Zn) | |
|---|---|---|---|---|---|
| Average number of crystals per stem section | 9.12 (± 0.4) | | 0 | 8.25 (± 0.46) | |
| Elemental composition of crystal (weight%) | Cryo | Dry | | Cryo | Dry |
| O | 75 | 61 | | 74.9 | 62.2 |
| Ca | 23.6 | 38.4 | | 23.1 | 36 |
| K | 1.4 | 0.6 | | 1.4 | 0.6 |
| Cd | 0 | 0 | | 0.6 | 1.2 |

EDX analysis was used to map the elemental distribution in stem cross sections (Fig. 3). This showed that the crystals were very high in calcium (Ca), confirming their identity as CaOx druse crystals. In plants exposed to Cd, Cd was co-localizing with the CaOx crystals (Fig. 3c). Although the Cd concentration was much lower compared to the Ca concentration, its presence in crystals was obvious and distinguishable from the distribution of other elements, such as potassium (K) (Fig. 3d). Elemental spectra were recorded from three areas within or outside a crystal to determine if Cd also accumulated outside the crystals. A representative sample of such analysis is shown in Figure 4. In none of the cases was Cd detected outside the crystals, confirming that the CaOx crystals were enriched in Cd. In stems, crystals were observed exclusively inside the vacuoles of pith cells, which were the only sites at which Cd was detected. The spectral analysis showed that CaOx crystals were mainly composed of oxygen (O) and Ca (carbon was excluded from the analysis because it was used for sample coating) (Fig. 4c). K was also detected at

low levels at the site of the crystal. However, K is likely to be present in the vacuole solution rather than the CaOx crystal, since it was also detected, at higher levels, in the vacuoles of cells without a crystal (Fig. 4c). Analyses of crystals in dry samples showed element concentrations as expected for CaOx crystals, with mainly O and Ca, and very little K (Table 1). The presence of Cd did not seem to have an effect on the concentration of the other elements studied.

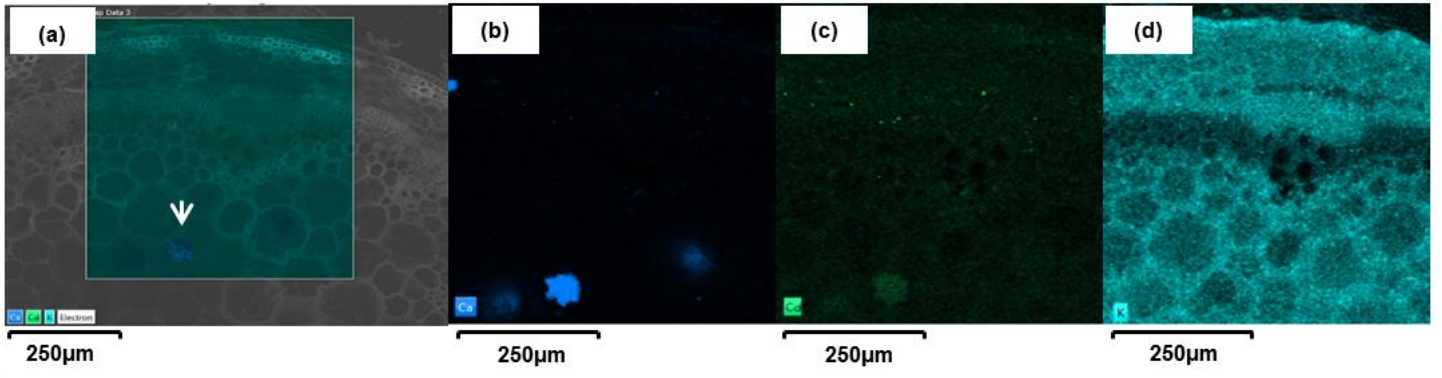



Figure 3 - Visualization of a *Gomphrena claussenii* stem section using scanning electron microscopy with the energy dispersive X-ray system (SEM-EDX). The section (a) was taken from the stem of a plant exposed to high cadmium for three weeks. The inset in (a) was used for element mapping; the arrow indicates a calcium oxalate crystal. Distribution of calcium (Ca, blue; b), cadmium (Cd, green; c) and potassium (K, green-blue; d) is shown.

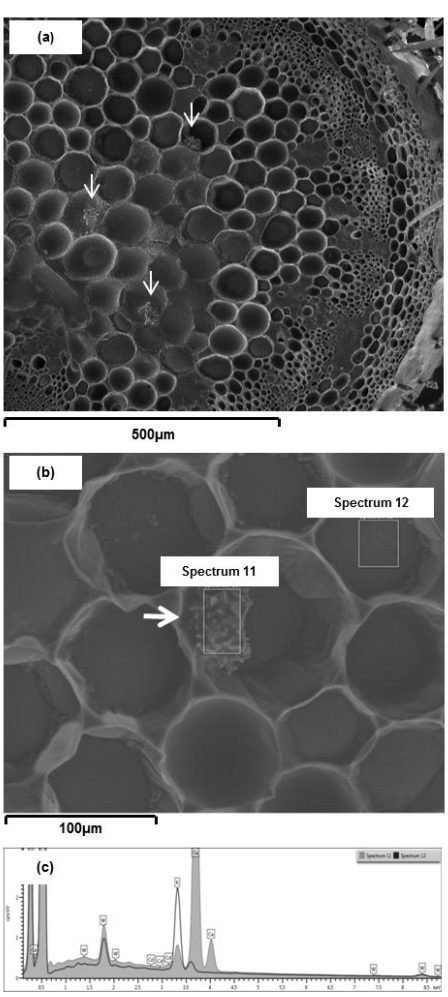
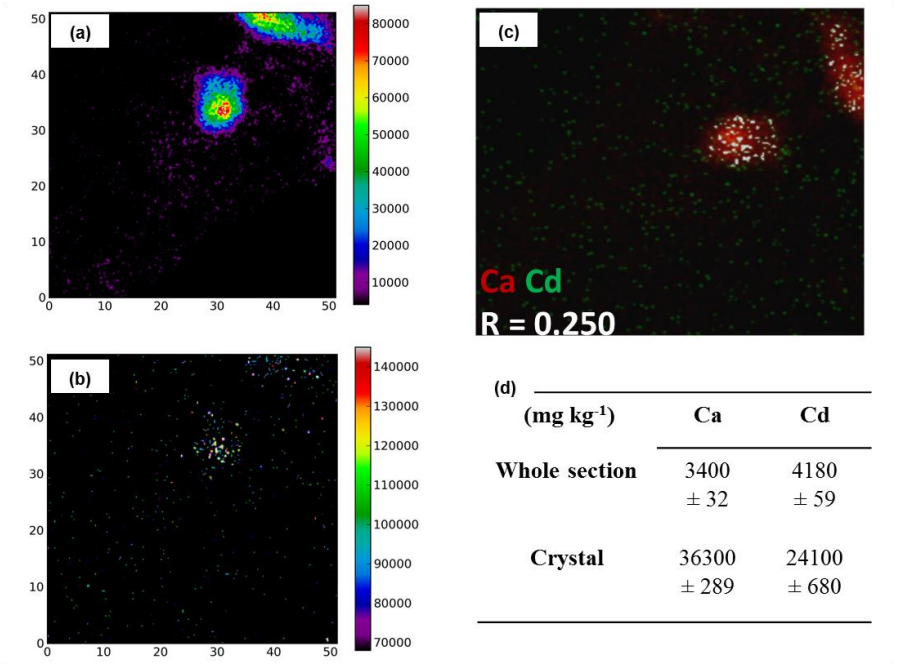



Figure 4 - Cryo stem sections of a *Gomphrena claussenii* plant exposed to high cadmium (Cd) at two magnifications (a, b). Arrows indicate calcium oxalate crystals. Two insets in (b), were used for element spectrum analysis, one covering a crystal (Spectrum 11), the other not (Spectrum 12). The two overlapping energy dispersive X-ray (EDX) spectrums are compared (c). Carbon (C), oxygen (O), calcium (Ca), tungsten (W), potassium (K) and Cd peaks were identified. Cd is only detected in spectrum 11, in which also Ca is much more prominent than in spectrum 12.

Crystals were also found in the mesophyll tissue of leaf sections of plants grown in control and Cd-exposed conditions (Fig. 2c). Most crystals were identified inside the spongy mesophyll cells. These crystals had a comparable composition as the ones found in the stem (data not shown), although the Cd concentration in leaf crystals of Cd-exposed plants was around half the Cd concentration of stem crystals, in line with the lower Cd concentration in leaves compared to stems (Fig. 1).

To confirm the co-localization of Cd with the CaOx crystals, Cd-exposed plants were analysed using micro-PIXE. In addition to the higher sensitivity, Cd was measured using the K-line, which circumvents the interference with K present in the samples. The quantitative distribution maps of Ca and Cd in a representative leaf section confirmed that indeed the CaOx crystals are enriched in Cd (Fig. 5).




(d)

| (mg kg$^{-1}$) | Ca | Cd |
|---|---|---|
| **Whole section** | 3400 ± 32 | 4180 ± 59 |
| **Crystal** | 36300 ± 289 | 24100 ± 680 |

Figure 5 - Quantitative spatial distributions of Ca (a) and Cd (b) in a leaf of Cd-exposed *Gomphrena claussenii*; merged distributions of Ca (red) and Cd (green) producing white co-localisation points (c); and concentrations of Ca and Cd (in mg kg–1; average ± SE) in the whole leaf cross-section and in the crystal (d). R, Pearson's correlation coefficient; the scan size is 50 × 50 μm.

No crystals were found in stems and only one crystal was found in one of the leaf sections of all sections of plants exposed to high Zn that were evaluated in the SEM-EDX, in line with what was previously observed with light microscopy. This means that the high Zn treatment reduced the formation of CaOx crystals virtually to zero in the *G. claussenii* plants. However, plants exposed to high Zn showed a clear Zn peak in cellular spectrums when examined by EDX (Fig. 6), indicating that Zn was accumulated in these cells. This peak was not seen in plants grown under control conditions (data not shown). Observation of individual cells indicated that Zn is accumulated inside the cell vacuole

(Fig. 6b,c). More specific comparisons between mesophyll and epidermis cells in leaves (Fig. 7a,b) supported the notion that Zn does not accumulate in a specific tissue. Similar results were found for stem, where Zn was also detected, but not limited to a specific tissue (Fig. 7c,d).

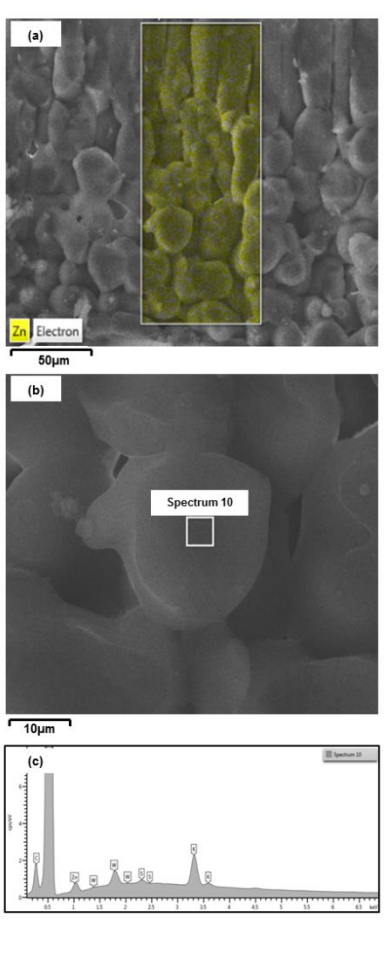



Figure 6 - Cryo leaf sections of a *Gomphrena claussenii* plant exposed to high zinc (Zn) (1000 µM $ZnSO_4$ during three weeks) at two magnifications (a, b). The inset in (b), was used for energy dispersive X-ray (EDX) element spectrum analysis (Spectrum 10). The spectrum covers the vacuole of a mesophyll cell. The elements identified in this area are represented by peaks in

the EDX spectrum (c). Carbon (C), oxygen (O), sulfur (S), tungsten (W), potassium (K) and Zn were identified.

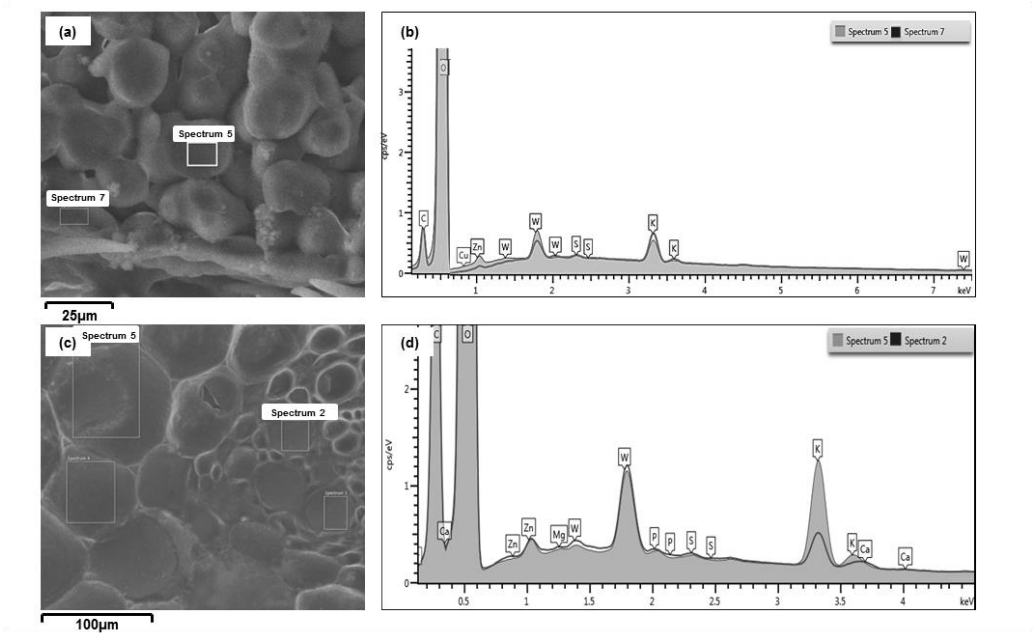



Figure 7 - Comparison of Zn concentrations in leaf (a and b) and stem (c and d) cells using SEM-EDX. Samples were taken from *Gomphrena claussenii* plants exposed to high Zn (1000 µM $ZnSO_4$ during three weeks). Epidermis and mesophyll cells were compared in leaf (spectra 5 and 7) (a). Pith and vascular bundle cells were compared in stem (spectra 5 and 2) (c). Overlapping elemental spectra are compared for leaf (b) and stem (d). Carbon (C), oxygen (O), sulfur (S), cupper (Cu), phosphorus (P), calcium (Ca), tungsten (W), potassium (K) and Zn were identified.

## Shoot oxalate levels increased upon Zn or Cd exposure

In order to find any evidence for specific primary metabolic compounds that increased in abundance upon Zn or Cd exposure and may thus be candidates for metal chelation, the polar metabolite composition of shoot and root tissue of control and metal-exposed plants was analysed by GC-TOF-MS combined with an untargeted metabolomics approach. 92 compounds were detected in this analysis, of which 26 possible metabolites

could be putatively identified (Table S1). These were mainly sugars, organic acids and amino acids.

The abundance of soluble sugars in *G. claussenii* depended on the Zn and Cd treatments. Sugars, such as sucrose, sorbose, glucose and fructose, accumulated in metal-exposed roots to almost twice the level found in roots of plants growing under control conditions (Fig. 8a). For shoots this was only the case upon Cd exposure (Fig. 8b).

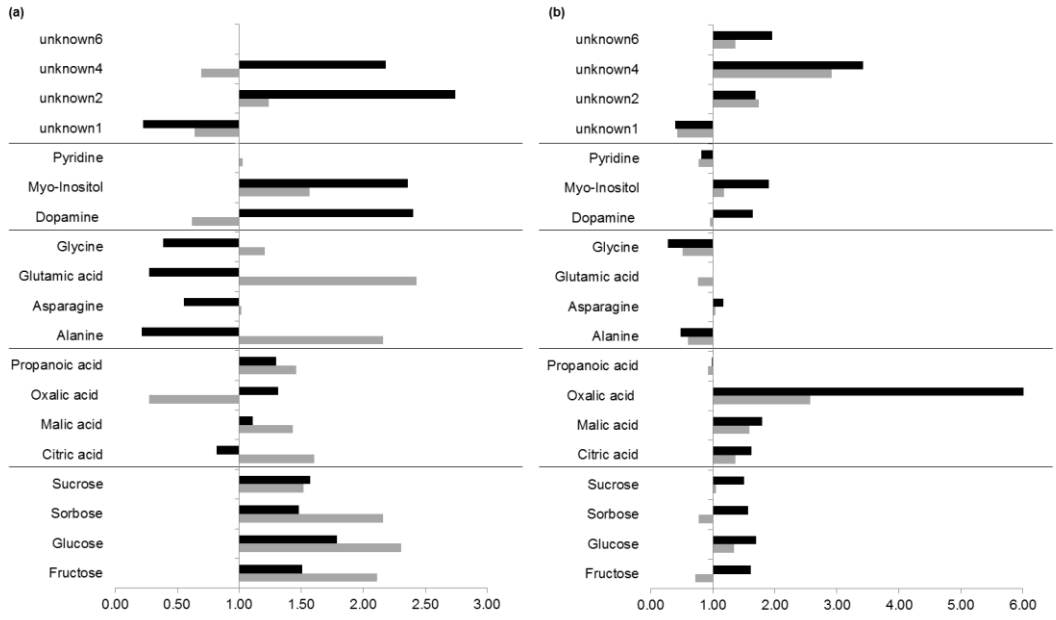



Figure 8 - Log2 values of fold changes for metabolites detected by GC-TOF-MS in roots (a) or shoots (b) of *Gomphrena claussenii* plants, comparing plants grown at control conditions (supplied with 2 μM $ZnSO_4$) to plants exposed to 1000 μM $ZnSO_4$ (grey bars) or to 100 μM $CdSO_4$ (in the presence of 2 μM $ZnSO_4$) (black bars).

The abundance of organic acids and amino acids in roots depended on the metal exposure treatment (Fig. 8a). Citrate, malate and propanoic acid all increased upon Zn exposure, while the abundance of oxalate decreased around four-fold. Such decrease was

not seen in roots of Cd-exposed plants. Zn exposure also increased the abundance of especially alanine and glutamic acid in roots, while upon Cd exposure, the concentrations of all identified amino acids (glycine, glutamic acid, asparagine and alanine) decreased two- to four-fold (Fig. 8a). Among the other identified metabolites, dopamine and myo-inositol increased over two-fold in response to Cd exposure. Four unknown metabolites were detected, of which two increased and one decreased substantially in roots upon Cd exposure.

The changes in organic acid and amino acid concentrations in response to Zn or Cd exposure were much more similar in shoots than in roots (Fig. 8b). Citrate, malate and oxalate were all higher in metal-exposed plants than in the control. Especially oxalate levels were induced, 2.6-fold by Zn exposure and 6.4-fold by Cd exposure. Considering that oxalate may be the major vacuolar chelator of Zn and Cd, the oxalate concentrations were quantified (Table 2). It showed that especially in response to Cd exposure, oxalate concentrations increased from 4.8 mg $g^{-1}$ in shoots of control plants to 18.4 mg $g^{-1}$ in shoots of plants exposed to Cd. The increase of oxalate levels coincided with a reduction in glycine levels, as could also be seen for roots, which is in agreement with the fact that both compounds have glyoxylate as a common precursor. The same unknown metabolites that were detected in roots showed a comparable response to Zn or Cd exposure in shoots as in roots exposed to Cd. It will be interesting to determine the nature of these metabolites, so they may be studied in more detail.

Table 2 - Concentrations of oxalic acid (in mg $g^{-1}$ and µmol $g^{-1}$ fresh weight) in roots and shoots of *G. claussenii* plants grown for three weeks in hydroponic solution containing 2 µM $ZnSO_4$ and thereafter continued to grow in these control conditions or exposed to high Zn (1000 µM $ZnSO_4$) or high Cd concentrations (100 µM $CdSO_4$, 2 µM $ZnSO_4$) for another three weeks. Averages of three replicates are given ± SE.

| | Control (2 µM Zn) | | 1000 µM Zn | | 100 µM Cd (2 µM Zn) | |
|---|---|---|---|---|---|---|
| | mg $g^{-1}$ | mmol $g^{-1}$ | mg $g^{-1}$ | mmol $g^{-1}$ | mg $g^{-1}$ | mmol $g^{-1}$ |
| Root | 3.6 (± 0.2) | 40 (± 2.2) | 3.4 (± 0.03) | 38 (± 0.3) | 3.9 (± 0.01) | 43 (± 0.1) |
| Shoot | 4.8 (± 0.28) | 53 (± 3.1) | 7.1 (± 1.7) | 79 (± 1.8) | 18.4 (± 2.1) | 204 (± 23) |

## DISCUSSION

In this research we confirmed the large Zn and Cd storage capacity of roots, stem and leaves of *G. claussenii* (Fig. 1a), thus supporting our previous results on this new metal bioindicator species (Villafort Carvalho *et al.*, 2013). Not only are the Zn and Cd concentrations of *G. claussenii* in the same range or even larger than those of the well-known Brassicaceae hyperaccumulator species *N. caerulescens* (Küpper & Kochian, 2010) and *A. halleri* (Zhao *et al.*, 2000) at high metal exposure, the metal content per plant is much higher than in these species, due to the much higher plant biomass of *G. claussenii*. Shoots, comprising stems and leaves, are responsible for storing 80% of the total Zn and Cd content per plant, a characteristic contributing substantially to the phytoremediation potential of this species. The high concentrations found in stems and leaves, which are at least half those of roots, indicate that restricting metals to the roots by limiting metal loading into the root xylem, is not part of the tolerance mechanism in *G. claussenii*.

Different from the known Brassicaceae metal accumulators is that *G. claussenii* did not evolve the typical hyperaccumulation characteristics of those species, which allow them to hyperaccumulate metals already at normal mineral supply, rather than at the high metal supply needed for Zn/Cd hyperaccumulation of *G. claussenii*. It therefore seems as if the Brassicaceae hyperaccumulators evolved an additional trait, i.e. preferential metal accumulation in leaves, which is not apparent in *G. claussenii*. The latter species is however extremely tolerant to Zn and Cd exposure (Villafort Carvalho *et al.*, 2013), perhaps even more tolerant than *N. caerulescens* or *A. halleri* (Assunção *et al.*, 2003a; Zhao *et al.*, 2006). The tolerance is both to high metal exposure in their growth medium as well as to high metal content in tissues. We did not find any indications that metals are stored in the apoplast of stem or leaf tissues, or on the plant surface. Instead, metal tolerance appears to reflect efficient sequestration of metals in vacuoles of root, stem and leaf cells. The high metal storage capacity of *G. claussenii* stems is also described for the Zn and Cd hyperaccumulator species *Sedum alfredii*, in which the metal concentrations in stems are even higher than in leaves (Tian *et al.*, 2009). Storage in stems may be a more attractive site than leaves, which are generally more metabolically active and in which vacuolar sequestration will put more demands on maintaining a very efficient mechanism to keep the accumulated metals within the vacuole. Proper sequestration will also be essential to prevent metal-instigated damage of the photosynthetic machinery in leaf parenchyma cells.

SEM coupled with EDX has been described as a suitable approach to evaluate the distribution of metals and metalloids in plants and to obtain an initial understanding of morphological changes and cellular accumulation of metals upon exposure (Lombi *et al.*, 2011). The high resolution and reasonable sensitivity (~100 mg $kg^{-1}$) make this a feasible technology to apply successfully for metal accumulator plants (Frey *et al.*, 2000; Zhao *et*

*al.*, 2000; Cosio *et al.*, 2005; Wójcik *et al.*, 2005). During sample preparation the temperature is an important factor, especially for metal analysis. Samples can be frozen (cryo) or prepared at ambient temperature (dry), which means they will be scanned with or without water, which can make a great difference for element concentrations. As observed for *G. claussenii* (Table 1) and also shown in other studies (Isaure *et al.*, 2006; Lombi *et al.*, 2011), cryo samples have a better resolution but a lower detection limit since water and therefore oxygen concentration is higher compared with dry samples, where concentrations are more accurate once elements are not diluted. To complement the results obtained by EDX, the micro-PIXE technique was applied because of its high elemental sensitivity (> 1 mg $kg^{-1}$) at moderate lateral resolution, which at JSI is currently limited to 0.7 μm. As imaging is more laborious, it seems most efficient to first use SEM-EDX, to provide a first impression, and subsequently use micro-PIXE, to obtain a detailed analysis of interesting observations, as we did.

Incorporation of metals in organic acid crystals, as we observed for Cd in *G. claussenii*, has been reported before for several metal sensitive plant species, such as *Silene vulgaris*, tobacco (*Nicotiana tabacum*), *Tamarix smyrnensis* and *Morus alba* (Franceschi & Schueren, 1986; Mazen & El Maghraby, 1997; Bringezu *et al.*, 1999; Choi *et al.*, 2001; Manousaki *et al.*, 2008; Katayama *et al.*, 2013). Specifically the role of CaOx crystals in relation to Cd accumulation has been studied in tobacco (Choi *et al.*, 2001; Choi & Harada, 2005; Isaure *et al.*, 2010). Tobacco forms these crystals at the leaf surface, which is effective for metal tolerance, but different from our observations in *G. claussenii*, and not contributing much to metal hyperaccumulation. A previous study comparing tobacco and *G. claussenii* under increasing concentrations of Cd, reported an increase in leaf Ca concentrations in tobacco, but a slight decrease of Ca for *G. claussenii* (Borin, 2010). This agrees with the assumption that in tobacco, the little Cd that is transported to the shoots

(compared to *G. claussenii*), can be effectively kept out of the cells and excreted to the leaf surface, incorporated in CaOx crystals. Despite the slight decrease in *G. claussenii* Ca concentrations, in terms of molar concentration ratios there will be a large excess (> 10-fold) of Ca compared to Cd and Cd is unlikely to interfere much with formation of CaOx crystals. Oxalic acid is always in large excess in terms of molar concentrations (Table 2).

Zn, unlike Cd, has not been found in CaOx crystals (Jáuregui-Zúñiga *et al.*, 2005; Todeschini *et al.*, 2011), which may explain why we do not detect Zn-enriched CaOx crystals in *G. claussenii*. Why the CaOx crystals disappear upon increasing Zn exposure is intriguing though. Since the Zn exposure level correlates well with the internal Zn concentration (Villafort Carvalho *et al.*, 2013), we speculate that when plants are exposed to increasing Zn supply, Zn will start to compete with Ca in binding to vacuolar oxalate. Different from the large excess of Ca compared to Cd upon Cd exposure, this is much less when compared to Zn upon Zn exposure. At high Zn exposure, the Zn shoot molar concentration is 67 μmol $g^{-1}$ (Fig. 1). Assuming a Ca concentration of around 10 g $kg^{-1}$ (Borin, 2010), corresponding to 250 μmol $g^{-1}$, it seems very likely that Zn will interfere with the formation of CaOx crystals.

While the tissue-specific and cellular localization of Zn and Cd has been studied for the Zn/Cd hyperaccumulator species *N. caerulescens, Noccaea praecox, A. halleri* and *S. alfredii* (Küpper *et al.*, 1999; Küpper *et al.*, 2000; Cosio *et al.*, 2005; Fukuda *et al.*, 2008; Vogel-Mikuš *et al.*, 2008a; Tian *et al.*, 2011), in none of these species, CaOx crystals were described, let alone their incorporation of Zn or Cd. It should be noted though, that Amaranthaceae species generally contain high levels of oxalate, frequently resulting in CaOx crystal formation (Siener *et al.*, 2006). Comparisons of *G. claussenii* with the aforementioned hyperaccumulator species indicated that although there are some similarities, there is no obvious commonly preferred tissue for metal sequestration among

these species. For instance, *A. halleri* preferentially accumulates Zn and Cd in the cells forming the base of trichomes (Hokura *et al.*, 2006; Fukuda *et al.*, 2008), while in *N. caerulescens* (Küpper *et al.*, 2004; Cosio *et al.*, 2005) and *N. praecox* (Vogel-Mikuš *et al.*, 2008a; Vogel-Mikuš *et al.*, 2008b) metals accumulate in the leaf epidermis. For *S. alfredii*, Zn was found to be mainly stored in the epidermis of stems and leaves, while Cd was mainly stored in the pith of stems and in the leaf mesophyll (Tian *et al.*, 2011). As in *G. claussenii*, Ca co-localized with Cd in *S. alfredii* (Tian *et al.*, 2011), but not with Zn (Tian *et al.*, 2009). In *G. claussenii*, Zn is not preferentially stored in the epidermis, as we find similar Zn levels in epidermis and parenchyma cells (Fig. 6). Although the tissue-specific distribution differs between species, Zn and Cd appear to be mostly confined to vacuoles in the examined species (Zhao *et al.*, 2000; Ma *et al.*, 2005), suggesting that the physiological and molecular mechanisms for vacuolar loading and confinement might be similar.

The increase in oxalic acid levels at the expense of glycine, especially prominent in shoots of *G. claussenii* upon high Cd exposure, is in line with an import role of this primary metabolite in chelating vacuolar Cd. Co-localization of Cd in CaOx crystals, as we observed, will contribute to better Cd sequestration, but we can only speculate if this is essential for Cd tolerance. Based on the size and occurrence of the CaOx crystals and the micro-PIXE data (Fig. 5), we estimate that roughly about 10-30% of the vacuolar Cd is associated with CaOx crystals, which seems substantial. For Zn tolerance, shoot oxalic acid levels do not increase much upon high Zn exposure, which means the Zn-chelation capacity by organic acids will also be supplied by malic and citric acid. Both of these compounds increased in both roots and shoots upon high Zn exposure (Fig. 8). The acidic pH of plant vacuoles allows organic acids to be efficient vacuolar metal chelators (Haydon & Cobbett, 2007). Zn is known to bind especially to malate and citrate in *A. halleri*, *N.*

*caerulescens* and *S. alfredii* (Ueno *et al.*, 2005; Yang *et al.*, 2006; Sarret *et al.*, 2009; Monsant *et al.*, 2011; Huguet *et al.*, 2012). Also in *S. alfredii* the malate concentrations increase in response to Zn exposure (Yang *et al.*, 2006).

The untargeted approach we used to detect organic acids also allowed the detection of additional primary metabolites (Fig. 8). Changes in the soluble sugar concentrations and disturbance of the source-sink relationships are well known to occur during abiotic stress (Rolland *et al.*, 2006). Specifically for toxic metal exposure, a decrease in chlorophyll content and therefore carbon assimilation, has been described as metal toxicity symptoms for metal sensitive plants (Alaoui-Sossé *et al.*, 2004; Maksymiec, 2007). Similarly, a reduction in sugar concentrations has been reported for roots (Costa & Spitz, 1997; Podazza *et al.*, 2006; Mahajan *et al.*, 2013), and at very high exposures, for shoots as well (Ci *et al.*, 2009; Nayek *et al.*, 2010). Plants tolerant to high metal exposure maintain photosynthesis rates and have an increase in soluble sugar concentrations in roots (Chinmayee *et al.*, 2012; He *et al.*, 2013) as we also found for *G. claussenii*. This increase in sugars can be due to a higher demand for energy by sink tissues, like roots and stems, to accommodate the high accumulation of Zn and Cd. Alternatively, this increase could also be an indirect effect of the response to metal-induced reactive oxygen species (ROS) production in plants, as has been found before to occur in plants exposed to biotic and abiotic stress conditions (Couee *et al.*, 2006; Rosa *et al.*, 2009; Keunen *et al.*, 2013).

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Table S1

List of possible primary metabolites identified in roots and shoots of *Gomphrena claussenii* and their relative concentrations during exposure to high zinc and cadmium. Significant values are presented in bold ($p \leq 0.05$).

# CHAPTER 4

## Exposure to high Zn or Cd supply hardly affects gene expression of the heavy metal bioindicator *Gomphrena claussenii*

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(Submitted to The Plant Journal)

## ABSTRACT

*Gomphrena claussenii* was recently described as the first Zn and Cd hypertolerant metallophyte plant species from South-America. Different from most Zn/Cd metallophytes, *G. claussenii* is a metal bioindicator rather than a metal hyperaccumulator species, displaying extreme metal tolerance, but exposure-dependent Zn and Cd hyperaccumulation. The transcriptional response of *G. claussenii* to high metal exposure was examined using RNA-seq and compared with the response of the closely related metal-sensitive species, *G. elegans*. Plants were grown under control conditions, and exposed to high, but realistic, cadmium and zinc concentrations in hydroponics. In total 18,633 orthologous transcript pairs were identified for *G. claussenii* and *G. elegans*. The latter species showed a strong transcriptional response to metal exposure, with more than 10 to 20 times more genes significantly changing expression in shoots (2953 vs. 240), respectively roots (13529 vs. 629) than in the metal-adapted *G. claussenii*. When comparing *G. claussenii* with *G. elegans* at each treatment, 3300 transcripts, 3006 under high Zn and under 706 under Cd exposure, were significantly differentially expressed (≥ 4-fold and $p \leq 0.05$). Many of these transcripts encode proteins involved in metal homeostasis or in (a)biotic stress response. The metal hypertolerance of *G. claussenii* appears to be a constitutive, species-specific, trait, relying on adaptations in metal homeostasis and general plant stress response.


