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Transcriptional regulation of genes involved in Zn transport after foliar Zn application to *Medicago sativa*

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One-sentence summary:

Upregulation of *ZIP2*, *NAS1* and *HMA4* and downregulation of *ZIP3* are associated with Zn sequestration and shoot-to-root translocation in *Medicago sativa* following foliar Zn biofortification

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34 **Author contributions**

35 EP and LE conceived the ideas; AC, EP, LE designed the methodology; AC and BM designed and
36 performed real-time PCR; AC and CM performed chemical analyses; AC and EP performed data
37 analysis; AC, EP, PJW, LE led the writing of the manuscript; all authors contributed critically to the
38 drafts and gave final approval for publication.

39

ABSTRACT

Zinc (Zn) is an essential micronutrient for both plants and animals, and Zn deficiency is one of the most widespread problems for agricultural production. Although many studies have been performed on the biofortification of staple crops with Zn, few studies have focused on forage crops. In this study the molecular mechanisms of Zn transport-related in *Medicago sativa* L. were investigated following foliar Zn applications aimed at increasing the accumulation of Zn in edible tissues. Zinc uptake and redistribution between shoot and root were determined following the application of six Zn doses to leaves (0, 0.01, 0.1, 0.5, 1, 10 mg Zn plant^{-1}). Twelve putative genes encoding proteins involved in Zn transport (*MsZIP*$_{1-7}$, *MsZIF1*, *MsMTP1*, *MsYSL1*, *MsHMA4* and *MsNAS1*) were identified and the changes in their expression following foliar Zn application were quantified using newly designed RT-qPCR assays. Shoot and root Zn concentration was increased following foliar Zn applications $\geq$ 0.1 mg plant^{-1}. Increased expression of *MsZIP2*, *MsHMA4* and *MsNAS1* in shoots, and of *MsZIP2* and *MsHMA4* in roots, was observed with the largest Zn dose. By contrast, *MsZIP3* was downregulated in shoots at Zn doses $\geq$ 0.1 mg plant^{-1}. Three functional modules were identified in the *M. sativa* response to foliar Zn application: genes involved in Zn uptake by cells, genes involved in vacuolar Zn sequestration and genes involved in Zn redistribution within the plant. These results will inform genetic engineering strategies aimed at increasing the efficiency of crop Zn biofortification.

KEY WORDS:

Zinc, micronutrient, biofortification, alfalfa (*Medicago sativa*), ZIP transporters, Nicotianamine, Metal Tolerance Protein (MTP), Yellow Stripe-Like Protein (YSL), Zinc Induced facilitators (ZIF), Heavy Metal transporters (HMA), cellular zinc homeostasis.

INTRODUCTION

Food quality is a key factor for human health (Geissler and Powers, 2017). For their well-being, humans require sufficient quantities of at least 18 mineral elements, which have specific physiological roles and are irreplaceable in the diet (White, 2016). Eight essential macronutrients (i.e., N, P, S, Ca, Mg, K, Na, Cl) are required in large amounts in the diet (> 100 mg day^{-1}), and 10 micronutrients are required in smaller amount (e.g., Zn, Fe, F, Mn). The main sources of these elements in the human diet include edible crops, animal products (e.g., meat, fish, eggs) and dairy products (e.g., milk, cheese, butter) as well as mineral supplements (Keen, 1990; Prasad, 2013; White, 2016). A large proportion of the world's population suffers from Zn related diseases (i.e., malabsorption syndrome, liver disease, chronic renal disease, sickle cell disease and other chronic diseases), since it relies on cereal-based diets with low Zn content due to poor soil Zn availability (WHO, 2005; Alloway, 2009; Prasad, 2013; Kumssa et al., 2015; Cakmak et al., 2017). Diversification of the human diet and biofortification of edible crops are therefore needed to alleviate Zn deficiency in humans. Similarly, increasing Zn concentrations in forage crops are important for maintaining livestock health and the quality of food products, which affect human health indirectly (McDonald et al., 2002; Ciccolini et al., 2017; Capstaff and Miller, 2018; Huma et al., 2019).

Zinc plays a major role as a co-factor of over 300 enzymes in plants and is an essential micronutrient (Broadley et al., 2007). Zinc is involved in various physiological functions, such as CO_2 fixation, protein synthesis, free radical capture, regulation of growth and development, and disease resistance (Sasaki et al., 1998; Broadley et al., 2007). Many structural motifs in transcriptional regulatory proteins are stabilized by Zn, such as Zn finger domains (Albert et al., 1998). Zinc deficiency reduces crop production, as does Zn excess (White and Pongrac, 2017). Excessive Zn^{2+} can compete with other cations in binding to enzymes and for transport across membranes, thereby impairing cellular activities (White and Pongrac, 2017). Thus, the uptake of Zn^{2+} by cells and its transport within the plant must be strictly regulated. Plant cells have evolved

several homeostatic mechanisms for avoiding Zn^{2+} toxicity when exposed to large Zn availability in their environment. These include the reduction of Zn influx to cells, the stimulation of Zn efflux from the cytosol, the sequestration of Zn in vacuoles, and the chelation of Zn by Zn binding ligands. In general, the concentration of Zn in plant tissues must be kept between 15 to 300 µg Zn g^{-1} dry matter (DM) to maintain cell structure and function (Broadley et al., 2012; White and Pongrac, 2017). Although tolerance to large tissue Zn concentrations varies among species (Alloway, 2008; White and Pongrac, 2017), Zn concentrations above 400-500 µg g^{-1} DM often cause toxicity symptoms including impaired root and shoot growth, chlorosis and necrosis of leaves, reduced photosynthesis, nutrient imbalance and ultimately loss of yield (Chaney, 1993; Broadley et al., 2007; Di Baccio et al., 2009; White and Pongrac, 2017).

The process of producing crops with greater mineral concentrations in edible tissues is called biofortification and provides a solution to the problem of mineral deficiencies in human and animal nutrition (White and Broadley, 2005). There are various approaches to Zn biofortification of edible crops, including agronomic strategies and conventional or transgenic breeding strategies. Agronomic biofortification aims to increase Zn concentrations in edible tissues through the application of Zn-fertilisers to the soil or to leaves. It is relatively inexpensive and efficient (Saltzman et al., 2013). Foliar application of Zn is generally more effective than the application of Zn fertilisers to soil, since Zn uptake by plant roots is often limited by the low solubility of Zn salts, its binding to organic substrates, and its immobilization in the microbial biomass (Gregory et al., 2017). Both agronomic and genetic biofortification strategies have been studied extensively in cereal staple crops, such as rice, wheat and maize, but less in legumes, such as beans, peas or lentils (White and Broadley, 2005, 2011; Rawat et al., 2013). An international program, the HarvestPlus Zinc Fertilizer Project, is exploring the potential of Zn fertilisers to enhance the yields and Zn concentrations in edible portions of staple crops in developing countries of Africa, Asia and South America (www.harvestzinc.org) (Cakmak, 2012), but this program does not include forage crops.

The natural direction of Zn flux in plants is from the soil via roots to the shoot and seeds (White

and Broadley, 2009). Various transport proteins and ligands that are responsible for Zn^{2+} uptake by roots and its transport and sequestration within the plant have been characterized (Olsen and Palmgreen, 2014; Caldelas and Weiss, 2017; White and Pongrac, 2017). Among these, ZRT-IRT-like Proteins (ZIPs), have been studied in several plants, including *Arabidopsis thaliana*, soybean (*Glycine max*), barley (*Hordeum vulgare*), barrel medic (*Medicago truncatula*) and rice (*Oryza sativa*) (Grotz et al., 1998; Zhao and Eide, 1996; López-Millán et al., 2004; Milner et al., 2013; Tiong et al., 2015). These proteins not only transport Zn^{2+} across membranes, but can also transport other transition metal cations, including Cd^{2+}, Fe^{3+}/Fe^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} (Grotz et al., 1998; Mäser et al., 2001, Eckhardt et al., 2001). Generally, the expression of ZIP genes is upregulated when plants become Zn deficient (Ramesh et al., 2003; Ishimaru et al., 2006; Eide et al., 1996), facilitating Zn influx to cells and movement of Zn between organs, and also when plants become Fe or Mn deficient (Bughio et al., 2002; Vert et al., 2002; Ishimaru et al., 2006; Pedas et al., 2008). Other proteins that transport Zn include the Metal Tolerance Proteins (MTPs), which function as cation/proton antiporters and are thought to transport Zn into vacuoles (Kolaj-Robin et al., 2015) and the Yellow Stripe-Like Proteins (YSLs), which transport the Zn-Nicotianamine complex (NA-Zn) and load Zn into the xylem and phloem (Curie et al., 2009). The Zinc Induced Facilitators (ZIFs) and the Heavy Metal transporters (HMAs) are implicated in Zn influx to vacuoles and to the xylem, respectively (Olsen and Palmgren, 2014). Zinc is chelated by organic molecules, such as the carboxylic acid, citric acid, and nicotianamine (NA) in plants (Sinclair and Krämer, 2012). Nicotianamine is a non-proteinogenic amino acid with a high affinity for Fe, Cu and Zn, and is involved in their homeostasis (Deinlein et al., 2012). Nicotianamine mediates the intercellular and interorgan movement of Zn and was found to enable Zn hyperaccumulation in *Arabidopsis halleri* and *Noccaea caerulescens* (Deinlein et al., 2012; Foroughi et al., 2014) In general the functions of these transporters have been studied by expressing them in yeast, but to understand how the various Zn transport proteins and chelates act together to maintain appropriate cytosolic and tissue Zn concentrations it is important to study the responses of an intact plant to

144 fluctuations in Zn supply.

145 In this study the transcriptional responses of genes encoding Zn transport-related processes
146 facilitating Zn uptake by cells, vacuolar sequestration and redistribution within the plant were
147 studied following foliar Zn application to the most productive and widely cultivated forage legume,
148 alfalfa (*Medicago sativa* L.). The study was designed to provide information on the molecular
149 responses to Zn biofortification of forage crops (Foyer et al., 2016; Capstaff and Miller, 2018). The
150 following hypotheses were tested: i) foliar application of Zn increases shoot and root Zn
151 concentrations, which results in changes in the expression of genes involved in Zn transport-related
152 processes to detoxify excess Zn; ii) genes encoding Zn transport-related processes are organized in
153 functional modules, that act in a concerted manner to redistribute Zn within the plant to maintain
154 non-toxic cytosolic and tissue Zn concentrations. Genes encoding putative Zn transport-related
155 processes were identified in alfalfa through phylogenetic comparisons and their likely roles are
156 discussed. Changes in the expression of these genes following foliar Zn application were
157 determined and the possible effects of these on the redistribution of Zn within cells and between
158 tissues are discussed. The knowledge gained from this study could help to optimize Zn
159 biofortification strategies when using foliar Zn fertilisers and to provide strategies for breeding
160 forage crops to addresses Zn deficiencies in livestock.