## INTRODUCTION

Only a few percent of all plant species are adapted to thrive on sites highly enriched in normally toxic heavy metals such as Cu, Ni, Pb, Cd or Zn. These metallophytes have

evolved mechanisms to overcome the toxic effects of these metals and the difficulty to acquire essential elements under nutritionally unfavourable conditions (Clemens, 2006; Verbruggen *et al.*, 2009). Two metal tolerance strategies are distinguished, with most species favouring a metal exclusion strategy to avoid excess metal uptake and others allowing metals to be taken up, but to sequester them efficiently or excrete them in order to avoid metal toxicity (Baker, 1987; Baker *et al.*, 1994; Wang *et al.*, 2009). Most of these hypertolerant species limit metal translocation to the shoots and store metals predominantly in the roots. A rare group of species has developed metal hyperaccumulation, in which metals are mainly accumulated to very high levels in the aboveground parts (Krämer, 2010; Hanikenne & Nouet, 2011; Lin & Aarts, 2012; van der Ent *et al.*, 2013). There is increasing evidence that hyperaccumulation of metals in shoots provides an ecophysiological advantage by providing increased plant defence against herbivores (Fones *et al.*, 2010; Kazemi-Dinan *et al.*, 2014).

Most metal hyperaccumulator species are adapted to nickel (Ni) exposure, and only a small group of zinc (Zn) and cadmium (Cd) hyperaccumulators has been described (Krämer, 2010). There is an interest in identifying and understanding Zn/Cd hyperaccumulators, for phytoremediation purposes. Phytoremediation is one of the most attractive biological techniques for environmental remediation that uses plants species with the ability to concentrate and tolerate high levels of metals (Pilon-Smits, 2005; Chaney *et al.*, 2007; Padmavathiamma & Li, 2007). Metal contamination is a serious environmental risk, not only to natural vegetation, but also to human health, especially regarding Cd, which is a known carcinogen (Clemens *et al.*, 2013). Recently it became clear that large parts of the rice-producing areas of China are contaminated with relatively low concentrations of Cd, much of which is bioavailable and ends up in the grain (Zhao *et*

*al.*, 2015). A proper phytoremediation crop to assist in removing these low Cd concentrations would be very useful to deal with this contamination.

The molecular knowledge behind metal accumulation in hyperaccumulators can contribute significantly to our understanding of plant metal homeostasis (Verbruggen *et al.*, 2009; Hassan & Aarts, 2011). Currently, most of the molecular knowledge related to Zn homeostasis and Cd tolerance in plants is restricted to the Brassicaceae family (Krämer, 2010). Two of these species are studied in more detail: *Arabidopsis halleri* and *Noccaea* (formerly *Thlaspi*) *caerulescens* (Baker & Whiting, 2002; Assunção *et al.*, 2003b; Krämer, 2010; Halimaa *et al.*, 2014; Pollard *et al.*, 2014).

Transcriptome analysis of non-model species has become a realistic possibility since the development of next-generation deep-coverage transcript sequencing (RNA-seq) (Wang *et al.*, 2009; Grabherr *et al.*, 2011). This allows detailed transcriptome analysis without the availability of a reference genome. So far this has provided valuable insights in the transcriptomes of metal hypertolerant/hyperaccumulating species such as *Psychotria gabriellae*, hyperaccumulating nickel (Ni) (Merlot *et al.*, 2014), *Sedum alfredii* (nowadays referred to as *S. plumbazincicola* (Gong, 2015)), adapted to high Zn and Cd (Gao *et al.*, 2013) and *Silene vulgaris*, tolerant to cupper (Cu) (Baloun *et al.*, 2014).

*Gomphrena claussenii*, a Brazilian species found growing at a Zn mining area, has been recently described as the first extreme Zn/Cd metallophyte from South-America (Villafort Carvalho *et al.*, 2013). The very high Zn/Cd tolerance and accumulation capacity under conditions of high exposure, together with its high biomass production, makes it an interesting species for phytoremediation and revegetation programs. In contrast with the other known Zn/Cd/Ni hyperaccumulators, *G. claussenii* shows a specific accumulation response. It is a metal bioindicator rather than a metal

hyperaccumulator species, which means the concentrations of accumulated Zn or Cd is more or less linearly related to the Zn or Cd concentrations plants are exposed to (Villafort Carvalho *et al.*, 2013). Next to being Zn and Cd hypertolerant, *G. claussenii* has the ability to accumulate these metals in shoots to similar levels to those found in hyperaccumulator species. A novel cellular mechanism of tolerance and accumulation was recently described for this species, when metal distribution analysis in *G. claussenii* revealed that Cd is preferentially found in calcium oxalate crystals, sequestering Cd in the vacuoles (Villafort Carvalho *et al.*, 2015).

Although the metal adaptation of *G. claussenii* has evolved independently of the metal adaptation in Brassicaceae species, we expect it involved a similar recruitment of highly expressed metal transporters to allow for the high Zn/Cd uptake from soil and translocation to the shoots. While the Zn/Cd sequestration chelators may be different, the metals are stored in leaf mesophyll vacuoles, expected to require similar transporters to accomplish this as previously found in Brassicaceae hyperaccumulators. We performed an RNA-seq transcriptomic comparison of *G. claussenii* and the related, non-metal-adapted, species *Gomphrena elegans* (Villafort Carvalho *et al.*, 2013) exposed to different Zn and Cd supplies, to further investigate this. Indeed, several metal transporters were differentially expressed between both species, suggesting at least partial convergent evolution of the metal hyperaccumulation characteristics of the Amaranthaceae *G. claussenii* and the Brassicaceae *N. caerulescens* and *A. halleri*.

# EXPERIMENTAL PROCEDURES

## Plant material and RNA preparation

Clones from single genotypes of Zn/Cd tolerant *Gomphrena claussenii* Moq. and the related non-metal-adapted *Gomphrena elegans* Mart. were cultivated hydroponically as described by (Villafort Carvalho *et al.*, 2013). In summary, cuttings were grown in 600-ml pots (one plant per pot), on modified Clark's nutrient solution buffered with a pH of 5.5 (Villafort Carvalho *et al.*, 2013). After three weeks of pre-culture in a quarter strength Clark's solution and high humidity, plants were exposed to half-strength solution containing either normal Zn supply (2 μM $ZnSO_4$), excess Zn (1000 μM $ZnSO_4$) or high Cd (50 μM $CdSO_4$; 2 μM $ZnSO_4$) for *G. claussenii*, resp. 100 μM $ZnSO_4$ or 5 μM $CdSO_4$; 2 μM $ZnSO_4$) for *G. elegans*. Nutrient solutions were replaced once a week. After four days of exposure, plants were separated in roots and shoots (stem and leaves), which were harvested and stored at –80 °C until further use.

## RNA-seq

RNA was extracted from roots and shoots separately using the RNeasy kit (Qiagen). For each condition three biological replicate samples of one plant were used for RNA sequencing on an Illumina HiSeq2000 platform. For each sample 1 ug of total RNA was used for library preparation. Samples were enriched for poly adenylated mRNA and processed directly according to TruSeq RNA protocol (Illumina Inc, San Diego CA, USA). mRNA was fragmented and further processed for end repair, adaptor ligation, cDNA synthesis and final library amplification following manufacturer's protocol (Illumina Inc, San Diego CA, USA). Final libraries were eluted in 30 μl elution buffer. Library quality was analysed using a Bioanalyzer 2100 DNA 7500 chip (Agilent Technologies) and

quantified on a Qubit quantitation platform using Quant-iT PicoGreen (Invitrogen, Life Technologies). Indexed libraries were equimolarly pooled and diluted to 6 pM for TruSeq Single Read v2 DNA clustering on two partial flow cell lanes using a cBot (Illumina Inc, San Diego CA, USA). Final sequencing was done on a HiSeq 2000 platform. Reads were split per sample by corresponding index read using CASAVA 1.8 software. The raw single-end reads were trimmed using PRINSEQ lite (Schmieder & Edwards, 2011) and assembled into contigs larger than 200 nucleotides (nt) using Trinity (Grabherr *et al.*, 2011) with the minimal kmer-coverage parameter set to 2. The transcriptomes were searched against the NCBI non-redundant (NR) protein database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) using BLASTX (Altschul *et al.*, 1997) with the e-value threshold parameter set to $10^{-5}$. In this study we only considered those transcripts for which the best BLASTX-hit was a plant-derived sequence. The BLASTX output was used for assigning gene ontology (GO) terms to the transcripts with the Blast2GO (Conesa *et al.*, 2005) program. Open reading frames (ORFs) were predicted using the ESTScan program (Iseli *et al.*, 1999). Prior to predicting ORFs, ESTScan was trained using a set of sequences that were constructed as follows: Exonerate (Slater & Birney, 2005) alignments were made for each transcript and its best BLASTX-hit. All transcripts were discarded for which the exonerate alignment did not include at least 98% of the best hit or the transcript was predicted to have frame shifts. Custom scripts were used to search for complete ORFs (from start to stop codon) on the transcripts for which exonerate alignment fulfilled the requirements. The final training set contained all transcripts with full ORFs. Pairwise orthologous transcripts for *G. claussenii* and *G. elegans* were identified by running Inparanoid (Remm *et al.*, 2001) on the predicted protein sequences.

## Expression analysis

To examine the transcriptional response, transcript reads were mapped back to the corresponding *de novo* assembled transcriptome using Bowtie 2 (Langmead & Salzberg, 2012). Transcript abundances, expressed in both count- and Reads Per Kilobase per Million mapped reads (RPKM)-values were determined using eXpress (Roberts & Pachter, 2013) with sequence bias correction. The count-based DESeq package (Anders & Huber, 2010) was used for identifying transcripts and genes in each of the individual species that were differentially expressed between control and treatment conditions within the species separately. NoiSeq (Tarazona *et al.*, 2011) was used to identify transcripts that are differentially expressed in the two species based on RPKM values. In order to compare the response of the individual species to the treatments we used the linear model functionality of DESeq. More specifically, the purpose of this analysis was to determine whether gene expression changes (fold changes) between control and treatments were significantly different in the two species. GO-term enrichment analyses were performed with the cytoscape (Shannon *et al.*, 2003) plugin BINGO (Maere *et al.*, 2005). Custom scripts were used to transform the Blast2GO output into annotation files suitable for BINGO.

## qRT-PCR

DNase treated RNA was converted to cDNA using iScript cDNA Synthesis (BioRad). 3 µl of five times diluted cDNA was used for real-time quantitative PCR. Reactions were performed on a CFX96 Touch Real-Time PCR detection system (BioRad) using iQ SYBR Green Supermix (BioRad). Primer sequences are available in Table S1.

# RESULTS

## Transcriptome sequences of *G. claussenii* and *G. elegans* are highly similar

*De novo* transcriptome sequences were generated for the Zn/Cd hypertolerant species *G. claussenii* and the related, non-tolerant, species *G. elegans*, using roots and shoots of plants exposed for four days to control, excess Zn and high Cd supply, leading to 684,437,403 reads for *G. claussenii* and 721,488,403 reads for *G. elegans* (Supplemental figure S1). Trimmed reads were assembled into 206,921 contigs with an average length of 2061nucleotide (nt) and an N50 (length for which the collection of all contigs of that length or longer contains at least half of the sum of the lengths of all contigs) of 1683 nt for *G. claussenii*, and 194,900 contigs of on average 2164 nt, and an N50 of 1763 nt for *G. elegans* (Table 1). In addition to the average and N50 lengths, the overall contig length distributions of both species are also very similar (Supplemental figure S2).

Both transcriptomes were annotated by BLASTX comparison to the NCBI non-redundant protein database. This resulted in 44% of the *G. claussenii* and 43% of *G. elegans* contigs having a significant BLASTX-hit. Further analyses regarding putative gene functions were restricted to those contigs that had a plant protein match, which means 65,637 contigs for *G. claussenii* and 66,830 for *G. elegans*. The best BLASTX-hit of the annotated transcriptome was used for a taxonomic comparison. Comparable results were observed for *G. claussenii* (Figure 1A) and *G. elegans* (Figure 1B), with both transcriptomes sharing most similarity with *Beta vulgaris*, also from the Amaranthaceae family.

The transcriptome annotation was refined through the assignment of GO terms using Blast2GO. In total, 52739 of *G. claussenii* and 53213 of *G. elegans* transcripts received GO term annotations. Comparison of the GO slim annotation revealed that with the exception of a few terms, the frequencies of the majority of GO slim terms are similar between the species (Supplemental figure S3).

To be able to identify orthologous genes, the transcriptomes of both species were compared. In total 18,633 pairs of contigs were found with best bi-directional blast hits, and these were considered to be orthologous. Approximately 87% of these orthologues had at least 95% identity (Supplemental figure S4a).

Table 1 - Summary of the *Gomphrena claussenii* and *Gomphrena elegans* transcriptome assembly. Raw sequence reads (total reads) were assembled into putative transcripts (total assembled contigs) using Trinity software. The N50 is the length for which the collection of all contigs of that length or longer contains at least half of the sum of the lengths of all contigs. Contig sequence length is in base pairs, bp.

| | total reads | total assembled contigs | N50 contig size (bp) | average contig size (bp) |
|---|---|---|---|---|
| G. claussenii | 684,437,403 | 206,921 | 1683 | 2061 |
| G. elegans | 721,488,403 | 194,900 | 1763 | 2164 |

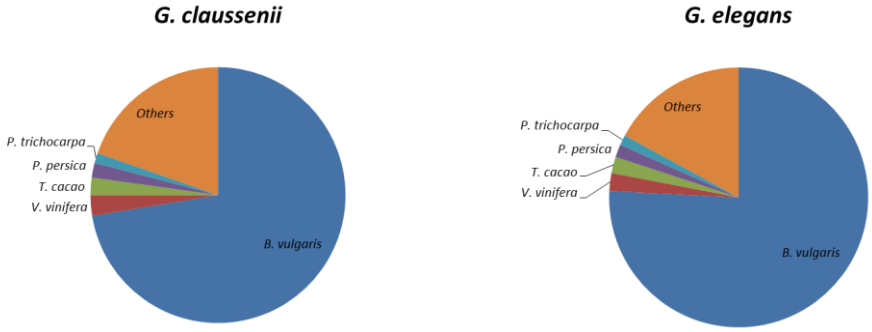


**Figure 1** - Taxonomic distribution of the *Gomhrena claussenii* (a) and *Gomphrena elegans* (b) transcripts according to best BlastX-hits. Beta vulgaris (Amaranthaceae), Vitis vinifera (Vitaceae), Theobroma cacao (Malvaceae), Prunus persica (Rosaceae) and Populus trichocarpa (Salicaceae) are the five most represented species.

## The *G. claussenii* transcriptome is less responsive to Zn and Cd compared with *G. elegans*

Zn exposure had a stronger effect on the number of differentially expressed transcripts in either species than Cd exposure and more transcripts were differentially expressed in roots than in shoots (Figure 2). *G. elegans* was more transcriptionally affected by either metal exposure treatment than *G. claussenii*, especially in roots, even though the Zn and Cd concentrations to which *G. claussenii* was exposed were much higher than the concentrations used for *G. elegans* exposure. Due to the high number of transcripts responsive to Zn and Cd, only transcripts with significant differences in expression ≥ 2-fold were further considered (Table S2).

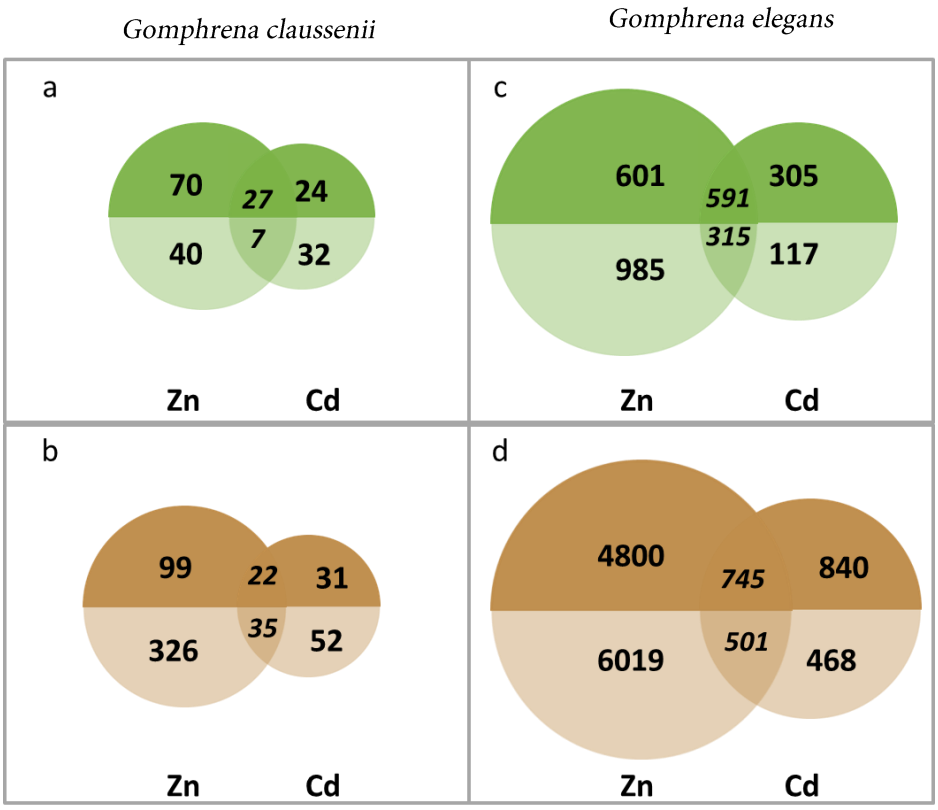


**Figure 2** - Summary of differentially expressed transcripts (DETs) of *Gomphrena claussenii* (a, b) and *Gomphrena elegans* (c, d) shoot (green) and root (brown) after exposure to high zinc (Zn; 1000, resp. 100 µM $ZnSO_4$) or cadmium (Cd; 50, resp. 5 µM $CdSO_4$ + 2 µM $ZnSO_4$) compared with control treatments (2 µM $ZnSO_4$, no Cd). Venn diagrams show the number of significantly ($p \leq 0.05$) DETs with induced (dark green/brown) or reduced (light green/brown) expression.

## Characterization of Zn and Cd responsive processes in *G. claussenii* and *G. elegans*

To better understand the functional characteristics of Zn and Cd responsive transcripts, a Gene Ontology (GO) enrichment analysis was performed and the processes over-represented in roots and shoot of both species upon exposure to Zn or Cd were identified (Figure 3). In *G. claussenii*, transcripts involved in DNA regulation showed a remarkable down-regulation in shoots, especially upon Cd treatment, whereas transcripts related with photosynthesis were induced by both treatments (Figure 3a). The metal exposures hardly evoked a stress response, which is in line with the upregulation of photosynthesis. Only on roots there is enrichment of cellular stress response transcripts upon Zn exposure. There is also little evidence of an adaptation in mineral nutrient homeostasis. Zn exposure reduced the response to Zn ions, and the transcripts related to biosynthesis and metabolism of nicotianamine (NA), a known divalent cation chelator. Cd on the other hand increased the representation of this type of transcripts. Both metal exposures increased the abundance of transcripts related to nitrate and inorganic anion transport in roots. The GO enrichment picture for *G. elegans* is drastically different (Figure 3b). Exposure to both metals led to a strong stress response, mainly in shoots, but for Cd also in roots. It also caused a drastic underrepresentation of photosynthesis related transcripts, in line with the increased stress response. Roots appeared to suffer especially from the Zn treatment, with reducing transcripts involved in cell wall organization and DNA or histone methylation, while increasing transcripts associated with catabolism and lipid oxidation. Both metal treatments reduced (an)ion transport in roots, the opposite of what happened in *G. claussenii.*

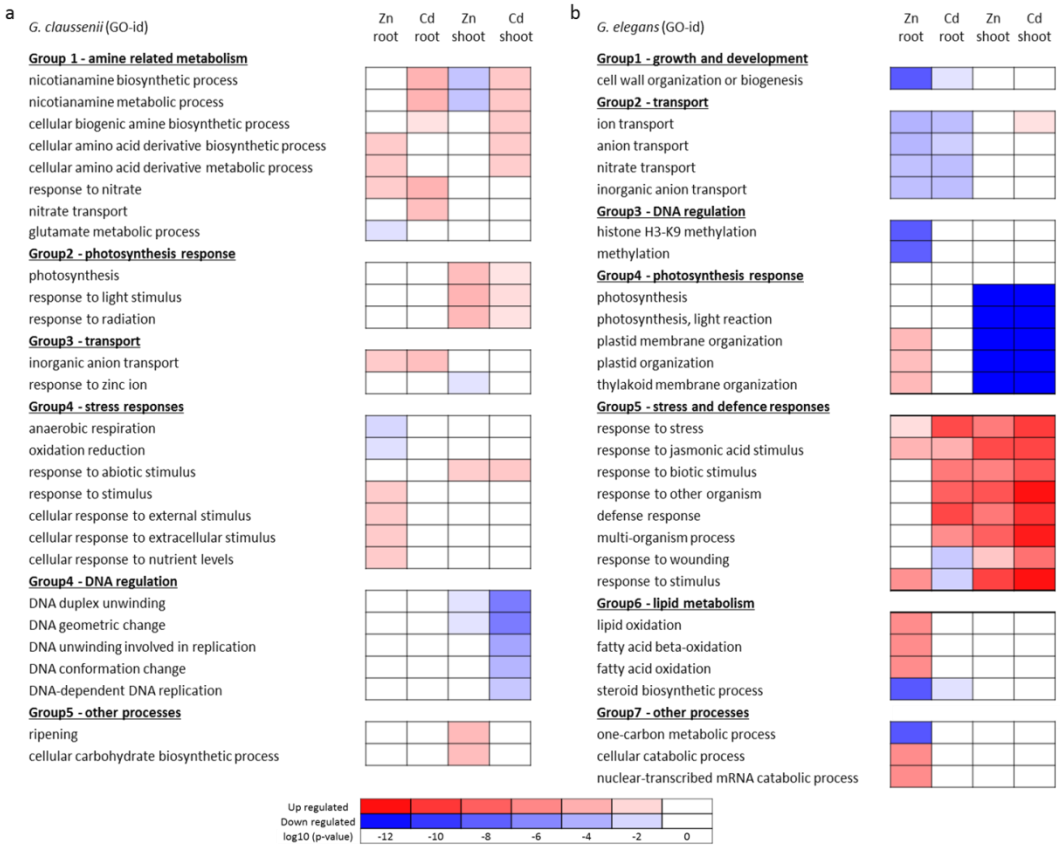


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**Figure 3** - Gene ontology (GO) enrichment analysis of *Gomphrena claussenii* (a) and *Gomphrena elegans* (b) root and shoot transcripts upon zinc (1000, resp. 100 µM $ZnSO_4$) or cadmium (50, resp. 5 µM $CdSO_4$ + 2 µM $ZnSO_4$) exposures. The most significant GO enriched biological processes (GO-id) are presented. A red-blue gradation indicates transcripts regulation direction, up-regulation (red) or down-regulation (blue).

## Transcripts with different regulation and expression levels in *G. claussenii* and *G. elegans* under Zn and/or Cd treatments

With so many more genes of the non-metal-adapted *G. elegans* responding to metal exposure than of the metal-adapted *G. claussenii*, the latter species may have its metal response genes constitutively expressed. Considering the high sequence similarity between *G. claussenii* and *G. elegans* transcripts, allowing the definition of orthologous transcript pairs, and considering the high correlation between orthologous pair transcripts expression for root and shoot samples in each treatment (Supplemental figure S4b), a direct comparison of expression of orthologous transcripts can justifiably be made. For this comparison, only transcripts with a significant ($p \leq 0.05$) and at least four-fold difference in expression between both species were considered. This meant 2639 root transcripts and 497 shoot transcripts to respond differentially to high Zn exposure (Table S3) and 524 root transcripts and 279 shoot transcripts to respond differentially to Cd exposure (Table S3).

When considering the most differentially expressed root (Table 2) and shoot transcripts (Table 3) in either of two species, there were indeed many that are strongly differentially expressed in *G. elegans*, but more or less constitutively expressed in *G. claussenii*. GO analysis of this class of transcripts indicated enrichment in transcripts encoding proteins functioning in cell proliferation or differentiation (e.g. involved in cell wall, lignin and xylan biosynthesis) and in amino acid transport and sugar response. Other transcripts found within this class, such as those encoding cytochrome P450 proteins, are related to stress or defence response. Two transcripts for metal homeostasis genes were found in the same class, encoding the iron reductase FRO and the copper-transporting P-type ATPase HMA5.

Table 2 - Root transcripts which are differentially expressed upon either high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5) when compared to control conditions (Zn2) in either *Gomphrena claussenii* (GC) or *Gomphrena elegans* (GE), and which are most differentially expressed when comparing both species. Differential expressions are indicated as ratios. Very small ratios (<0.01) are indicated as 1 divided by X (1/X). For ease of interpretation, the most similar *Arabidopsis thaliana* gene for each contig ID (Contig) is indicated with the gene name (Name), gene code (Code) and putative protein function. Gene ontology (GO) annotation is provided for biological process. Significant values are presented in bold (p≤0.05). Significant differences between the two species for the transcript expression are in grey. Contigs with lower counts were not detected, ND.

| Name | Code | Putative function | GO annotation | Zn1000/ Zn2GC | Zn100/ Zn2 GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|
| BXL1 | AT5G49360 | Beta-xylosidase 1 | Developmental processes | 2.23 | 1780 | 0.64 | 3.73 | 17793 |
| | AT4G22310 | Uncharacterized protein family | Electron transport or energy pathways; transport | 1.76 | 645 | 0.46 | 19.2 | 17352 |
| BCAT-2 | AT1G10070 | Branched-chain amino acid transaminase 2 | Other biological processes | 1.16 | 624 | 0.48 | 3.12 | 8597 |
| | AT5G42830 | HXXXD-type acyl-transferase family protein | Biological processes unknown | 0.34 | 312 | 0.21 | 29.9 | 5117 |
| PSK4 | AT3G49780 | Phytosulfokine 4 precursor | Developmental processes | 0.33 | 255 | 0.69 | 11.5 | 20828 |
| | AT1G08510 | Fatty acyl-ACP thioesterases B | Other metabolic processes; other cellular processes | 1.17 | 244 | 0.83 | 0.82 | 7850 |
| BXL1 | AT5G49360 | Beta-xylosidase 1 | Developmental processes | 0.80 | 222 | 0.86 | 1.03 | 3350 |
| TBL38 | AT1G29050 | Trichome birefringence-like 38 | Biological processes unknown | 0.65 | 160 | 0.55 | 1.63 | 18589 |
| | AT3G59480 | Pfkb-like carbohydrate kinase family protein | Biological processes unknown | 0.59 | 125 | 0.30 | 0.29 | 12898 |
| | AT1G67980 | Caffeoyl-coa 3-O-methyltransferase | Other metabolic processes; other cellular processes | 0.77 | 57.6 | 0.77 | 200 | 18337 |
| | AT3G28960 | Transmembrane amino acid transporter family protein | Transport | 1.81 | 49.5 | 1.29 | 271 | 7666 |
| | AT5G11170 | DEAD/DEAH box RNA helicase family protein | Other biological processes | 1/552 | 35.2 | 0.60 | 1.31 | 8801 |
| CYP51G1 | AT1G11680 | Cytochrome p450 51g1 | Developmental processes | 0.06 | 33.8 | 1.37 | 4.33 | 5799 |
| CNGC13 | AT4G01010 | Cyclic nucleotide-gated channel 13 | Response to abiotic or biotic stimulus; | 0.94 | 30.7 | 0.80 | 249 | 5542 |


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| Name | Code | Putative function | GO annotation | Zn1000/ Zn2GC | Zn100/ Zn2 GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|
| | | | signal transduction; response to stress | | | | | |
| | AT4G03460 | Ankyrin repeat family protein | Biological processes unknown | 0.84 | 26.2 | 0.75 | 83.3 | 7914 |
| | - | - | Biological processes unknown | 0.02 | 26.0 | 1.14 | 2.75 | 18336 |
| | AT1G68300 | Adenine nucleotide alpha hydrolases-like superfamily protein | Response to abiotic or biotic stimulus | 0.03 | 19.4 | 1.18 | 1.32 | 14399 |
| | AT3G15980 | Coatomer, beta' subunit | Cell organization and biogenesis; protein metabolism | 2.02 | 15.9 | 1.79 | 468 | 10650 |
| CYP72A15 | AT3G14690 | Cytochrome P450, family 72, subfamily A, polypeptide 15 | Biological processes unknown | 2.69 | 12.4 | 1.79 | 381 | 4524 |
| | AT5G51890 | Peroxidase superfamily protein | Biological processes unknown | 1.17 | 0.05 | 1.12 | 0.11 | 19483 |
| LOX2 | AT3G45140 | Lipoxygenase 2 | Response to stress; cell organization and biogenesis; response to abiotic or biotic stimulus | 1.58 | 0.05 | 1.36 | 0.13 | 20051 |
| | AT3G43660 | Vacuolar iron transporter (VIT) family protein | Biological processes unknown | 1.06 | 0.03 | 1.10 | 0.10 | 13459 |
| | AT2G41190 | Transmembrane amino acid transporter family protein | Transport | 4.84 | 0.02 | 2.89 | 0.69 | 7060 |
| FRO2 | AT1G01580 | Ferric reduction oxidase 2 | Response to abiotic or biotic stimulus; transport | 0.70 | 1/111 | 1.35 | 0.03 | 7438 |
| RNS3 | AT1G26820 | Ribonuclease 3 | Other biological processes | 1.46 | 1/129 | 0.89 | 0.40 | 18463 |
| EXPA12 | AT3G15370 | Expansin 12 | Cell organization and biogenesis | 1.68 | 1/173 | 0.95 | 0.62 | 11546 |
| | AT4G12530 | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein | Transport | 0.87 | 1/190 | 0.90 | 0.46 | 16862 |
| | AT5G57035 | U-box domain-containing protein kinase family protein | Biological processes unknown | 0.73 | 1/210 | 0.83 | 0.55 | 17800 |
| MRP4 | AT2G47800 | Multidrug resistance-associated protein 4 | Response to abiotic or biotic stimulus; response to stress; transport | 1.00 | 1/214 | 0.99 | 0.48 | 3047 |
| | AT4G09810 | Nucleotide-sugar transporter family protein | Transport | 1.08 | 1/239 | 1.12 | 0.63 | 21140 |
| RNS1 | AT2G02990 | Ribonuclease 1 | Response to stress; transport | 1.12 | 1/243 | 0.93 | 0.33 | 11949 |
| | AT3G56230 | BTB/POZ domain-containing protein | Other metabolic processes; other cellular processes | 1.16 | 1/250 | 1.08 | 0.38 | 12081 |
| | AT5G05340 | Peroxidase superfamily protein | Biological processes unknown | 1.47 | 1/257 | 1.88 | 0.60 | 11027 |
| | AT3G09925 | Pollen Ole e 1 allergen | Biological processes unknown | 0.67 | 1/289 | 0.79 | 0.65 | 14573 |

| Name | Code | Putative function | GO annotation | Zn1000/ Zn2GC | Zn100/ Zn2 GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|
| | AT5G25940 | Early nodulin-related | Other biological processes | 0.48 | 1/352 | 0.60 | 1.37 | 18283 |
| OMT1 | AT5G54160 | O-methyltransferase 1 | Response to stress | 0.53 | 1/362 | 0.88 | 0.43 | 7404 |
| XTH16 | AT3G23730 | Xyloglucan endotransglucosylase/hydrolase 16 | Biological processes unknown | 0.78 | 1/412 | 0.71 | 0.78 | 13094 |
| | AT5G53050 | Alpha/beta-Hydrolases superfamily protein | Biological processes unknown | 1.28 | 1/479 | 1.07 | 2.37 | 9574 |
| BCS1 | AT3G50930 | Cytochrome BC1 synthesis | Response to abiotic or biotic stimulus; response to stress | 0.62 | 11.4 | 1.01 | 249 | 8547 |
| | AT5G52390 | PAR1 protein | Biological processes unknown | 4.67 | 5.30 | 1.29 | 177 | 13047 |
| CYP83B1 | AT4G31500 | Cytochrome P450, family 83, subfamily B, polypeptide 1 | Response to abiotic or biotic stimulus; response to stress; developmental processes | 1.19 | 0.96 | 0.77 | 112 | 8734 |
| GER3 | AT5G20630 | Germin 3 | Electron transport or energy pathways; protein metabolism; response to abiotic or biotic stimulus | 0.78 | 1.29 | 0.40 | 59.3 | 13521 |
| GLR2.1 | AT5G27100 | Glutamate receptor 2.1 | Response to abiotic or biotic stimulus | 0.53 | 1.24 | 0.45 | 47.7 | 1294 |
| BCB | AT5G20230 | Blue-copper-binding protein | Response to abiotic or biotic stimulus; response to stress; transport | 0.09 | 0.42 | 0.20 | 30.5 | 16342 |
| | AT4G17660 | Protein kinase superfamily protein | Biological processes unknown | 0.32 | 0.32 | 0.23 | 22.8 | 17057 |
| ACA1 | AT3G63380 | Atpase E1-E2 type family protein | Response to abiotic or biotic stimulus; signal transduction; response to stress; transport | 0.06 | 1.27 | 0.09 | 15.0 | 2981 |
| | - | - | Biological processes unknown | 0.29 | 0.24 | 8.46 | 0.24 | 6122 |
| | AT3G60370 | FKBP-like peptidyl-prolyl cis-trans isomerase family protein | Electron transport or energy pathways; protein metabolism; transcription DNA-dependent | 0.93 | 0.55 | 0.94 | 0.09 | 13321 |
| | - | - | Biological processes unknown | 1.29 | 0.58 | 1.18 | 0.09 | 16549 |
| CRK25 | AT4G05200 | Cysteine-rich RLK 25 | Response to stress; transport | 0.84 | 1.13 | 1.12 | 0.04 | 7238 |
| | AT3G47570 | Leucine-rich repeat protein kinase family protein | Signal transduction; protein metabolism | ND | ND | 0.94 | 0.04 | 3924 |
| | AT3G20810 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | Transcription, DNA-dependent | 0.94 | 2.14 | 0.61 | 0.03 | 12418 |

| Name | Code | Putative function | GO annotation | Zn1000/ Zn2GC | Zn100/ Zn2 GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|
| TPS21 | AT5G23960 | Terpene synthase 21 | Response to abiotic or biotic stimulus | ND | ND | 0.81 | 0.01 | 9170 |
| RHM1 | AT1G78570 | Rhamnose biosynthesis 1 | Response to abiotic or biotic stimulus; signal transduction; transport | 1/274 | 1.61 | 0.29 | 0.78 | 1753 |
| | AT5G61170 | Ribosomal protein S19 family protein | Protein metabolism | 1/547 | 0.90 | 0.50 | 0.65 | 12069 |

Table 3 - Shoot transcripts which are differentially expressed upon either high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5) when compared to control conditions (Zn2) in either *Gomphrena claussenii* (GC) or *Gomphrena elegans* (GE), and which are most differentially expressed when comparing both species. Differential expressions are indicated as ratios. Very small ratios (<0.01) are indicated as 1 divided by X (1/X). For ease of interpretation, the most similar *Arabidopsis thaliana* gene for each contig ID (Contig) is indicated with the gene name (Name), gene code (Code) and putative protein function. Gene ontology (GO) annotation is provided for biological process. Significant values are presented in bold (p≤0.05). Significant differences between the two species for the transcript expression are in grey. Contigs with lower counts were not detected, ND.

| Name | Code | Putative function | GO annotation | Zn1000/ Zn2 GC | Zn100/ Zn2 GE | Cd50/ Zn2 GC | Cd5/ Zn2 GE | Contig |
|---|---|---|---|---|---|---|---|---|
| OMT1 | AT5G54160 | O-methyltransferase 1 | Response to stress | 1.02 | 236 | 0.88 | 68.6 | 7217 |
| HDG2 | AT1G05230 | Homeodomain GLABROUS 2 | Developmental processes; cell organization and biogenesis; transcription DNA-dependent | 1.10 | 181 | 1.04 | 51.0 | 19156 |
| CRK8 | AT4G23160 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 8 | Biological processes unknown | 1.18 | 135 | 0.79 | 311 | 17171 |
| ALDH2C4 | AT3G24503 | Aldehyde dehydrogenase 2C4 | Response to abiotic or biotic stimulus; response to stress; transport | 1.08 | 62.9 | 0.83 | 13.3 | 4134 |
| GPT2 | AT1G61800 | Glucose-6-phosphate/phosphate translocator 2 | Response to abiotic or biotic stimulus; transport | 0.55 | 49.8 | 0.87 | 6.32 | 8932 |
| | AT4G17660 | Protein kinase superfamily protein | Biological processes unknown | 0.70 | 46.8 | 0.97 | 77.1 | 17057 |
| | AT5G01250 | Alpha 1,4-glycosyltransferase family protein | Biological processes unknown | 0.82 | 36.8 | 0.83 | 10.08 | 14964 |
| CV | AT2G25625 | Unknown protein | Biological processes unknown | 0.73 | 35.0 | 1.25 | 24.1 | 17708 |
| ALN | AT4G04955 | Allantoinase | Response to stress | 0.69 | 28.8 | 0.53 | 3.02 | 3436 |


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| Name | Code | Putative function | GO annotation | Zn1000/ Zn2 GC | Zn100/ Zn2 GE | Cd50/ Zn2 GC | Cd5/ Zn2 GE | Contig |
|---|---|---|---|---|---|---|---|---|
| GSTU19 | AT1G78380 | Glutathione S-transferase TAU 19 | Electron transport or energy pathways; protein metabolism | 0.96 | 25.9 | 1.08 | 65.4 | 13065 |
| CNGC14 | AT2G24610 | Cyclic nucleotide-gated channel 14 | Other biological processes | 1.08 | 25.3 | 1.11 | 24.2 | 12741 |
| | AT5G01740 | Nuclear transport factor 2 (NTF2) family protein | Biological processes unknown | 0.57 | 22.6 | 1.24 | 6.29 | 14956 |
| ALD1 | AT2G13810 | AGD2-like defense response protein 1 | Response to abiotic or biotic stimulus; signal transduction; response to stress | 0.80 | 17.2 | 1.02 | 57.5 | 6268 |
| AKS2 | AT1G05805 | Basic helix-loop-helix (bHLH) DNA-binding superfamily protein | Transcription, DNA-dependent; other cellular processes; other metabolic processes | 0.12 | 12.2 | 0.33 | 2.98 | 11832 |
| | AT3G47570 | Leucine-rich repeat protein kinase family protein | Signal transduction; protein metabolism | 0.73 | 8.50 | 0.79 | 29.7 | 1292 |
| GAD1 | AT5G17330 | Glutamate decarboxylase | Developmental processes; other biological processes | 0.07 | 5.40 | 0.64 | 4.12 | 19389 |
| | - | - | Biological processes unknown | 0.77 | 0.18 | 0.47 | 0.06 | 15564 |
| AHA4 | AT3G47950 | H(+)-atpase 4 | Response to stress; DNA or RNA metabolism; other biological processes | 1.44 | 0.13 | 1.33 | 0.14 | 8831 |
| HA7 | AT3G60330 | H(+)-atpase 7 | Biological processes unknown | 1.25 | 0.12 | 1.33 | 0.17 | 8287 |
| | AT2G01275 | RING/FYVE/PHD zinc finger superfamily protein | Biological processes unknown | 1.97 | 0.06 | 0.95 | 0.30 | 13476 |
| | AT5G05140 | Transcription elongation factor (TFIIS) family protein | Response to abiotic or biotic stimulus; response to stress | 1.16 | 0.05 | 1.11 | 0.26 | 9414 |
| | AT1G29500 | SAUR-like auxin-responsive protein family | Other biological processes | 1.30 | 0.05 | 1.24 | 0.17 | 16912 |
| | AT5G20050 | Protein kinase superfamily protein | Other biological processes; transport | 1.64 | 0.04 | 1.15 | 0.28 | 7010 |
| | AT5G53050 | Alpha/beta-Hydrolases superfamily protein | Biological processes unknown | 1.07 | 0.03 | 1.06 | 1.66 | 13567 |
| | AT1G03020 | Thioredoxin superfamily protein | Developmental processes | 0.91 | 0.02 | 1.10 | 0.21 | 17797 |

| Name | Code | Putative function | GO annotation | Zn1000/ Zn2 GC | Zn100/ Zn2 GE | Cd50/ Zn2 GC | Cd5/ Zn2 GE | Contig |
|---|---|---|---|---|---|---|---|---|
| RBCS1A | AT1G67090 | Ribulose bisphosphate carboxylase small chain 1A | Response to abiotic or biotic stimulus; response to stress | 1.28 | 1/74 | 1.58 | 0.35 | 20017 |
| EXPB2 | AT1G65680 | Expansin B2 | Cell organization and biogenesis; other cellular processes | 4.53 | 1/74.8 | 1.46 | 0.39 | 11782 |
| | AT1G03020 | Thioredoxin superfamily protein | Developmental processes | 1.09 | 1/92.8 | 1.77 | 0.18 | 17728 |
| | AT2G06500 | Hat family dimerisation domain | Biological processes unknown | 0.47 | 1/98.5 | 0.44 | 0.50 | 21016 |
| CPK9 | AT3G20410 | Calmodulin-domain protein kinase 9 | Protein metabolism; other cellular processes; response to abiotic or biotic stimulus; signal transduction; response to stress; other biological processes; cell organization and biogenesis; other metabolic processes; transport | 1.17 | 1/116 | 0.98 | 0.17 | 20616 |
| WRKY40 | AT1G80840 | WRKY DNA-binding protein 40 | Response to abiotic or biotic stimulus; signal transduction; response to stress | 3.35 | 21.7 | 2.08 | 124 | 9568 |
| | AT2G27420 | Cysteine proteinases superfamily protein | Protein metabolism | 0.91 | 2.32 | 0.81 | 32.02 | 11391 |
| NQR | AT3G27890 | NADPH:quinone oxidoreductase | Electron transport or energy pathways; response to abiotic or biotic stimulus; signal transduction | 1.02 | 13.4 | 0.97 | 22.5 | 13415 |
| | AT3G28960 | Transmembrane amino acid transporter family protein | Transport | 1.74 | 33.3 | 0.91 | 19.9 | 7666 |
| | AT4G16260 | Glycosyl hydrolase superfamily protein | Response to abiotic or biotic stimulus; response to stress | 0.73 | 2.44 | 0.83 | 19.9 | 9992 |
| HMA5 | AT1G63440 | Heavy metal atpase 5 | Response to stress | 0.76 | 3.57 | 0.34 | 7.59 | 5311 |
| CYP716A1 | AT5G36110 | Cytochrome P450, family 716, subfamily A, polypeptide 1 | Biological processes unknown | 0.32 | 0.23 | 1.61 | 0.18 | 6400 |
| ARR9 | AT3G57040 | Response regulator 9 | Signal transduction; transcription,DNA-dependent; developmental processes | ND | ND | 3.55 | 0.14 | 14884 |



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| Name | Code | Putative function | GO annotation | Zn1000/ Zn2 GC | Zn100/ Zn2 GE | Cd50/ Zn2 GC | Cd5/ Zn2 GE | Contig |
|---|---|---|---|---|---|---|---|---|
| CSD2 | AT2G28190 | Copper/zinc superoxide dismutase 2 | Response to abiotic or biotic stimulus; response to stress | 1.55 | 0.30 | 0.60 | 0.07 | 13579 |
| | AT5G02800 | Protein kinase superfamily protein | Signal transduction; transcription,DNA-dependent | 1.00 | 1.37 | 1.02 | 0.06 | 7346 |
| BGLU46 | AT1G61820 | Beta glucosidase 46 | Other metabolic processes; other cellular processes | 1.08 | 0.27 | 1.05 | 0.05 | 7305 |
| COL5 | AT5G57660 | CONSTANS-like 5 | Response to abiotic or biotic stimulus; transcription,DNA-dependent; response to stress | 32.5 | 0.98 | 1.21 | 1.28 | 10349 |
| PIN2 | AT5G57090 | Auxin efflux carrier family protein | Response to abiotic or biotic stimulus; developmental processes; cell organization and biogenesis | 13.3 | 0.52 | 5.98 | 0.75 | 2959 |
| | AT1G03020 | Thioredoxin superfamily protein | Developmental processes | 5.21 | 0.28 | 7.00 | 0.46 | 17320 |
| RH39 | AT4G09730 | Rh39 | Cell organization and biogenesis | 1/81 | 0.55 | 1/697 | 0.64 | 4679 |
| MUS81 | AT4G30870 | Restriction endonuclease, type II-like superfamily protein | Protein metabolism; transcription,DNA-dependent; response to stress | 1/85.4 | 1.03 | 0.94 | 1.42 | 3770 |
| WAK5 | AT1G21230 | Wall associated kinase 5 | Protein metabolism; other cellular processes; other metabolic processes | 1/118 | 0.56 | 0.47 | 0.90 | 9719 |

Genes that are consistently higher expressed in *G. claussenii* compared with *G. elegans* in any of the three treatments, could also be involved in high metal adaptation, as has previously been observed in *A. halleri* and *N caerulescens* (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006; Weber *et al.*, 2006; van de Mortel *et al.*, 2008). In total 1054 transcripts, 679 in roots and 507 in shoots, were significantly higher expressed (≥ 4 fold; $p \leq 0.05$) in *G. claussenii* than in *G. elegans* (Table S4). No biological function could be assigned to 308 of these transcripts, and only 58 showed differential expression in *G. claussenii* when comparing treatments. From this large set of transcripts a selection was made of 75 transcripts for which the potential function indicates an involvement with metal tolerance adaptation (Table 4 and 5).

Many of these transcripts encode proteins involved in (a)biotic stress response such as enzymes in flavonoid and anthocyanin biosynthesis, cytochrome P450s, glutathione S-transferases, (ascorbate) peroxidases and a blue-copper-binding protein. Transcripts associated with zinc (*ZIP1, ZIP3, IRT3, NAS1, NAS4, HMA2, YSL1, PAP23, TMN7*), iron (*FIT, FRO2, FRO7, EIN3, BGLU42*), nitrogen (*AMT1;2, NRT2, NR2*), potassium (*HAK5, KUP3*), sulfate (*SULTR3;4, SULTR1;3*) and phosphate homeostasis (*PHT1;7, PAP26*) are found in this group. Other identified transcripts encode proteins involved with transcriptional regulation such as the two bHLH-transcription factors, one of which is involved in circadian clock regulation, which have the largest difference in expression between the two species.

Table 4 - Selection of transcripts which are more highly expressed in *Gomphrena claussenii* (GC) roots compared to *Gomphrena elegans* (GE) upon control (Zn2), high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5). Differential expressions are indicated as ratios. Very small ratios (<0.01) are indicated as 1 divided by X (1/X). For ease of interpretation, the most similar *Arabidopsis thaliana* gene for each contig ID (Contig) is indicated with the gene name (Name), gene code (Code) and putative protein function. Gene ontology (GO) annotation is provided for biological process. Significant differences between the two species (probability ≥0.95) or two conditions (p≤0.05) for the transcript expression are indicated in bold. Contig with lower counts were not detected, ND.

| Name | Code | Putative function | GO annotation | Zn2GC/ Zn2 GE | Zn1000GC/ Zn100 GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AVA-P2 | AT1G19910 | Atpase, F0/V0 complex, subunit C protein | Biological processes unknown | 470 | 5.64E+15 | 265 | 1.13 | ND | 1.15 | ND | 20570 |
| | - | - | Biological processes unknown | 2.07E+17 | 6.88E+09 | 6.19E+19 | 1.03 | ND | 1.05 | ND | 19890 |
| CYP71A22 | AT3G48310 | Cytochrome P450, family 71, subfamily A, polypeptide 22 | Biological processes unknown | 1.89E+05 | 6.61E+08 | 4296 | 0.87 | ND | 1.08 | ND | 5916 |
| | - | - | Biological processes unknown | 1.24E+06 | 4.10E+06 | 1.46E+12 | 1.16 | ND | 1.06 | ND | 21005 |
| SUS4 | AT3G43190 | Sucrose synthase 4 | Signal transduction; response to abiotic or biotic stimulus; response to stress | 110 | 5.65E+05 | 132 | 1.26 | ND | 0.97 | ND | 21170 |
| CYP81D8 | AT4G37370 | Cytochrome P450, family 81, subfamily D, polypeptide 8 | Other biological processes; response to abiotic or biotic stimulus; response to stress | 8.40 | 993 | 20.1 | 1.30 | 1/81 | 0.93 | 0.37 | 5284 |
| USPL1 | AT1G49320 | Unknown seed protein like 1 | Developmental processes | 428 | 944 | 434 | 1.29 | ND | 1.00 | ND | 16926 |
| MLP43 | AT1G70890 | MLP-like protein 43 | Other cellular processes; transport | 10.1 | 630 | 13.6 | 0.94 | 1/53.2 | 0.66 | 0.47 | 16383 |
| CYP76C4 | AT2G45550 | Cytochrome P450, family 76, subfamily C, polypeptide 4 | Biological processes unknown | 164 | 526 | 368 | 0.97 | 0.36 | 1.03 | 0.44 | 5423 |
| EDR2 | AT4G19040 | Enhanced disease resistance 2 | Biological processes unknown | 6983 | 512 | 588 | 1.03 | ND | 1.04 | ND | 14824 |
| CPK20 | AT2G38910 | Calcium-dependent protein kinase 20 | Cell organization and biogenesis; protein metabolism | 61.2 | 298 | 101 | 1.00 | ND | 1.06 | ND | 20837 |
| GPT2 | AT1G61800 | Glucose-6-phosphate/phosphate translocator 2 | Other biological processes; response to abiotic or biotic stimulus | 27.4 | 294 | 44.2 | 0.48 | 0.05 | 1.01 | 0.58 | 8932 |
| | AT5G51890 | Peroxidase superfamily protein | Biological processes unknown | 8.93 | 293 | 86.3 | ND | 0.04 | ND | 0.11 | 19483 |
| APX3 | AT4G35000 | Ascorbate peroxidase 3 | Other biological processes; response to stress | 5.45 | 207 | 4.30 | 0.93 | 0.03 | 0.93 | 1.14 | 20678 |
| | AT5G48850 | Tetratricopeptide repeat (TPR)-like superfamily protein | Response to stress; other cellular processes | 12.2 | 154 | 1.29 | 7.94 | 2.99 | 2.41 | 86.79 | 19030 |

| Name | Code | Putative function | GO annotation | Zn2GC/ Zn2 GE | Zn1000GC/ Zn100 GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BSMT1 | AT3G11480 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein | Other biological processes; response to abiotic or biotic stimulus; other cellular processes | 19.6 | 127 | 65.8 | 4.74 | 9.34 | 0.81 | 2.46 | 7175 |
| MLP43 | AT1G70890 | MLP-like protein 43 | Other cellular processes; transport | 61.6 | 112 | 28.6 | 1.11 | 0.77 | 0.90 | 1.85 | 15163 |
| FRO2 | AT1G01580 | Ferric reduction oxidase 2 | Other biological processes; response to abiotic or biotic stimulus; transport | 4.32 | 107 | 188 | 0.68 | 1/116 | 1.32 | 0.03 | 7438 |
| | AT5G39580 | Peroxidase superfamily protein | Response to abiotic or biotic stimulus; response to stress; transport | 2.94 | 96.8 | 5.03 | 2.46 | 0.04 | 2.47 | 1.29 | 13264 |
| RS2Z | AT3G53500 | RNA-binding (RRM/RBD/RNP motifs) family protein with retrovirus zinc finger-like domain | Protein metabolism; transcription,DNA-dependent | 3188 | 94.0 | 190 | 0.89 | ND | 0.92 | ND | 20323 |
| | AT4G01070 | UDP-Glycosyltransferase superfamily protein | Response to stress; other biological processes; cell organization and biogenesis | 14.2 | 87.0 | 57.4 | 0.80 | ND | 0.80 | ND | 20554 |
| SULTR3; 4 | AT3G15990 | Sulfate transporter 3; 4 | Biological processes unknown | 1.92 | 82.9 | 3.15 | 1.07 | 0.03 | 1.14 | 0.65 | 16716 |
| | AT3G43850 | Unknown protein | Biological processes unknown | 16.5 | 68.8 | 69.1 | 1.34 | 0.38 | 1.18 | 0.26 | 13812 |
| HAK5 | AT4G13420 | High affinity K+ transporter 5 | Transport | 3.44 | 61.5 | 2.50 | 0.60 | 0.04 | 0.69 | 0.89 | 1793 |
| NRT2:1 | AT1G08090 | Nitrate transporter 2:1 | Developmental processes; other biological processes; transport | 2.18 | 53.6 | 16.2 | 0.98 | 0.05 | 1.52 | 0.19 | 3908 |
| CYP72A15 | AT3G14690 | Cytochrome P450, family 72, subfamily A, polypeptide 15 | Biological processes unknown | 181 | 45.0 | 0.73 | 2.76 | 14.4 | 1.81 | 428.15 | 4524 |
| | AT3G43660 | Vacuolar iron transporter (VIT) family protein | Biological processes unknown | 1.12 | 42.0 | 11.1 | 1.02 | 0.03 | 1.10 | 0.11 | 13459 |
| HSL1 | AT1G28440 | HAESA-like 1 | Protein metabolism; response to abiotic or biotic stimulus; signal transduction | 16.1 | 40.6 | 22.1 | 1.18 | 0.58 | 1.14 | 0.81 | 827 |
| TMN7 | AT3G13772 | Transmembrane nine 7 | Other metabolic processes; other cellular processes | 224 | 33.3 | 207 | 0.97 | ND | 0.98 | ND | 19525 |
| | AT3G14470 | NB-ARC domain-containing disease resistance protein | Response to abiotic or biotic stimulus; response to stress | 52.7 | 28.1 | 36.2 | 1.10 | ND | 1.09 | ND | 19432 |
| | AT5G03455 | Rhodanese/Cell cycle control phosphatase superfamily protein | Protein metabolism | 34.1 | 26.0 | 39.8 | 0.99 | 1.58 | 0.99 | 0.80 | 16745 |
| PAPA26 | AT5G34850 | Purple acid phosphatase 26 | Other biological processes; response to abiotic or biotic stimulus; response to stress | 1.78 | 24.5 | 4.39 | 1.32 | 0.12 | 1.48 | 0.57 | 4886 |

| Name | Code | Putative function | GO annotation | Zn2GC/ Zn2 GE | Zn1000GC/ Zn100 GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| NAS4 | AT1G56430 | Nicotianamine synthase 4 | Developmental processes; cell organization and biogenesis; transport | 2.47 | 23.8 | 5.42 | 0.56 | 0.07 | 1.85 | 0.78 | 20014 |
| TRFL2 | AT1G07540 | TRF-like 2 | Biological processes unknown | 88.9 | 22.7 | 1.26E+05 | 1.19 | ND | 1.16 | ND | 16638 |
| UREG | AT2G34470 | Urease accessory protein G | Other metabolic processes | 13.2 | 22.2 | 11.9 | 0.95 | 0.65 | 1.03 | 1.04 | 11109 |
| GSTU8 | AT3G09270 | Glutathione S-transferase TAU 8 | Protein metabolism; response to stress | 13.0 | 21.7 | 1.01 | 2.49 | 1.77 | 1.36 | 15.95 | 12753 |
| | AT3G21560 | UDP-Glycosyltransferase superfamily protein | Response to abiotic or biotic stimulus | 3.51 | 20.5 | 5.68 | 2.22 | 0.44 | 2.13 | 1.21 | 17117 |
| | AT4G02330 | Plant invertase/pectin methylesterase inhibitor superfamily | Response to abiotic or biotic stimulus; response to stress; transport | 6.59 | 20.2 | 5.74 | 1.77 | 0.68 | 2.18 | 2.39 | 3658 |
| NHX2 | AT3G05030 | Sodium hydrogen exchanger 2 | Transport | 6.60 | 20.2 | 6.87 | 0.91 | 0.36 | 0.97 | 0.87 | 3838 |
| NAS1 | AT5G04950 | Nicotianamine synthase 1 | Response to stress; developmental processes; cell organization and biogenesis | 2.95 | 18.0 | 6.16 | 0.54 | 0.09 | 1.83 | 0.80 | 20503 |
| FIT1 | AT2G28160 | FER-like regulator of iron uptake | Response to stress; transcription,DNA-dependent | 5.97 | 18.0 | 10.8 | 1.60 | 0.67 | 1.39 | 0.73 | 12743 |
| GSTU19 | AT1G78380 | Glutathione S-transferase TAU 19 | Electron transport or energy pathways; protein metabolism; other cellular processes | 15.5 | 16.2 | 1.04 | 1.69 | 1.93 | 0.93 | 13.31 | 13065 |
| GSTF8 | AT2G47730 | Glutathione S-transferase phi 8 | Response to abiotic or biotic stimulus; response to stress | 23.2 | 16.1 | 1.75 | 1.31 | 2.24 | 0.97 | 11.73 | 20458 |
| | AT1G18210 | Calcium-binding EF-hand family protein | Biological processes unknown | 4.46 | 15.8 | 5.60 | 0.49 | 0.16 | 0.85 | 0.56 | 18831 |
| ZIP2 | AT5G59520 | ZRT/IRT-like protein 2 | Other biological processes; response to stress; other cellular processes; transport | 4.28 | 15.5 | 5.84 | 0.94 | 0.32 | 1.19 | 0.82 | 8413 |
| GSTU25 | AT1G17180 | Glutathione S-transferase TAU 25 | Other biological processes | 12.2 | 14.9 | 3.17 | 1.50 | 1.61 | 0.86 | 3.29 | 12472 |
| PAP3 | AT1G14700 | Purple acid phosphatase 3 | Biological processes unknown | 25.5 | 14.4 | 25.7 | 0.29 | ND | 3.55 | ND | 14218 |
| AATP1 | AT5G40010 | AAA-atpase 1 | Other cellular processes; response to abiotic or biotic stimulus; signal transduction | 8.91 | 14.0 | 2.54 | 0.95 | 0.73 | 0.90 | 2.93 | 5438 |
| RPS13A | AT4G00100 | Ribosomal protein S13A | Developmental processes; cell organization and biogenesis; protein metabolism | 8.44 | 12.8 | 4.40 | 3.37 | 2.78 | 1.31 | 2.38 | 16326 |

| Name | Code | Putative function | GO annotation | Zn2GC/ Zn2 GE | Zn1000GC/ Zn100 GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AMT1; 2 | AT1G64780 | Ammonium transporter 1; 2 | Response to abiotic or biotic stimulus; transport | 4.20 | 12.5 | 4.95 | 0.87 | 0.36 | 0.88 | 0.71 | 4521 |
| SULTR1; 3 | AT1G22150 | Sulfate transporter 1; 3 | Biological processes unknown | 0.27 | 11.0 | 0.39 | 3.81 | 0.11 | 3.82 | 2.46 | 3499 |
| BGLU42 | AT5G36890 | Beta glucosidase 42 | Response to stress; other cellular processes | 29.0 | 9.66 | 51.0 | 1.12 | ND | 1.10 | ND | 21086 |
| | AT3G05640 | Protein phosphatase 2C family protein | Other biological processes; response to abiotic or biotic stimulus; response to stress | 10.0 | 9.50 | 96.5 | 1.73 | 2.37 | 2.14 | 0.21 | 8144 |
| GSTU7 | AT2G29420 | Glutathione S-transferase tau 7 | Other biological processes | 37.3 | 9.27 | 2.46 | 1.04 | 5.19 | 1.06 | 15.19 | 12334 |
| SPS2F | AT5G11110 | Sucrose phosphate synthase 2F | Response to abiotic or biotic stimulus; response to stress; developmental processes | 28.6 | 9.26 | 20.1 | 1.19 | 4.66 | 1.09 | 1.52 | 19866 |
| MLP43 | AT1G70890 | MLP-like protein 43 | Other cellular processes; transport | 11.1 | 8.35 | 20.4 | 1.52 | 2.52 | 1.08 | 0.57 | 20375 |
| GSTU19 | AT1G78380 | Glutathione S-transferase TAU 19 | Electron transport or energy pathways; protein metabolism | 15.9 | 7.54 | 1.01 | 1.79 | 4.74 | 1.18 | 17.32 | 12089 |
| CML41 | AT3G50770 | Calmodulin-like 41 | Transport | 11.3 | 7.51 | 1.65 | 1.08 | 1.95 | 1.06 | 6.69 | 15301 |
| EIN3 | AT3G20770 | Ethylene insensitive 3 family protein | Protein metabolism; response to abiotic or biotic stimulus; signal transduction | 14.3 | 7.08 | 16.5 | 1.02 | 2.54 | 1.11 | 0.91 | 1912 |
| HMA2 | AT4G30110 | Heavy metal atpase 2 | Other biological processes | 5.25 | 6.66 | 7.13 | 1.51 | 1.49 | 1.17 | 0.83 | 876 |
| | AT1G76140 | Prolyl oligopeptidase family protein | Protein metabolism; transport | 43.3 | 8.40 | 55.3 | 0.02 | 0.15 | 3.90 | 2.94 | 1722 |
| | AT1G35460 | Basic helix-loop-helix (bHLH) DNA-binding superfamily protein | Transcription,DNA-dependent | 5.94 | 12.9 | 23.9 | 0.43 | 0.26 | 0.51 | 0.12 | 20192 |
| PAP8 | AT2G01890 | Purple acid phosphatase 8 | Other biological processes; transport | 19.2 | 8.83 | 23.4 | 0.21 | ND | 3.50 | ND | 19132 |
| ZIP3 | AT2G32270 | Zinc transporter 3 precursor | Developmental processes; other biological processes; response to stress | 28.6 | 0.28 | 19.1 | 0.03 | 3.85 | 0.87 | 1.16 | 18607 |
| PAP22 | AT3G52820 | Purple acid phosphatase 22 | Transcription,DNA-dependent; response to stress | 5.31 | 4.09 | 7.63 | 1.63 | 2.32 | 1.37 | 0.86 | 17381 |
| HMA2 | AT4G30110 | Heavy metal atpase 2 | Other biological processes | 3.85 | 2.50 | 6.43 | 0.81 | 1.59 | 0.83 | 0.47 | 1225 |
| ZIP1 | AT3G12750 | Zinc transporter 1 precursor | Other biological processes; response to stress; transport | 3.94 | 17.7 | 5.60 | 0.28 | 0.08 | 2.49 | 1.69 | 10699 |
| KUP3 | AT3G02050 | K+ uptake transporter 3 | Response to stress; transport | 4.56 | 2.75 | 4.68 | 1.05 | 2.14 | 0.92 | 0.82 | 1887 |
| IRT3 | AT1G60960 | Iron regulated transporter 3 | Response to abiotic or biotic stimulus; other cellular processes; response to | 3.99 | 3.54 | 4.37 | 0.18 | 0.25 | 1.09 | 0.97 | 8335 |

| Name | Code | Putative function | GO annotation | Zn2GC/ Zn2 GE | Zn1000GC/ Zn100 GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | stress; transport | | | | | | | | |
| | AT3G14880 | Function unknown | Response to abiotic or biotic stimulus | 90.0 | 26.8 | 23.8 | 0.16 | ND | 0.45 | ND | 14142 |
| | AT5G51160 | Ankyrin repeat family protein | Other biological processes; response to stress; transport | 23.2 | 47.0 | 4.22 | 0.36 | 0.22 | 0.65 | 3.29 | 9626 |
| | AT5G05340 | Peroxidase superfamily protein | Biological processes unknown | 20.4 | 2.85 | 0.72 | 0.99 | 8.04 | 0.96 | 24.51 | 13788 |
| | AT1G01490 | Heavy metal transport/detoxification superfamily protein | Biological processes unknown | 17.9 | 6.86 | 0.81 | 0.61 | 1.92 | 0.72 | 15.01 | 19724 |
| GCH | AT5G64300 | GTP cyclohydrolase II | Developmental processes | 6.76 | 4.17 | 1.41 | 0.46 | 0.93 | 0.40 | 1.78 | 3421 |
| ZIP1 | AT3G12750 | Zinc transporter 1 precursor | Other biological processes; response to stress | 5.99 | 0.92 | 4.23 | 0.06 | 0.50 | 0.87 | 1.18 | 8806 |

Table 5 - Selection of transcripts which are more highly expressed in *Gomphrena claussenii* (GC) shoots compared to *Gomphrena elegans* (GE) upon control (Zn2), high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5). Differential expressions are indicated as ratios. Very small ratios (<0.01) are indicated as 1 divided by X (1/X). For ease of interpretation, the most similar *Arabidopsis thaliana* gene for each contig ID (Contig) is indicated with the gene name (Name), gene code (Code) and putative protein function. Gene ontology (GO) annotation is provided for biological process. Significant differences between the two species (probability ≥0.95) or two conditions (p≤0.05) for the transcript expression are indicated in bold. Contig with lower counts were not detected, ND.