161

162 **RESULTS**

163

164 **Shoot and root Zn concentrations**

165 The application of Zn to leaves did not modify shoot or root biomass and all *M. sativa* plants
166 had root nodules (data not shown). However, Zn concentrations in both shoots and roots were
167 strongly affected by foliar Zn application ($F_{(5,17)}$=32.61, P<0.001; $F_{(5,\ 17)}$=28.53, P<0.001;
168 respectively) (Fig. 1). A foliar Zn application of 0.01 mg Zn plant^{-1} produced a shoot Zn
169 concentration similar to that of the control (no-Zn addition), but shoot Zn concentrations were

increased progressively by larger doses (0.1 < 0.5/1 < 10 mg Zn plant^{-1}), from more than threefold to 35-fold more than that of the control (Fig. 1). Foliar applications of 0.01, 0.1 and 0.5 mg Zn plant^{-1} did not produce root Zn concentrations greater than that of the control treatment, but foliar doses of 1 and 10 mg Zn plant^{-1} increased root Zn concentrations to threefold and 11-fold more than the control treatment, respectively. Shoot and root Zn contents were also strongly affected by foliar Zn application ($F_{(5,17)}$=53.73, P<0.001; $F_{(5, 17)}$=32.45, P<0.001; respectively) and their responses to increasing foliar Zn applications followed the corresponding Zn concentrations (Supplemental Fig. S1).

Phylogenetic analysis

Phylogenetic analysis of the coding sequences of the *ZIP* genes revealed several distinct clades (Supplemental Fig S2). One clade contained sequences for *MsZIP2* and *MsZIP7*, which were similar to each other. In addition, the sequence of *MsZIP2* was closely related to those of *MtZIP2* and *GmZIP1-ZIP2* and the sequence of *MsZIP7* was closely related to those of *MtZIP7* and *AtZIP11*. Another clade contained the sequences of *MsZIP1*, *MsZIP3*, *MsZIP5* and *MsZIP6*. The sequence of *MsZIP1* clustered with that of *MtZIP1*. Sequences of *MsZIP3* and *MsZIP5* were similar to each other and clustered with the corresponding sequences for *M. truncatula* genes (Supplemental Fig. S2). Sequences for *MsZIP1*, *MsZIP3* and *MsZIP5* were closely related to each other, whereas that of *MsZIP6* formed a separate cluster with the sequences of *MtZIP6* and *AtZIP12*. The sequence of *MsZIP4* was distant from the sequences of other *M. sativa ZIPs* and formed a cluster with the sequences of *MtZIP4* and *AtZIP4*.

Phylogenetic analyses of the coding sequences of the other genes related to Zn transport processes revealed that they were all similar to their *M. truncatula* counterparts. As regards *ZIF*, the sequence of *MsZIF1* clustered with the sequences of *MtZIF1* and *GmZIF1* (Supplemental Fig. S3a). As regards *MTP*, the sequence of *MsMTP1* formed a cluster with *MtMTP1* and *GmMTP1* and was also related to *AtMTP1* and *AtMTPA1* (Supplemental Fig. S3b). Similarly, the sequence of *MsYSL1*

196 was most similar to those of *MtYSL1* and *GmYSL1* (Supplemental Fig. S3c) and the sequence of
197 *MsHMA4* was most similar to those of *MtHMA4* and *GmHMA4* (Supplemental Fig. S3d). Finally,
198 the sequence of *MsNAS1* was closely related to those of *MtNAS* and *GmNAS* (Supplemental Fig.
199 S3e).
200
201 **Gene expression analysis**
202 The expression of *MsZIP3* was significantly downregulated at foliar doses of 0.1, 1 and 10 mg
203 Zn plant^{-1} ($F_{(3, 11)}$ = 28.46, P<0.01) (Fig. 2). By contrast, the expression of *MsZIP2* was significantly
204 upregulated at the largest dose of 10 mg Zn plant^{-1} ($F_{(3, 11)}$ = 5.59, P<0.05). The expression of
205 *MtZIP1*, *MtZIP5* and *MtZIP6* in shoots was not significantly affected by foliar Zn application,
206 although a general trend towards downregulation with increasing foliar Zn doses was observed. The
207 expression of *MsZIP4* and *MsZIP7* in shoots was unaffected by foliar Zn application. The
208 expression of *MsZIP2* was significantly upregulated in roots at the largest foliar dose of 10 mg Zn
209 plant^{-1} ($F_{(3, 11)}$=9.26, P<0.01 (Fig. 2). In roots, *ZIP* genes were not significantly affected by foliar Zn
210 application, although a general trend of *MtZIP1*, *MtZIP3*, *MtZIP5* and *MtZIP7* towards upregulation
211 with increasing foliar Zn doses was observed. Of the other genes related to Zn transport processes,
212 the expression of *MsHMA4* was significantly upregulated in both shoots ($F_{(3, 11)}$ = 115.29, P<0.01)
213 and roots ($F_{(3, 11)}$ = 14.23, P<0.01) following the application of 1 and 10 mg Zn plant^{-1} (shoots:
214 +63% and +424%, respectively; roots: +86% and +66%, respectively; Fig. 3). In shoots, the
215 expression of *MsHMA4* was about 3-fold higher following a dose of 10 mg Zn plant^{-1} than
216 following a dose of 1 mg Zn plant^{-1}, whereas the expression of *MsHMA4* in roots was similar when
217 1 or 10 mg Zn plant^{-1} was applied. The expression of *MsNAS1* was also significantly upregulated
218 ($F_{(3, 11)}$ = 6.46, P< 0.05) at the largest foliar Zn dose (10 mg plant^{-1}), whereas its expression in roots
219 was unaltered following foliar Zn application (Fig. 3). In shoots, *MsYSL1* and *MsZIF1* were not
220 significantly affected by foliar Zn application, although there was a trend towards upregulation of
221 the expression with increasing Zn doses, while the expression of *MsMTP1* remained unaltered

following the application of Zn (Fig. 3). Finally, in roots, *MsMTP1* and *MsZIF1* were not significantly affected by foliar Zn application, although there was a trend towards upregulation of the expression with increasing Zn doses, whereas the expression of *MsYSL1* remained unchanged (Fig. 3).

Using correlation analysis to reveal functional modules of genes whose expression is co-regulated in plants, three functional modules for ZIP gene co-expression were observed (Fig. 4a; $r >$ 0.6). In the first functional module, the expression of *MsZIP1*, *MsZIP3*, *MsZIP5* and *MsZIP6* in shoots were all strongly correlated. Although the expression of *MsZIP7* in shoots was also placed in this module, its expression was poorly correlated with the expression of other *ZIP* genes in either shoots or roots. The second functional module comprised correlations between the expression of *MsZIP4* in shoots and roots, and the expression of *MsZIP1*, *MsZIP3*, *MsZIP4*, *MsZIP5* and *MsZIP6* in roots. The third functional module comprised correlations in the expression of *MsZIP2* in shoots and roots and *MsZIP7* in roots. With respect to the expression of the other genes involved in Zn transport related processes, there was a strong divergence in their expression patterns in shoots and roots (Fig. 4b). In the root, the expression of *MsNAS1*, *MsYSL1, MsZIF1* and *MsMTP1* were strongly correlated (Fig. 4b). The expression of *MsYSL1*, *MsZIF1* and *MsMTP1* in roots showed good correlations with the expression of *MsMTP1* and *MsZIF1* in shoots. In the shoot the expression of *MsYSL1*, *MsMTP1* and *MsZIF1* were all strongly correlated. Also in shoots, the expression of *MsNAS1* and *MsHMA4* were well correlated and showed a good correlation with the expression of *MsMTP1* and *MsZIF1* in shoots. Interestingly, the expression of *MsNAS1* strongly correlated with that of *MsYSL1* in shoots.

PERMANOVA showed that the expression of *ZIP* genes was significantly affected by foliar Zn application dose and differed between shoots and roots, which explained 29% and 23% of the total variance, respectively (Table 1). The expression of other genes related to Zn transport processes that were studied (*MsZIF1*, *MsNAS1*, *MsHMA4*, *MsYSL1* and *MsMTP1*) were also affected by foliar Zn application dose and the organ examined. Zinc application dose explained 17% of the total variance,

248 while plant organ explained 19%. PERMANOVA on all studied genes highlighted a significant
249 effect of Zn application dose, plant organ and their interaction on gene expression, explaining 68%
250 of the total variance.
251
252 **DISCUSSION**
253
254 **Plant Zn nutritional status after foliar Zn application**
255 The critical leaf concentration for Zn deficiency approximates 15–20 μg Zn g^{-1} dry weight and
256 the critical leaf concentration for Zn toxicity approximates 400-500 μg Zn g^{-1} (Broadley et al.,
257 2012; White and Pongrac, 2017). Before foliar Zn application, the alfalfa plants used in the
258 experiments reported here were probably Zn deficient, since their shoot Zn concentrations were
259 below the critical leaf concentration for Zn deficiency (Fig. 1). After the application of the lowest
260 foliar Zn dose (0.01 mg $plant^{-1}$) plants probably remained Zn deficient (7.6 μg Zn g^{-1} dry weight),
261 but all other foliar Zn doses increased Zn concentrations in shoots above the critical concentration
262 for Zn deficiency (Fig. 1). Plants treated with 0.1 mg Zn $plant^{-1}$ probably had an optimal Zn status
263 for plant growth, whereas plants treated with 0.5 and 1 mg Zn $plant^{-1}$ had shoot Zn concentrations
264 close to the toxicity threshold. When a foliar dose of 10 mg Zn $plant^{-1}$ was applied, shoot Zn
265 concentrations greatly exceeding the threshold for Zn toxicity (Fig. 1). Plants often exhibit
266 characteristic visual symptoms of Zn deficiency and Zn toxicity when these occur (Broadley et al.,
267 2012; White and Pongrac, 2017), but five days after foliar Zn application no visual symptoms of Zn
268 deficiency or toxicity, nor differences in plant biomass, were observed among plants receiving
269 contrasting foliar Zn doses (data not shown). Foliar Zn doses larger than 0.1 mg Zn $plant^{-1}$ resulted
270 in incremental increases in the Zn concentration of roots (Fig. 1), despite Zn having limited
271 mobility in the phloem (White and Broadley, 2011; White, 2012). This observation suggests that
272 roots can act as a sink for Zn applied to leaves, thereby mitigating excessive Zn accumulation in
273 shoot tissues. In previous work, foliar application of Zn was shown to increase Zn concentration in

phloem-fed tissues, such as fruits, seed, and tubers (Cakmak, 2004, 2008; Cakmak et al., 2010; White et al., 2017). The shoot to root Zn concentration ratio shifted from values below one in conditions of Zn deficiency (0.4) to values greater than one in Zn-replete or Zn-intoxicated plants (1.3 - 3.2) (Fig. 1). When the plants are Zn deficient the recirculation of Zn between organs via the xylem and phloem is required to meet minimal growth demands and the application of foliar Zn to Zn deficient plants must be effectively redistributed within the plant (Erenoglou et al., 2011; Sinclair and Kramer, 2012), whereas when excessive foliar Zn is applied, Zn must be chelated in the cytoplasm, sequestered in the vacuole and redistributed via the phloem or xylem to other organs to avoid toxicity (White and Pongrac, 2017).