| Name | Code | Putative function | GO annotation | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | - | - | Biological processes unknown | 5.30E+10 | 1.19E+11 | 1.86E+08 | 1.19 | ND | 1.07 | ND | 21005 |
| | AT1G15910. | XH/XS domain-containing protein | DNA or RNA metabolism | 3.33E+13 | 1.79E+10 | 2.41E+05 | 1.11 | ND | 1.32 | ND | 21036 |
| | AT4G32870 | Polyketide cyclase/dehydrase and lipid transport superfamily protein | Biological processes unknown | 5.39E+06 | 7.42E+09 | 1.64E+11 | 1.32 | ND | 1.15 | ND | 17644 |
| | - | - | Biological processes unknown | 6.49E+05 | 2.78E+08 | 1.20E+07 | 1.20 | ND | 1.48 | ND | 20691 |
| | AT5G59210 | Myosin heavy chain-related | Transport | 1.03E+07 | 8.35E+07 | 2.40E+05 | 1.40 | ND | 0.99 | ND | 20055 |
| FBH1 | AT1G35460 | Basic helix-loop-helix (bHLH) DNA-binding superfamily protein | Transcription,DNA-dependent | 3.24E+10 | 1.03E+07 | 2.01E+07 | 0.95 | ND | 1.09 | ND | 20192 |
| FMN/FHY | AT4G21470 | Riboflavin kinase/FMN hydrolase | Transcription,DNA-dependent; response to stress | 127 | 2.43E+06 | 2.90E+07 | 1.17 | ND | 1.15 | ND | 18189 |
| GSTU9 | AT5G62480 | Glutathione S-transferase tau 9 | Other biological processes | 7.95E+09 | 1.70E+06 | 238 | 0.97 | ND | 0.92 | ∞ | 12604 |
| EDR2 | AT4G19040 | Enhanced disease resistance 2 | Biological processes unknown | 1.97E+04 | 2.16E+05 | 1.02E+05 | 1.05 | ND | 1.01 | ND | 14824 |
| BGLU42 | AT5G36890 | Beta glucosidase 42 | Response to stress; other cellular processes | 4482 | 4.26E+03 | 6139 | 1.29 | ND | 1.29 | ND | 20809 |
| ATHB13 | AT1G69780 | Homeobox-leucine zipper protein family | Developmental processes; transcription, DNA-dependent | 2050 | 3805 | 3.46E+04 | 1.20 | ND | 0.97 | ND | 19528 |
| | AT4G11740 | Ubiquitin-like superfamily protein | Transport | 501 | 574 | 2.33E+05 | 0.94 | ND | 1.07 | ND | 18705 |
| | AT1G20340 | Cupredoxin superfamily protein | Protein metabolism; other cellular processes; response to abiotic or biotic stimulus | 519 | 501 | 652 | 1.01 | ND | 0.97 | ND | 17711 |

| Name | Code | Putative function | GO annotation | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AT5G05140 | Transcription elongation factor (TFIIS) family protein | Other biological processes; response to abiotic or biotic stimulus | 21.4 | 439 | 80.3 | 1.15 | 0.05 | 1.11 | 0.26 | 9414 |
| | AT3G57800 | Basic helix-loop-helix (bHLH) DNA-binding superfamily protein | Transcription,DNA-dependent | 917 | 416 | 7863 | 0.79 | ND | 0.77 | ND | 13140 |
| CYP87A2 | AT1G12740 | Cytochrome P450, family 87, subfamily A, polypeptide 2 | Response to stress; other cellular processes; transport | 1143 | 295 | 294 | 1.24 | 0.91 | 1.12 | 0.63 | 5018 |
| CPK20 | AT2G38910 | Calcium-dependent protein kinase 20 | Cell organization and biogenesis; protein metabolism | 184 | 263 | 230 | 1.00 | ND | 0.93 | ND | 20837 |
| | AT4G01070 | UDP-Glycosyltransferase superfamily protein | Response to stress; other biological processes; cell organization and biogenesis | 5.26E+08 | 255 | 2.50E+10 | 1.13 | ND | 1.28 | ND | 9946 |
| TMN7 | AT3G13772 | Transmembrane nine 7 | Other metabolic processes; other cellular processes | 168 | 180 | 167 | 1.10 | ND | 0.99 | ND | 19525 |
| CYP89A6 | AT1G64940 | Cytochrome P450, family 87, subfamily A, polypeptide 6 | Biological processes unknown | 409 | 157 | 167 | 1.00 | 2.20 | 0.97 | 2.05 | 5602 |
| | AT5G17050 | UDP-glucosyl transferase 78D2 | Other biological processes; response to abiotic or biotic stimulus | 85.8 | 109 | 74.2 | 1.31 | ND | 1.04 | ND | 5956 |
| | AT5G21990 | Tetratricopeptide repeat (TPR)-like superfamily protein | Protein metabolism; transport | 104 | 83.2 | 91.9 | 1.06 | 1.16 | 0.95 | 1.01 | 5462 |
| CYP83B1 | AT4G31500 | Cytochrome P450, family 83, subfamily B, polypeptide 1 | Response to abiotic or biotic stimulus; response to stress; developmental processes | 85.4 | 76.8 | 523 | 1.34 | ND | 1.00 | 3.05 | 8734 |
| UGT85A2 | AT1G22360 | UDP-glucosyl transferase 85A2 | Biological processes unknown | 34.6 | 76.5 | 20.1 | 2.69 | 0.99 | 1.72 | 2.54 | 7984 |
| FRO7 | AT5G49740 | Ferric reduction oxidase 7 | Electron transport or energy pathways; other metabolic processes; other cellular processes | 46.8 | 70.4 | 33.7 | 2.26 | 1.29 | 1.61 | 1.96 | 1688 |
| | AT1G29500 | SAUR-like auxin-responsive protein family | Other biological processes | 2.71 | 62.4 | 18.3 | 1.29 | 0.05 | 1.25 | 0.16 | 16912 |
| GSTF8 | AT2G47730 | Glutathione S-transferase phi 8 | Response to abiotic or biotic stimulus; response to stress | 136 | 62.2 | 27.8 | 0.82 | 1.53 | 0.82 | 3.47 | 18543 |
| BCS1 | AT3G50930 | Cytochrome BC1 synthesis | Other biological processes; response to abiotic or biotic stimulus | 627 | 59.9 | 89.3 | 1.03 | 9.98 | 1.29 | 8.90 | 8547 |
| | AT1G73280 | Serine carboxypeptidase-like 3 | Protein metabolism | 148 | 50.7 | 53.2 | 1.30 | ND | 0.94 | ND | 20629 |

| Name | Code | Putative function | GO annotation | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| OMT1 | AT5G54160 | O-methyltransferase 1 | Response to stress | 430 | 48.2 | 19.9 | 0.89 | 6.95 | 0.90 | 17.5 | 10205 |
| | AT5G03455 | Rhodanese/Cell cycle control phosphatase superfamily protein | Other biological processes; protein metabolism | 49.8 | 46.9 | 61.3 | 0.96 | 0.86 | 1.04 | 0.76 | 16745 |
| | AT3G05640 | Protein phosphatase 2C family protein | Other biological processes; response to abiotic or biotic stimulus; response to stress | 32.3 | 42.7 | 30.2 | 1.45 | 0.91 | 1.28 | 1.20 | 8144 |
| ARF11 | AT2G46530 | Auxin response factor 11 | Biological processes unknown | 7.05 | 40.2 | 7.43 | 1.01 | 0.15 | 1.06 | 0.93 | 17939 |
| | AT4G09810 | Nucleotide-sugar transporter family protein | Transport | 7.95 | 38.9 | 11.4 | 0.91 | 0.16 | 1.05 | 0.65 | 21140 |
| GRP2 | AT4G13850 | Glycine-rich RNA-binding protein 2 | Response to abiotic or biotic stimulus; response to stress; transcription,DNA-dependent | 11.2 | 36.4 | 8.65 | 0.72 | 0.18 | 0.83 | 0.93 | 17454 |
| | AT5G33340 | Eukaryotic aspartyl protease family protein | Response to abiotic or biotic stimulus; protein metabolism; response to stress | 348 | 35.3 | 20.4 | 0.82 | 6.68 | 0.95 | 14.1 | 6584 |
| | AT1G73270 | Serine carboxypeptidase-like 6 | Protein metabolism | 47.0 | 30.1 | 188 | 1.36 | ND | 1.27 | ND | 19861 |
| SULTR3;4 | AT3G15990 | Sulfate transporter 3;4 | Biological processes unknown | 22.7 | 23.6 | 13.0 | 1.08 | 0.87 | 1.04 | 1.61 | 2531 |
| LOX2 | AT3G45140 | Lipoxygenase 2 | Response to stress; other biological processes | 37.4 | 20.5 | 11.2 | 1.17 | 1.79 | 1.13 | 3.28 | 6074 |
| HMA2 | AT4G30110 | Heavy metal atpase 2 | Other biological processes | 279 | 17.0 | 24.5 | 0.94 | 13.13 | 0.97 | 9.73 | 1225 |
| BCB | AT5G20230 | Blue-copper-binding protein | Response to abiotic or biotic stimulus; response to stress | 136 | 14.8 | 11.8 | 0.65 | 4.87 | 1.00 | 9.61 | 16342 |
| CCOAMT | AT1G67980 | Caffeoyl-coa 3-O-methyltransferase | Other metabolic processes; other cellular processes | 35.8 | 13.3 | 5.84 | 1.26 | 2.79 | 1.20 | 6.40 | 12388 |
| WRKY40 | AT1G80840 | WRKY DNA-binding protein 40 | Response to abiotic or biotic stimulus; signal transduction; response to stress | 62.0 | 13.2 | 8.11 | 3.05 | 12.0 | 2.74 | 18.0 | 9774 |
| PR1 | AT2G14610 | Pathogenesis-related gene 1 | Response to abiotic or biotic stimulus; signal transduction; response to stress | 38.4 | 12.7 | 1.55 | 0.87 | 2.30 | 0.92 | 20.5 | 16047 |
| OEP37 | AT2G43950 | Chloroplast outer envelope protein 37 | Transport | 32.4 | 12.7 | 80.9 | 1.05 | 2.17 | 1.02 | 0.34 | 18789 |
| YSL1 | AT4G24120 | YELLOW STRIPE like 1 | Developmental processes; other biological processes; transport | 4.45 | 12.5 | 3.11 | 0.68 | 0.20 | 0.74 | 0.91 | 2306 |

| Name | Code | Putative function | GO annotation | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| EXL2 | AT5G64260 | EXORDIUM like 2 | Other biological processes | 15.9 | 12.3 | 5.70 | 1.00 | 1.09 | 1.15 | 2.83 | 11197 |
| CYP76C7 | AT3G61040 | Cytochrome P450, family 76, subfamily C, polypeptide 7 | Other metabolic processes; other cellular processes | 94.9 | 12.1 | 4.73 | 1.21 | 8.23 | 1.32 | 23.1 | 10202 |
| PNP-A | AT2G18660 | Plant natriuretic peptide A | Electron transport or energy pathways; response to abiotic or biotic stimulus | 67.8 | 11.3 | 8.92 | 0.79 | 4.20 | 0.94 | 6.18 | 17516 |
| HMA2 | AT4G30110 | Heavy metal atpase 2 | Other biological processes | 10.4 | 9.66 | 10.3 | 1.09 | 0.98 | 0.96 | 0.84 | 876 |
| GLN1;2 | AT1G66200 | Glutamine synthase clone F11 | Electron transport or energy pathways; response to abiotic or biotic stimulus; response to stress | 11.8 | 8.88 | 11.2 | 0.84 | 1.03 | 0.91 | 0.90 | 20338 |
| | AT1G01490 | Heavy metal transport/detoxification superfamily protein | Biological processes unknown | 23.2 | 8.65 | 7.45 | 1.29 | 3.46 | 1.49 | 4.59 | 19724 |
| CYSD2 | AT5G28020 | Cysteine synthase D2 | Other metabolic processes; other cellular processes | 4.81 | 8.48 | 1.75 | 1.50 | 0.72 | 1.34 | 3.20 | 20796 |
| NR2 | AT1G37130 | Nitrate reductase 2 | Developmental processes; response to abiotic or biotic stimulus | 2.30 | 7.28 | 2.17 | 1.21 | 0.32 | 1.21 | 1.12 | 831 |
| NAS4 | AT1G56430 | Nicotianamine synthase 4 | Developmental processes | 13.87 | 7.02 | 5.95 | 0.61 | 1.01 | 1.45 | 2.98 | 10477 |
| ATNCL | AT1G53210 | Sodium/calcium exchanger family protein | Response to stress; other biological processes | 12.5 | 6.29 | 11.5 | 0.65 | 1.05 | 0.88 | 0.83 | 6261 |
| HDGL2 | AT1G05230 | Homeodomain GLABROUS 2 | Developmental processes; cell organization and biogenesis; transcription,DNA-dependent | 812 | 5.96 | 21.6 | 1.09 | 128 | 1.02 | 36.30 | 19156 |
| PAP23 | AT4G13700 | Purple acid phosphatase 23 | Biological processes unknown | 17.7 | 7.39 | 13.5 | 1.18 | 2.33 | 1.30 | 1.50 | 3842 |
| | AT4G28730 | Glutaredoxin family protein | Other metabolic processes; cell organization and biogenesis | 6.83 | 7.76 | 9.56 | 1.02 | 0.77 | 1.13 | 0.71 | 14650 |
| ZIP1 | AT3G12750 | Zinc transporter 1 precursor | Other biological processes; response to stress | 12.7 | 1.78 | 5.94 | 0.07 | 0.43 | 1.55 | 2.88 | 10699 |
| PIP1,4 | AT4G00430 | Plasma membrane intrinsic protein 1;4 | Response to abiotic or biotic stimulus | 5.14 | 3.99 | 4.79 | 1.21 | 1.38 | 1.24 | 1.17 | 10390 |
| NAP10 | AT1G63270 | Non-intrinsic ABC protein 10 | Transport | 7.01E+05 | 2.83 | 4.67 | 0.92 | ∞ | 1.00 | ∞ | 12698 |
| GSTF8 | AT2G47730 | Glutathione S-transferase phi 8 | Response to abiotic or biotic stimulus | 235 | 81.95 | 28.1 | 0.44 | 1.01 | 0.62 | 4.49 | 13446 |

| Name | Code | Putative function | GO annotation | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AT4G17660 | Protein kinase superfamily protein | Biological processes unknown | 64.1 | 2.56 | 2.15 | 0.70 | 55.9 | 0.95 | 94.63 | 17057 |
| | AT5G07990 | Cytochrome P450 superfamily protein | Response to abiotic or biotic stimulus | 32.1 | 2.56 | 2.71 | 1.02 | 11.0 | 0.93 | 9.65 | 4977 |
| | AT1G32900 | UDP-Glycosyltransferase superfamily protein | Response to abiotic or biotic stimulus | 18.6 | 2.26 | 3.95 | 1.60 | 12.3 | 1.31 | 5.75 | 9197 |
| | AT1G01490 | Heavy metal transport/detoxification superfamily protein | Biological processes unknown | 16.4 | 2.57 | 2.67 | 0.84 | 4.48 | 1.00 | 5.35 | 17977 |
| GSTU9 | AT5G62480 | Glutathione S-transferase tau 9 | Other biological processes | 12.7 | 1.16 | 1.29 | 0.52 | 4.97 | 0.66 | 5.80 | 16066 |
| | AT4G16260 | Glycosyl hydrolase superfamily protein | Response to abiotic or biotic stimulus; response to stress | 11.4 | 2.87 | 0.41 | 0.73 | 2.39 | 0.83 | 19.82 | 9992 |
| ZIP1 | AT3G12750 | Zinc transporter 1 precursor | Other biological processes; response to stress | 10.7 | 1.01 | 3.84 | 0.03 | 0.24 | 1.87 | 4.60 | 7379 |
| | AT1G01490 | Heavy metal transport/detoxification superfamily protein | Biological processes unknown | 9.96 | 3.64 | 3.47 | 1.20 | 2.70 | 1.30 | 3.23 | 17991 |
| | AT1G60960 | Iron regulated transporter 3 | Response to abiotic or biotic stimulus | 8.39 | 0.74 | 3.22 | 0.12 | 1.14 | 1.38 | 3.18 | 8335 |
| NRAMP6 | AT1G15960 | NRAMP metal ion transporter 6 | Other cellular processes; transport | 6.22 | 1.79 | 1.45 | 1.10 | 3.21 | 0.95 | 3.58 | 4046 |
| | AT4G16260 | Glycosyl hydrolase superfamily protein | Response to abiotic or biotic stimulus; response to stress | 5.56 | 1.10 | 1.55 | 0.88 | 3.70 | 0.94 | 2.96 | 8983 |
| PHT1;4 | AT2G38940 | Phosphate transporter 1;4 | Other biological processes; transport | 5.49 | 0.85 | 1.23 | 0.99 | 5.61 | 0.96 | 3.90 | 20924 |

## Transcripts differentially expressed upon high Zn and/or Cd treatment in *G. claussenii*

Next to genes constitutively highly expressed in *G. claussenii* indicative of a profilactively active metal tolerance mechanism, also transcripts exclusively differentially regulated in *G. claussenii* when compared to *G. elegans* upon metal treatment might be associated with tolerance to or accumulation of Zn and Cd. Comparing the expression of transcripts between control and high Zn or Cd treatment 131 *G. claussenii* root transcripts were significantly differentially expressed (≥4-fold; $p \leq 0.05$) (Tables S5) from which 81 were not differentially expressed upon metal treatment in *G. elegans* (Table 6). Similarly, 42 out of 47 transcripts were exclusively differentially expressed in *G. claussenii* shoots upon either Zn or Cd exposure (Table S5, Table 7).

In *G. claussenii* roots, the group of down-regulated transcripts contains many stress and defence response transcripts (Table 6). Within this group, there are three transcripts that belong to the ZIP family of Zn transporter genes, encoding orthologues of ZIP1, ZIP3 and IRT3. One of the upregulated transcripts is similar to *SDI1*, a gene normally induced by sulfur deficiency. Among the shoot transcripts with the highest expression under metal treatments when compared with the control treatment (Table 7), there are two encoding transcription factors, *COL5* and *WRKY40*, respectively involved in flowering initiation and pathogen response. Also *PCR2*, encoding a metal transporter, was higher expressed in shoots, especially upon Cd treatment. Other known metal homeostasis genes, such those for the zinc transporters ZIP1, ZIP3 and IRT3 and for ferretin (FER1) had a lower expression under high Zn (Table 7)

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Table 6 - Root transcripts which are differentially expressed upon either high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5) when compared to control conditions (Zn2) in *Gomphrena claussenii* (GC) and are not regulated in *Gomphrena elegans* (GE). Differential expressions are indicated as ratios. Very small ratios (<0.01) are indicated as 1 divided by X (1/X). For ease of interpretation, the most similar *Arabidopsis thaliana* gene for each contig ID (Contig) is in dated with the gene name (Name), gene code (Code) and putative protein function. Significant values are presented in bold (p≤0.05). Significant differences between the two species for the transcript expression are highlighted in grey. Contig with lower counts were not detected, ND.

| Name | Code | Putative function | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|
| | AT3G49260 | IQ-domain 21 | 32.6 | 0.66 | 8.73 | 0.80 | 9278 |
| | AT4G17390 | Ribosomal protein L23/L15e family protein | 19.0 | 3.46 | 0.50 | 0.54 | 14581 |
| | AT4G28780 | GDSL-like Lipase/Acylhydrolase superfamily protein | 17.9 | 0.45 | 4.88 | 1.27 | 9948 |
| PMT5 | AT3G18830 | Polyol/monosaccharide transporter 5 | 14.7 | 1.20 | 8.18 | 1.80 | 4602 |
| RPI2 | AT2G01290 | Ribose-5-phosphate isomerase 2 | 13.5 | 1.25 | 5.50 | 1.06 | 10755 |
| | AT1G73970 | Unknown protein | 9.50 | ND | 1.59 | ND | 9525 |
| SDI1 | AT5G48850 | Tetratricopeptide repeat (TPR)-like superfamily protein | 7.86 | 2.65 | 2.38 | 77.5 | 19030 |
| PDCB3 | AT1G18650 | Plasmodesmata callose-binding protein 3 | 7.54 | 0.53 | 5.45 | 0.82 | 15630 |
| | AT5G17030 | UDP-glucosyl transferase 78D3 | 6.77 | ND | 0.83 | ND | 11910 |
| | AT1G56710 | Pectin lyase-like superfamily protein | 5.54 | 1.80 | 4.84 | 0.57 | 5101 |
| | AT4G10265 | Wound-responsive family protein | 5.09 | ND | 2.39 | ND | 19044 |
| BSMT1 | AT3G11480 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein | 4.67 | 8.81 | 0.81 | 2.35 | 7175 |
| ACC2 | AT1G01480 | 1-amino-cyclopropane-1-carboxylate synthase 2 | 4.48 | ND | 1.72 | ND | 20060 |
| SDI1 | AT5G48850 | Tetratricopeptide repeat (TPR)-like superfamily protein | 4.02 | 1.81 | 2.15 | 2.09 | 9924 |
| | AT4G37560 | Acetamidase/Formamidase family protein | 0.23 | 1.09 | 0.48 | 0.60 | 6422 |

| Name | Code | Putative function | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|
| | AT5G49690 | UDP-Glycosyltransferase superfamily protein | 0.23 | 2.97 | 0.46 | 1.38 | 6746 |
| RGLG2 | AT5G14420 | RING domain ligase2 | 0.23 | 0.78 | 0.22 | 0.64 | 8671 |
| | AT2G05940 | Protein kinase superfamily protein | 0.22 | 1.39 | 0.60 | 3.39 | 8187 |
| | AT3G47570 | Leucine-rich repeat protein kinase family protein | 0.22 | 0.87 | 0.42 | 21.7 | 1292 |
| EXLA1 | AT3G45970 | Expansin-like A1 | 0.21 | 1.19 | 0.33 | 4.44 | 13283 |
| PAP8 | AT2G01890 | Purple acid phosphatase 8 | 0.21 | ND | 3.56 | ND | 19132 |
| | AT2G26530 | Protein of unknown function | 0.20 | 0.50 | 0.45 | 1.12 | 7392 |
| | AT4G27220 | NB-ARC domain-containing disease resistance protein | 0.20 | 0.58 | 0.65 | 1.28 | 195 |
| | AT3G20395 | RING/U-box superfamily protein | 0.19 | ND | 0.37 | ND | 13514 |
| PGP21 | AT3G62150 | P-glycoprotein 21 | 0.19 | 0.17 | 0.32 | 0.06 | 19530 |
| | - | - | 0.19 | 2.83 | 3.12 | 0.90 | 13862 |
| | - | - | 0.19 | 0.56 | 0.44 | 1.31 | 20826 |
| | AT2G27660 | Cysteine/Histidine-rich C1 domain family protein | 0.19 | 0.08 | 0.21 | 0.37 | 10223 |
| IRT3 | AT1G60960 | Iron regulated transporter 3 | 0.18 | 0.25 | 1.12 | 0.96 | 8335 |
| | - | - | 0.18 | 0.32 | 0.28 | 4.76 | 15111 |
| GER3 | AT5G20630 | Germin 3 | 0.16 | 1.12 | 0.19 | 0.76 | 13009 |
| | AT3G14880 | Unknown | 0.16 | ND | 0.46 | ND | 14142 |
| | AT4G10265 | Wound-responsive family protein | 0.15 | 3.13 | 0.58 | 2.15 | 18940 |
| HSP17.6II | AT5G12020 | 17.6 kda class II heat shock protein | 0.15 | 4.38 | 0.77 | 12.9 | 17221 |
| CRK8 | AT4G23160 | Cysteine-rich RLK (RECEPTOR-like protein kinase) 8 | 0.15 | 2.08 | 0.67 | 0.95 | 4098 |
| LUP2 | AT1G78960 | Lupeol synthase 2 | 0.15 | 1.02 | 0.56 | 0.93 | 2995 |
| | AT2G45910 | U-box domain-containing protein kinase family protein | 0.14 | ND | 0.13 | ND | 19519 |
| | AT5G13400 | Major facilitator superfamily protein | 0.14 | 2.59 | 0.56 | 0.73 | 6852 |

| Name | Code | Putative function | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|
| GEX3 | AT5G16020 | Gamete-expressed 3 | 0.13 | 0.58 | 1.00 | 3.32 | 3825 |
| | AT2G45910 | U-box domain-containing protein kinase family protein | 0.13 | 0.32 | 0.11 | 0.62 | 10363 |
| | AT5G17230 | Phytoene synthase | 0.13 | 7.33 | 0.03 | 0.66 | 11067 |
| | - | - | 0.12 | ND | 0.14 | ND | 20953 |
| | AT1G02960 | Unknown protein | 0.11 | 0.68 | 0.68 | 1.11 | 5322 |
| GAD1 | AT5G17330 | Glutamate decarboxylase | 0.11 | 0.49 | 0.18 | 1.56 | 19389 |
| MSL10 | AT5G12080 | Mechanosensitive channel of small conductance-like 10 | 0.11 | 0.28 | 0.37 | 3.17 | 14609 |
| GAD1 | AT5G17330 | Glutamate decarboxylase | 0.11 | 0.32 | 0.21 | 1.14 | 7890 |
| MSL10 | AT5G12080 | Mechanosensitive channel of small conductance-like 10 | 0.11 | 0.34 | 0.45 | 3.10 | 3389 |
| | AT4G17260 | Lactate/malate dehydrogenase family protein | 0.11 | 0.18 | 0.22 | 0.76 | 16224 |
| | AT4G38260 | Protein of unknown function | 0.10 | 0.42 | 0.38 | 0.33 | 12376 |
| | AT4G04980 | Unknown protein | 0.10 | 0.54 | 0.19 | 0.47 | 8846 |
| | AT4G29050 | Concanavalin A-like lectin protein kinase family protein | 0.10 | 0.00 | 0.94 | 0.35 | 6477 |
| | AT2G15220 | Plant basic secretory protein (BSP) family protein | 0.09 | 1.16 | 0.44 | 1.63 | 11963 |
| CHIA | AT5G24090 | Chitinase A | 0.08 | 1/143 | 0.09 | 1.31 | 10368 |
| | AT3G19010 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | 0.08 | 0.58 | 0.43 | 1.15 | 10153 |
| ZIP1 | AT3G12750 | Zinc transporter 1 precursor | 0.06 | 0.48 | 0.88 | 1.16 | 8806 |
| ACA12 | AT3G63380 | Atpase E1-E2 type family protein | 0.06 | 1.27 | 0.09 | 15.0 | 2981 |
| | AT4G16720 | Ribosomal protein L23/L15e family protein | 0.05 | ND | 2.31 | ND | 16238 |
| ZIP3 | AT2G32270 | Zinc transporter 3 precursor | 0.03 | 3.59 | 0.85 | 1.16 | 18607 |
| | AT2G21590 | Glucose-1-phosphate adenylyltransferase family protein | 0.02 | ∞ | 0.20 | ND | 16946 |
| RHM1 | AT1G78570 | Rhamnose biosynthesis 1 | 1/274 | 1.61 | 0.29 | 0.78 | 1753 |

| Name | Code | Putative function | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|
| | AT5G61170 | Ribosomal protein S19e family protein | 1/547 | 0.90 | 0.50 | 0.65 | 12069 |
| | AT2G31610 | Ribosomal protein S3 family protein | 1/2127 | ND | 0.62 | ND | 13255 |
| | - | - | 0.00 | 2.57 | 280 | 4.45 | 11722 |
| | - | - | 0.30 | ND | 9.25 | ND | 15792 |
| | AT4G24340 | Phosphorylase superfamily protein | 2.74 | 1.49 | 6.52 | 0.76 | 9874 |
| MSD1 | AT3G10920 | Manganese superoxide dismutase 1 | 0.63 | 2.42 | 5.38 | 0.75 | 11367 |
| bZIP2 | AT2G18160 | Basic leucine-zipper 2 | 1.83 | ND | 4.65 | ND | 14488 |
| | AT4G24710 | P-loop containing nucleoside triphosphate hydrolases superfamily protein | 0.86 | 0.49 | 0.22 | 0.93 | 20358 |
| GSTU9 | AT5G62480 | Glutathione S-transferase tau 9 | 0.29 | 0.14 | 0.21 | 0.54 | 13356 |
| CYP76C1 | AT2G45560 | Cytochrome P450, family 76, subfamily C, polypeptide 1 | 0.39 | 0.07 | 0.20 | 1.22 | 17987 |
| | - | - | 0.14 | 1.58 | 0.18 | 1.49 | 12729 |
| | AT2G16980 | Major facilitator superfamily protein | 1.02 | 3.59 | 0.16 | 0.78 | 7913 |
| NIP5;1 | AT4G10380 | NOD26-like intrinsic protein 5;1 | 0.62 | 1/77.1 | 0.15 | 0.29 | 12039 |
| | AT3G22640 | Cupin family protein | 0.38 | 30.3 | 0.13 | 0.62 | 9230 |
| BCB | AT5G20230 | Blue-copper-binding protein | 0.29 | 0.08 | 0.09 | 0.42 | 15984 |
| | - | - | 1.20 | 0.10 | 0.06 | 1.46 | 12692 |
| CYP76C1 | AT2G45560 | Cytochrome P450, family 76, subfamily C, polypeptide 1 | 0.41 | ND | 0.03 | ND | 18317 |
| CYP76C7 | AT3G61040 | Cytochrome P450, family 76, subfamily C, polypeptide 7 | 0.35 | ND | 0.03 | ND | 14766 |
| | AT2G26695 | Ran BP2/NZF zinc finger-like superfamily protein | 0.37 | 0.34 | 0.02 | 0.43 | 17288 |
| | AT3G54510 | Early-responsive to dehydration stress protein (ERD4) | ND | ND | 1/50.9 | ND | 12404 |
| CYP76C5 | AT1G33730 | Cytochrome P450, family 76, subfamily C, polypeptide 5 | 0.37 | ∞ | 1/159 | ∞ | 18611 |

Table 7 - Shoot transcripts which are differentially expressed upon either high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5) when compared to control conditions (Zn2) in *Gomphrena claussenii* (GC) and are not regulated in *Gomphrena elegans* (GE). Differential expressions are indicated as ratios. Very small ratios (<0.01) are indicated as 1 divided by X (1/X). For ease of interpretation, the most similar *Arabidopsis thaliana* gene for each contig ID (Contig) is indicated with the gene name (Name), gene code (Code) and putative protein function. Significant values are presented in bold (p≤0.05). Significant differences between the two species for the transcript expression are highlighted in grey. Contig with lower counts were not detected, ND.

| Name | Code | Putative function | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|
| COL5 | AT5G57660 | CONSTANS-like 5 | **32.5** | 0.98 | 1.21 | 1.28 | 10349 |
| WRKY40 | AT1G80840 | WRKY DNA-binding protein 40 | **16.6** | 3.32 | **10.7** | 3.40 | 16234 |
| PIN2 | AT5G57090 | Auxin efflux carrier family protein | **13.3** | 0.52 | 5.98 | 0.75 | 2959 |
| | AT3G13610 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | **7.18** | 1.15 | 2.94 | 0.44 | 7713 |
| | AT1G03020 | Thioredoxin superfamily protein | **5.21** | 0.28 | **7.00** | 0.46 | 17320 |
| | AT1G05260 | Peroxidase superfamily protein | **4.77** | 0.86 | **6.60** | 0.40 | 9495 |
| | AT1G48450 | Protein of unknown function | **4.74** | 0.98 | 3.04 | 1.06 | 6988 |
| SDI1 | AT5G48850 | Tetratricopeptide repeat (TPR)-like superfamily protein | **4.44** | 3.15 | 1.71 | 0.57 | 19030 |
| RFL1 | AT1G12210 | RPS5-like 1 | **4.30** | 0.65 | 1.54 | 1.05 | 939 |
| FER1 | AT5G01600 | Ferretin 1 | **0.24** | 0.61 | 0.28 | 0.26 | 12959 |
| WNK3 | AT3G48260 | With no lysine (K) kinase 3 | **0.20** | 0.87 | 0.58 | 0.95 | 1788 |
| ZIP3 | AT2G32270 | Zinc transporter 3 precursor | **0.18** | 1.21 | 1.25 | 5.23 | 18607 |
| | AT5G19890 | Peroxidase superfamily protein | **0.14** | 0.78 | **0.21** | 1.51 | 8677 |
| | AT5G36160 | Tyrosine transaminase family protein | **0.12** | 1.82 | 1.24 | 2.92 | 6662 |
| IRT3 | AT1G60960 | Iron regulated transporter 3 | **0.12** | 1.12 | 1.38 | 3.13 | 8335 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| PAP8 | AT2G01890 | Purple acid phosphatase 8 | 0.12 | ND | 0.82 | ND | 19132 |
| XBCP3 | AT1G09850 | Xylem bark cysteine peptidase 3 | 0.11 | 1.43 | 0.22 | 1.37 | 4301 |
| PAP3 | AT1G14700 | Purple acid phosphatase 3 | 0.08 | 0.41 | 1.64 | 1.37 | 14218 |
| ZIP1 | AT3G12750 | Zinc transporter 1 precursor | 0.07 | 0.44 | 1.56 | 2.89 | 10699 |
| | AT4G34950 | Major facilitator superfamily protein | 0.07 | 0.52 | 0.73 | 1.14 | 3512 |
| WAK5 | AT1G21230 | Wall associated kinase 5 | 0.06 | 1.15 | 0.42 | 2.38 | 2473 |
| | AT4G09730 | Rh39 | 1/81 | 0.55 | 1/697 | 0.64 | 4679 |
| MUS81 | AT4G30870 | Restriction endonuclease, type II-like superfamily protein | 1/85.4 | 1.03 | 0.94 | 1.42 | 3770 |
| WAK5 | AT1G21230 | Wall associated kinase 5 | 1/118 | 0.56 | 0.47 | 0.90 | 9719 |
| | AT1G76140 | Prolyl oligopeptidase family protein | 1/313 | 0.18 | 1.39 | 10.8 | 1722 |
| | AT3G45710 | Major facilitator superfamily protein | 12.8 | 4.58 | 20.9 | 1.32 | 3612 |
| BIP2 | AT5G42020 | Heat shock protein 70 (Hsp 70) family protein | 0.77 | 1.42 | 12.2 | 2.17 | 13400 |
| GER3 | AT5G20630 | Germin 3 | 1.22 | 1.22 | 6.23 | 1.78 | 15073 |
| | AT5G07850 | HXXXD-type acyl-transferase family protein | 4.62 | 0.00 | 5.50 | 0.29 | 5464 |
| PCR2 | AT1G14870 | Plant cadmium resistance 2 | 3.59 | ∞ | 5.14 | ∞ | 14940 |
| COX2 | ATMG00160 | Cytochrome oxidase 2 | 0.61 | 1.29 | 0.17 | 1.50 | 10652 |
| | AT1G19530 | Unknown protein | 0.58 | 0.25 | 0.17 | 0.97 | 16521 |
| | AT2G38870 | Serine protease inhibitor, potato inhibitor I-type family protein | 0.51 | 0.48 | 0.14 | 3.41 | 19576 |
| | AT5G20400 | 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | 0.30 | 0.88 | 0.10 | 1.02 | 11501 |
| | AT1G15380 | Lactoylglutathione lyase / glyoxalase I family protein | 0.44 | 2.37 | 0.09 | 0.39 | 15180 |
| | AT3G04200 | Rmlc-like cupins superfamily protein | 1.01 | 0.62 | 0.08 | 0.51 | 14094 |
| | - | - | 0.26 | 1.33 | 0.08 | 0.85 | 8664 |
| | AT3G15670 | Late embryogenesis abundant protein (LEA) family protein | 0.24 | ND | 0.07 | ND | 18008 |
| | AT1G28590 | GDSL-like Lipase/Acylhydrolase superfamily protein | 0.00 | ND | 0.04 | ND | 7285 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | AT2G38870 | Serine protease inhibitor, potato inhibitor I-type family protein | 0.26 | 0.76 | 0.03 | 16.2 | 19654 |
| | AT3G59850 | Pectin lyase-like superfamily protein | 0.00 | 0.18 | 0.02 | 0.23 | 8375 |
| MSL6 | AT1G78610 | Mechanosensitive channel of small conductance-like 6 | 0.92 | 0.78 | 0.02 | 0.00 | 1276 |

## Verification of Zn homeostasis and Cd tolerance related transcripts

Genes involved in Zn homeostasis and Cd tolerance have already been identified for other plant species, especially for the model species *Arabidopsis thaliana* (Verbruggen *et al.*, 2009; Sinclair & Krämer, 2012). Transcripts homologous to those genes in *G. claussenii* and *G. elegans* are encoding proteins potentially involved in Zn and Cd tolerance and accumulation. Based on annotation, orthologous transcripts of both species were investigated and homologues of *A. thaliana* genes previously identified to have a role in Zn and Cd tolerance were selected. In total 23 transcripts were identified to encode proteins involved in metal uptake, chelation, vacuolar transport and xylem loading (Table 8). A small subset of these transcripts, predicted to encode proteins involved in metal homeostasis processes were selected (orthologues of the *A. thaliana IRT3*, *NRAMP2*, *FRD3*, *FRO2*, *NAS1* and *NAS4* genes) to evaluate their expression levels under metal exposure, including additional treatments of 100 µM $ZnSO_4$ and 1000 µM $ZnSO_4$ + 50 µM $CdSO_4$ (*G. claussenii*) and 100 µM $ZnSO_4$ + 5 µM $CdSO_4$ (*G. elegans*) by real-time quantitative (q) RT-PCR (Figure 4). Expression levels determined by qRT-PCR were comparable to those determined by RNAseq for both *G. claussenii* and *G. elegans* (Supplemental figure S5), confirming the quality and accuracy of the RNA-seq data. The gene expression upon exposures of either species to the combined high Zn/high Cd treatments resembled more the high Zn than the high Cd treatment, perhaps except for the *NAS1* root expression in *G. claussenii* (Figure 4).

Several transcripts were found to be most similar to *ZIP* genes, *ZIP1*, *ZIP6*, *ZIP7*, *ZIP11* and *IRT3*, potentially encoding Zn transporters (Table 8). While several *ZIP* genes were higher expressed in *G. claussenii* than in *G. elegans*, their expression was generally reduced upon high Zn exposure. Upon Cd exposure, the *ZIP* expression in *G. elegans*

shoots was upregulated, which was not very prominent in *G. claussenii*. The transcript most similar to *MTP1*, encoding a metal transporter involved in vacuolar Zn sequestration, was much higher expressed in *G. claussenii* than in *G. elegans*, especially under control conditions. Of the four *NRAMP* genes, a gene most similar to *NRAMP6* was much higher expressed in *G. elegans* than in *G. claussenii*, while the homologue for *NRAMP2* had for all conditions a higher expression level in *G. claussenii*. The *NRAMP1* transcript was down-regulated in roots by Cd treatment in both species and lower expressed in *G. claussenii* compared with *G. elegans* in shoots. Two of the three transcripts coding for NA synthase were constitutively highly expressed in roots (both most similar to*NAS4*) and only one in shoots (*NAS1*) of *G. claussenii* when compared with *G. elegans*. Transcripts homologous to *HMA2* had a much higher expression in *G. claussenii* especially for shoots. Root *FRD3* expression in *G. claussenii* did not respond to metal-exposure, while in *G. elegans* its expression was reduced upon Zn exposure. *FRO2* was mainly expressed in roots, constitutively in *G. claussenii* whereas in *G. elegans* it was significantly reduced by Zn. The transcript encoding the Fe-deficiency Induced Transcription factor 1 (FIT1), was higher expressed in *G. claussenii* than in *G. elegans* roots under all three metal conditions. Additionally the transcript homologous to *FRO7* had also a much higher expression in *G. claussenii* roots and shoots.

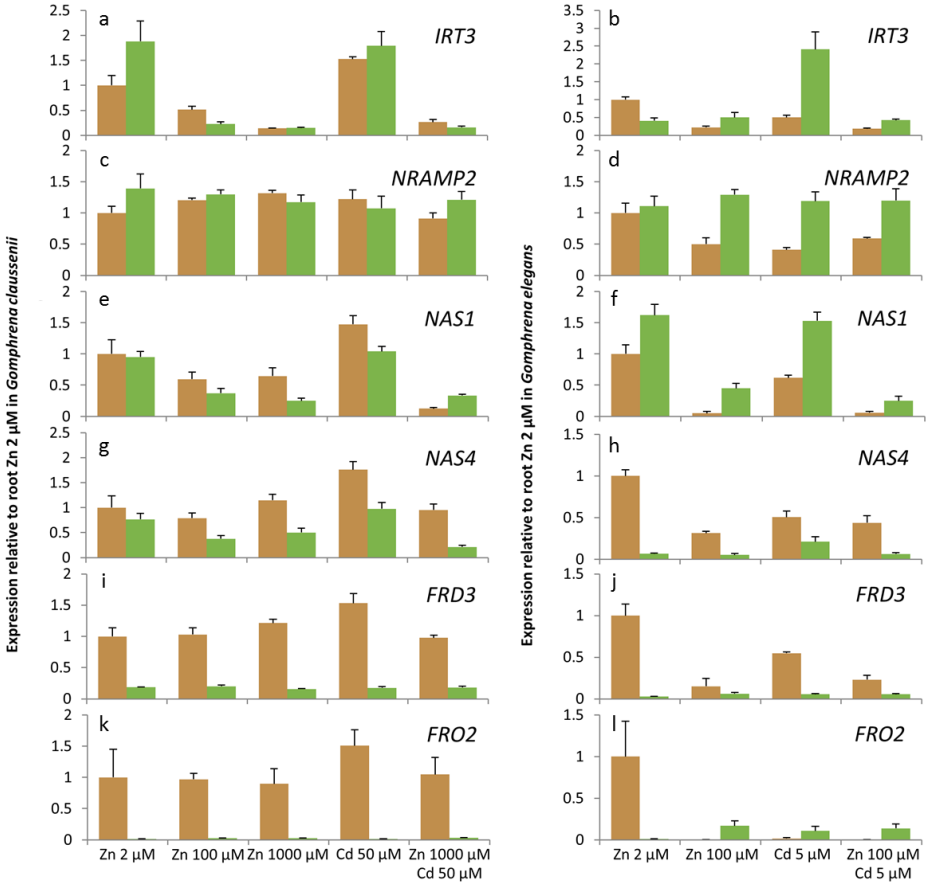


Figure 4 - Relative expressions of a selected set of metal homeostasis genes of *Gomphrena claussenii* (a, c, e, g, i and k) and *Gomphrena elegans* (b, d, f, h, j and l) as determined by real-time quantitative RT-PCR for the orthologues of *Arabidopsis thaliana* genes *IRT3* (a,b), *NRAMP2* (c,d), *NAS1* (e,f),. *NAS4* (g,h), *FRD3* (i,j) and *FRO2* (k,l). Relative expression is determined under control (Zn 2 µM), high zinc (Zn 100 µM and Zn 1000 µM), high cadmium and high zinc (Zn 1000 µM Cd 50 µM or Zn 100 µM Cd 5 µM) and high cadmium (Cd 50 µM or Cd 5 µM) treatments in root (brown) and shoot (green) tissues. The relative gene expression is normalized to a constitutively expressed gene, *UBP6*. Mean values with standard errors are shown.

Table 8 - *Gomphrena claussenii* (GC) or *Gomphrena elegans* (GE) contig IDs (Contig) most similar to *A. thaliana* genes known to be involved in mineral homeostasis. Differential expressions are indicated as ratios for either species comparisons (GC/GE) or treatment comparisons (e.g. Zn1000/Zn2). Very small ratios (<0.01) are indicated as 1 divided by X (1/X). Exposures are high zinc (1000 or 100 µM $ZnSO_4$; Zn1000 or Zn100) or cadmium exposure (50 or 5 µM $CdSO_4$ at 2 µM $ZnSO_4$; Cd50 or Cd5), and are compared to control conditions (2 µM $ZnSO_4$; Zn2). Significant differences between the two species (p ≥0.90) or two conditions (p≤0.05) for the transcript expression are indicated in bold. Contig with lower counts were not detected, ND.

| Name | Code | Putative Function | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|
| Roots | | | | | | | | | | |
| ZIP1 | AT3G12750 | zinc transporter 1 precursor | 5.99 | 0.92 | 4.23 | 0.06 | 0.48 | 0.88 | 1.16 | 8806 |
| ZIP1 | AT3G12750 | zinc transporter 1 precursor | 1.53 | 1.79 | 3.04 | 0.28 | 0.29 | 2.10 | 0.98 | 7379 |
| ZIP1 | AT3G12750 | zinc transporter 1 precursor | 3.94 | 17.7 | 5.60 | 0.28 | 0.08 | 2.48 | 1.64 | 10699 |
| ZIP6 | AT2G30080 | ZIP metal ion transporter family | 1.10 | 2.48 | 1.60 | 1.05 | 0.56 | 1.21 | 0.78 | 10751 |
| ZIP7 | AT2G04032 | zinc transporter 7 precursor | 0.06 | 0.30 | 0.13 | ND | 0.27 | ND | 0.36 | 13675 |
| ZIP11 | AT1G55910 | zinc transporter 11 precursor | 0.76 | 1.92 | 0.34 | 1.17 | 0.56 | 1.19 | 2.47 | 9017 |
| IRT3 | AT1G60960 | iron regulated transporter 3 | 3.99 | 3.54 | 4.37 | 0.18 | 0.25 | 1.12 | 0.96 | 8335 |
| MTP1 | AT2G46800 | zinc transporter of Arabidopsis thaliana | 64743 | 45.8 | 19.0 | 1.17 | ND | 0.84 | ND | 14596 |
| MTP8 | AT3G58060 | Cation efflux family protein | 2.19 | 8.61 | 1.69 | 1.11 | 0.35 | 0.93 | 1.15 | 6292 |
| MTP8 | AT3G58060 | Cation efflux family protein | 0.31 | 1.91 | 0.59 | 0.51 | 0.10 | 0.86 | 0.41 | 17216 |
| MTP11 | AT2G39450 | Cation efflux family protein | 4.47 | 4.94 | 6.27 | 0.99 | 1.10 | 1.16 | 0.77 | 7551 |
| MTP11 | AT2G39450 | Cation efflux family protein | 1.06 | 1.67 | 1.16 | 1.12 | 0.86 | 1.14 | 0.97 | 16423 |
| NRAMP1 | AT1G80830 | natural resistance-associated macrophage protein 1 | 1.89 | 1.30 | 3.41 | 0.63 | 1.12 | 0.42 | 0.22 | 12867 |
| NRAMP2 | AT1G47240 | natural resistance-associated macrophage protein 2 | 4.10 | 5.05 | 4.11 | 1.07 | 1.07 | 1.07 | 1.00 | 4972 |

| Name | Code | Putative Function | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|
| NRAMP6 | AT1G15960 | natural resistance-associated macrophage protein 6 | 0.13 | 0.06 | 0.13 | 1.83 | 5.09 | 1.60 | 1.49 | 4046 |
| NRAMP6 | AT1G15960 | natural resistance-associated macrophage protein 6 | 1/102 | 1/775 | 1/541 | ND | 1.01 | ND | 0.20 | 14355 |
| NAS1 | AT5G04950 | nicotianamine synthase 1 | 2.95 | 18.0 | 6.16 | 0.54 | 0.10 | 1.84 | 0.84 | 20503 |
| NAS4 | AT1G56430 | nicotianamine synthase 4 | 1.29 | 3.36 | 3.48 | 0.96 | 0.44 | 1.59 | 0.55 | 10477 |
| NAS4 | AT1G56430 | nicotianamine synthase 4 | 2.47 | 23.8 | 5.42 | 0.55 | 0.07 | 1.83 | 0.79 | 20014 |
| HMA2 | AT4G30110 | heavy metal atpase 2 | 5.25 | 6.66 | 7.13 | 1.53 | 1.48 | 1.18 | 0.82 | 876 |
| HMA2 | AT4G30110 | heavy metal atpase 2 | 3.85 | 2.50 | 6.43 | 0.82 | 1.56 | 0.83 | 0.46 | 1225 |
| HMA2 | AT4G30110 | heavy metal atpase 2 | 1.78 | 3.11 | 2.43 | 0.99 | 0.69 | 0.93 | 0.64 | 15333 |
| FIT1 | AT2G28160.1 | FER-like regulator of iron uptake | 5.97 | 10.8 | 18.0 | 1.39 | 0.73 | 1.60 | 0.67 | 12743 |
| FRO2 | AT1G01580 | ferric reduction oxidase 2 | 4.32 | 107 | 188 | 0.68 | 1/116 | 1.32 | 0.03 | 7438 |
| FRO7 | AT5G49740.1 | ferric reduction oxidase 7 | 6.82 | 8.25 | 7.59 | 1.09 | 0.78 | 1.14 | 1.14 | 1688 |
| FRD3 | AT3G08040.2 | MATE efflux family protein | 1.01 | 5.99 | 0.83 | 1.28 | 0.24 | 1.32 | 1.58 | 3923 |
| FRD3 | AT3G08040.2 | MATE efflux family protein | 0.89 | 4.78 | 1.19 | 1.22 | ND | 1.05 | ND | 12199 |
| Shoot | | | | | | | | | | |
| ZIP1 | AT3G12750 | zinc transporter 1 precursor | 10.7 | 3.84 | 3.84 | 0.03 | 0.23 | 1.86 | 4.51 | 7379 |
| ZIP1 | AT3G12750 | zinc transporter 1 precursor | 3.41 | 1.34 | 1.34 | 0.27 | 1.04 | 1.24 | 2.76 | 8806 |
| ZIP1 | AT3G12750 | zinc transporter 1 precursor | 12.7 | 5.94 | 5.94 | 0.07 | 0.44 | 1.56 | 2.89 | 10699 |
| ZIP6 | AT2G30080 | ZIP metal ion transporter family | 4.02 | 4.32 | 4.32 | 0.88 | 1.52 | 0.94 | 0.77 | 10751 |
| ZIP7 | AT2G04032 | zinc transporter 7 precursor | 1.95 | 0.36 | 0.36 | ND | 3.93 | ND | 6.98 | 13675 |
| ZIP11 | AT1G55910 | zinc transporter 11 precursor | 2.32 | 0.48 | 0.48 | 0.84 | 5.53 | 1.02 | 4.28 | 9017 |
| IRT3 | AT1G60960 | iron regulated transporter 3 | 8.39 | 3.22 | 3.22 | 0.12 | 1.12 | 1.38 | 3.13 | 8335 |
| MTP1 | AT2G46800 | zinc transporter of Arabidopsis thaliana | 163 | 20.6 | 20.6 | 1.17 | ND | 1.04 | ND | 14596 |
| MTP8 | AT3G58060 | Cation efflux family protein | 0.84 | 0.23 | 0.23 | 0.78 | 2.44 | 0.76 | 2.41 | 6292 |
| MTP8 | AT3G58060 | Cation efflux family protein | 0.91 | 0.97 | 0.97 | 2.05 | ND | 1.28 | 1.00 | 17216 |

| Name | Code | Putative Function | Zn2 GC/ Zn2 GE | Zn1000GC/ Zn100GE | Cd50GC/ Cd5 GE | Zn1000/ Zn2 GC | Zn100/ Zn2GE | Cd50/ Zn2GC | Cd5/ Zn2GE | Contig |
|---|---|---|---|---|---|---|---|---|---|---|
| MTP11 | AT2G39450 | Cation efflux family protein | 3.69 | 3.21 | 3.21 | 1.17 | 1.02 | 0.94 | 0.95 | 7551 |
| MTP11 | AT2G39450 | Cation efflux family protein | 1.43 | 0.75 | 0.75 | 0.76 | 1.18 | 0.80 | 1.35 | 16423 |
| NRAMP1 | AT1G80830 | natural resistance-associated macrophage protein 1 | 0.07 | 0.03 | 0.03 | 0.80 | 0.81 | 0.77 | 1.83 | 12867 |
| NRAMP2 | AT1G47240 | natural resistance-associated macrophage protein 2 | 3.91 | 3.49 | 3.49 | 1.02 | 1.04 | 0.95 | 0.93 | 4972 |
| NRAMP6 | AT1G15960 | natural resistance-associated macrophage protein 6 | 6.22 | 1.45 | 1.45 | 1.10 | 3.22 | 0.96 | 3.57 | 4046 |
| NRAMP6 | AT1G15960 | natural resistance-associated macrophage protein 6 | 1/501026 | 1/1.06E+12 | 1/1.06E+12 | ND | 0.93 | ND | 1.86 | 14355 |
| NAS1 | AT5G04950 | nicotianamine synthase 1 | 1.67 | 1.95 | 1.95 | 0.31 | 0.20 | 1.35 | 1.02 | 20503 |
| NAS4 | AT1G56430 | nicotianamine synthase 4 | 13.9 | 5.95 | 5.95 | 0.61 | 1.02 | 1.45 | 2.95 | 10477 |
| NAS4 | AT1G56430 | nicotianamine synthase 4 | 1.70 | 1.97 | 1.97 | 0.28 | 0.21 | 1.37 | 1.04 | 20014 |
| HMA2 | AT4G30110 | heavy metal atpase 2 | 10.4 | 10.3 | 10.3 | 1.09 | 0.99 | 0.96 | 0.84 | 876 |
| HMA2 | AT4G30110 | heavy metal atpase 2 | 279 | 24.5 | 24.5 | 0.94 | 13.0 | 0.97 | 9.53 | 1225 |
| HMA2 | AT4G30110 | heavy metal atpase 2 | 18.0 | 6.94 | 6.94 | 0.77 | 0.92 | 0.68 | 1.61 | 15333 |
| FIT1 | AT2G28160.1 | FER-like regulator of iron uptake | 1.12 | 0.52 | 0.90 | 1.23 | 2.35 | 0.76 | 0.80 | 12743 |
| FRO2 | AT1G01580 | ferric reduction oxidase 2 | 10.9 | 0.66 | 1.3 | 1.3 | 19.1 | 1.45 | 10.5 | 7438 |
| FRO7 | AT5G49740.1 | ferric reduction oxidase 7 | 46.8 | 33.7 | 70.4 | 1.61 | 1.96 | 2.26 | 1.29 | 1688 |
| FRD3 | AT3G08040.2 | MATE efflux family protein | 1.81 | 0.56 | 1.12 | 0.63 | 1.44 | 0.92 | 1.36 | 3923 |
| FRD3 | AT3G08040.2 | MATE efflux family protein | 2.81 | 1.69 | 1.79 | 1.10 | ND | 1.16 | ND | 12199 |

## DISCUSSION

We investigated the molecular basis of metal tolerance in *G. claussenii* using RNA-Seq transcriptome sequencing technology to determine gene expression in the roots and shoots of plants exposed to high levels of Zn and Cd. We compared this species to *G. elegans*, a closely related Zn/Cd-sensitive and -non-accumulating species, to identify genes that might be involved in Zn/Cd hypertolerance and hyperaccumulation, and to understand the bioindicator accumulation response.

Prior to this study, nucleotide sequence data were unavailable for either of these non-model species. The molecular analysis of *G. claussenii* and *G. elegans* therefore required the generation of *de novo* transcriptome data using the cost-effective Illumina technology. Accordingly, we sequenced and assembled 205,921 contigs for *G. claussenii* and 194,900 for *G. elegans*. Unsurprisingly, most of the annotated reads were homologous to sequences from *Beta vulgaris*, which is the only species from the family Amaranthaceae with a complete published genome sequence (Dohm *et al.*, 2014). Together, these transcriptomes provided a comprehensive dataset that was essential for our subsequent investigation, and this dataset remains a useful resource for future studies involving *Gomphrena* species. The dataset adds a substantial body of genomic information to the comparatively underexplored Amaranthaceae family. It will also contribute to our molecular understanding of metal bioindicator species, given that *G. claussenii* is the first Zn/Cd bioindicator species that has been investigated at the genomic and transcriptomic levels.

The transcriptomic analysis of metal-tolerant species typically involves the comparison of gene expression data with a closely related non-accumulator species, e.g.

*N. caerulescens* and *A. thaliana* (van de Mortel *et al.*, 2006; van de Mortel *et al.*, 2008), *N. caerulescens* and *Thlaspi arvense* (Hammond *et al.*, 2006), or *A. halleri* and *A. thaliana* (Talke *et al.*, 2006; Weber *et al.*, 2006). To ensure the comparison of *G. claussenii* and *G. elegans* was appropriate, we first needed to establish their phylogenetic relationship. We found that *G. claussenii* and *G. elegans* were sufficiently closely related for valid molecular comparisons, with approximately 87% of sequence pairs sharing at least 95% nucleotide identity. We observed striking differences between *G. claussenii* and *G. elegans* in terms of their response to metals, with *G. elegans* showing a stronger transcriptional response to metal exposure than *G. claussenii*. For example, stress-related transcripts – e.g. the cytochrome P450, WRKY transcription factor, glutamate decarboxylase, and glutathione-S-transferase families – were more responsive to Zn and/or Cd in *G. elegans* but were constitutively expressed at high levels in *G. claussenii*. More specifically, genes involved in sulfur assimilation and glutathione metabolism, such as ATP sulfurylase 1 (*APS1*) and glutathione-S-transferase, are known to play an important role in Cd tolerance (Gallego *et al.*, 2012; Jozefczak *et al.*, 2015). These transcripts were constitutively expressed in *G. claussenii* but strictly regulated by Cd exposure in *G. elegans*, suggesting that different defence mechanisms are used in each species.

Genes involved in photosynthesis also showed different responses in the two species. Toxic levels of Zn and/or Cd can directly affect photosynthesis by inhibiting chlorophyll synthesis and promoting chloroplast degradation, causing symptoms of chlorosis (Chaney, 1993; Gallego *et al.*, 2012). At the cellular level especially Cd is known to induce an oxidative stress response through reactive oxygen species (ROS) production, which has a strong negative effect on chloroplast status and subsequent inhibition of photosynthesis (Cuypers *et al.*, 2010). The mild regulation of these genes in *G. claussenii* indicates its capacity to maintain photosynthesis despite high internal Zn and Cd levels,

whereas the strong suppression of this group of genes in *G. elegans* reveals an acute response to metal toxicity, typical of a non-Cd-tolerant species.

*G. claussenii* shares a constitutively lower level of stress-response gene expression with hyperaccumulator species, compared with sensitive species such as *G. elegans*. Overall, *G. claussenii* shows a physiological response broadly similar to that of Brassicaceae hyperaccumulator species (Figure 5). This species has enhanced metal uptake in the roots and enhanced metal storage capacity. There is no evidence of enhanced root to shoot transport, which is seen in Brassicaceae hyperaccumulators. The transcriptome data are in line with the physiological observations. Many genes that were previously found to be highly expressed in hyperaccumulators, almost irrespective of metal exposure levels, are also higher, and constitutively, expressed in *G. claussenii* compared to *G. elegans*. However, especially the lack of hyperaccumulation of Zn and Cd at low exposure levels makes that this species is different from hyperaccumulators, suggesting that *G. claussenii* has an intermediate phenotype between metal sensitive and hyperaccumulator species, supporting the initial observations that it displays a bioindicator response.

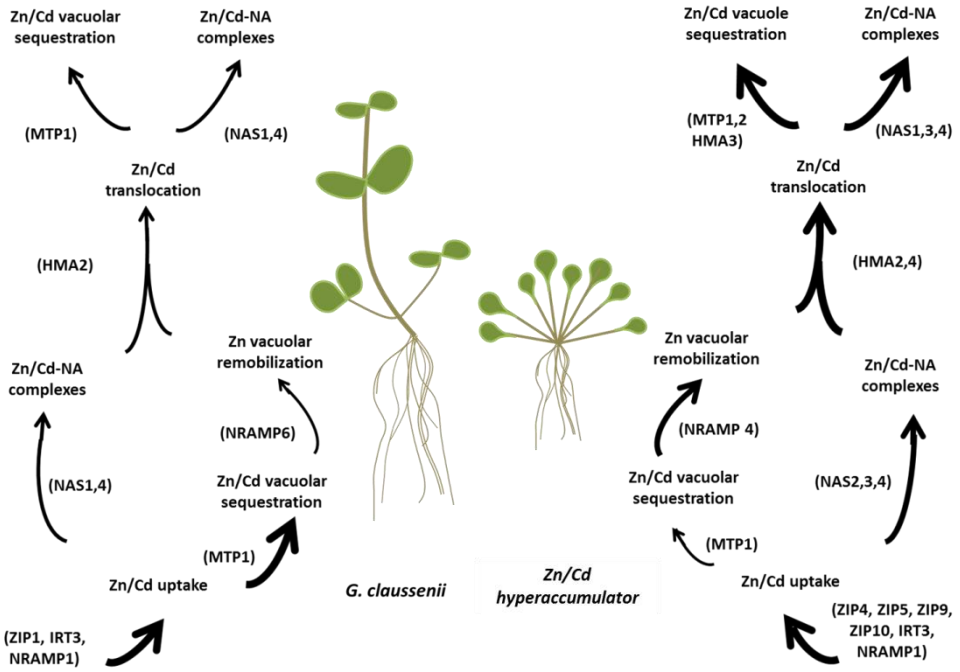


Figure 5 - Molecular mechanism of tolerance and accumulation in *Gomphrena claussenii* and a classical Zn/Cd metal hyperaccumulator such as *Noccaea caerulescens*. Arrow width represents the expression levels of genes involved in metal uptake, chelation, vacuole sequestration, vacuole remobilization and translocation. Expression levels in hyperaccumulator species are from Verbruggen et al. (2009).

Potassium levels directly affect the osmotic balance of plant cells and therefore mediate turgor pressure and expansion (Maathuis, 2009). Three genes encoding potassium transporters were found to be expressed more strongly in *G. claussenii* than *G. elegans*, namely *HAK5* (Nieves-Cordones et al., 2010), *ZIFL2* (Remy *et al.*, 2015) and *NHX2* (Barragan *et al.*, 2012). The higher expression levels in *G. claussenii* indicate that Zn and Cd tolerance influences potassium homeostasis. Higher external potassium supply was shown to alleviate Zn toxicity in peach seedlings (Song *et al.*, 2015). Moreover, higher expression of potassium transporter genes, including *HAK5*, were also observed in *N.*

*caerulescens* roots when compared to *A. thaliana* (van de Mortel *et al.*, 2006). This all indicates that modulating potassium homeostasis through upregulation of potassium transporters is an important strategy for Zn tolerance in plants. A high potassium concentration was suggested to protect plants photosynthesis and antioxidative defence system during high Zn exposure (Song *et al.*, 2015), but the exact mechanism of this protective effect is not yet clear.

Tolerance to excess Zn and Cd is directly linked to Fe homeostasis because increased competition of Zn or Cd with Fe regarding uptake often leads to Fe deficiency in plants. Surprisingly, the *FRO2* transcript was down regulated in *G. elegans* roots exposed to excess Zn or Cd (Figure 4H and Table 8). In other metal sensitive species, such as *A. thaliana*, Zn and Cd excess causes Fe deficiency resulting in the induction of genes involved in Fe uptake (van de Mortel *et al.*, 2008).

In tolerant species such as *N. caerulescens* (van de Mortel *et al.*, 2006) and *A. halleri* (Shanmugam *et al.*, 2011), genes involved in Fe homeostasis are not significantly modulated by Zn and Cd. Accordingly, we found that *G. claussenii FIT1, FRO2, FRO7* and *FRD3* were more or less constitutively expressed, and Fe levels in the shoot were not affected by Zn and Cd, as previously reported by Villafort Carvalho *et al.* (2013). An important transcription factor known to drive upregulation of Fe uptake genes during Fe deficiency, is FIT1 (Bauer *et al.*, 2007). *FIT1* is higher expressed in *G. claussenii* than *G. elegans* roots (Table 8), which could explain the much higher root expression of *FRO2* in *G. claussenii*, but it does not respond much to either excess Zn or Cd. It was also not detected as a major Fe deficiency regulator in *N. caerulescens*, in which instead another Fe-deficiency responsive bHLH transcription factor, bHLH100, was found to be most responsive and differentially regulated (van de Mortel *et al.*, 2008). We did not find a suitable orthologue for bHLH100 in *Gomphrena*, but this could be due to the

phylogenetic distance between the species. *FRO7* is thought to encode a chloroplast specific ferric reductase (Jeong *et al.*, 2008). Especially in shoots, *FRO7* is expressed at higher levels in *G. claussenii* than *G. elegans*, confirming the requirement of more Fe in chloroplasts to counteract the negative effect of Zn or Cd accumulation on Fe uptake and translocation to the leaves. Our data also revealed which *G. claussenii* genes potentially control metal tolerance and accumulation in this species.

We especially examined genes representing families already known to be involved in Zn homeostasis and Cd tolerance (Table 8). Most important of these are the ZIP Zn uptake proteins. Among the *ZIP* transcripts identified in *G. claussenii*, *ZIP1*, *ZIP7*, *ZIP11* and *IRT3* are thought to be involved in Zn homeostasis (Lin *et al.*, 2009; Assunção *et al.*, 2010; Milner *et al.*, 2013). *ZIP1* and *IRT3* are plasma membrane transporters involved in metal uptake. In Zn hyperaccumulators, *ZIP1* is constitutively expressed at low levels and is not thought to play an important role in Zn accumulation (van de Mortel *et al.*, 2006; Weber *et al.*, 2006), but may be needed for proper Zn uptake under excess Cd (van de Mortel *et al.*, 2008). The *G. claussenii* orthologue of *ZIP1* was also induced by Cd exposure, and suppressed by high levels of Zn, which suggests that the *G. claussenii* ZIP1 protein plays a role in Zn uptake but may also transport Cd at low affinity. *IRT3* encodes a Zn transporter which is expressed in roots and shoots, induced by Zn deficiency in non-accumulator species (Lin *et al.*, 2009). It resembles *ZIP4*, for which we did not find a *Gomphrena* orthologue. Under control conditions, *IRT3* is expressed at higher levels in *G. claussenii* than *G. elegans*, similar to the situation when comparing *A. halleri* and *A. thaliana*. Overexpressing *IRT3* in *A. thaliana* enhanced Zn accumulation in the shoots (Lin *et al.*, 2009). This gene is also expressed at much higher levels in *N. caerulescens* (in which the gene is named *ZNT2*) and *A. halleri* compared to *A. thaliana* (van de Mortel *et al.*, 2006; Lin *et al.*, 2009) or *Thlaspi. arvense* (Assunção *et al.*, 2001).