Foliar Zn application alters the expression of genes involved in Zn transport-related processes

Despite several genes encoding Zn transporters having been identified in plants, and the encoded proteins characterized, the mechanisms of Zn uptake and transport in alfalfa are still largely unknown. However, the recently sequenced alfalfa genome has allowed the discovery of genes involved in Zn uptake and distribution within this species (O'Rourke et al., 2015). In the present study, 12 putative genes encoding proteins likely to be involved in processes related to Zn transport in the plant (*MsZIP*$_{1\text{-}7}$, *MsZIP1*, *MsMTP1*, *MsYSL1*, *MsHMA4* and *MsNAS1*) were identified and their expression in shoots and roots quantified following foliar Zn applications (Fig. 2; Fig. 3). The expression of genes encoding proteins involved in Zn uptake by cells, vacuolar sequestration and redistribution within the plant responded differently to increasing foliar Zn dose. The expression of *MsZIP2*, *MsHMA4* and *MsNAS1* in shoots was increased by increasing foliar Zn dose, while only the expression of *MsZIP2* and *MsHMA4* in roots were upregulated by increasing foliar Zn dose (Fig. 2; Fig. 3). By contrast, *MsZIP3* was downregulated in shoots when foliar Zn doses $\geq$ 0.1 mg Zn plant^{-1} were applied (Fig. 2). These changes in gene expression might produce a reduction in Zn uptake capacity (but not necessarily a reduction in actual Zn uptake since this will also be related to apoplastic Zn concentration) by cells in both the shoot and root (by reducing Zn

300 influx through *MsZIP3* and increasing Zn efflux through *MsHMA4*), chelation of Zn using Zn-NA
301 in the shoot for Zn-detoxification and phloem transport, and greater recirculation of Zn within the
302 plant in both the phloem and xylem (by increasing NA concentrations and *MsZIP2* and *MsHMA4*
303 activities), and are, therefore, consistent with the observation that increasing foliar Zn dose
304 increases both shoot and root Zn concentrations.
305
306 **Regulation of genes encoding ZIP transporters**
307 The influx and efflux of Zn across the plasma membrane of plant cells must be tightly
308 controlled to allow optimal cell functioning and hence to ensure normal plant growth and
309 development (Sinclair and Krämer, 2012). The expression of only two of the seven *ZIP* genes
310 studied, *MsZIP2* and *MsZIP3*, showed statistically significant responses to foliar Zn application
311 (Fig. 2). The expression of *MsZIP2* was significantly upregulated in both shoots and roots in
312 response to the largest dose of foliar Zn applied (10 mg Zn plant^{-1}). It is likely that this dose is toxic
313 to both shoot and root cells. The relative induction in the expression of *MsZIP2* was greatest in
314 roots. The phylogenetic analysis of ZIP transporters revealed that *MsZIP2* is closely related to
315 *MtZIP2* and *AtZIP2* (Supplemental Fig. S6). Thus, *MsZIP2* is probably located in the plasma
316 membrane performing similar functions to *MtZIP2* and *AtZIP2*. Burleigh et al. (2003) reported that
317 *M. truncatula* plants grown with adequate soil Zn availability expressed *MtZIP2* in roots and stems,
318 but not in leaves. The expression of *MtZIP2* in roots increased with increasing Zn fertiliser
319 applications to soil, with the greatest expression being found at toxic Zn doses (Burleigh et al.,
320 2003). Similarly, Milner et al. (2013) found that the expression of *AtZIP2* was ~10-fold higher in
321 roots than shoots in Zn-replete *Arabidopsis thaliana* plants and that Zn deficiency reduced the
322 expression of *AtZIP2* in both roots and shoots. The localization of *ZIP2* at the plasma membrane
323 was observed in both *M. truncatula* (Burleigh et al., 2003) and *A. thaliana* (Milner et al., 2013). The
324 expression of *AtZIP2* was localized to the stele of the root (Milner et al., 2013), supporting a role of
325 *AtZIP2* in long distance transport of Zn between roots and shoots. It is possible that the increased

326 expression of *MsZIP2* observed in our study when plants experience Zn toxicity might be a
327 detoxification strategy, either through storing excess Zn in xylem parenchyma cells or recirculating
328 Zn in the xylem.

329 The expression of *MsZIP3* was significantly downregulated in shoots following the foliar
330 application of Zn (Fig. 2). The ZIP3 transporter is thought to mediate Zn influx to the cell from the
331 apoplast (Sinclair and Kramer, 2012). Therefore, the downregulation of *MsZIP3* in shoots of plants
332 receiving more Zn is consistent with the ability of plant cells to control their Zn uptake to effect
333 cytoplasmic Zn homeostasis. Reduced expression of *MsZIP3* in plants with a greater Zn supply is
334 also in agreement with previous studies of *M. truncatula* and *A. thaliana* (Grotz et al., 1998; Lopéz-
335 Millán et al., 2004). However, although *AtZIP3* could restore growth to a Zn-uptake defective yeast
336 (Milner et al., 2013), *MtZIP3* was not found to be able to restore the growth of a Zn-uptake
337 defective yeast in Zn-limited media, although it did restore the growth of a Fe-uptake defective
338 yeast in Fe-limited media (López-Millán et al., 2004). Thus, the MsZIP3 transporter could have a
339 higher affinity for Fe than Zn. In *O. sativa ZIP3* gene is expressed in the xylem parenchyma and
340 transfer cells and might be responsible for unloading transition metal cations from the xylem to the
341 parenchyma in plants receiving an excessive Zn supply (Sasaki et al., 2015). The role of OsZIP3 in
342 unloading Zn from the vascular tissues, suggests that the reduced expression of *MsZIP3* in shoots of
343 *M. sativa* receiving an excessive foliar Zn dose might be a detoxification strategy to reduce Zn
344 uptake by shoot cells.

345 The observation that foliar Zn applications had no effect on the expression of ZIP genes, except
346 *MsZIP2* and *MsZIP3* (Fig. 2), might be explained by the roles of ZIP proteins in the transport of
347 other transition metals. For example, evidence of Cu and Mn transport by *ZIP4* were provided
348 through yeast complementation studies (Wintz et al., 2003; López-Millán et al., 2004). Moreover,
349 applying the same technique, a role of *ZIP6* was highlighted in the transport of Fe by López-Millán
350 et al. (2004), whereas Wintz et al. (2003) did not find any involvement of *ZIP6* in the transport of
351 Cu, Zn or Fe. Although the changes in the expression of *MsZIP1*, *MsZIP5* and *MsZIP6* following

352 foliar Zn application were not statistically significant, changes in their expression in shoots were
353 positively correlated with changes in the expression of *MsZIP3*, showing a general trend for them to
354 be downregulated following foliar Zn application and suggesting that these four ZIPs might act as a
355 functional module in the shoot (Fig. 4). By contrast, the expression of *MsZIP1, MsZIP3, MSZIP4*,
356 and *MsZIP5* were positively correlated in roots, suggesting that these genes behave as a functional
357 module in roots (Fig. 4).
358
359 **Regulation of genes encoding other Zn transport-related processes**
360 The expression of *MsHMA4*, which is implicated in Zn redistribution within the plant (Hussain
361 et al., 2004; Sinclair et al., 2018), was increased in both shoots and roots of plants whose shoot Zn
362 concentration suggested they were close to, or experiencing, Zn toxicity (Fig. 3). The significant
363 upregulation of *MsHMA4* following foliar application of ≥ 1 mg Zn plant^{-1} might be related to the
364 removal of excess Zn from both shoots and roots. This interpretation is consistent with the role of
365 HMA4 in *A. thaliana* and in the metal hyperaccumulators *Arabidopsis halleri* and *Noccea*
366 *caerulescens* (Baker and Whiting, 2002; Hussain et al., 2004; Hanikenne et al., 2008; Ò Lochlainn
367 et al., 2011; White and Pongrac, 2017), in which greater expression of *HMA4* results in greater Zn
368 flux to the xylem and Zn translocation to transpiring leaves. However, the phylogenetic similarity of
369 *MsHMA4* to *MtHMA4* and, particularly, to *AtHMA5* (Supplemental Fig. S3d) suggest a role in Cu
370 transport (Andrés-Colás et al., 2006; Sankaran et al., 2009; Hermand et al., 2014). This implication
371 of the latter observation is unclear.
372 Since Zn^{2+} concentrations are low in the alkaline phloem sap, the transport of most Zn in the
373 phloem is as Zn ligand complexes, such as zinc-nicotianamine (NA-Zn) (Deshpande et al., 2018).
374 Nicotianamine is the main Zn chelate in phloem transport and is also important for Zn sequestration
375 in vacuoles (Deinlein et al., 2012), and tolerance of excessive Zn uptake (Aarts et al., 2014).
376 Nicotianamine concentrations generally correlate with those of *NAS* transcripts, and for this reason
377 *NAS* expression can be used as a proxy for NA content (Talke et al., 2006; Haydon et al., 2012).