As one of the major metal ligand in plants, NA has been described to play a fundamental role in Fe and Zn homeostasis, controlling the intercellular distribution and long-distance transport of these metals (Haydon *et al.*, 2012; Hofmann, 2012; Clemens *et al.*, 2013). The essential role of nicotianamine synthase (NAS) in Zn hyperaccumulation has recently been demonstrated in *A. halleri*, where the reduction of NA levels in the roots, caused by suppressing NAS expression, resulted in lower levels of Zn accumulation in the leaves (Deinlein *et al.* (2012); (Tsednee *et al.*, 2014). Both *A. halleri* and *N. caerulescens* express *NAS* genes at higher levels than non-accumulator species (Talke *et al.*, 2006; van de Mortel *et al.*, 2006). In *G. claussenii* and *G. elegans*, we identified orthologues of *A. thaliana NAS1* and *NAS4*. In *G. claussenii*, the high-level expression of *NAS4* in the roots and *NAS1* in the shoots (Fig 4, Table 8) strongly suggests that NA has a similar function in this species as in the Brassicaceae Zn/Cd hyperaccumulators.

Metal tolerance proteins (MTPs), or Cation Diffusion Facilitators (CDFs) are involved in the efflux of metal ions from the cytoplasm(Gustin *et al.*, 2011). MTP1 is localized on the tonoplast, and it transports metals into the vacuole thus contributing to Zn accumulation and tolerance (Drager *et al.*, 2004). Five copies of *MTP1* are found in the *A. halleri* genome, conferring stronger tolerance to high Zn levels than the single copy gene present in *A. thaliana* (Shahzad *et al.*, 2010). High levels of *MTP1* expression have also been reported in other Zn hyperaccumulators such as *N. caerulescens* and *N. goesingense* (Assunção *et al.*, 2001; Gustin *et al.*, 2009). Among several transcripts with a putative role in Zn and Cd tolerance, *MTP1* was expressed at the highest levels in *G. claussenii* in comparison with *G. elegans*. This suggests that MTP1 has a very important role in *G. claussenii*, and considering its function in vacuolar Zn loading, it is expected to be a major contributor to Zn tolerance. MTP8 and MTP11 are other members of the CDF family, but they are associated with Mn, rather than Zn, homeostasis in *A. thaliana*, rice,

cucumber and barley (Delhaize *et al.*, 2007; Chen *et al.*, 2013; Migocka *et al.*, 2014; Pedas *et al.*, 2014). These genes have also been suggested to affect metal homeostasis in *N. caerulescens* and *A. halleri* (Krämer *et al.*, 2007) due to their high expression levels (Hammond *et al.*, 2006; Talke *et al.*, 2006; van de Mortel *et al.*, 2008). Recently, Halimaa *et al.* (2014) reported that both genes show accession-dependent expression levels in *N. caerulescens*, and suggested that MTP8 is associated with Cd accumulation whereas MTP11 is associated with Zn accumulation. *MTP8* and *MTP11* are both constitutively expressed at higher levels in *G. claussenii* than in *G. elegans* roots, indicating that indeed both proteins may be involved in Zn and Cd accumulation also in *G. claussenii*.

The ATPase transporters HMA4 and HMA2 play a major role in Zn and Cd translocation by controlling xylem loading (Hussain *et al.*, 2004; Wong & Cobbett, 2009). Especially HMA4 is considered to be the major factor facilitating high root to shoot translocation of Zn and Cd in hyperaccumulator species (Hanikenne *et al.*, 2008; Ó Lochlainn *et al.*, 2011; Craciun *et al.*, 2012). Recently, Tan *et al.* (2013) showed that the overexpression of the wheat HMA2 in rice increased Zn/Cd translocation from roots to shoot. In *N. caerulescens*, another ATPase gene from the same $P_{1B}$ subfamily, *HMA3*, was shown to encode an important tonoplast transporter, contributing strongly to vacuolar sequestration of Cd (Ueno *et al.*, 2011). Three pairs of transcripts annotated as *HMA2* were detected in *G. claussenii* and *G. elegans* whereas *HMA3* and *HMA4* orthologues were not identified. These three transcripts were constitutively expressed, at high levels, in *G. claussenii*, especially in shoots, in line with the high constitutive expression found for HMA3 and HMA4 in the Brassicaceae hyperaccumulators. Despite the best similarity to *HMA2*, the three transcripts may actually be orthologues of *HMA2*, *HMA3* and *HMA4*, or perform at least similar functions. In any case, it seems likely that also in *G. claussenii*, the $P_{1B}$ subfamily of ATPases contribute to Zn and Cd translocation and tolerance.

Other genes presumed to be involved in metal translocation include *NRAMP1*, encoding a manganese (Mn) transporter that can transport Cd and thus contribute to Cd accumulation (Takahashi *et al.*, 2011; Halimaa *et al.*, 2014; Milner *et al.*, 2014). Although the *NRAMP1* transcript was downregulated in both *G. claussenii* and *G. elegans* in response to Cd, it was expressed at much higher levels in *G. claussenii*. Another *NRAMP* gene identified was most similar to *NRAMP6*. The expression was very different from the expression of *NRAMP1*, in that one of the *NRAMP6* transcripts was expressed at much lower levels in *G. claussenii* than *G. elegans*, especially in the shoots. The function of NRAMP6 is still unclear. In *A. thaliana* it appears to act as an intracellular transporter that can mobilize Cd but not Zn or Fe (Cailliatte *et al.*, 2009). The overexpression of *NRAMP6* in *A. thaliana* causes Cd hypersensitivity, whereas loss-of-function *nramp6* mutants are more Cd tolerant. The reduced expression of *NRAMP6* in *G. claussenii*, would therefore be expected to contribute to Cd accumulation and tolerance. However, Halimaa *et al.* (2014) reported the highest expression of *NcNRAMP6* in the *N. caerulescens* Cd hyperaccumulating accession 'Ganges', which is hard to interpret. Still, NRAMP6 may act as a vacuolar efflux pump for Cd (Halimaa *et al.*, 2014) and like NRAMP3/4 it may be involved in metal remobilization (Oomen *et al.*, 2009).

In conclusion, comparative transcriptome analysis of the metal hypertolerant bioindicator species *G. claussenii* and the non-accumulator *G. elegans* suggests a new intermediate phenotype in Zn and Cd tolerance. Our results indicate that genes previously known to be involved in Zn homeostasis and Cd tolerance also support the adaptation of *G. claussenii* to metals, but these genes are regulated in a unique manner, especially those required for metal translocation (Figure 5). We propose that *G. claussenii* has evolved a molecular mechanism for metal tolerance distinct from those of both non-accumulator species and metal hyperaccumulators.

## ACKNOWLEDGEMENTS

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## SUPPLEMENTAL FIGURES

Figure S1 – Transcriptome set-up and sequence results.

Figure S2 – *G. claussenii* and *G. elegans* orthologue comparison.

Figure S3 - Comparison of qRT-PCR and in silico RNA-seq.

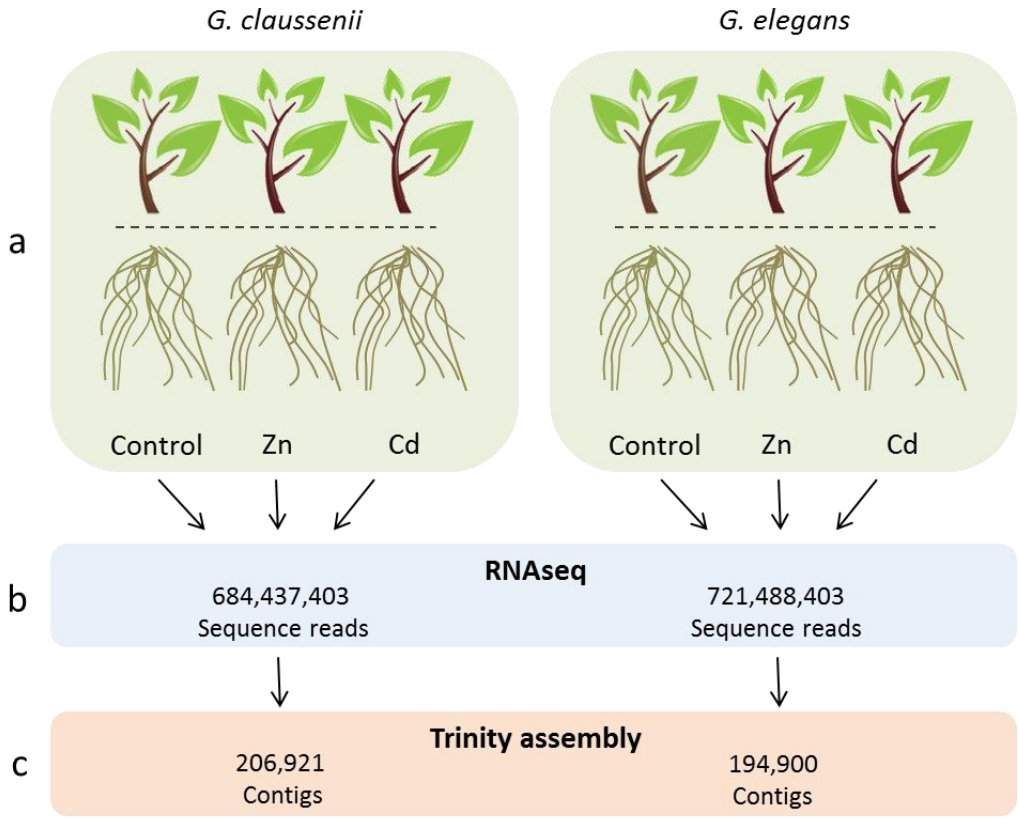


Figure S1 – Experimental set-up for RNA-seq sampling with shoot and root samples from *Gomphrena claussenii* and *Gomphrena elegans* after four days of exposure to control (2 µM of $ZnSO_4$), high zinc (1000 µM Zn for *G. claussenii* and 100 µM Zn for *G. elegans*) or cadmium (50 µM for *G. claussenii* and 5 µM for *G. elegans* with 2 µM of Zn) treatments (a). Raw sequence reads of each species obtained from the Illumina HiSeq2000 platform (b) and assembly of these into contigs using Trinity (c).

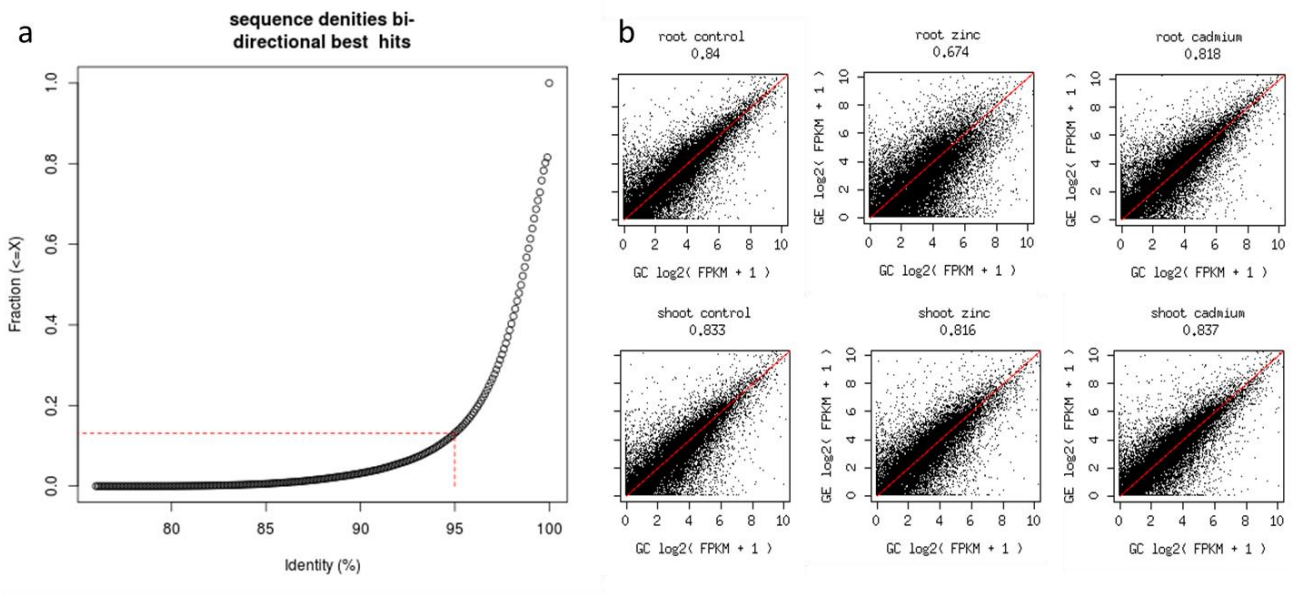


Figure S2 - Cumulative distribution plot demonstrating the high overall sequence identity between *G. claussenii* and *G. elegans* orthologous pairs (a). Scatterplots of log2 (1 + RPKM) (Reads Per Kilobase per Million mapped reads) expression levels of orthologous *G. claussenii* and *G. elegans* sequences (b).

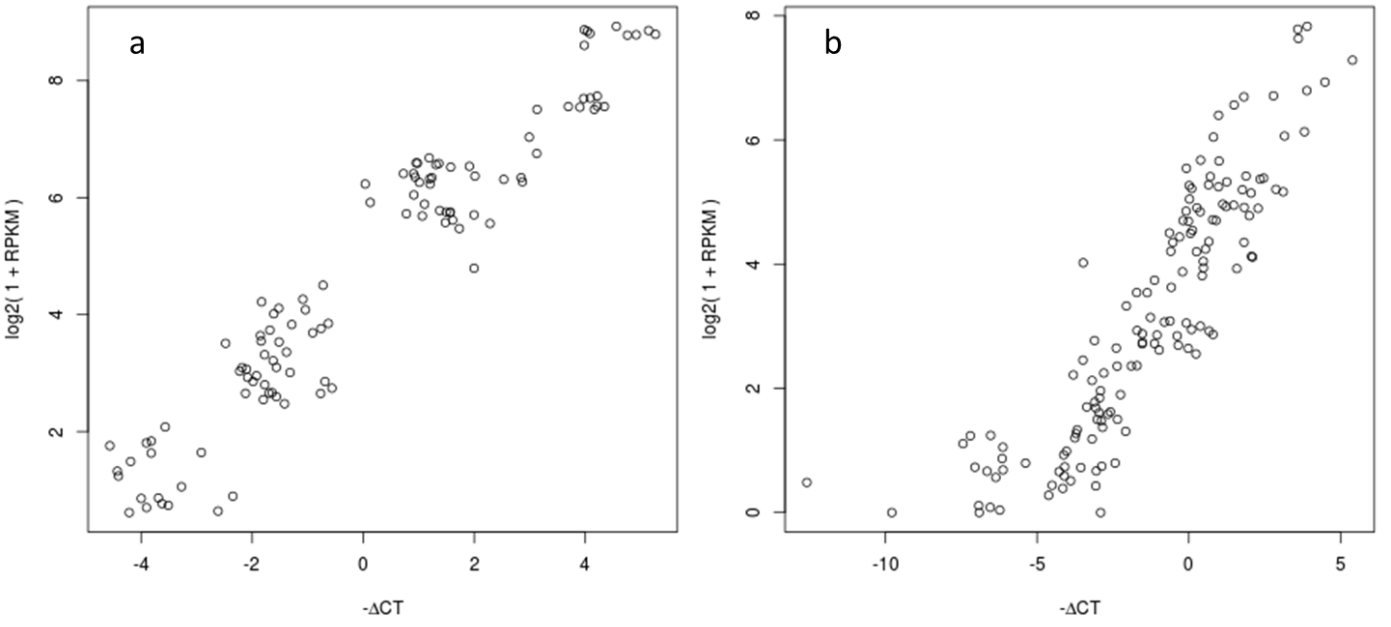


Figure S3 - Comparison of qRT-PCR and in silico RNA-seq expression levels in *Gomphrena claussenii* (a) and *Gomphrena elegans* (b). Delta Ct (cycle threshold) (ΔCT) values were obtained by subtracting the Ct values of target genes by those of UBP6. In silico expression levels are given as log2 (1 + RPKM) (Reads Per Kilobase per Million mapped reads). The Ct values are shown for the IRT3, NRAMP2, NAS1, NAS4, FRD3 and FRO2 homologues in all experimental samples (in total 108 data points). The overall spearman-rank correlation coefficient between the qRT-PCR and in silico expression values is 0.92 for *G. claussenii* and 0.91 for *G. elegans*.

## SUPPLEMENTAL TABLES

All the supplemental tables can be accessed online in the Dropbox page created for this thesis in the folder correspondent to this chapter using the following link and user information:

Link: https://www.dropbox.com/home

Email: villafortcarvalhothesis@gmail.com

Password: gomphrena

# CHAPTER 5

## General discussion

Hyperaccumulator plant species have a remarkable capacity to tolerate and accumulate metals. Research has focused on the physiological and molecular basis of this phenomenon and the use of such plants for environmental remediation. Metal homeostasis has been investigated primarily in the model plant species *Arabidopsis thaliana*, but valuable data have also been acquired by studying the model hyperaccumulator species *Noccaea caerulescens* and *Arabidopsis halleri* (Verbruggen *et al.*, 2009; Lin & Aarts, 2012; Leitenmaier & Küpper, 2013). Most of our knowledge therefore reflects the analysis of a small group of species, the best studied of which belong to the Brassicaceae family (Krämer, 2010). However, advances in the analysis of genomes and gene expression now make it much easier to study non-model metal-tolerant species, adding to the body of data relating to metal tolerance mechanisms.

This thesis describes the investigation of metal tolerance in *Gomphrena claussenii*, a newly discovered metal hypertolerant and bioindicator species. The great capacity of this species for metal accumulation prompted us to explore the molecular basis of these traits. Our research revealed unique mechanisms of Zn and Cd tolerance, with potential applications in phytoremediation.

## The application of *G. claussenii* in phytoremediation

Industrial activities such as mining and smelting cause continuous metal contamination. Whereas most soils contain 10–100 mg $kg^{-1}$ of Zn and less than 1 mg $kg^{-1}$ of Cd, polluted soils often contain more than 1000 mg $kg^{-1}$ of Zn and 50 mg $kg^{-1}$ of Cd (Kabata-Pendias & Mukherjee, 2007b; Nagajyoti *et al.*, 2010). Such contamination is usually addressed through conventional physical and chemical remediation techniques, such as soil excavation, soil washing or burning, oxidation and vitrification (Wu, G *et al.*,

2010). These are expensive and environmentally hazardous processes, whereas metal hyperaccumulator plant species provide an opportunity to remediate soils inexpensively in an environmentally beneficial manner (Chaney *et al.*, 2007). The cultivation of metal-tolerant plants in metal-polluted areas reduces the risk of issues such as soil erosion and further contamination due to water percolation. The vegetation can be used to stabilize metals in the soil, a technique known as phytostabilisation, or to remove metals by harvesting the plant biomass, a technique known as phytoextraction (Pilon-Smits, 2005; Chaney *et al.*, 2007; Padmavathiamma & Li, 2007).

The low biomass yield of most hyperaccumulator species limits the amount of metal that can be sequestered from the soil, restricting the practical implementation of phytoextraction strategies (Chaney *et al.*, 2007; van Nevel *et al.*, 2007; Maestri *et al.*, 2010). An ideal species for phytoextraction should combine high metal tolerance and high metal accumulation with high biomass production. No species with all three properties have yet been identified in nature, so two alternative strategies have been considered: breed or genetically engineer hyperaccumulator species to increase their biomass production, or genetically engineer high-biomass species to increase their metal accumulation and tolerance (Chaney *et al.*, 2007). Both strategies are challenging and neither has been achieved thus far.

We investigated the newly-discovered metal hypertolerant species *Gomphrena claussenii*, which is native to South America and was found growing on a Zn mining site in Brazil. This metal accumulator species has significant potential for phytoremediation applications. We evaluated the physiological effects of excess Zn and Cd in *G. claussenii* compared to the related non-tolerant species *G. elegans* (Chapter 2). We found that, unlike classical hyperaccumulator species, *G. claussenii* produces large amounts of biomass and yet accumulates as much Zn and Cd in the shoot tissues as typical

hyperaccumulators. It also has a high capacity for regeneration, which is favourable for phytoextraction. *G. claussenii* tolerates more than 10 times the levels of Zn and Cd than normal species such as *G. elegans* (Chapter 2) and accumulates up to 9.3 g Zn $kg^{-1}$ dry weight (0.9%) and 1.3g Cd $kg^{-1}$ dry weight (0.1%) in the shoot tissues (Chapter 2) (Villafort Carvalho *et al.*, 2013).

The evaluation of metal concentrations in different plant organs indicated that *G. claussenii* shoots contain 80% of the total Zn and Cd content of the entire plant (Chapter 3), which facilitates phytoextraction. The analysis of metal concentrations in *G. claussenii* root, leaf and stem tissues indicated that the stem plays an important role in metal accumulation (Chapter 3) (Villafort Carvalho *et al.*, 2015). From a plant perspective, stems are attractive tissues for metal sequestration because they have low metabolic activity. The storage of metals in the stem has been reported in the Zn and Cd hyperaccumulator species *Sedum alfredii* (Tian *et al.*, 2009).

Four functional categories have been proposed to classify plants according to their metal responses: "regular", excluder, bioindicator and hyperaccumulator (McGrath *et al.*, 2000; van der Ent *et al.*, 2013). Based on the observed metal accumulation characteristics, *G. claussenii* does not fit within any of these pre-determined categories. It does not match the strict definition of a Zn and Cd hyperaccumulator because it accumulates higher concentrations of these metals in its roots compared to the shoots. Furthermore, whereas classical hyperaccumulator species sequester metals even from soils with low metal concentrations (Bert *et al.*, 2002; Assunção *et al.*, 2003a; van der Ent *et al.*, 2013), *G. claussenii* displays a metal bioindicator response (Chapter 2) (Villafort Carvalho *et al.*, 2013). However, its ability to accumulate high concentrations of metal distinguishes *G. claussenii* from classical bioindicator species. We therefore propose the new classification of **metal hyperbioindicator** to describe the unique properties of *G. claussenii* (Figure 1).

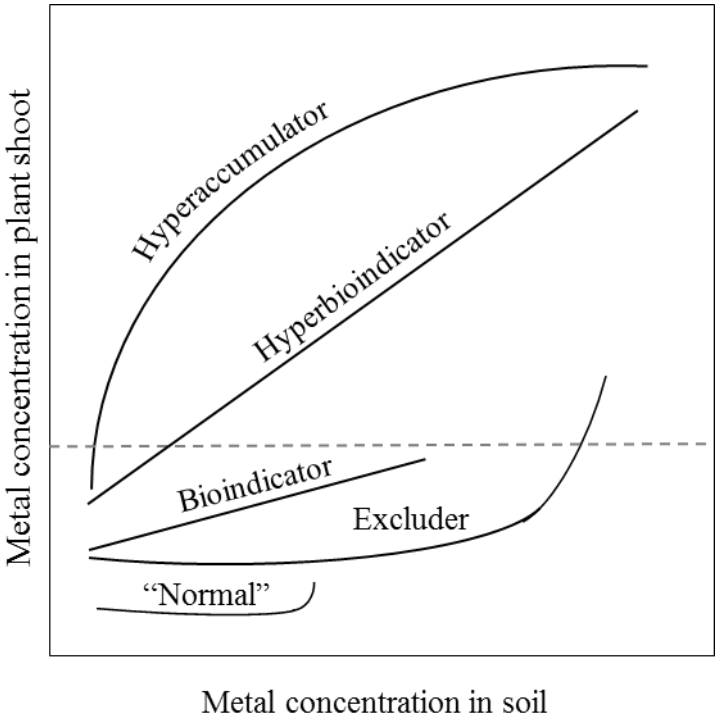


Figure 1- Conceptual response mechanisms of metal accumulation in plant shoots. Adapted from McGrath *et al.* (2000) and van der Ent *et al.* (2013). The dotted line indicates the hyperaccumulator threshold.

The metal accumulation response in hyperaccumulators has been explained by the elemental defence hypothesis, which states that high internal metal concentrations represent an ecological adaptation to resist pathogens and deter herbivores (Pollard & Baker, 1997). Most metal hyperaccumulator species have been described as obligate or endemics from serpentine (ultramafic) soils (Pollard *et al.*, 2014) therefore the majority of hyperaccumulators are nickel (Ni) tolerant. Compared with serpentine soils there are few sites naturally enriched in Zn which explains the lower abundance of Zn and Cd accumulator species. Moreover most Zn and Cd hyperaccumulator species, including *N. caerulescens* and *A. halleri* are facultative hyperaccumulators which mean that these

species have evolved the hyperaccumulator trait in non-metalliferous soils and moved later to a metal enriched site. These hyperaccumulator species can therefore accumulate metals even if the environmental concentration is low. The low level of metal accumulation under normal environmental conditions indicates that *G. claussenii* may have evolved in response to selection pressures distinct from those affecting hyperaccumulator species. Thus far, *G. claussenii* has been identified solely at three metal-enriched sites (Senna *et al.*, 210; Forzza *et al.*, 2010), suggesting it may be endemic to metalliferous soils. If *G. claussenii* evolved strictly as an adaptation to metal-rich environments it would not need to accumulate metals under normal environmental conditions, which would explain its bioindicator response. Geological studies in the Vazante region, where *G. claussenii* was identified and collected, support this hypothesis (Júnior *et al.*, 2008). The Vazante area has high Zn concentrations near the soil surface, declining with soil depth and this metal-enriched environment has not been produced by anthropogenic activities. *G. claussenii* physiological response together with soil metal information from this site indicates that *G. claussenii* has evolved a unique molecular mechanism for metal tolerance distinct from those of both non-accumulator species and metal hyperaccumulators.

## Metal accumulation and tolerance strategies in *G. claussenii*

To ensure metal homeostasis in environments with high metal concentrations, most plant species attempt to avoid the accumulation of surplus metal (Lin & Aarts, 2012). They usually prevent excess metal uptake by producing root exudates that chelate metal ions in the soil. Metal hyperaccumulator species use a different strategy based on metal sequestration and chelation within the plant tissues (Leitenmaier & Küpper, 2013).

The compartmentalisation of Zn and Cd in cells with low metabolic activity, such as trichomes, epidermis and stem pith cells, achieves metal tolerance in hyperaccumulator species such as *A. halleri*, *N. caerulescens*, *N. praecox* and *S. alfredii* (recently renamed as *S. plumbizincicola* (Wu *et al.*, 2013) (Küpper *et al.*, 2004; Cosio *et al.*, 2005; Hokura *et al.*, 2006; Fukuda *et al.*, 2008; Vogel-Mikuš *et al.*, 2008a; Vogel-Mikuš *et al.*, 2008b; Tian *et al.*, 2011). A specific metal compartmentalisation strategy was also identified in *G. claussenii*, based on Cd sequestration in vacuolar calcium oxalate (CaOx) crystals (Chapter 3). The incorporation of Cd into CaOx crystals in *G. claussenii* plants exposed to excess Cd was confirmed by metal distribution analysis and the higher oxalic acid concentration in the shoots. This tolerance mechanism has previously been investigated in metal-sensitive species such as tobacco (Choi *et al.*, 2001; Choi & Harada, 2005; Isaure *et al.*, 2010). The CaOx crystals in tobacco are excreted at the leaf surface, a useful metal tolerance strategy that does not require a metal hyperaccumulator phenotype. In *G. claussenii*, the crystals are retained internally.

The incorporation of Cd into CaOx crystals is not used as a tolerance mechanism by any of the known Cd hyperaccumulator species. In terms of phytoremediation strategies, this novel tolerance mechanism in *G. claussenii* may represent an efficient way to store high concentrations of Cd in the shoot tissues. The storage of Cd in crystals may also facilitate the disposal of plants biomass after harvesting and could even be exploited for metal recovery.

Interestingly, separate and distinct mechanisms of Zn and Cd accumulation have been described in hyperaccumulator species such as *N. caerulescens* (Vogel-Mikuš et al., 2008a), *S. alfredii* (Tian *et al.*, 2009; Tian *et al.*, 2011) and *G. claussenii* (Chapter 3) (Villafort Carvalho *et al.*, 2015). Despite their chemical similarity, Zn and Cd can interact with different metal chelators. In *G. claussenii*, Cd and Ca are distributed with similar

profiles but the reverse profile is observed for Zn, a phenomenon previously reported in *S. alfredii* (Tian et al., 2011). This difference was demonstrated by the ability of Zn to interfere with the formation of CaOx crystals (Chapter 3). Zn was not incorporated into CaOx crystals, but higher levels of Zn reduced the number of crystals we detected. However, metabolomic analysis (Chapter 3) indicated that both Zn and Cd are associated with oxalate in *G. claussenii*. These data suggest that when *G. claussenii* plants are exposed to high levels of Zn, the Zn-oxalate levels increase enough to reduce Ca-oxalate and disturb the formation of Ca-oxalate crystals. Because Cd is much less abundant than Ca in plants, the concentration of Ca-oxalate remains high enough to precipitate and include Cd-oxalate in the crystals.

Despite the differing metal distribution profiles in *G. claussenii* and hyperaccumulator species, both Zn and Cd are predominantly stored in the vacuole (Zhao *et al.*, 2000; Ma *et al.*, 2005). The efficiency of metal chelation by different compounds depends on the pH in each subcellular compartment. For example, the acidic vacuolar environment favours the chelation of metals by organic acids, and this is an important metal tolerance mechanism in several plant species (Haydon & Cobbett, 2007). Together with oxalate, malate and citrate play key roles in the chelation of Zn in hyperaccumulator species (Yang *et al.*, 2006; Sarret *et al.*, 2009; Monsant *et al.*, 2011) and their high concentrations in *G. claussenii* suggest a similar role (Chapter 3).

Nicotianamine (NA) is other important metal chelator in plants, with key roles in iron (Fe), Zn, copper (Cu) and manganese (Mn) homeostasis (Clemens *et al.*, 2013). In metal hyperaccumulator species, NA may facilitate Zn translocation and accumulation in shoot tissues (Clemens *et al.*, 2013). Transcriptome analysis has shown that NA is a potential metal chelator in *G. claussenii*. Nicotianamine synthase (NAS) is required for NA biosynthesis. *N. caerulescens* and *A. halleri* express *NAS* genes constitutively at higher

levels than the non-accumulator *A. thaliana* (Talke *et al.*, 2006; van de Mortel *et al.*, 2006). NA may facilitate Zn hyperaccumulation in *A. halleri,* where the depletion of NA in the roots by suppressing *NAS* expression inhibits the accumulation of Zn in the leaves (Deinlein *et al.*, 2012). *NAS* orthologues corresponding to *NAS1* and *NAS4* in *A. thaliana* were identified in *G. claussenii* (Chapter 4). The constitutive expression of *NAS* genes in *G. claussenii* at higher levels than in *G. elegans* suggests that NA may promote metal tolerance and accumulation in *G. claussenii.* These data also suggest that metal chelation is not a simple process but may require several compounds that bind specific metals.

## The molecular mechanism of metal hypertolerance in *G. claussenii*

Comparative transcriptomics and related approaches have increased our understanding of the molecular basis of metal homeostasis in plants related to *A. thaliana* (Hammond *et al.*, 2006; Weber *et al.*, 2006; van de Mortel *et al.*, 2008; Verbruggen *et al.*, 2009; Hanikenne & Nouet, 2011; Lin & Aarts, 2012; Halimaa *et al.*, 2014). However, the advent of advanced sequencing methods has facilitated the study of non-model species with similar tools, increasing the number of metal tolerant species that can be investigated at the molecular level (Verbruggen *et al.*, 2013).

Genes involved in metal tolerance and accumulation have been identified by comparing the transcriptomes of related metal-tolerant and metal-sensitive species, such as *N. caerulescens* and *A. thaliana* (van de Mortel *et al.*, 2006; van de Mortel *et al.*, 2008), or *A. halleri* and *A. thaliana* (Weber *et al.*, 2006). Similarly, we compared the transcriptomes of *G. claussenii* and *G. elegans* (Chapter 4). A common feature in all of these studies was that exposure to high concentrations of metals caused the induction of several genes in the metal sensitive species that were constitutively expressed in the

hyperaccumulator species. Because *G. claussenii* has a bioindicator-like response to metal accumulation, we were surprised to observe the constitutive expression of genes that are induced by metals in *G. elegans*. *G. claussenii* may have evolved strictly in a metal-rich environment. If so, the constitutive high-level expression of these genes will be an adaptive response.

In plants, metal homeostasis involves extensive interaction and crosstalk among the regulatory mechanisms controlling individual metal species (Clemens, 2006; Puig & Penarrubia, 2009; Merchant, 2010). Therefore, Zn and Cd tolerance in *G. claussenii* is anticipated to directly affect other metals. Several transcripts representing metal transporters were expressed at higher levels in *G. claussenii* than *G. elegans* (Chapter 4), including potassium, phosphate, sulfate, nitrate and iron transporters.