Accordingly, in the work reported here the increased expression of *MsNAS1* in shoots following the application of ≥ 1 mg Zn plant^{-1} (Fig. 3) probably reflects the role of NA in Zn detoxification through its sequestration within vacuoles and its redistribution from shoot to root after excessive foliar Zn applications. This observation is consistent with Deshpande et al. (2018), who found that the expression of *NAS2* in the durum wheat (*Triticum durum* Desf.) increased following foliar Zn application and reports that *NAS* expression is constitutively high in plants that hyperaccumulate Zn (Becher et al., 2004; Weber et al., 2004; Haydon et al., 2012; White and Pongrac, 2017).

Homologs of *MsMTP1* and *MsZIF1* were previously found to encode transporters loading Zn and NA into the vacuoles of *Thlaspi geosingense* and *A. thaliana* cells, respectively (Gustin et al., 2009; Haydon et al., 2012). Unexpectedly, the expression of these genes was unaffected by foliar Zn application (Fig. 3). This observation suggests that the proteins encoded by these genes might not contribute to Zn detoxification in *M. sativa*. Nevertheless, only *MsZIF1* of all the genes studied here showed a trend towards increased expression in roots with increasing foliar Zn dose (Fig. 3), which might indicate a role in detoxification of excess Zn in roots through its sequestration with NA in the vacuole. The high correlation between the expression of *MsMTP1* and *MsZIF1* in both shoots and roots suggests that they might constitute a functional module and act synergistically to sequester Zn in the vacuole (Fig. 4), as supported by other studies (Gustin et al., 2009; Haydon et al., 2012; Sharma et al., 2016).

In *A. thaliana*, *AtYSL1* has a role in the long-distance transport of the NA-Zn complex and in loading Zn into seeds (Jean et al., 2005; Curie et al., 2009). For this reason, an increase in the expression of *MtYSL1* was expected to occur in parallel with the increased expression of *MsNAS1* in shoots. However, the expression of *MsYSL1* did not show any significant change in shoots or roots in response to foliar Zn application, although there was a trend towards greater *MsYSL1* expression in shoots with increasing foliar Zn doses (Fig. 3). In addition, the high correlation in the expression of *MsNAS1* and *MsYSL1* in shoots in response to foliar Zn applications (Fig. 4) supports the expectation that these genes are components of a functional module affecting the long-distance

404 transport of Zn in the plant, as it was previously highlighted in *A. thaliana* by Pita-Barbosa et al.
405 (2019).

406 The responses of gene expression to foliar Zn applications suggest three functional modules
407 that effect cytoplasmic Zn homeostasis through Zn transport related processes in *M. sativa*: genes
408 involved in Zn influx to cells (shoots: *MsZIP1, MsZIP5*, and *MsZIP6;* roots: *MsZIP1, MSZIP4*,
409 *MsZIP5*, and *MsZIP6*), genes involved in Zn sequestration in the vacuole (shoots and roots:
410 *MsMTP1* and *MsZIF1*) and genes involved in Zn redistribution within the plant (shoots and roots:
411 *MsHMA4* and *MsYSL1*).

412 In conclusion, this is the first study to characterise the expression of genes related to Zn
413 transport processes following foliar Zn application to a forage legume and provides new molecular
414 insights to the responses of Zn transport related processes to foliar Zn applications. A significant
415 increase in the expression of *MsZIP2* as foliar Zn doses increase suggests the detoxification of
416 excess Zn through the accumulation of Zn in xylem parenchyma cells. A decrease in the expression
417 of *MsZIP3* as foliar Zn doses increase suggests a reduction in the Zn influx capacity of shoot cells
418 to reduce Zn uptake. An increase in the expression of *MsHMA4* in roots and shoots as foliar Zn
419 doses increase suggests an increase in the transport of Zn in the xylem when plants are subject to Zn
420 toxicity, while an increase in the expression of *MsNAS1* in the shoot suggests the chelation of
421 excess Zn in the shoot, enabling Zn sequestration in vacuoles or the redistribution of Zn to roots via
422 the phloem. The elucidation of three functional modules of genes involved in (a) Zn influx to cells,
423 (b) sequestration of Zn in the vacuole and (c) redistribution of Zn within the plant are fundamental
424 to understanding the molecular mechanisms of cytoplasmic Zn homeostasis and might inform the
425 selection of appropriate genotypes enabling greater Zn accumulation in edible portions or increased
426 tolerance of Zn in the environment.

427

428 **MATERIALS AND METHODS**

429

430 **Plant growth and experimental design**

431 Surface sterilized seeds of alfalfa (*M. sativa* L.) were germinated on moist sterilized silica sand
432 (1-4 mm size) in a climatic chamber at 24/21 °C day/night temperature, 16/18 h light/dark cycle and
433 200 µmol photons m^{-2} s^{-1}. After two weeks of growth, three seedlings were transplanted to 1500 mL
434 volume pots, filled with sterilized silica sand (number of pots 18) and *Sinorhizobium meliloti* was
435 supplied as a filtrate to all plants to ensure that the plants produced nodules in all treatments. A
436 Hoagland nutrient solution lacking Zn (Li et al., 2013) was used to fertilize the plants, with 10 mL
437 solution being applied every week. After two months of growth, plants were treated with one of six
438 doses of Zn (0, 0.01, 0.1, 0.5, 1 and 10 mg Zn $plant^{-1}$) (three replicates per dose). Six $ZnSO_4 \cdot 7H_2O$
439 solutions of 0, 0.05, 0.5, 2.5, 5, 50 g Zn L^{-1} were prepared to supply these doses. A drop of Tween
440 20 detergent was added to the six solutions to break the surface tension of the leaves and enhance
441 Zn uptake. Zinc was applied to the middle leaf laminae of the three plants in each pot as twenty 10
442 µl-droplets. The experiment was arranged in a fully randomized design, with three replicates for
443 each Zn dose. The shoots and roots of the plants were harvested separately five days after Zn
444 application. At harvest, 1 mM $CaCl_2$ solution and water were used to remove any residual Zn from
445 the leaf surface (Yilmaz et al., 2017). Shoot and root fresh weight was measured, whereas shoot and
446 root dry weight was determined on subsamples after oven drying at 70°C to constant weight.
447
448 **Measurement of zinc concentrations**
449 Approximately 100 mg of shoot or root dry biomass were carefully weighed and mineralized in
450 a microwave medium pressure digestor (Milestone Start D, FKV Srl, Torre Boldone, Italy) with 7
451 mL of 69% HNO_3 and 2 mL of 30% H_2O_2 (ultrapure grade). Zinc concentration in the resulting
452 solutions was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES)
453 using an Optima 8000 spectrometer (Perkin Elmer, Waltham, MA, USA), following the procedure
454 of Nölte (2003).
455

Gene selection and design and validation of new RT-qPCR assay

Seven genes encoding putative ZRT-IRT-like proteins (ZIP) were selected for investigation (i.e., *ZIP1-7*) (Fig. 5). The selection was based on information gathered by Burleigh et al. (2003) and López-Millán et al. (2004) on the expression of genes encoding Zn transporters in the model legume *Medicago truncatula* and on the structure of the neighbor joining (NJ) tree built using available ZIP sequences of several plant species. Five more genes, whose products are involved in Zn transport-related processes (Olsen and Palmgren, 2014), were also chosen for investigation based on information gathered by other authors and on sequence similarity with other plant species. The *NAS1* gene encoding nicotianamine synthase (NAS) was chosen because this enzyme synthesizes nicotianamine (NA), which is involved in long-distance Zn transport (Curie et al., 2009) (Fig. 5). The *HMA4* gene, which encodes a transmembrane P-type ATPase heavy metal transporter, was chosen because this transporter loads Zn into the xylem in roots for its transport to shoots (Palmer and Guerinot, 2009) (Fig. 5). The *MTP1* gene, which encodes a transporter of the CDF family, was selected because this transporter is implicated in the sequestration of excess Zn in the vacuole (Desbrosses-Fonrouge et al., 2005; Gustin et al., 2009) (Fig. 5). The *ZIF1* gene, which encodes the Zn-induced facilitator 1 transporter, was chosen because it transports NA into the vacuole to chelate vacuolar Zn (Haydon and Cobbett, 2007; Haydon et al., 2012) (Fig. 5). The *YSL1* gene, which encodes a transporter of Zn-NA complexes, was chosen because it is implicated in Zn loading and transport of Zn in the phloem (Palmer and Guerinot, 2009) (Fig. 5). To standardize the expression of genes encoding Zn transport-related processes, two reference genes were selected: actin (*ACT*) and elongation factor 1-α (*EF1-α*) (Nicot et al., 2005).

Using the draft genome sequence of alfalfa in the Alfalfa Gene Index and Expression Atlas Database (AEGD) (O'Rourke et al., 2015; http://plantgrn.noble.org/AGED/index.jsp), homologous gene sequences of *M. sativa* were retrieved by BLASTn similarity searches using the gene sequences of *M. truncatula*. The chosen genes for *M. sativa* were named *MsZIP1-7* for the seven *ZIP* genes and *MsNAS1*, *MsHMA4*, *MsZIF1*, *MsYSL1*, *MsMTP1* for the other selected genes. The

two reference genes were named *MsACT-101* and *MsEF1-α*. The gene sequences and their annotations have been deposited in NCBI under the Submission # 2338923.

Forward and reverse new PCR primers for the 12 Zn transport-related genes and the two reference genes suitable for SYBR® Green II RT-qPCR assays (Biorad, USA) were designed (Table 2). The Primer-BLAST online tool in the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers. The newly designed RT-qPCR assays are suitable for both *M. sativa* and *M. truncatula*. The length of the fragment, the Sanger sequences of the PCR amplicons (Table 2) and the single melting temperature peaks confirmed the specificity of the new RT-qPCR assays (Supplemental Fig. S4). Sanger sequencing was performed on PCR amplicons of three cDNA samples (Supplemental Material and Methods S1). Examples of electropherograms of the sequences are reported in Supplemental Fig. S5. The sequences of the obtained PCR amplicons have been deposited in NCBI the Submission # 2338930. Amplification efficiencies (E) in the range of 96.1-111.0% are evidence of accurate quantification, while the coefficients of correlation (R^2>0.998) indicate high precision of measurements across concentration ranges of at least 3-4 orders of magnitude (Table 2; Supplemental Fig. S6). The concentration ranges over which the relationship between the relative fluorescence and the logarithm of the concentration is linear, and the precision of quantification (standard curves) as reflected in the coefficient of correlation (R^2), were determined using three independent 10-fold serial dilutions of a cDNA sample of *M. sativa*. The accuracy of quantification was determined by the efficiency (E) of each qPCR amplification, using the equation $E = [10^{-1/S} - 1] \times 100$, where S is the slope of the standard curve. The evaluation of the reference genes based on the cycle threshold (Ct) values made us choose the actin gene (*MsACT-101*) for quantifying relative gene expression in the shoots and the elongation factor 1-α (*MsEF1-α*) gene for quantifying relative gene expression in roots (Supplemental Fig. S7a,b). This choice was based on the observations that there was no statistical difference in the expression of the reference genes in tissues following foliar Zn

507 applications and that *MsACT-101* and *MsEF1-α* showed the smallest overall variation in the shoot
508 and root, respectively (Supplemental Fig. S7c,d).
509
510 **RNA extraction and gene expression analysis**
511 Total RNA was extracted from 50 mg subsamples of fresh shoot and root tissue, using the
512 RNeasy Mini Kit (Qiagen, Hilden, Germany). The extractions were performed from tissues of
513 plants treated with the foliar Zn doses that produced a significant increase in Zn concentration in
514 shoots (0.1, 1 and 10 mg Zn plant^{-1}) and the control plants to which no foliar Zn had been applied
515 (24 RNA extractions). Any DNA in the RNA extracts was removed by a DNase treatment
516 (Promega, USA). The purity of the RNA extracts was verified by spectroscopic light absorbance
517 measurements at 230 nm, 260 nm and 280 nm using the NanoDrop 2000 (Termo Scientific,
518 Worchesre, MA, USA) (Desjardins and Conkin, 2010). The integrity and approximate
519 concentration of the extracted RNA was determined by electrophoresis of the RNA extracts in a 1%
520 agarose gel containing Sybr Safe (Invitrogen, Carlsbad, CA). One microgram of total RNA was
521 reverse transcribed to complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Biorad,
522 Hercules, California) in a 20 µL reaction volume. The RT-qPCRs for gene expression analysis were
523 run as three technical replicates with a final reaction volume of 20 µL, containing 10 µL of SYBR
524 Green Supermix (Biorad), 5 µL of 100-fold diluted cDNA, and 0.4 µM final concentrations of the
525 gene-specific PCR primers on a CFX Connect Real-Time System thermal cycler (Biorad, Hercules,
526 California). The qPCR conditions were 95°C for 3', followed by 40 cycles of 95° C for 5', and 60°
527 C for 30''. A dissociation curve of each reaction was performed (65° C to 95° C, 0.5° C increment
528 every 5'') to check that PCR amplified only one product. The most suitable reference gene for
529 relative gene expression analysis was determined by comparing the expression levels of the
530 reference genes *MsACT-101* and *MsEF1-α* across all cDNA samples. Relative gene expression was
531 calculated using the double standardisation (ΔΔCq) method that requires a reference gene and a
532 control treatment (Livak and Schmittgen, 2001).

533

534 **Bioinformatic and statistical analyses**

535

536 A BLAST search was performed in the Alfalfa Gene Index and Expression Atlas database using
537 the *ZIP1-7*, *ZIF1*, *MTP1*, *YSL1*, *HMA4* and *NAS* coding sequences from *M. truncatula*. This
538 allowed the identification of gene sequences encoding potential metal transporters and chelators in
539 the whole *M. sativa* genome. The sequences obtained were aligned with the corresponding
540 sequences from *M. truncatula* and the length of the *M. sativa* genes were determined after removing
541 the external unaligned nucleotides. The *M. sativa* and *M. truncatula ZIP* gene sequences were also
542 aligned with those of other plant species (*A. thaliana, G. max, H. vulgare, O. sativa, Triticum*
543 *aestivum*, and *Zea mays)* obtained from a search of GenBank. Similarly, the *M. sativa* and *M.*
544 *truncatula* gene sequences of *ZIF1*, *MTP1*, *YSL1*, *HMA4* and *NAS* were aligned with their
545 corresponding sequences of other plant species (*A. thaliana, G. max, H. vulgare, O. sativa, T.*
546 *aestivum*, and *Z. mays*) obtained from a search of GenBank. Sequence alignments were performed
547 using the algorithm ClustalW in MEGA X (Kumar et al., 2018). Phylogenetic comparisons were
548 performed to infer the putative roles of the selected *M. sativa* Zn transport-related proteins. The
549 phylogenetic trees were inferred by Neighbor-Joining (NJ) analysis (Saitou et al., 1987) in MEGA
550 X and the evolutionary distances were calculated using the p-distance method (Nei and Kumar,
551 2000). Branch support bootstrap values were derived from 500 bootstrap replicates. The
552 phylograms were drawn by MEGA X and edited using Adobe Illustrator CC 2017.

553 The effect of the application of the foliar Zn on tissue Zn concentration and on the expression
554 of the selected genes was analysed in shoots and roots separately by one-way analysis of variance
555 (ANOVA), followed by a Tukey-B test in the case of significance of the response to foliar Zn
556 application. When required, gene expression data were log-transformed to meet the ANOVA
557 assumptions. The data displayed graphically are the means and associated standard errors of the
558 untransformed raw data. All statistical analyses were performed using the software package SPSS

559 version 21.0 (SPSS Inc., Chicago, IL, USA). Permutational analysis of variance (PERMANOVA;
560 Anderson, 2001) was used to test the effect of foliar Zn application and plant organ (shoot and root)
561 on the expression of the seven *ZIP* genes and of the other five genes encoding Zn transport-related
562 processes separately. In addition, the PERMANOVA was performed on the expression of all the
563 genes together. The response data matrices were standardised by sample, and total and then
564 Euclidean distances were calculated among samples. *P*-values were calculated using the Monte-
565 Carlo test (Anderson and Braak, 2003). Since PERMANOVA is sensitive to differences in
566 multivariate location and dispersion, analysis of homogeneity of multivariate dispersion
567 (PERMDISP; Anderson, 2006) was performed to check the homogeneity of dispersion among
568 groups. The analyses were performed using PRIMER 7 and PERMANOVA+ software (Clarke and
569 Gorley, 2015). Finally, heatmaps were constructed to illustrate correlations in expression among
570 *ZIP*s and among other genes encoding Zn transport-related processes using the R package ggplot2
571 (Wickham, 2011), using the average linkage clustering of the Pearson correlations calculated from
572 relative gene expression following foliar Zn application.
573
574 **Supplemental Materials**
575
576 **Supplemental Figure S1.** Shoot and root zinc (Zn) content of alfalfa (*Medicago sativa* L.) five
577 days after application of six doses of Zn to leaves.
578
579 **Supplemental Figure S2.** Neighbor-Joining phylogenetic tree of *ZIP* gene sequences of *Medicago*
580 *sativa*, *Medicago truncatula* and other plant species.
581
582
583 **Supplemental Figure S3.** Neighbor-Joining phylogenetic trees of sequences of Zinc Induced
584 Facilitator (*ZIF*), Metal Tollerance Protein (*MTP*), Yellow Stripe Like protein (*YSL*), Heavy Metal

585 Transporter (*HMA*) and Nicotianamine Synthase (*NAS*) genes of *Medicago sativa*, *Medicago*
586 *truncatula* and other plant species.
587
588 **Supplemental Figure S4.** Melting curve analysis of the qPCR products obtained by the newly
589 designed pair of primers.
590
591 **Supplemental Figure S5.** Electropherograms of PCR products obtained by the newly designed
592 primers for *MsZIP*$_{1\text{-}7}$ transporters and for other Zn related genes.
593
594 **SupplementalFigure S6.** Standard curves for the newly designed qPCR primer pairs.
595
596 **Supplemental Figure S7.** Cycle threshold value of the reference genes, *actin 101* (*MsACT-101*)
597 and *elongation factor 1-α* (*MsEFf1-α*) in shoots and roots for the no-Zn addition control and the
598 three Zn doses.
599
600 **Supplemental Material and Methods S1.** PCR amplification conditions.
601
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607 support in real-time RT-PCR.
608
609 **Tables**
610

Table 1 Permutation analyses of variance (PERMANOVAs) on the effect of application of three doses of zinc (Zn) (0.1, 1 and 10 mg Zn plant^{-1}) and plant compartment (shoot and root) on the expression of seven *MsZIP* genes and separately on the expression of other five genes (*MsZIF1*, *MsNAS1*, *MsHMA4*, *MsYSL1* and *MsMTP1*) (see Table 1, Fig. 1). A PERMANOVA was also performed on the response of all the genes. The analysis of homogeneity of multivariate dispersion (PERMDISP) was also performed. The studied plant was alfalfa (*Medicago sativa* L.). The analysis included also no-Zn addition control. Gene relative expression was studied on a total of 24 experimental units, corresponding to three replicates per each level of treatment.

Response variables	Explanatory variables	Zn application (Zn)	Plant compartment (Comp)	Zn x Comp	Residual
ZIP genes	Pseudo F	5.56	8.16	1.76	
	P(perm)	**0.002**	**0.001**	0.082	
	Explained variance (%)	29.1	22.9	9.7	38.3
	PERMDISP *P*(perm)	**0.005**	0.766		
Other genes	Pseudo F	3.06	5.59	1.76	
	P(perm)	**0.007**	**0.015**	0.1	
	Explained variance (%)	17.3	19.35	12.78	50.55
	PERMDISP *P*(perm)	0.412	0.852		
All genes	Pseudo F	4.27	10.49	3.41	
	P(perm)	**0.001**	**0.001**	**0.003**	
	Explained variance (%)	17.3	25.2	25.6	31.9
	PERMDISP *P*(perm)	0.152	**0.030**		

611

Table 2 Gene name, forward and reverse sequences of fourteen newly designed primer pairs for the quantification of the expression of genes of alfalfa (*Medicago sativa*), encoding proteins involved in cellular zinc (Zn) influx and efflux and Zn chelation (see **Fig. 1**). Two reference genes (i.e., *MsACT-101* and *MsEF1-*□□ □were also designed. The length of the amplicons, the primer amplification Efficiency (%) and R^2 of the standard curve are indicated (see **Fig. S3**). The reference sequences are indicated by the accession number of the *Medicago truncatula* sequences and by the contig number of the *M. sativa* sequences. The primers were designed using the National Center for Biotechnology Information *PRIMER Blast* online tool.