The regulation of Zn and Fe in plants involves overlapping biological processes (Palmer & Guerinot, 2009; Shanmugam *et al.*, 2013). Therefore, Fe deficiency is a direct consequence of excess Zn and Cd exposure in most plant species (Clemens, 2006). For non-tolerant species such as *A. thaliana*, the Fe deficiency genes *IRT1*, *IRT2* and *FRO2* are induced in the presence of excess Zn (Shanmugam *et al.*, 2011). In contrast, these Fe homeostasis genes show a constitutive low level of expression in *A. halleri*, and other genes including *IRT3* may maintain the high levels of Fe in this species (Shanmugam *et al.*, 2011). *G. claussenii* may employ a different strategy to maintain Fe levels when exposed to excess Zn and Cd, e.g. the constitutive high-level expression of *FIT1*, *FRO2*, *FRO7* and *IRT3* (Chapter 4). These results suggest that while *A. halleri* maintains a low activity of the Fe acquisition transporters, *G. claussenii* evolved a steadily high activity of Fe homeostasis genes.

The gene encoding the high-affinity potassium transporter HAK5 (Nieves-Cordones *et al.*, 2010) is expressed at higher levels in the roots of *G. claussenii* than *G. elegans* (Chapter 4). This gene is also expressed at high levels in the roots of *N. caerulescens* (van de Mortel *et al.*, 2006). Because potassium is essential in plants and also the most abundant cation in plant cells, it plays important roles in turgor pressure, cell expansion, plant movement and stomatal opening (Maathuis, 2009). The *ZIFL2* and *NHX2* genes encoding two further potassium transporters were also strongly expressed in *G. claussenii*. The role of the plasma membrane transporter ZIFL2 may be to remove potassium from the roots (Remy *et al.*, 2015). *ZIFL2* is expressed at different levels in different *N. caerulescens* accessions suggesting a role in Zn and Cd tolerance (Halimaa *et al.*, 2014). NHX2 translocates potassium from the cytosol to the vacuole, and thus plays an essential role in cell expansion and the maintenance of turgor (Barragan *et al.*, 2012). Potassium plays an important role in abiotic stress (Wang *et al.*, 2013) so the regulation of potassium-related genes may be required to maintain potassium at sufficient levels for effective stress responses. More specifically, higher external potassium supply was shown to alleviate Zn toxicity in peach seedlings (Song *et al.*, 2015). The expression profiles of *HAK5*, *ZIFL2* and *NHX2* genes suggest that Zn and Cd accumulation in *G. claussenii* has an impact on potassium homeostasis. Interestingly, higher expression of potassium transporter genes, including *HAK5* were also observed in *N. caerulescens* roots compared to *A. thaliana* (van de Mortel *et al.*, 2006). These results indicate that a sufficient potassium concentration through higher regulation of potassium transporters is an important strategy for Zn tolerant plants. High potassium concentration was suggested to protect plants photosynthesis and antioxidative defence system during high Zn exposure (Song *et al.*, 2015).

Our *G. claussenii* transcriptome analysis also identified a number of transcripts homologous to specific genes involved in Zn homeostasis and Cd tolerance (Chapter 4). *G. claussenii* has a molecular response broadly similar to hyperaccumulator species but further responses that are unique to this species. The high-level expression of ZIP genes such as *ZIP1* and *IRT3* promotes efficient metal uptake by the roots. Metal chelation by NA then facilitates Zn transport in the roots. MTP1 allows the vacuole to store large amounts of Zn and Cd in the roots and shoots. The low-level expression of NRAMP6 limits metal distribution within the cell and the expression of HMA2 and NRAMP1 ensures the moderate translocation of Zn and Cd to the shoot. Metal remobilization and translocation are two crucial steps for metal accumulation in the shoots of metal hyperaccumulator species, but unlike classical species *G. claussenii* has a higher metal concentration in the roots and therefore a lower rate of metal remobilization and translocation.

Concluding, the research described in this thesis shows that *G. claussenii* tolerates very high levels of Zn and Cd, and may be highly suitable for phytoremediation applications, although field experiments will be needed to confirm the feasibility of practical deployment. We found that *G. claussenii* has a unique strategy for Cd accumulation compared to hyperaccumulator species. Further analysis of zinc accumulation in *G. claussenii* may improve our understanding of the metal sequestration mechanism in this species which may be useful for biofortification programs in Amaranthaceae crops, e.g. spinach (*Spinacia oleracea*), beet (*Beta vulgaris*), quinoa (*Chenopodium quinoa*) and Amaranth species. Several *G. claussenii* genes required for Zn and Cd translocation and vacuolar accumulation (such as HMAs, NAs and MTPs) were identified as promising candidates for follow up studies for their function in metal homeostasis and accumulation. Such studies could involve targeted gene silencing in *G.*

*claussenii*, which would require the development of genetic transformation strategies for this species, or the overexpression of these transcripts in model species such as *A. thaliana* or *N. tabacum*. The separate regulation of two components of metal homeostasis (translocation and vacuolar accumulation) results in a moderate level of metal translocation and simultaneous high-level accumulation, distinguishing *G. claussenii* from other metal tolerant species. The data presented in this thesis will encourage further research into the ecological and genetic characteristics of *G. claussenii* to determine which selection pressures favoured the evolution of hypertolerance and bioindicator traits. The validation of *G. claussenii* metal homeostasis genes and the investigation of their functions as a component of Zn and Cd tolerance will confirm the mechanisms underlying the *G. claussenii* hyperbioindicator response.

# REFERENCES

**Abràmoff MD, Magalhães PJ, Ram SJ. 2004.** Image processing with ImageJ. *Biophotonics International* **11**: 36-42.

**Alaoui-Sossé B, Genet P, Vinit-Dunand F, Toussaint ML, Epron D, Badot PM. 2004.** Effect of copper on growth in cucumber plants (*Cucumis sativus*) and its relationships with carbohydrate accumulation and changes in ion contents. *Plant Science* **166**: 1213-1218.

**Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997.** Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25**(17): 3389-3402.

**Anders S, Huber W. 2010.** Differential expression analysis for sequence count data. *Genome Biology* **11**(10).

**Assunção AGL, Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO. 2003a.** Differential metal-specific tolerance and accumulation patterns among *Thlaspi caerulescens* populations originating from different soil types. *New Phytologist* **159**(2): 411-419.

**Assunção AGL, Martins PDAC, De Folter S, Vooijs R, Schat H, Aarts MGM. 2001.** Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* **24**(2): 217-226.

**Assunção AGL, Schat H, Aarts MGM. 2003b.** Thlaspi caerulescens, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159**(2): 351-360.

**Assunção AGL, Schat H, Aarts MGM. 2010.** Regulation of the adaptation to zinc deficiency in plants. *Plant Signaling and Behavior* **5**(12): 1553–1555.

**Baker AJ, Brooks RR. 1989.** Terrestrial higher plants which hyper-accumulate metallic elements - a review of their distribution, ecology and phytochemistry. *Biorecovery* **1**: 81-123.

**Baker AJ, Ernst WHO, van der Ent A, Malaisse F, Ginocchio R 2010.** Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. In: Batty LC ed. Ecology of Industrial Pollution: Cambridge University Press

**Baker AJM. 1987.** Metal tolerance. *New Phytologist* **106**: 93-111.

**Baker AJM, McGrath SP, Sidoli CMD, Reeves RD. 1994.** The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. *Resources, Conservation and Recycling* **11**(1-4): 41-49.

**Baker AJM, Whiting SN. 2002.** In search of the Holy Grail - A further step in understanding metal hyperaccumulation? *New Phytologist* **155**(1): 1-4.

**Baloun J, Nevrtalova E, Kovacova V, Hudzieczek V, Cegan R, Vyskot B, Hobza R. 2014.** Characterization of the *HMA7* gene and transcriptomic analysis of candidate genes for copper tolerance in two *Silene vulgaris* ecotypes. *Journal of Plant Physiology* **171**(13): 1188-1196.

**Barragan V, Leidi EO, Andres Z, Rubio L, De Luca A, Fernandez JA, Cubero B, Pardo JM. 2012.** Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in Arabidopsis. *Plant Cell* **24**(3): 1127-1142.

**Bauer P, Ling HQ, Guerinot ML. 2007.** FIT, the FER-LIKE IRON DEFICIENCY INDUCED TRANSCRIPTION FACTOR in Arabidopsis. *Plant Physiology and Biochemistry* **45**(5): 260-261.

**Bert V, Bonnin I, Saumitou-Laprade P, De Laguérie P, Petit D. 2002.** Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **155**(1): 47-57.

**Borin ALDC. 2010.** *Fitorremediação de cádmio e zinco por Amaranthaceae.* . Universidade Federal de Lavras Lavras.

**Boyd RS. 2007.** The defense hypothesis of elemental hyperaccumulation: Status, challenges and new directions. *Plant and Soil* **293**(1-2): 153-176.

**Bringezu K, Lichtenberger O, Leopold I, Neumann D. 1999.** Heavy metal tolerance of *Silene vulgaris*. *Journal of Plant Physiology* **154**: 536-546.

**Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. 2007.** Zinc in plants. *New Phytologist* **173**(4): 677-702.

**Broadley MR, Willey NJ, Wilkins JC, Baker AJM, Mead A, White PJ. 2001.** Phylogenetic variation in heavy metal accumulation in angiosperms. *New Phytologist* **152**(1): 9-27.

**Brown SL, Chaney RL, Angle JS, Baker AJ. 1995.** Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. *Soil Science Society of America Journal* **59**(1): 125-133.

**Cailliatte R, Lapeyre B, Briat JF, Mari S, Curie C. 2009.** The NRAMP6 metal transporter contributes to cadmium toxicity. *Biochemical Journal* **422**(2): 217-228.

**Carreno-Quintero N, Acharjee A, Maliepaard C, Bachem CWB, Mumm R, Bouwmeester H, Visser RGF, Keurentjes JJB. 2012.** Untargeted metabolic quantitative trait loci analyses reveal a relationship between primary metabolism and potato tuber quality. *Plant Physiology* **158**(3): 1306-1318.

**Chaney R 1993.** Zinc phytotoxicity. Zinc in soils and plants: Springer, 135-150.

**Chaney RL, Angle JS, Broadhurst CL, Peters Ca, Tappero RV, Sparks DL. 2007.** Improved understanding of hyperaccumulation yields commercial phytoextraction and phytomining technologies. *Journal of Environmental Quality* **36**: 1429-1423.

**Chao DY, Silva A, Baxter I, Huang YS, Nordborg M, Danku J, Lahner B, Yakubova E, Salt DE. 2012.** Genome-Wide Association Studies Identify Heavy Metal ATPase3 as the Primary Determinant of Natural Variation in Leaf Cadmium in *Arabidopsis thaliana*. *PLoS Genet* **8**(9): e1002923.

**Chen Z, Fujii Y, Yamaji N, Masuda S, Takemoto Y, Kamiya T, Yusuyin Y, Iwasaki K, Kato S-i, Maeshima M. 2013.** Mn tolerance in rice is mediated by MTP8. 1, a member of the cation diffusion facilitator family. *Journal of Experimental Botany* **64**(14): 4375-4387.

**Chinmayee MD, Mahesh B, Pradesh S, Mini I, Swapna TS. 2012.** The assessment of phytoremediation potential of invasive weed *Amaranthus spinosus* L. *Appl Biochem Biotechnol* **167**: 1550-1559.

**Choi YE, Harada E. 2005.** Roles of calcium and cadmium on Cd-containing intra- and extracellular formation of Ca crystals in tobacco. *Journal of Plant Biology* **48**: 113-119.

**Choi YE, Harada E, Wada M, Tsuboi H, Morita Y, Kusano T, Sano H. 2001.** Detoxification of cadmium in tobacco plants: Formation and active excretion of crystals containing cadmium and calcium through trichomes. *Planta* **213**: 45-50.

**Ci D, Jiang D, Dai T, Jing Q, Cao W. 2009.** Effects of cadmium on plant growth and physiological traits in contrast wheat recombinant inbred lines differing in cadmium tolerance. *Chemosphere* **77**: 1620-1625.

**Clark RB. 1975.** Characterization of phosphatase of intact maize roots. *Journal of Agricultural and Food Chemistry* **23**(3): 458-460.

**Clemens S. 2001.** Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212**(4): 475-486.

**Clemens S. 2006.** Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **88**(11): 1707-1719.

**Clemens S, Deinlein U, Ahmadi H, Horeth S, Uraguchi S. 2013.** Nicotianamine is a major player in plant Zn homeostasis. *Biometals* **26**: 623-632.

**Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. 2005.** Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**(18): 3674-3676.

**Cornu JY, Deinlein U, Horeth S, Braun M, Schmidt H, Weber M, Persson DP, Husted S, Schjoerring JK, Clemens S. 2015.** Contrasting effects of nicotianamine synthase knockdown on zinc and nickel tolerance and accumulation in the zinc/cadmium hyperaccumulator *Arabidopsis halleri*. *New Phytol* **206**(2): 738-750.

**Cosio C, DeSantis L, Frey B, Diallo S, Keller C. 2005.** Distribution of cadmium in leaves of *Thlaspi caerulescens*. *Journal of Experimental Botany* **56**(412): 765-775.

**Costa G, Spitz E. 1997.** Influence of cadmium on soluble carbohydrates, free amino acids, protein content of in vitro cultured *Lupinus albus*. *Plant Science* **128**(2): 131-140.

**Couee I, Sulmon C, Gouesbet G, El Amrani A. 2006.** Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. *Journal of Experimental Botany* **57**(3): 449-459.

**Craciun AR, Meyer CL, Chen J, Roosens N, De Groodt R, Hilson P, Verbruggen N. 2012.** Variation in HMA4 gene copy number and expression among *Noccaea caerulescens* populations presenting different levels of Cd tolerance and accumulation. *Journal of Experimental Botany* **63**(11): 4179-4189.

**Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Nair AR, Munters E, Artois TJ, et al. 2010.** Cadmium stress: An oxidative challenge. *Biometals* **23**(5): 927-940.

**Deinlein U, Weber M, Schmidt H, Rensch S, Trampczynska A, Hansen TH, Husted S, Schjoerring JK, Talke IN, Krämer U, et al. 2012.** Elevated nicotianamine levels in *Arabidopsis halleri* roots play a key role in zinc hyperaccumulation. *Plant Cell* **24**(2): 708-723.

**Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardson AE. 2007.** A role for the *AtMTP11* gene of Arabidopsis in manganese transport and tolerance. *The Plant Journal* **51**(2): 198-210.

**Dickinson NM, Baker AJM, Doronila A, Laidlaw S, Reeves RD. 2009.** Phytoremediation of inorganics: Realism and synergies. *International Journal of Phytoremediation* **11**(2): 97-114.

**Dohm JC, Minoche AE, Holtgräwe D, Capella-Gutiérrez S, Zakrzewski F, Tafer H, Rupp O, Sörensen TR, Stracke R, Reinhardt R. 2014.** The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). *Nature* **505**(7484): 546-549.

**Drager DB, Desbrosses-Fonrouge AG, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P, Krämer U. 2004.** Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *Plant Journal* **39**(3): 425-439.

**Ernst WHO. 2006.** Evolution of metal tolerance in higher plants. *Forest Snow and Landscape Research* **80**(3): 251-274.

**Ferreira EO, Dias DA. 2004.** Phytochemical investigation of *Gomphrena claussenii* Moq. *Biochemical Systematics and Ecology* **32**(9): 823-827.

**Filho SR, Viana MB 2011.** Gestão da água: o desafio do zinco em Vazante-MG. In: Fernandes FRC, Enriqez MR, Alamino RCJ eds. Recursos Minerais & Sustentabilidade Territorial. Rio de Janeiro: CETEM/MCTI, 333-360.

**Fones H, Davis CAR, Rico A, Fang F, Smith JAC, Preston GM. 2010.** Metal hyperaccumulation armors plants against disease. *PLoS Pathogens* **6**(9).

**Forzza RC, M. LP, Costa A, Carvalho AA, Peixoto AL, Walter BMT, Bicudo C, Zappi D, Costa DP, Lleras E, et al. 2010.** *Catálogo de plantas e fungos do Brasil*. Rio de Janeiro: Jardim Botânico do Rio de Janeiro.

**Franceschi VR, Schueren AM. 1986.** Incorporation of strontium into plant calcium oxalate crystals. *Protoplasma* **130**(2-3): 199-205.

**Frey B, Keller C, Zierold K, Schulin R. 2000.** Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* **23**: 675-687.

**Fukuda N, Hokura A, Kitajima N, Terada Y, Saito H, Abe T, Nakai I. 2008.** Micro X-ray fluorescence imaging and micro X-ray absorption spectroscopy of cadmium hyper-accumulating plant, *Arabidopsis halleri* ssp. gemmifera, using high-energy synchrotron radiation. *Journal of Analytical Atomic Spectrometry* **23**(8): 1068.

**Gallego SM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD, Benavides MP. 2012.** Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany* **83**: 33-46.

**Gao J, Sun L, Yang X, Liu JX. 2013.** Transcriptomic analysis of cadmium stress response in the heavy metal hyperaccumulator *Sedum alfredii* Hance. *PLoS One* **8**(6): e64643.

**Ginocchio R, Baker AJM. 2004.** Metallophytes in Latin America: A remarkable biological and genetic resource scarcely known and studied in the region. *Revista Chilena de Historia Natural* **77**(1): 185-194.

**Gong JM 2015**. Transcriptomic analysis of cadmium hyperaccumulation and hypertolerance in *Sedum plumbizincicola*. *Plant and Animal Genome XXIII Conference*: Plant and Animal Genome.

**Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al. 2011.** Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **29**(7): 644-652.

**Gustafsson JP 2000.** Visual MINTEq.In högskolan KT.

**Gustin JL, Loureiro ME, Kim D, Na G, Tikhonova M, Salt DE. 2009.** MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. *Plant Journal* **57**(6): 1116-1127.

**Gustin JL, Zanis MJ, Salt DE. 2011.** Structure and evolution of the plant cation diffusion facilitator family of ion transporters. *BMC Evolutionary Biology* **11**(1).

**Halimaa P, Lin YF, Ahonen VH, Blande D, Clemens S, Gyenesei A, Haikio E, Karenlampi SO, Laiho A, Aarts MGM, et al. 2014.** Gene expression differences between *Noccaea caerulescens* ecotypes help to identify candidate genes for metal phytoremediation. *Environ Sci Technol* **48**(6): 3344-3353.

**Hall JL. 2002.** Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53**(366): 1-11.

**Hammond JP, Bowen HC, White PJ, Mills V, Pyke KA, Baker AJ, Whiting SN, May ST, Broadley MR. 2006.** A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes. *New Phytologist* **170**(2): 239-260.

**Hanikenne M, Nouet C. 2011.** Metal hyperaccumulation and hypertolerance: A model for plant evolutionary genomics. *Current Opinion in Plant Biology* **14**(3): 252-259.

**Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U. 2008.** Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature* **453**(7193): 391-395.

**Hassan Z. 2013.** *Functional analysis of zinc hyperaccumulation related genes of Noccaea (Thlaspi) caerulescens for phytoremediation purposes.* PhD, Wageningen University.

**Hassan Z, Aarts MGM. 2011.** Opportunities and feasibilities for biotechnological improvement of Zn, Cd or Ni tolerance and accumulation in plants. *Environmental and Experimental Botany* **72**(1): 53-63.

**Haydon MJ, Cobbett CS. 2007.** Transporters of ligands for essential metal ions in plants. *New Phytologist* **174**(3): 499-506.

**Haydon MJ, Kawachi M, Wirtz M, Hillmer S, Hell R, Krämer U. 2012.** Vacuolar nicotianamine has critical and distinct roles under iron deficiency and for zinc sequestration in Arabidopsis. *Plant Cell* **24**(2): 724-737.

**He J, Ma C, Ma Y, Li H, Kang J, Liu T, Polle A, Peng C, Luo ZB. 2013.** Cadmium tolerance in six poplar species. *Environmental Science and Pollution Research* **20**(1): 163-174.

**Hoagland DR, Arnon DI. 1940.** Crop production in artificial culture solutions and in soils with special reference to factors influencing yields and absorption of inorganic nutrients. *Soil Science* **50**: 463-485.

**Hofmann NR. 2012.** Nicotianamine in zinc and iron homeostasis. *Plant Cell* **24**(2): 373.

**Hokura A, Onuma R, Kitajima N, Terada Y, Saito H, Abe T, Yoshida S, Nakai I. 2006.** 2-D X-ray fluorescence imaging of cadmium hyperaccumulating plants by using High-energy Synchrotron Radiation X-ray Microbeam. *Chemistry Letters* **35**(11): 1246-1247.

**Hörger AC, Fones HN, Preston GM. 2013.** The current status of the elemental defense hypothesis in relation to pathogens. *Frontiers in Plant Science* **4**(OCT).

**Huguet S, Bert V, Laboudigue A, Barthès V, Isaure M-P, Llorens I, Schat H, Sarret G. 2012.** Cd speciation and localization in the hyperaccumulator *Arabidopsis halleri*. *Environmental and Experimental Botany* **82**: 54-65.

**Huitson SB, Macnair MR. 2003.** Does zinc protect the zinc hyperaccumulator *Arabidopsis halleri* from herbivory by snails? *New Phytologist* **159**(2): 453-459.

**Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS. 2004.** P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in arabidopsis. *Plant Cell* **16**(5): 1327-1339.

**Iqbal M, Nawaz I, Hassan Z, Hakvoort HWJ, Bliek M, Aarts MGM, Schat H. 2013.** Expression of HMA4 cDNAs of the zinc hyperaccumulator *Noccaea caerulescens* from endogenous NcHMA4 promoters does not complement the zinc-deficiency phenotype of the *Arabidopsis thaliana* hma2hma4 double mutant. *Frontiers in Plant Science* **4**.

**Isaure MP, Fayard B, Sarret G, Pairis S, Bourguignon J. 2006.** Localization and chemical forms of cadmium in plant samples by combining analytical electron microscopy and X-ray spectromicroscopy. *Spectrochimica Acta Part B: Atomic Spectroscopy* **61**(12): 1242-1252.

**Isaure MP, Sarret G, Harada E, Choi YE, Marcus MA, Fakra SC, Geoffroy N, Pairis S, Susini J, Clemens S, et al. 2010.** Calcium promotes cadmium elimination as vaterite grains by tobacco trichomes. *Geochimica et Cosmochimica Acta* **74**(20): 5817-5834.

**Iseli C, Jongeneel CV, Bucher P 1999**. ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. *International Conference on Intelligent Systems for Molecular Biology*. 138-148.

**Jáuregui-Zúñiga D, Angeles Ferrer M, Calderón AA, Muñoz R, Moreno A. 2005.** Heavy metal stress reduces the deposition of calcium oxalate crystals in leaves of *Phaseolus vulgaris*. *Journal of Plant Physiology* **162**(10): 1183-1187.

**Jeong J, Cohu C, Kerkeb L, Pilon M, Connolly EL, Guerinot ML. 2008.** Chloroplast Fe(III) chelate reductase activity is essential for seedling viability under iron limiting conditions. *Proceedings of the National Academy of Sciences of the United States of America* **105**(30): 10619-10624.

**Jhee EM, Boyd RS, Eubanks MD. 2005.** Nickel hyperaccumulation as an elemental defense of *Streptanthus polygaloides* (Brassicaceae): influence of herbivore feeding mode. *New Phytologist* **168**(2): 331-344.

**Jiang RF, Ma DY, Zhao FJ, McGrath SP. 2005.** Cadmium hyperaccumulation protects *Thlaspi caerulescens* from leaf feeding damage by thrips (*Frankliniella occidentalis*). *New Phytologist* **167**(3): 805-814.

**Jozefczak M, Bohler S, Schat H, Horemans N, Guisez Y, Remans T, Vangronsveld J, Cuypers A. 2015.** Both the concentration and redox state of glutathione and ascorbate influence the sensitivity of Arabidopsis to cadmium. *Annals of Botany* DOI: 10.1093/aob/mcv075

**Júnior MB, de Mello JWV, Ernesto C, Schaefer G, Dussin TM, Júnior JA. 2008.** Distribuição e formas de ocorrência de zinco em solos no município de Vazante-MG. *Revista Brasileira de Ciência do Solo* **32**(5): 2183-2194.

**Kabata-Pendias A, Mukherjee A 2007a.** Plants. Trace elements from soil to human. Berlin Heidelberg: Springer, 57-65.

**Kabata-Pendias A, Mukherjee A 2007b.** Trace elements of Group 12. Trace elements from soil to human. Berlin Heidelberg: Springer, 283-319.

**Katayama H, Banba N, Sugimura Y, Tatsumi M, Kusakari S, Oyama H, Nakahira A. 2013.** Subcellular compartmentation of strontium and zinc in mulberry idioblasts in relation to phytoremediation potential. *Environmental and Experimental Botany* **85**: 30-35.

**Kazemi-Dinan A, Thomaschky S, Stein RJ, Krämer U, Muller C. 2014.** Zinc and cadmium hyperaccumulation act as deterrents towards specialist herbivores and impede the performance of a generalist herbivore. *New Phytologist* **202**(2): 628-639.

**Keunen E, Peshev D, Vangronsveld J, W VDE, Cuypers A. 2013.** Plant sugars are crucial players in the oxidative challenge during abiotic stress: extending the traditional concept. *Plant, Cell and Environment* **36**: 1242-1255.

**Krämer U. 2010.** Metal hyperaccumulation in plants. *Annu Rev Plant Biol* **61**: 517-534.

**Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC. 1996.** Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**(6566): 635-638.

**Krämer U, Talke IN, Hanikenne M. 2007.** Transition metal transport. *FEBS letters* **581**(12): 2263-2272.

**Küpper H, Kochian LV. 2010.** Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator, *Thlaspi caerulescens* (Ganges population). *New Phytologist* **185**(1): 114-129.

**Küpper H, Lombi E, Zhao FJ, McGrath SP. 2000.** Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. *Planta* **212**(1): 75-84.

**Küpper H, Mijovilovich A, Meyer-Klaucke W, Kroneck PM. 2004.** Tissue- and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by x-ray absorption spectroscopy. *Plant Physiology* **134**(2): 748-757.

**Küpper H, Zhao FJ, McGrath SP. 1999.** Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **119**(1): 305-312.

**Langmead B, Salzberg SL. 2012.** Fast gapped-read alignment with Bowtie 2. *Nature Methods* **9**(4): 357-359.

**Lasat MM, Baker AJM, Kochian LV. 1996.** Physiological characterization of root $Zn^{2+}$ absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of Thlaspi. *Plant Physiology* **112**(4): 1715-1722.

**Leitenmaier B, Küpper H. 2013.** Compartmentation and complexation of metals in hyperaccumulator plants. *Frontiers in Plant Science* **4**: 374.

**Lin Y-F. 2014.** *An evolutionary perspective on differential regulation of zinc and cadmium homeostasis genes in Arabidopsis thaliana and Noccaea caerulescens.* PhD, Wageningen University.

**Lin YF, Aarts MGM. 2012.** The molecular mechanism of zinc and cadmium stress response in plants. *Cellular and Molecular Life Sciences* **69**(19): 3187-3206.

**Lin YF, Liang HM, Yang SY, Boch A, Clemens S, Chen CC, Wu JF, Huang JL, Yeh KC. 2009.** Arabidopsis IRT3 is a zinc-regulated and plasma membrane localized zinc/iron transporter. *New Phytologist* **182**(2): 392-404.

**Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR. 2006.** Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature Protocols* **1**(1): 387-396.

**Lochlainn SO, Bowen HC, Fray RG, Hammond JP, King GJ, White PJ, Graham NS, Broadley MR. 2011.** Tandem quadruplication of HMA4 in the zinc (Zn) and cadmium (Cd) hyperaccumulator *Noccaea caerulescens*. *PLoS One* **6**(3): e17814.

**Lombi E, Scheckel KG, Kempson IM. 2011.** In situ analysis of metal(loid)s in plants: State of the art and artefacts. *Environmental and Experimental Botany* **72**(1): 3-17.

**Lommen A. 2009.** Metalign: Interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. *Analytical Chemistry* **81**(8): 3079-3086.

**Lyubenova L, Pongrac P, Vogel-Mikuš K, Mezek GK, Vavpetič P, Grlj N, Regvar M, Pelicon P, Schröder P. 2013.** The fate of arsenic, cadmium and lead in *Typha latifolia*: A case study on the applicability of micro-PIXE in plant ionomics. *J Hazard Mater* **248-249**(1): 371-378.

**Ma JF, Ueno D, Zhao FJ, McGrath SP. 2005.** Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Planta* **220**(5): 731-736.

**Maathuis FJ. 2009.** Physiological functions of mineral macronutrients. *Current Opinion in Plant Biology* **12**(3): 250-258.

**Macnair MR, Smith SE, Cumbes QJ. 1993.** Heritability and distribution of variation in degree of copper tolerance in *Mimulus guttatus* at Copperopolis, California. *Heredity* **71**(5): 445-455.

**Maere S, Heymans K, Kuiper M. 2005.** BiNGO: A cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* **21**(16): 3448-3449.

**Maestri E, Marmiroli M, Visioli G, Marmiroli N. 2010.** Metal tolerance and hyperaccumulation: Costs and trade-offs between traits and environment. *Environmental and Experimental Botany* **68**(1): 1-13.

**Mahajan P, Singh HP, Batish DR, Kohli RK. 2013.** Cr(VI) imposed toxicity in maize seedlings assessed in terms of disruption in cabohydrate metabolism. *Biol Trace Elem Res* **156**: 316-322.

**Maksymiec W. 2007.** Signaling responses in plants to heavy metal stress. *Acta Physiologiae Plantarum* **29**(3): 177-187.

**Manousaki E, Kadukova J, Papadantonakis N, Kalogerakis N. 2008.** Phytoextraction and phytoexcretion of Cd by the leaves of *Tamarix smyrnensis* growing on contaminated non-saline and saline soils. *Environ Res* **106**(3): 326-332.

**Marchioretto MS, Senna L, Siqueira JC 2010.** Amaranthaceae. In: Editorial AJE ed. Catálogo de plantas e fungos do Brasil. Rio de Janeiro, 590-596.

**Mari S, Lebrun M 2006.** Metal immobilization: Where and how? In: Tamas MJ, Enrico eds. Molecular Biology of Metal Homeostasis and Detoxification Springer Berlin Heidelberg, 274-298.

**Marques APGC, Rangel AOSS, Castro PML. 2009.** Remediation of heavy metal contaminated soils: Phytoremediation as a potentially promising clean-up technology. *Critical Reviews in Environmental Science and Technology* **39**: 622-654.

**Marschner H, Marschner P. 2012.** *Marschner's mineral nutrition of higher plants*: Academic press.

**Mary V, Ramos MS, Gillet C, Socha AL, Giraudat J, Agorio A, Merlot S, Clairet C, Kim SA, Punshon T, et al. 2015.** Bypassing iron storage in endodermal vacuoles rescues the iron mobilization defect in the natural resistance associated-macrophage protein3 natural resistance associated-macrophage protein4 double mutant. *Plant Physiology* **169**(1): 748-759.

**Mazen AMA, El Maghraby OMO. 1997.** Accumulation of cadmium, lead and strontium, and a role of calcium oxalate in water hyacinth tolerance. *Biologia Plantarum* **40**(3): 411-417.

**McGrath S, Dunham S, Correll R 2000.** Potential for phytoextraction of zinc and cadmium from soils using hyperaccumulator plants. In: Terry N, Bañuelos GS eds. Phytoremediation of contaminated soil and water: Lewis Publishers: New York, NY, USA, 109-128.

**Merchant SS. 2010.** The elements of plant micronutrients. *Plant Physiology* **154**(2): 512-515.

**Merlot S, Hannibal L, Martins S, Martinelli L, Amir H, Lebrun M, Thomine S. 2014.** The metal transporter PgIREG1 from the hyperaccumulator *Psychotria gabriellae* is a candidate gene for nickel tolerance and accumulation. *Journal of Experimental Botany* **65**(6): 1551-1564.

**Mickelbart MV, Hasegawa PM, Bailey-Serres J. 2015.** Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nature Reviews Genetics*.

**Migocka M, Papierniak A, Maciaszczyk-Dziubińska E, Poździk P, Posyniak E, Garbiec A, Filleur S. 2014.** Cucumber metal transport protein MTP8 confers increased tolerance to manganese when expressed in yeast and *Arabidopsis thaliana*. *Journal of Experimental Botany*: eru295.

**Mills RF, Francini A, da Rocha PSF, Baccarini PJ, Aylett M, Krijger GC, Williams LE. 2005.** The plant P 1B-type ATPase AtHMA4 transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *FEBS letters* **579**(3): 783-791.

**Milner MJ, Mitani-Ueno N, Yamaji N, Yokosho K, Craft E, Fei Z, Ebbs S, Clemencia Zambrano M, Ma JF, Kochian LV. 2014.** Root and shoot transcriptome analysis of two ecotypes of *Noccaea caerulescens* uncovers the role of NcNramp1 in Cd hyperaccumulation. *Plant Journal* **78**(3): 398-410.

**Milner MJ, Seamon J, Craft E, Kochian LV. 2013.** Transport properties of members of the ZIP family in plants and their role in Zn and Mn homeostasis. *Journal of Experimental Botany* **64**(1): 369-381.

**Monsant AC, Kappen P, Wang Y, Pigram PJ, Baker AJM, Tang C. 2011.** In vivo speciation of zinc in *Noccaea caerulescens* in response to nitrogen form and zinc exposure. *Plant and Soil* **348**(1-2): 167-183.