Gene*	Reference sequence (Accession number and contig number)	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	Efficiency (%)	R^2
MsZIP1	AY339054†/ 19855‡	ATGATTAAAGCCT TCGCGGC	TCTGCTGGAACTT GTTTAGAAGG	233	99.8	0.999
MsZIP2	AY007281/8 2450	AGCCCAATTGGCG TAGGAAT	ACAGCAACACCAA AAAGCACA	215	99.3	0.999
MsZIP3	AY339055/3 3860	TGGTGTGATTTTG GCAACCG	TGACGGACCCGAA GAAACAG	325	104.9	0.999
MsZIP4	XM_003603 101/92651	GGAGGGTGCATTT CTCAAGC	AGCAATGCCTGTT CCAATGC	108	97.1	0.999
MsZIP5	XM_013605 712/66451	TGAAGGCATGGG ACTTGGAA	CCAGCTGAAGCTG CATTGAA	192	99.3	0.998
MsZIP6	AY339058/9 668	CTTGGCGACACGT TCAATCC	CCACAAGTCCCGA AAAGGGA	188	106.0	0.998
MsZIP7	AY339059/6 2098	GGCTTGTGCTGGT TATTTGAT	TTTCCATGCGTCT GCTTTTGT	310	96.1	0.999
MsZIF1	XM_003601 836/59165	TGCCTGCATTTGG TTACCG	CTGCAGCTTCCAC ATTGTCAG	77	105.9	0.999
MsHMA4	XM_003626 900/19210	TGCTCAACTTGCC AAAGCAC	GGAATGAACCATC CCAGCCA	111	108.9	0.999
MsYSL1	XM_024781 439/4892	CAAGAAGCAAGT GCATGGGT	TCCACAGTCTTCTT TGCCTGAG	94	111.0	0.999
MsMTP1	FJ389717/67 347	TGCAGCATTTGCC ATCTCCT	TGCATAGAAACCA AAGCACCA	114	104.5	0.999
MsNAS1	XM_003594 705/61146	GCTAGCTTGGCTG AAGATTGG	AGATACAAAGCAC TCGGAGACA	87	100.5	0.999
MsACT-101	XM_003593 074/89028	TCTCTGTATGCCA GTGGACG	TCTGTTAAATCAC GCCCAGCA	140	102.4	0.999
MsEF1- –	XM_003618 727/56897	CCACAGACAAGC CCCTCAG	TCACAACCATACC GGGCTTC	114	100.2	0.999

*See Fig. 1 for the full names of the genes

† NCBI - GenBank Accession number - https://www.ncbi.nlm.nih.gov/genbank/

‡ AGED - The Alfalfa Gene Index and Expression Atlas Database - http://plantgrn.noble.org/AGED/

612

Figure legends

Figure 1. Zinc (Zn) concentrations in shoots and roots of alfalfa (*Medicago sativa*) five days after the application of Zn doses of 0, 0.01, 0.1, 0.5, 1 or 10 mg Zn plant^{-1} to leaves. Means ± standard error of three replicates are shown. Differences among the applied Zn doses were tested separately for shoot and root by one-way analysis of variance. Different letters denote significant differences in Zn concentrations in shoots and roots independently, according to Tukey-B honestly test (P < 0.05).

Figure 2. Relative expression of seven transmembrane zinc (Zn) transporter genes (*MsZIP1-7*) five days after the application of Zn doses of 0, 0.1, 1 or10 mg Zn plant^{-1} to leaves of alfalfa (*Medicago sativa*). Means ± standard error of three replicates are shown. The expression levels were calculated relative to reference genes (*MsACT-101* for shoot and *MsEF1-α* for root) and to the control (0 mg Zn plant^{-1}). The broken line denotes the threshold between up- and down-regulation relative to the control. Differences in the expressions of each gene after different Zn doses were tested separately for shoot and root by one-way analysis of variance. Different letters denote significant differences among Zn doses, according to Tukey-B test (P < 0.05). The full names of the genes are reported in the legend of Figure 1.

Figure 3. Relative expression of genes related to Zn transport processes (*MsZIF1*, *MsHMA4*, *MsYSL1*, *MsMTP1* and *MsNAS1*) five days after the application of Zn doses of 0, 0.1, 1 and 10 mg Zn plant^{-1} to leaves of alfalfa (*Medicago sativa*). Means ± standard error of three replicates are shown. The expression levels were calculated relative to reference genes (*MsACT-101* for shoot and *MsEF1-α* for root) and to the control (0 mg Zn plant^{-1}). The broken line denotes the threshold between up- and down-regulation relative to the control. Differences in the expression of each gene at the different Zn doses were tested separately for shoot and root by one-way analysis of variance.

639 Different letters denote significant differences among Zn doses, according to Tukey-B test ($P <$
640 0.05). The full names of the genes are reported in the legend of Figure 1.
641
642 **Figure 4.** Heatmaps reporting the correlations between the differences in expression of genes
643 related to zinc (Zn) transport processes in alfalfa (*Medicago sativa*) after foliar application of Zn
644 doses of 0.1, 1 and 10 mg Zn plant^{-1} relative to a control dose of 0 mg Zn plant^{-1}. The similarity in
645 the degree of correlation in fold-change of gene expression to Zn application relative to the control
646 is based on the average linkage clustering of the Pearson correlations (r). In the clustering trees the
647 genes are indicated in brown for roots and in green for shoots, while the ranks of correlations of the
648 heatmap are indicated by color intensity (r 0 to 1: from low to strong intensity of green). Seven
649 genes encoding transmembrane Zn transporter (*MsZIP*$_{1\text{-}7}$) (a); four genes encoding cellular Zn
650 transporters (including vacuolar transporters) (*MsZIF1*, *MsHMA4*, *MsYSL1* and *MsMTP1*) and a
651 gene encoding a nicotianamine synthase (*MsNAS1*) (b).
652
653 **Figure 5.** Suggested model for the roles of putative genes encoding proteins involved in Zinc (Zn)
654 transport-related processes in alfalfa (*Medicago sativa*). The sites of action in the plant (i.e., root
655 cytoplasm, rc; root vacuole, rv; xylem and apoplast, X/A; phoelm, P; leaf cytoplasm, lc; leaf
656 vacuole, lv) and the element (E) fluxes ($K_{1\text{-}13}$) are reported. The concentration of the element is
657 indicated in each site [E]. The scheme synthetizes information across studies in various plants. Gene
658 abbreviations: *ZIP*, Zrt-/Irt-like Protein; *NAS*, Nicotianamine synthase; *ZIF*, Zinc-Induced
659 Facilitator; *MTP*, Metal Transporter Protein; *HMA*, P_{1B}-type Heavy Metal ATPase; *YSL*, Yellow
660 Stripe Like Protein; *ZIP*? indicates a generic *ZIP*; free diffusion: diffusion through leaf epidermis;
661 stomata: absorption through stomata. Plant abbreviations: Mt, *Medicago truncatula*; At,
662 *Arabidopsis thaliana*; Os, *Oryza sativa*. References: [a]López-Millán et al., 2004; [b]Milner et al., 2013;
663 [c]Aarts, 2014; [d]Clemens et al., 2013; [e]Curie et al., 2009; [f]Haydon et al., 2012; [g]Desbrosses-Fonrouge
664 et al., 2005; [h]Hussain et al., 2004; [i]Palmer and Guerinot, 2009; [j]Burleigh et al., 2003; [k]Sasaki et al.,

665 2015; [l]lFageria et al., 2009.

666

667 **LITERATURE CITED**

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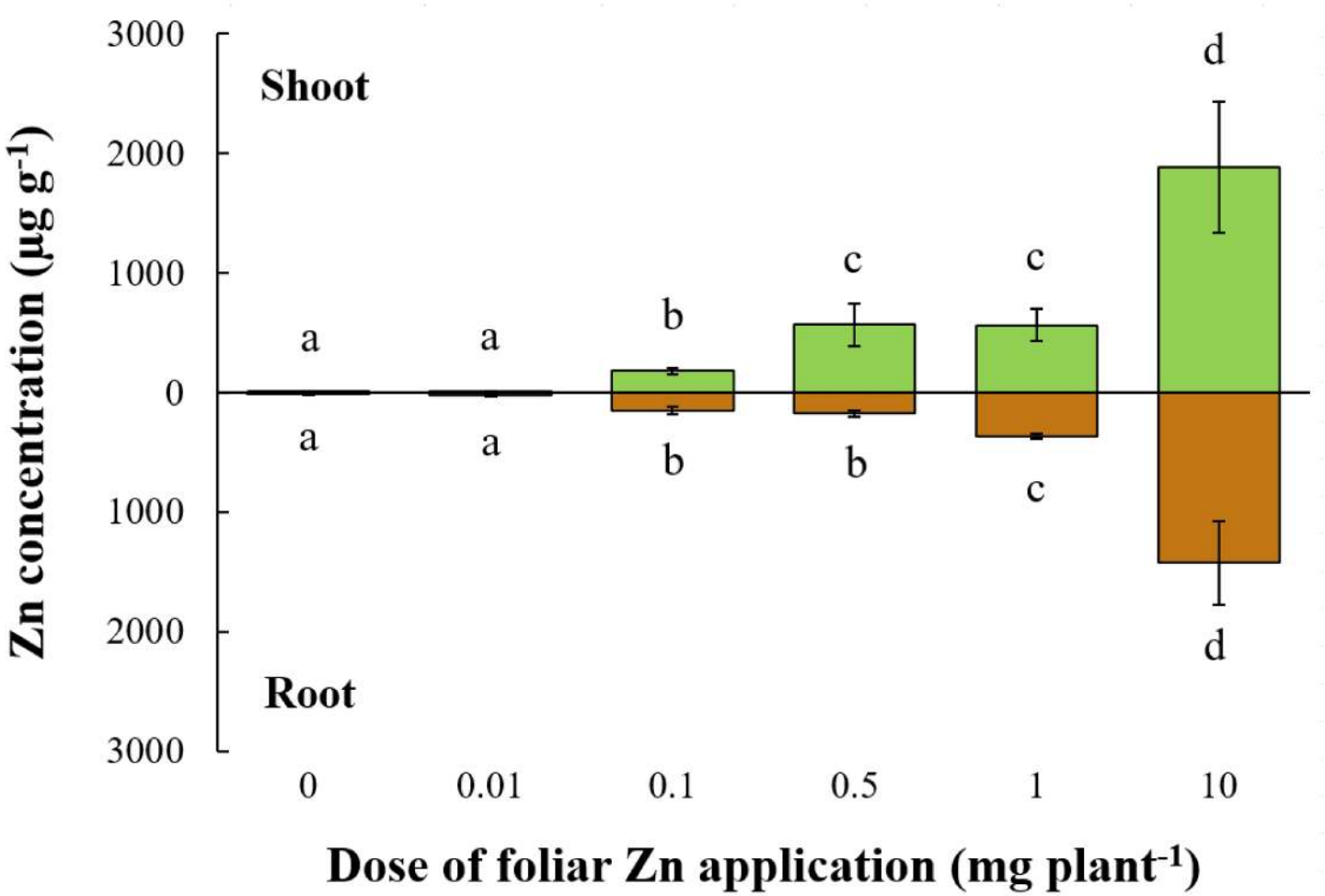


Figure 1. Zinc (Zn) concentrations in shoots and roots of alfalfa (*Medicago sativa*) five days after the application of Zn doses of 0, 0.01, 0.1, 0.5, 1 or 10 mg Zn plant^{-1} to leaves. Means ± standard error of three replicates are shown. Differences among the applied Zn doses were tested separately for shoot and root by one-way analysis of variance. Different letters denote significant differences in Zn concentrations in shoots and roots independently, according to Tukey-B honestly test ($P < 0.05$).

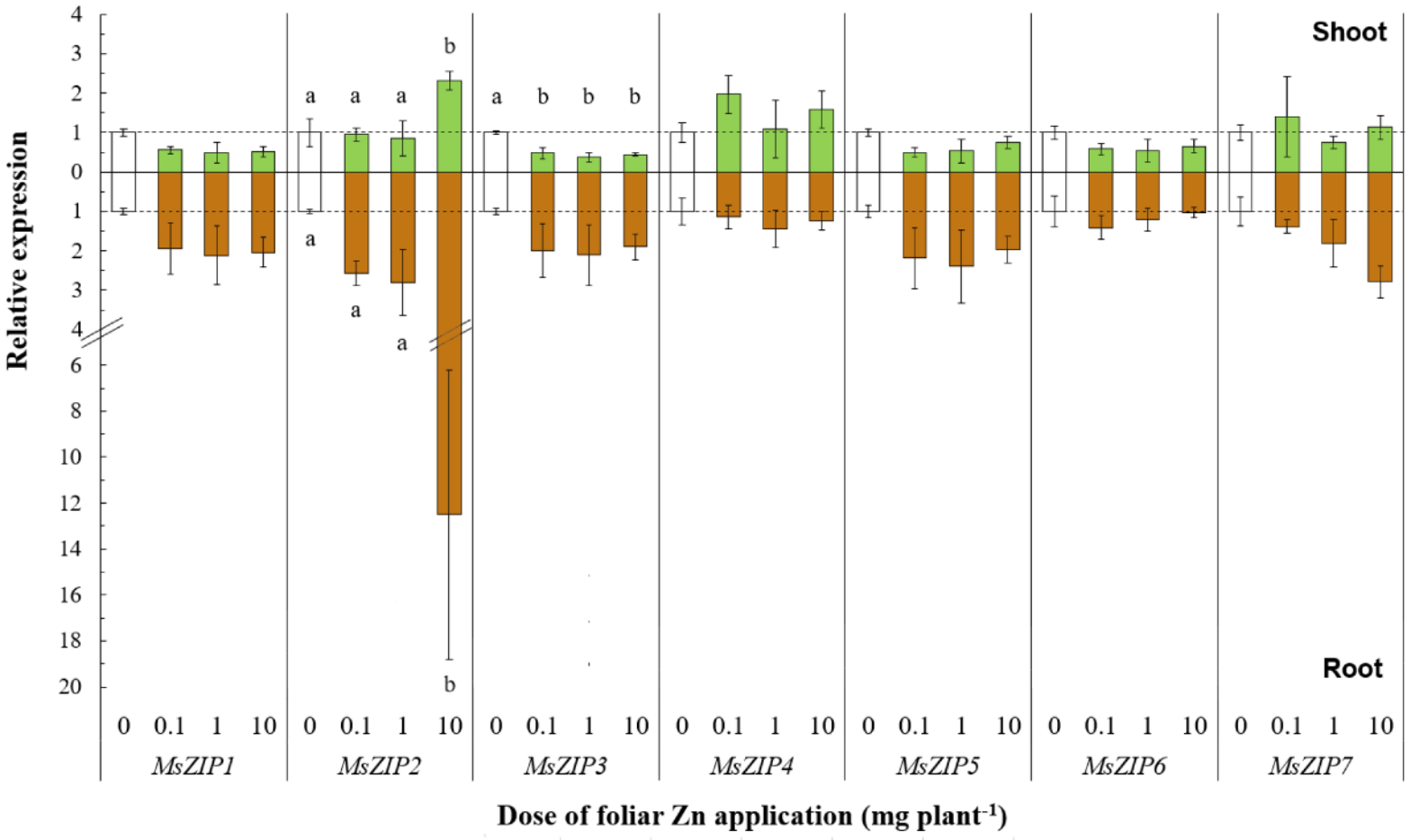


Figure 2. Relative expression of seven transmembrane zinc (Zn) transporter genes (*MsZIP1-7*) five days after the application of Zn doses of 0, 0.1, 1 or 10 mg Zn plant^{-1} to leaves of alfalfa (*Medicago sativa*). Means ± standard error of three replicates are shown. The expression levels were calculated relative to reference genes (*MsACT-101* for shoot and *MsEF1-α* for root) and to the control (0 mg Zn plant^{-1}). The broken line denotes the threshold between up- and down-regulation relative to the control. Differences in the expressions of each gene after different Zn doses were tested separately for shoot and root by one-way analysis of variance. Different letters denote significant differences among Zn doses, according to Tukey-B test ($P < 0.05$).

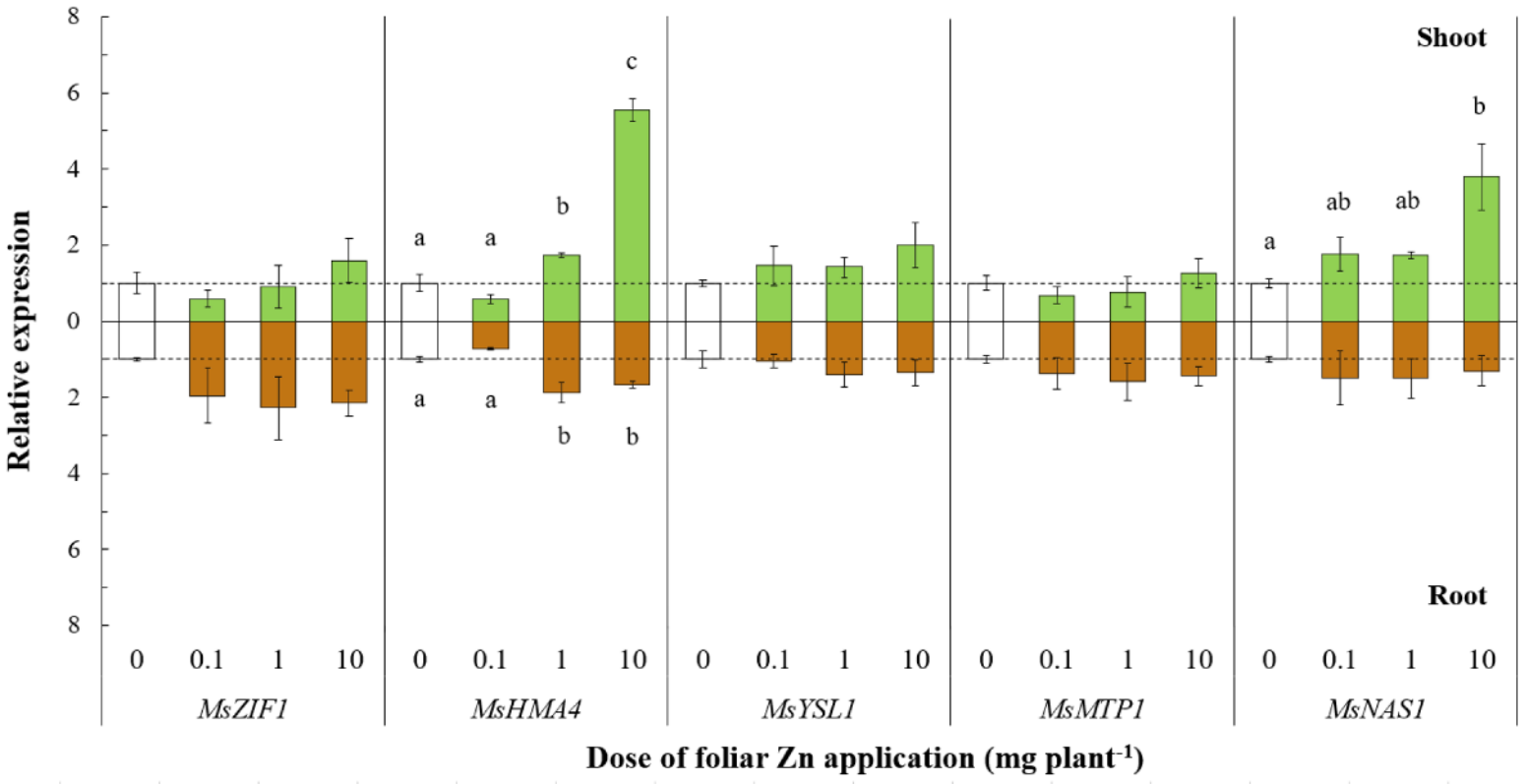


Figure 3. Relative expression of genes related to Zn transport processes (*MsZIF1*, *MsHIMA4*, *MsYSL1*, *MsMTP1* and *MsNAS1*) five days after the application of Zn doses of 0, 0.1, 1 or 10 mg Zn plant^{-1} to leaves of alfalfa (*Medicago sativa*). Means ± standard error of three replicates are shown. The expression levels were calculated relative to reference genes (*MsACT-101* for shoot and *MsEF1-α* for root) and to the control (0 mg Zn plant^{-1}). The broken line denotes the threshold between up- and down-regulation relative to the control. Differences in the expression of each gene at the different Zn doses were tested separately for shoot and root by one-way analysis of variance. Different letters denote significant differences among Zn doses, according to Tukey-B test ($P < 0.05$).