**Mussury RM, Scalon SPQ, Gomes AA, Barros SSUd. 2006.** Caracterização morfoanatômica de plântulas de *Gomphrena elegans* Mart. (Amaranthaceae). *Acta Scientiarum - Biological Sciences* **28**: 87-93.

**Nagajyoti PC, Lee KD, Sreekanth TVM. 2010.** Heavy metals, occurrence and toxicity for plants: a review. *Environmental Chemistry Letters* **8**(3): 199-216.

**Nayek S, Gupta S, Saha RN. 2010.** Metal accumulation and its effects in relation to biochemical response of vegetables irrigated with metal contaminated water and wastewater. *J Hazard Mater* **178**(1-3): 588-595.

**Ni WZ, Yang XE, Long XX. 2004.** Comparative studies on zinc tolerance and accumulation between two ecotypes of *Sedum alfredii* Hance in southeastern China. *Journal of Plant Nutrition* **27**(4): 627-635.

**Nieves-Cordones M, Aleman F, Martinez V, Rubio F. 2010.** The *Arabidopsis thaliana* HAK5 K+ transporter is required for plant growth and K+ acquisition from low K+ solutions under saline conditions. *Mol Plant* **3**(2): 326-333.

**Ó Lochlainn S, Bowen HC, Fray RG, Hammond JP, King GJ, White PJ, Graham NS, Broadley MR. 2011.** Tandem quadruplication of HMA4 in the zinc (Zn) and cadmium (Cd) hyperaccumulator *Noccaea caerulescens*. *PLoS One* **6**(3).

**Oomen RJ, Wu J, Lelievre F, Blanchet S, Richaud P, Barbier-Brygoo H, Aarts MGM, Thomine S. 2009.** Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **181**(3): 637-650.

**Padmavathiamma PK, Li LY. 2007.** Phytoremediation technology: Hyper-accumulation metals in plants. *Water, Air, and Soil Pollution* **184**(1-4): 105-126.

**Palmer CM, Guerinot ML. 2009.** Facing the challenges of Cu, Fe and Zn homeostasis in plants. *Nat Chem Biol* **5**(5): 333-340.

**Pedas P, Stokholm MS, Hegelund JN, Ladegård AH, Schjoerring JK, Husted S. 2014.** Golgi localized barley MTP8 proteins facilitate Mn transport. *PLoS One* **9**(12): e113759.

**Pelicon P, Podaru NC, Vavpetič P, Jeromel L, Ogrinc Potocnik N, Ondračka S, Gottdang A, Mous DJM. 2014.** A high brightness proton injector for the Tandetron accelerator at Jožef Stefan Institute. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms* **332**(0): 229-233.

**Pilon-Smits E. 2005.** Phytoremediation. *Annu Rev Plant Biol* **56**: 15-39.

**Pineau C, Loubet S, Lefoulon C, Chalies C, Fizames C, Lacombe B, Ferrand M, Loudet O, Berthomieu P, Richard O. 2012.** Natural variation at the FRD3 MATE transporter locus reveals cross-talk between Fe homeostasis and Zn tolerance in *Arabidopsis thaliana*. *PLoS Genet* **8**(12): e1003120.

**Podazza G, Rosa M, Gonzalez JA, Hilal M, Prado FE. 2006.** Cadmium induces changes in sucrose partitioning, invertase activities, and membrane functionality in roots of Rangpur lime (*Citrus limonia* L. Osbeck). *Plant Biology* **8**(5): 706-714.

**Pollard AJ, Baker AJM. 1997.** Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* **135**(4): 655-658.

**Pollard AJ, Reeves RD, Baker AJM. 2014.** Facultative hyperaccumulation of heavy metals and metalloids. *Plant Science* **217-218**: 8-17.

**Pongrac P, Vogel-Mikuš K, Jeromel L, Vavpetič P, Pelicon P, Kaulich B, Gianoncelli A, Eichert D, Regvar M, Kreft I. 2013.** Spatially resolved distributions of the mineral elements in the grain of tartary buckwheat (*Fagopyrum tataricum*). *Food Research International* **54**(1): 125-131.

**Pongrac P, Zhao FJ, Razinger J, Zrimec A, Regvar M. 2009.** Physiological responses to Cd and Zn in two Cd/Zn hyperaccumulating Thlaspi species. *Environmental and Experimental Botany* **66**(3): 479-486.

**Puig S, Penarrubia L. 2009.** Placing metal micronutrients in context: transport and distribution in plants. *Current Opinion in Plant Biology* **12**(3): 299-306.

**Quinn CF, Freeman JL, Reynolds RJ, Cappa JJ, Fakra SC, Marcus MA, Lindblom SD, Quinn EK, Bennett LE, Pilon-Smits EA. 2010.** Selenium hyperaccumulation offers protection from cell disruptor herbivores. *BMC ecology* **10**(1): 19.

**Raij BV, Gheyi HR, Bataglia OC 2001.** Determinação da condutividade elétrica e de cátions solúveis em extratos aquosos de solos. In: Raij BV, Andrade JCD, Cantarella H, Quaggio J eds. Análise Química para Avaliação da Fertilidade de Solos Tropicais.

**Rascio N, Navari-Izzo F. 2010.** Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? *Plant Science* **180**(2): 169-181.

**Reeves R. 1992.** The hyperaccumulation of nickel by serpentine plants. *The vegetation of ultramafic (serpentine) soils*: 253-277.

**Reeves RD. 1988.** Nickel and zinc accumulation by species of Thlaspi L., Cochlearia L., and other genera of the Brassicaceae. *Taxon*: 309-318.

**Reeves RD, Baker AJM, Becquer T, Echevarria G, Miranda ZJG. 2007.** The flora and biogeochemistry of the ultramafic soils of Goiás state, Brazil. *Plant and Soil* **293**: 107-119.

**Remm M, Storm CEV, Sonnhammer ELL. 2001.** Automatic clustering of orthologs and in-paralogs from pairwise species comparisons. *Journal of Molecular Biology* **314**(5): 1041-1052.

**Remy E, Cabrito TR, Batista RA, Teixeira MC, Sa-Correia I, Duque P. 2015.** The major facilitator superfamily transporter ZIFL2 modulates cesium and potassium homeostasis in Arabidopsis. *Plant and Cell Physiology* **56**(1): 148-162.

**Richau KH, Kozhevnikova AD, Seregin IV, Vooijs R, Koevoets PL, Smith JAC, Ivanov VB, Schat H. 2009.** Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **183**(1): 106-116.

**Roberts A, Pachter L. 2013.** Streaming fragment assignment for real-time analysis of sequencing experiments. *Nature Methods* **10**(1): 71-73.

**Rolland F, Baena-Gonzalez E, Sheen J. 2006.** Sugar sensing and signaling in plants: Conserved and novel mechanisms. *Annu Rev Plant Biol* **57**: 675-709.

**Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M, Prado FE. 2009.** Soluble sugars-metabolism, sensing and abiotic stress a complex network in the life of plants. *Plant Signaling and Behavior* **4**(5): 388-393.

**Sarret G, Willems G, Isaure MP, Marcus MA, Fakra SC, Frérot H, Pairis S, Geoffroy N, Manceau A, Saumitou-Laprade P. 2009.** Zinc distribution and speciation in *Arabidopsis halleri* x *Arabidopsis lyrata* progenies presenting various zinc accumulation capacities. *New Phytologist* **184**(3): 581-595.

**Schat H 1999.** Plant responses to inadequate and toxic micronutrient availability: General and nutrient-specific mechanisms. Plant Nutrition—Molecular Biology and Genetics: Springer, 311-326.

**Schat H, Ten Bookum WM. 1992.** Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68**: 219-229.

**Schmieder R, Edwards R. 2011.** Quality control and preprocessing of metagenomic datasets. *Bioinformatics* **27**(6): 863-864.

**Senna LR, Giulietti AM, Rapini A. 210.** Flora da Bahia: Amaranthaceae-Amaranthoideae e Gomphrenoideae. *Sitientibus, Série Ciências Biológicas* **10**(1): 3.

**Shahzad Z, Gosti F, Frerot H, Lacombe E, Roosens N, Saumitou-Laprade P, Berthomieu P. 2010.** The five AhMTP1 zinc transporters undergo different

evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *PLoS Genet* **6**(4): e1000911.

**Shanmugam V, Lo JC, Wu CL, Wang SL, Lai CC, Connolly EL, Huang JL, Yeh KC. 2011.** Differential expression and regulation of iron-regulated metal transporters in *Arabidopsis halleri* and *Arabidopsis thaliana*--the role in zinc tolerance. *New Phytologist* **190**(1): 125-137.

**Shanmugam V, Lo JC, Yeh KC. 2013.** Control of Zn uptake in *Arabidopsis halleri:* a balance between Zn and Fe. *Frontiers in Plant Science* **4**: 281.

**Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003.** Cytoscape: A software Environment for integrated models of biomolecular interaction networks. *Genome Research* **13**(11): 2498-2504.

**Sharma SS, Dietz KJ. 2006.** The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *Journal of Experimental Botany* **57**(4): 711-726.

**Siener R, Hönow R, Seidler A, Voss S, Hesse A. 2006.** Oxalate contents of species of the Polygonaceae, Amaranthaceae and Chenopodiaceae families. *Food Chemistry* **98**(2): 220-224.

**Sinclair SA, Krämer U. 2012.** The zinc homeostasis network of land plants. *Biochim Biophys Acta* **1823**(9): 1553-1567.

**Slater GSC, Birney E. 2005.** Automated generation of heuristics for biological sequence comparison. *BMC Bioinformatics* DOI: 10.1186/1471-2105-6-31

**Solé VA, Papillon E, Cotte M, Walter P, Susini J. 2007.** A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. *Spectrochimica Acta B* **62**: 63-68.

**Song ZZ, Duan CL, Guo SL, Yang Y, Feng YF, Ma RJ, Yu ML. 2015.** Potassium contributes to zinc stress tolerance in peach (*Prunus persica*) seedlings by enhancing photosynthesis and the antioxidant defense system. *Genetics and Molecular Research* **14**(3): 8338-8351.

**Strehmel N, Hummel J, Erban A, Strassburg K, Kopka J. 2008.** Retention index thresholds for compound matching in GC-MS metabolite profiling. *Journal of Chromatography B* **871**(2): 182-190.

**Takahashi R, Ishimaru Y, Senoura T, Shimo H, Ishikawa S, Arao T, Nakanishi H, Nishizawa NK. 2011.** The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. *Journal of Experimental Botany* **62**(14): 4843-4850.

**Talke IN, Hanikenne M, Krämer U. 2006.** Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in

metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **142**(1): 148-167.

**Tan J, Wang J, Chai T, Zhang Y, Feng S, Li Y, Zhao H, Liu H, Chai X. 2013.** Functional analyses of TaHMA2, a P(1B)-type ATPase in wheat. *Plant Biotechnol J* **11**(4): 420-431.

**Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A. 2011.** Differential expression in RNA-seq: A matter of depth. *Genome Research* **21**(12): 2213-2223.

**Tian S, Lu L, Labavitch J, Yang X, He Z, Hu H, Sarangi R, Newville M, Commisso J, Brown P. 2011.** Cellular sequestration of cadmium in the hyperaccumulator plant species *Sedum alfredii*. *Plant Physiology* **157**(4): 1914-1925.

**Tian SK, Lu LL, Yang XE, Labavitch JM, Huang YY, Brown P. 2009.** Stem and leaf sequestration of zinc at the cellular level in the hyperaccumulator *Sedum alfredii*. *New Phytologist* **182**(1): 116-126.

**Tikunov YM, Laptenok S, Hall RD, Bovy A, de Vos RCH. 2012.** MSClust: A tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. *Metabolomics* **8**(4): 714-718.

**Todeschini V, Lingua G, D'Agostino G, Carniato F, Roccotiello E, Berta G. 2011.** Effects of high zinc concentration on poplar leaves: A morphological and biochemical study. *Environmental and Experimental Botany* **71**(1): 50-56.

**Tsednee M, Yang SC, Lee DC, Yeh KC. 2014.** Root-secreted nicotianamine from *Arabidopsis halleri* facilitates zinc hypertolerance by regulating zinc bioavailability. *Plant Physiology* **166**(2): 839-852.

**Ueno D, Ma JF, Iwashita T, Zhao FJ, McGrath SP. 2005.** Identification of the form of Cd in the leaves of a superior Cd-accumulating ecotype of *Thlaspi caerulescens* using 113Cd-NMR. *Planta* **221**(6): 928-936.

**Ueno D, Milner MJ, Yamaji N, Yokosho K, Koyama E, Clemencia Zambrano M, Kaskie M, Ebbs S, Kochian LV, Ma JF. 2011.** Elevated expression of TcHMA3 plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *Plant Journal* **66**(5): 852-862.

**Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF. 2010.** Gene limiting cadmium accumulation in rice. *Proceedings of the National Academy of Sciences* **107**(38): 16500-16505.

**USEPA 1995.** Test Methods for Evaluating Solid Waste, Physical/Chemical Methods. Washington: United States Environmental Protection Agency.

**van de Mortel JE, Almar Villanueva L, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM. 2006.** Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and

the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **142**(3): 1127-1147.

**van de Mortel JE, Schat H, Moerland PD, Ver Loren van Themaat E, van der Ent S, Blankestijn H, Ghandilyan A, Tsiatsiani S, Aarts MGM. 2008.** Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* **31**(3): 301-324.

**van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H. 2013.** Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant and Soil* **362**((1-2)): 319-334.

**van der Ent A, Baker AJM, Reeves RD, Pollard AJ, Schat H. 2015.** Commentary: Toward a more physiologically and evolutionarily relevant definition of metal hyperaccumulation in plants. *Frontiers in Plant Science* **6**: 554.

**van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JA, Hooykaas PJ. 1999.** Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiology* **119**(3): 1047-1056.

**van Nevel L, Mertens J, Oorts K, Verheyen K. 2007.** Phytoextraction of metals from soils: how far from practice? *Environmental Pollution* **150**(1): 34-40.

**Verbruggen N, Hanikenne M, Clemens S. 2013.** A more complete picture of metal hyperaccumulation through next-generation sequencing technologies. *Frontiers in Plant Science* **4**: 388.

**Verbruggen N, Hermans C, Schat H. 2009.** Molecular mechanisms of metal hyperaccumulation in plants. *New Phytologist* **181**(4): 759-776.

**Villafort Carvalho MT, Amaral DC, Guilherme LRG, Aarts MGM. 2013.** *Gomphrena claussenii*, the first South American metallophyte species with indicator-like Zn and Cd accumulation and extreme metal tolerance. *Frontiers in Plant Science* DOI: 10.3389/fpls.2013.00180

**Villafort Carvalho MT, Pongrac P, Mumm R, van Arkel J, Aelst A, Jeromel L, Vavpetič P, Pelicon P, Aarts MGM. 2015.** *Gomphrena claussenii*, a novel metal-hypertolerant bioindicator species, sequesters cadmium, but not zinc, in vacuolar oxalate crystals. *New Phytologist* DOI: 10.1111/nph.13500

**Vogel-Mikuš K, Pongrac P, Pelicon P, Vavpetič P, Povh B, Bothe H, Regvar M. 2009.** *Micro-PIXE analysis for localization and quantification of elements in roots of mycorrhizal metal-tolerant plants*: Springer.

**Vogel-Mikuš K, Regvar M, Mesjasz-Przybylowicz J, Przybylowicz WJ, Simcic J, Pelicon P, Budnar M. 2008a.** Spatial distribution of cadmium in leaves of metal

hyperaccumulating *Thlaspi praecox* using micro-PIXE. *New Phytologist* **179**(3): 712-721.

**Vogel-Mikuš K, Simcic J, Pelicon P, Budnar M, Kump P, Necemer M, Mesjasz-Przybylowicz J, Przybylowicz WJ, Regvar M. 2008b.** Comparison of essential and non-essential element distribution in leaves of the Cd/Zn hyperaccumulator *Thlaspi praecox* as revealed by micro-PIXE. *Plant, Cell and Environment* **31**(10): 1484-1496.

**Wang M, Zheng Q, Shen Q, Guo S. 2013.** The Critical Role of Potassium in Plant Stress Response. *International Journal of Molecular Sciences* **14**(4): 7370-7390.

**Wang Z, Gerstein M, Snyder M. 2009.** RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics* **10**(1): 57-63.

**Weber M, Trampczynska A, Clemens S. 2006.** Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd2+-hypertolerant facultative metallophyte *Arabidopsis halleri*. *Plant, Cell and Environment* **29**(5): 950-963.

**Wójcik M, Vangronsveld J, D'Haen J, Tukiendorf A. 2005.** Cadmium tolerance in *Thlaspi caerulescens*: II. Localization of cadmium in *Thlaspi caerulescens*. *Environmental and Experimental Botany* **53**(2): 163-171.

**Wong CK, Cobbett CS. 2009.** HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*. *New Phytologist* **181**(1): 71-78.

**Wu C, Liao B, Wang SL, Zhang J, Li JT. 2010.** Pb and Zn accumulation in a Cd-hyperaccumulator (*Viola baoshanensis*). *International Journal of Phytoremediation* **12**(6): 574-585.

**Wu G, Kang H, Zhang X, Shao H, Chu L, Ruan C. 2010.** A critical review on the bio-removal of hazardous heavy metals from contaminated soils: issues, progress, eco-environmental concerns and opportunities. *J Hazard Mater* **174**(1-3): 1-8.

**Wu J, Zhao FJ, Ghandilyan A, Logoteta B, Guzman MO, Schat H, Wang X, Aarts MGM. 2009.** Identification and functional analysis of two ZIP metal transporters of the hyperaccumulator *Thlaspi caerulescens*. *Plant and Soil* **325**(1-2): 79-95.

**Wu LH, Liu YJ, Zhou SB, Guo FG, Bi D, Guo XH, Baker AJM, Smith JAC, Luo YM. 2013.** Sedum plumbizincicola X.H. Guo et S.B. Zhou ex L.H. Wu (Crassulaceae): A new species from Zhejiang Province, China. *Plant Systematics and Evolution* **299**(3): 487-498.

**Yang X, Li T, Yang J, He Z, Lu L, Meng F. 2006.** Zinc compartmentation in root, transport into xylem, and absorption into leaf cells in the hyperaccumulating species of *Sedum alfredii* Hance. *Planta* **224**(1): 185-195.

**Yang XE, Ye HB, Long XX, He B, He ZL, Stoffella PJ, Calvert DV. 2004.** Uptake and accumulation of cadmium and zinc by *Sedum alfredii* Hance at different Cd/Zn supply levels. *Journal of Plant Nutrition* **27**(11): 1963-1977.

**Zhao FJ, Jiang RF, Dunham SJ, McGrath SP. 2006.** Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*. *New Phytologist* **172**(4): 646-654.

**Zhao FJ, Lombi E, Breedon T, McGrath SP. 2000.** Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. *Plant, Cell and Environment* **23**(5): 507-514.

**Zhao FJ, Ma Y, Zhu YG, Tang Z, McGrath SP. 2015.** Soil contamination in China: Current status and mitigation strategies. *Environmental Science and Technology* **49**(2): 750-759.

**Zhao FJ, McGrath SP. 2009.** Biofortification and phytoremediation. *Current Opinion in Plant Biology* **12**(3): 373-380.

**Zhou W, Qiu B. 2005.** Effects of cadmium hyperaccumulation on physiological characteristics of *Sedum alfredii* Hance (Crassulaceae). *Plant Science* **169**(4): 737-745.

# SUMMARY

## *Gomphrena claussenii*, a Zn and Cd hyperbioindicator species

A small group of plant species called metallophytes have evolved the ability to grow in highly metal-enriched soils that are toxic to other plants. Some of these metal-tolerant species have evolved the ability to accumulate high levels of metals or metalloids, such as nickel (Ni), zinc (Zn), arsenic (As), cadmium (Cd) and lead (Pb). Important progress towards understanding the physiological and molecular basis of metal and metalloid homeostasis in plants has been made by studying these metal hyperaccumulator species, which may also be useful for as the basis of phytoremediation technologies in which plants are used to stabilize or extract metals from soil. *Gomphrena claussenii* Moq. (Amaranthaceae), is a previously uncharacterized plant species which grows in the metal-rich soils of a Zn mining area in the state of Minas Gerais, Brazil. This thesis describes the investigation of *G. claussenii* to determine the molecular basis of its ability to tolerate and accumulate Zn and Cd.

**Chapter 2** presents a detailed comparative investigation of the physiological impact of Zn and Cd exposure on *G. claussenii* and the closely-related non-tolerant species *G. elegans* Mart. growing in soil or in hydroponic conditions. The impact of Zn/Cd in each species was determined by measuring growth characteristics such as biomass and root elongation. It was found that *G. claussenii* plants growing in the field in the Zn mining area accumulated up to 10434 μg Zn and 96 μg Cd per gram of shoot dry weight. Under hydroponic conditions, *G. claussenii* tolerated up to 3000 μM Zn and up to 100 μM Cd, showing only slight metal toxicity symptoms at the highest concentrations and no significant decrease in biomass or root length. In contrast, *G. elegans* showed significant toxicity symptoms at 100 μM Zn and 5 μM Cd. It was also found that both

species accumulated more Zn and Cd in roots than shoots and that metal accumulation in *G. claussenii* showed a bioindicator-like response. Finally, the concentrations of other minerals such as Fe and Mn were not affected by Zn/Cd in *G. claussenii* shoots but declined dramatically in *G. elegans* in the presence of Zn/Cd. Taken together, these results indicated that *G. claussenii* is extremely tolerant to Zn and Cd and accumulates high levels of these metals in shoots, making it potentially valuable for phytoremediation applications.

**Chapter 3** addresses the distribution of Zn/Cd in *G. claussenii* stem and leaf tissues, and metabolic profiles were used to investigate the involvement of metabolites in the sequestration of Zn/Cd. *G. claussenii* plants were exposed to high concentrations of Zn/Cd and analysed by scanning electron microscopy using energy dispersive X-ray (SEM-EDX) and micro-proton-induced X-ray emission (micro-PIXE) technologies. We also investigated the dynamic profiles of primary metabolites in roots and shoots exposed to high levels of Zn/Cd to identify potential ligands for these metals. We observed the presence of abundant calcium oxalate (CaOx) crystals in the stem and leaf tissues of *G. claussenii* plants exposed to control and high levels of Cd, but intriguingly the number of crystals declined in the presence of Zn. Cd was shown to co-localize with calcium (Ca) in the CaOx crystals, indicating that Cd sequestration in vacuolar CaOx crystals in *G. claussenii* is part of a tolerance mechanism to deal with excess Cd accumulation. Furthermore, citrate, malate and oxalate levels all increased in the shoots of *G. claussenii* exposed to Zn/Cd suggesting these organic acids are involved in metal chelation and contribute to metal tolerance.

**Chapter 4** focuses on the molecular genetic aspects of hypertolerance in *G. claussenii*. The comparative transcriptomics analysis of *G. claussenii* and *G. elegans* was used to identify genes potentially responsible for the adaptation of *G. claussenii* to high

Zn/Cd exposure. The transcriptional response of both species to high Zn/Cd concentrations was investigated by RNA-Seq analysis. Transcript sequences were annotated, and differential expression induced by Zn/Cd exposure was analysed in *G. claussenii* and *G. elegans* roots and shoots. Orthologous transcript pairs were identified between both species, allowing the direct comparison of gene expression profiles. *G. elegans* showed a stronger transcriptional response to metal exposure than *G. claussenii*, featuring the significant modulation of 10–20 times as many genes. Many of these transcripts encode proteins involved in metal homeostasis or stress responses. Metal hypertolerance in *G. claussenii* therefore appears to be a constitutive expression trait, based on adaptations in the metal homeostasis and general stress response.

**Chapter 5** summarizes and evaluates the knowledge gained by the investigation set out in this thesis, focusing on the relevance of the information obtained from *G. claussenii* and its contribution to our current understanding of metal hypertolerance. It is also discussed the benefits of *G. claussenii* for phytoremediation applications, as well as its potential for future research activities.

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I am also thankful for the time I could share with other Brazilians in Wageningen. I would like to thank specially Ana Carolina, Debora, Renake, Manuela, Anibal, Marcos, Anderson, Zane, Paulo, Ana Paula, Nilma, Felipe e Marina. Your friendship warmed up the cold and cloudy days in the Netherlands.

Since Wageningen is an extremely international city I had the privilege to made many international friends. First, I am thankful for every friend we made in ICF. In special I would like to heartily thank our friends Miguel and Romi, Gerson and Maiga, Peter and Yuni, Gerwin and Phillipa, Daniel and Yelica for being part of our lives. I am also grateful to Bernhard, Karen, Pablo, Pamela, Red, Ellen and Enid for their company and friendship during these years.

Reconheco que o apoio e amor da minha familia, mesmo dos que estavam longe, foi indispensavel para a conclusao dessa etapa. Agradeco aos meus pais, Wagner e Maria Jose, por todo o carinho e dedicacao durante esses anos. Mesmo com a grande distancia e saudade, voces sempre nos apoiaram, suportaram e incentivaram nesse tempo. A presenca e o cuidado de voces fez o nosso tempo na Holanda muito mais felizes. Muito obrigada! Agradeco ao meu irmao Iori pela sua amizade e por ser tao amoroso. Sou grata pelo tempo que passamos juntos nesses anos, suas visitas alegraram o nosso tempo e diminuiram a nossa saudade. Agradeco a Tuquinha pelo carinho mesmo estando longe. Muito obrigado aos queridos amigos que durante os anos que estivemos na Holanda nos presentearam com as suas companhias.

Amor, thank you for giving up everything and going with me. Without your support and courage I think we would never get in The Netherlands. Thank you for backing me up always and for being my best friend. You were there when I presented my first seminar, you were there when I need help in the experiments, you were there when I rehearse my presentations so many times and you were always there to read my papers and check the English. We had a lot of challenges during these past years and I am grateful I was not alone. We also had lifetime experiences. In the end it was a great journey. I am very happy we went the two of us and came back the three of us. I am also thankful to my little son Joao who came and showed me that I was capable to love more than I knew. Amo muito voces dois!

## Curriculum Vitae

Mina Tomaz Villafort Carvalho was born on 30 May, 1987 in Belo Horizonte, Brazil. After obtaining her high school degree from "Cnec Cei" in Sete Lagoas, Brazil she moved to Lavras in 2005 to start her undergraduate studies at the Federal University of Lavras at the faculty of Biology. During her bachelor course she was involved on plant biotechnology research projects. In 2009 she started the master degree program of Soil Science at the Federal University of Lavras, Brazil. Her MSc thesis focused on the evaluation of *Gomphrena claussenii* Zn and Cd phytoextraction capacity.

In 2010, Mina moved to the Netherlands and started her PhD at the Laboratory of Genetics at Wageningen University under the supervision of Prof. Dr. Maarten Koornneef and Dr. Mark Aarts. Her PhD research focused on investigating the physiological and molecular genetic adaptation of *Gomphrena claussenii* under high zinc and cadmium exposure.

# List of publications

Villafort Carvalho MT, Amaral DC, Guilherme LRG, Aarts MGM. 2013. *Gomphrena claussenii*, the first South American metallophyte species with indicator-like Zn and Cd accumulation and extreme metal tolerance. *Frontiers in Plant Science* DOI: 10.3389/fpls.2013.00180

Villafort Carvalho MT, Pongrac P, Mumm R, van Arkel J, Aelst A, Jeromel L, Vavpetič P, Pelicon P, Aarts MGM. 2015. *Gomphrena claussenii*, a novel metal-hypertolerant bioindicator species, sequesters cadmium, but not zinc, in vacuolar oxalate crystals. *New Phytologist*. 208(3):763-75. DOI: 10.1111/nph.13500

# Education Statement of the Graduate School

## Experimental Plant Sciences

| | |
|---|---|
| Issued to: | Mina Tomaz Villafort Carvalho |
| Date: | 11 February 2016 |
| Group: | Laboratory of Genetics |
| University: | Wageningen University & Research Centre |

**1) Start-up phase** date

- ► **First presentation of your project**
  Molecular genetic and physiological characterization
  of Cd and Zn accumulation in *Gomphrena claussenii* Feb 28, 2011
- ► **Writing or rewriting a project proposal**
  Molecular genetic and physiological characterization
  of the adaptation of *Gomphrena claussenii* to Cd and Zn exposure Feb 2011
- ► **Writing a review or book chapter**
- ► **MSc courses**
  Plant Plasticity and Adaptation PPH 30806 Nov-Dec 2010
  Genetic Analysis, Tools and Concepts GEN 30306 Sep-Oct 2011
  Plant Biotechnology GEN-20806 Nov-Dec 2012
- ► **Laboratory use of isotopes**

*Subtotal Start-up Phase* *13.5 credits**

**2) Scientific Exposure**

- ► **EPS PhD student days**
  PhD student day 2012, Amsterdam Nov 30, 2012
  PhD student day 2013, Leiden Nov 29, 2013
- ► **EPS theme symposia**
  Theme 3 'Metabolism and Adaptation' 2011 Feb 07, 2011
  Theme 4 'Genome Biology' 2011, Dec 09, 2011

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| | Theme 3 'Metabolism and Adaptation' 2013 | Mar 22, 2013 |
| | Theme 3 'Metabolism and Adaptation' 2014, | Mar 11, 2014 |
| ► | **NWO Lunteren days and other National Platforms** | |
| | NWO-ALW 'Experimental Plant Sciences', Lunteren (NL) | Apr 04-05, 2011 |
| | NWO-ALW 'Experimental Plant Sciences', Lunteren (NL) | Apr 02-03, 2012 |
| | NWO-ALW 'Experimental Plant Sciences', Lunteren (NL) | Apr 14-15, 2014 |
| ► | **Seminars (series), workshops and symposia** | |
| | Plant Science Seminar Series | 2010/2014 |
| | Seminar: 'Illumina Sequencing' | Dec 07, 2011 |
| | Seminar: 'Chlorophyll Fluorescens as a powerful sensor' | Mar 01, 2011 |
| | Seminar: 'Genetic modification for iron biofortification and drought tolerance in rice' | Jun 29, 2012 |
| | Seminar: 'Plant versus virus: defense, counter defense and counter counter defense' | Oct 10, 2012 |
| | Plant Genome Mining (CBSG Bioinformatics Workshop) | Dec 13, 2012 |
| | Seminar: 'Multiple-stress management: what can we learn from plants?' | Dec 20, 2012 |
| | Mini-symposium 'Writing for high impact journals' | Aug 02, 2013 |
| | Wageningen PhD Symposium | Dec 10, 2013 |
| | Symposium: Plant Metabolomics | Dec 12, 2013 |
| | Seminar: 'Multifunctional sugars' | Dec 13, 2013 |
| ► | **Seminar plus** | |
| ► | **International symposia and congresses** | |
| | Plan Growth, Nutrition and Environment Interactions, Vienna (Austria) | Feb 18-21, 2012 |
| | Plant Abiotic Stress Tolerance II, Vienna (Austria) | Feb 22-25, 2012 |
| | International Plant Nutrition Colloquium, Istanbul (Turkey) | Aug 19-22, 2013 |
| ► | **Presentations** | |
| | Poster: Plan Growth, Nutrition and Environment Interactions | Feb 18-21, 2012 |
| | Presentation: EPS Theme3 "Metabolism and Adaptation", Amsterdam | Mar 22, 2013 |
| | Presentation: International Plant Nutrition Colloquium, Istanbul | Aug 19-22, 2013 |

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|---|---|---|
| | Presentation: ALW meeting 'Experimental Plant Sciences', Lunteren - | Apr 14-15, 2014 |
| ► | **IAB interview** | |
| | Meeting with a member of the International Advisory Board of EPS | Nov 14, 2012 |
| ► | **Excursions** | |
| | *Subtotal Scientific Exposure* | *14.6 credits** |

**3) In-Depth Studies**

| | | |
|---|---|---|
| ► | **EPS courses or other PhD courses** | |
| | Bioinformatics: a User's Approach | Aug 27-31, 2012 |
| | The Power of RNA-seq | Jun 05-07, 2013 |
| | Transcription Factors and Transcriptional Regulation | Dec 17-19, 2013 |
| | School of Plant Metallomics | Jan 27-31, 2014 |
| ► | **Journal club** | |
| | Member of a literature diiscussion group of Plant Genetics | 2010-2014 |
| ► | **Individual research training** | |
| | *Subtotal In-Depth Studies* | 7.9 credits* |

**4) Personal development**

| | | |
|---|---|---|
| ► | **Skill training courses** | |
| | Information Literacy including EndNote Introduction | Apr 12-13, 2011 |
| | Competence Assessments | Jan 18-Feb 17, 2011 |
| | Techniques for writing and presenting a scientific paper | Oct 15-18, 2013 |
| | ExPectationS (EPS Career day 2010) | Nov 19, 2010 |
| | ExPectationS (EPS Career day 2011) | Nov 18, 2011 |
| ► | **Organisation of PhD students day, course or conference** | |
| | Organizer of Plant Genetics Literature Discussion | Sep 2012-Feb 2014 |
| ► | **Membership of Board, Committee or PhD council** | |
| | *Subtotal Personal Development* | *4.2 credits** |

**TOTAL NUMBER OF CREDIT POINTS*** **40.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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