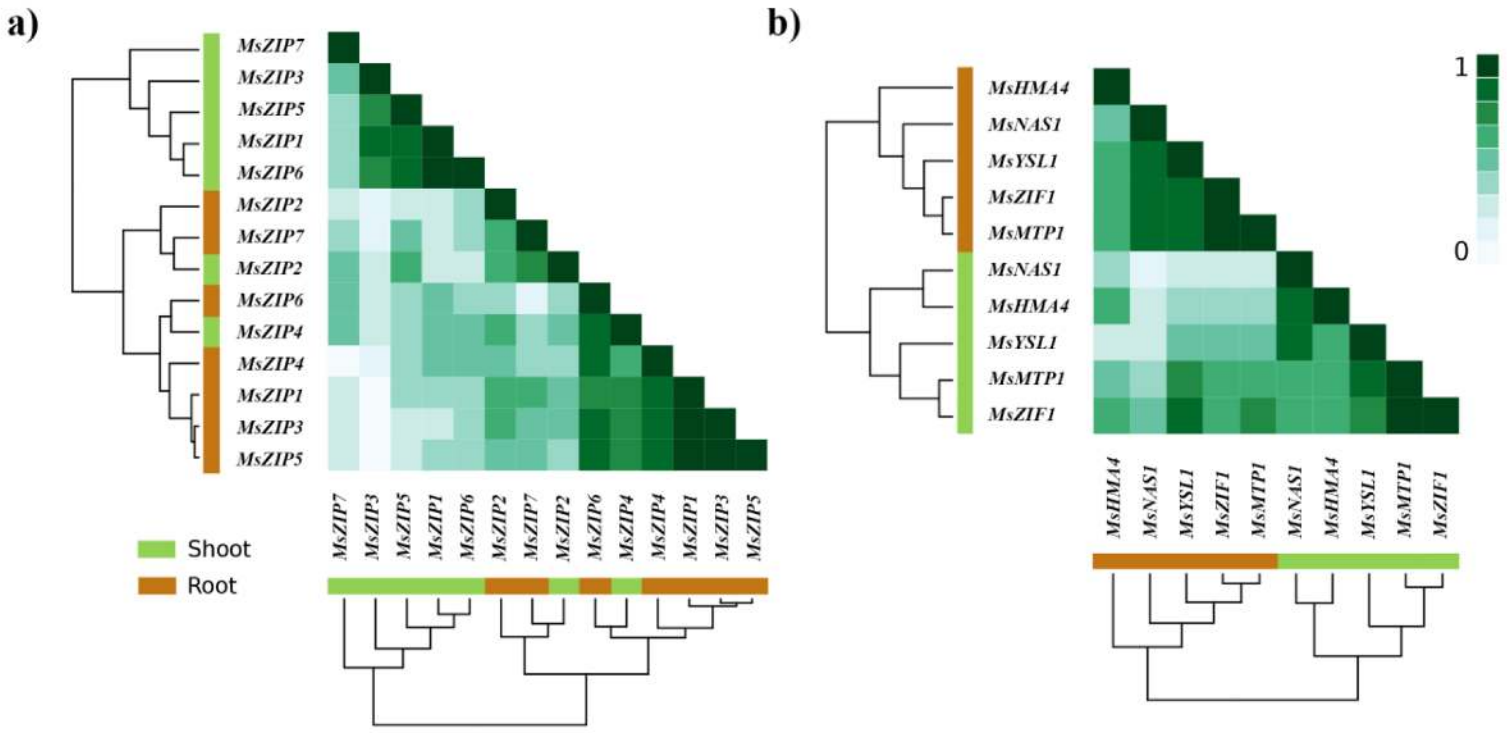
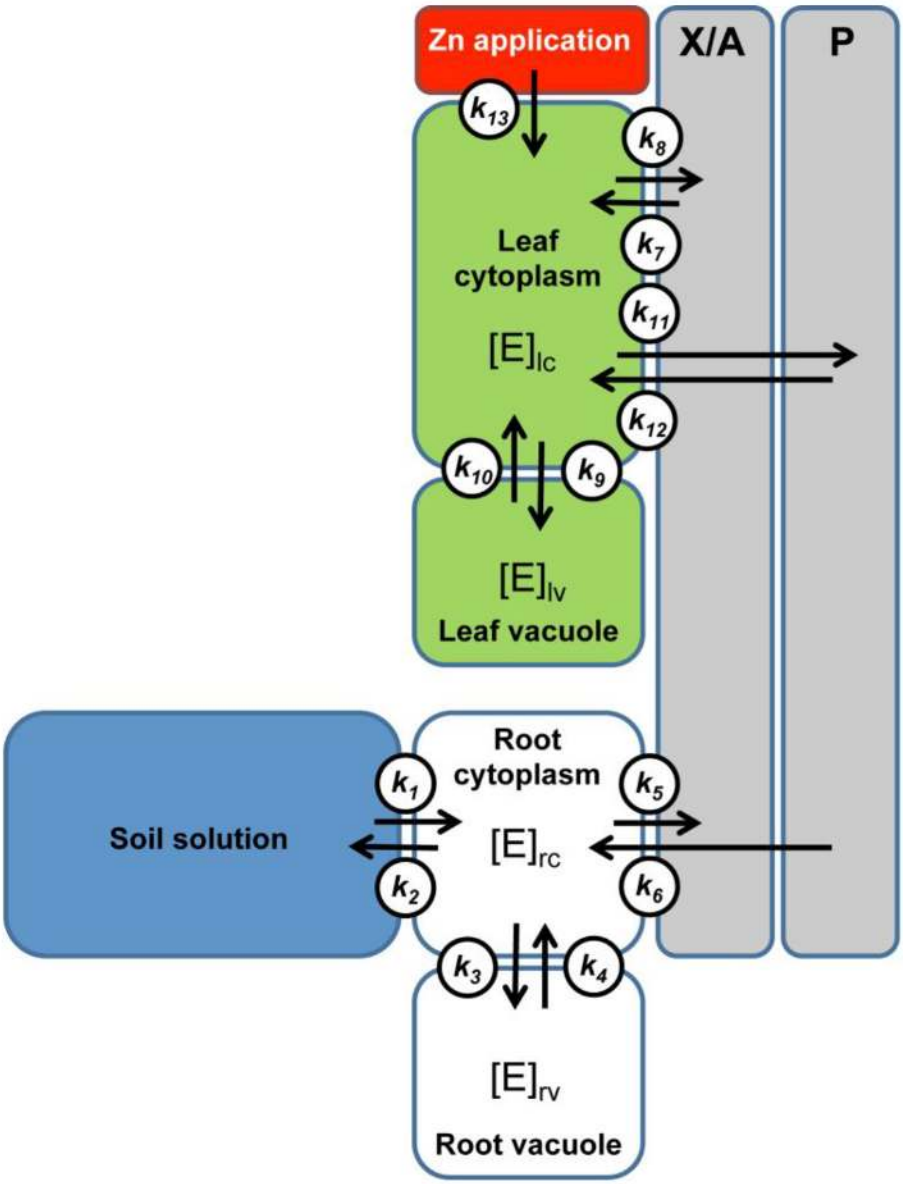


Figure 4. Heatmaps reporting the correlations between the differences in expression of genes related to zinc (Zn) transport processes in alfalfa (*Medicago sativa*) after foliar application of Zn doses of 0.1, 1 or 10 mg Zn plant^{-1} relative to a control dose of 0 mg Zn plant^{-1}. The similarity in the degree of correlation in fold-change of gene expression to Zn application relative to the control is based on the average linkage clustering of the Pearson correlations (r). In the clustering trees the genes are indicated in brown for roots and in green for shoots, while the ranks of correlations of the heatmap are indicated by color intensity (r 0 to 1: from low to strong intensity of green). Seven genes encoding transmembrane Zn transporter (*MsZIP*$_{1\text{-}7}$) (a); four genes encoding cellular Zn transporters (including vacuolar transporters) (*MsZIF1*, *MsHMA4*, *MsYSL1* and *MsMTP1*) and a gene encoding a nicotianamine synthase (*MsNAS1*) (b).



Flux	Putative genes
k_1	*MtZIP1*[a], *MtZIP4*[a], *MtZIP5*[a], *MtZIP6*[a], *Mt(At)ZIP7*[a,b]
k_2	*NAS1*[c]
k_3	*NAS1*[d,e], *AtZIF1*[f], *AtMTP1*[g]
k_4	*AtZIP1*[b]
k_5	*NAS1*[d,e], *AtHMA4*[h], *AtYSL1*[e,i], *MtZIP2*[j]
k_6	*MtZIP1*[a], *Mt(Os)ZIP3*[a,k], *MtZIP4*[a], *MtZIP5*[a], *MtZIP6*[a], *Mt(At)ZIP7*[a,b]
k_7	*MtZIP1*[a], *Mt(Os)ZIP3*[a,k], *MtZIP4*[a], *MtZIP5*[a], *MtZIP6*[a], *Mt(At)ZIP7*[a,b]
k_8	*AtHMA4*[h], *MtZIP2*[j]
k_9	*NAS1*[d,e], *AtZIF1*[f], *AtMTP1*[g]
k_{10}	*AtZIP1*[b]
k_{11}	*NAS1*[d,e], *AtYSL1*[e,i]
k_{12}	?
k_{13}	Free diffusion[l], stomata[l], *ZIP*?

Figure 5. Suggested model for the roles of putative genes encoding proteins involved in Zinc (Zn) transport-related processes in alfalfa (*Medicago sativa*). The sites of action in the plant (i.e., root cytoplasm, rc; root vacuole, rv; xylem and apoplast, X/A; phoelm, P; leaf cytoplasm, lc; leaf vacuole, lv) and the element (E) fluxes ($K_{1\text{-}13}$) are reported. The concentration of the element is indicated in each site [E]. The scheme synthetizes information across studies in various plants. Gene abbreviations: *ZIP*, Zrt-/Irt-like Protein; *NAS*, Nicotianamine synthase; *ZIF*, Zinc-Induced Facilitator; *MTP*, Metal Transporter Protein; *HMA*, P_{1B}-type Heavy Metal ATPase; *YSL*, Yellow Stripe Like Protein; *ZIP*? indicates a generic *ZIP*; free diffusion: diffusion through leaf epidermis; stomata: absorption through stomata. Plant abbreviations: Mt, *Medicago truncatula*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*. References: [a]López-Millán et al., 2004; [b]Milner et al., 2013; [c]Aarts, 2014; [d]Clemens et al., 2013; [e]Curie et al., 2009; [f]Haydon et al., 2012; [g]Desbrosses-Fonrouge et al., 2005; [h]Hussain et al., 2004; [i]Palmer and Guerinot, 2009; [j]Burleigh et al., 2003; [k]Sasaki et al., 2015; [l]Fageria et al., 2009.

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