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Correlation between phenotype and proteome in the Ni hyperaccumulator *Noccaea caerulescens* subsp. *caerulescens*

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ABSTRACT

The metallicolous population *Noccaea caerulescens* subsp. *caerulescens* (formerly *Thlaspi caerulescens*) of Monte Prinzera (MP) (Italy) is a well-known example of a hyperaccumulator. MP has a serpentine soil in which concentrations of Ni can be as high as 2500 mg kg^{-1}. The absence of *N. caerulescens* in the nearby non-ophiolitic soils along with its growth and reproductive traits, make this population an interesting model to study adaptation to extreme environments. Different phenotypes of *N. caerulescens* were identified in sub-sites differing in their geo-morphology. Differences in morphological and reproductive traits of plants were found through multivariate statistical analysis and were correlated with soil parameters, such as pH, organic matter content, water and Ni content. Phenotypic variability was correlated at the molecular level with proteome variation. Transcriptional regulators and signal transduction factors were differentially abundant in the sub-populations. We hypothesise that these proteomic variations may be relevant for adaptation of *N. caerulescens* to specific micro-habitats, at the same time revealing a general trade-off between plants and environment in terms of costs and benefits.

1. Introduction

Management of sites contaminated by metals is clearly important for environmental protection (Dickinson et al., 2009) but metals are present at high concentrations in ophiolitic rocks all over the world (Coleman, 1977; Coleman and Jove, 1992). The ophiolites are natural sites characterised by high concentrations of heavy metals. The rare endemic plant species often inhabiting ophiolites constitute important model species to investigate how a living organism can adapt to ecological stress (Baker et al., 2010). Monte Prinzera (MP) is a Natural Reserve located in the Tosco-Emilian Appennins (Italy). Its geo-physical condition allows the occurrence of some plant species endemic to these substrates such as *Alyssum bertolonii*, *Biscutella montanina*, *Linaria supina* and *Noccaea caerulescens* (Lombini et al., 1998). Metal tolerance and accumulation traits of these plants are common to more than 500 other plant species (Baker et al., 2000; Pollard et al., 2002; Krämer, 2010). *N. caerulescens* is widespread in central Europe and it is considered a facultative metallophyte (Escarrè et al., 2000). It is a member of the Brassicaceae family, with a high genomic syntheny to *Arabidopsis thaliana*. *N. caerulescens* is considered a model species for tolerance and accumulation of metals such as Zn, Cd Pb and Ni. It can be considered a "green rat" in terms of studying the genetic and molecular basis of plant–environment interactions (Escarrè et al., 2000; Assunção et al., 2003a). Living in metalliferous soils, it is more tolerant and accumulates more metals (up to 30 g kg^{-1} Zn, 14 g kg^{-1} of Cd and 47 g kg^{-1} of Ni) compared to plants living in non-metalliferous soils. The metal tolerance and accumulation of different accessions of *N. caerulescens* found all over Europe, is specific to the metal concentration in the soil of origin (Schat et al., 2000; Lombi et al., 2002; Assunção et al., 2003a). For this reason, Escarrè et al. (2000) suggested that *N. caerulescens* should be separated in different ecotypes or sub-species.

Studies on *N. caerulescens* have focused on the molecular and physiological mechanisms involved in metal accumulation (Milner and Kochian, 2008) and on the genetic variation among natural populations (Assunção et al., 2003b; Basic et al., 2006). Expression levels and functions of genes linked to uptake, translocation and sequestration of metals have been compared between *N. caerulescens* and the non-accumulator *A. thaliana* (Milner and Kochian, 2008). High-throughput transcriptonic studies showed how the hyper-expression of a set of metal and non-metal related genes contributed to build up the accumulator phenotype (Assunção et al., 2001; van de Mortel et al., 2008; Milner and Kochian, 2008), but its molecular bases are still unknown.

In different *N. caerulescens* accessions, a significant level of genetic polymorphysms was found in both target and non-target

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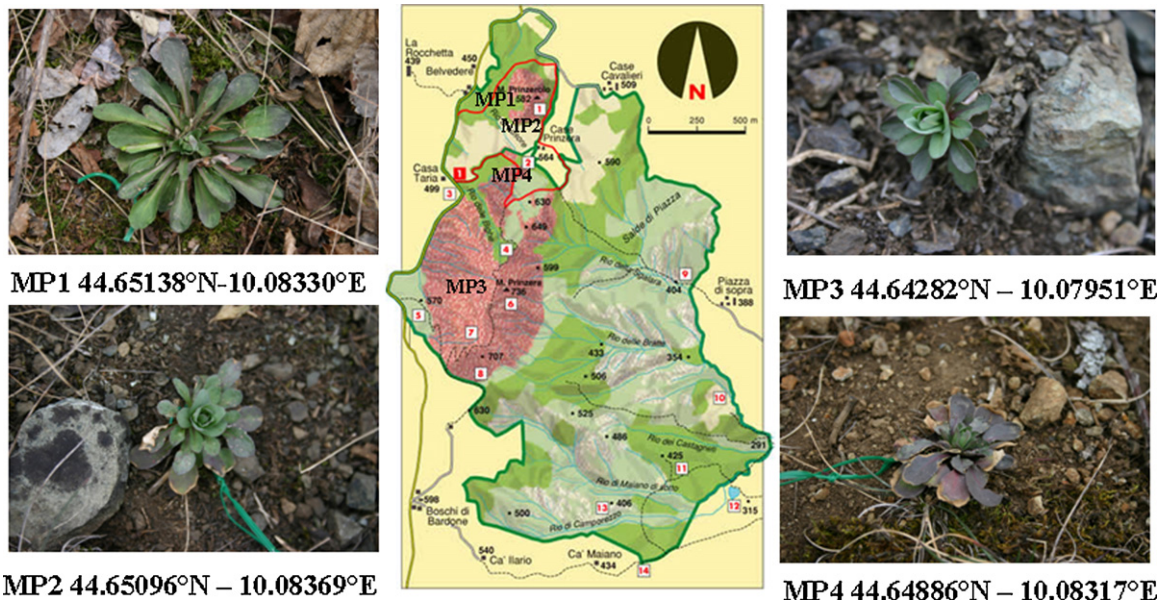

Fig. 1. Representative examples of differences in morphology in *Noccaea caerulescens* plants growing on Monte Prinzera Natural Reserve (Parma Province, Italy). MP1, MP2, MP3, MP4 correspond to the four sub-sites indicated in Section 2.1.

genes (Basic et al., 2006; Besnard et al., 2009). The degree of polymorphism might also be influenced by the environmental context in which this species lives through an increase in mutation rates and in recombination frequencies, as it was demonstrated in other organisms (Hoffmann and Parsons, 1991; Gregorius and Kleinschmit, 1999), but the genotypic plasticity of this species should not be considered the only driving force in its adaptation. Phenotypic plasticity is another process which may favour the growth of plants in extreme environments. Phenotypic plasticity is the ability of an organism with a given genotype to modulate its phenotype in response to environmental conditions (Sultan, 2000). Phenotypic plasticity can affect both intra-population and intra-species variation and cause the phenotypic variability observed within populations (Pfennig et al., 2010). Recently, several authors discussed the role of phenotypic plasticity within- and between-metal adapted populations like *N. caerulescens* (Basic and Besnard, 2006; Basic et al., 2006; Jiménez-Ambriz et al., 2006; Dechamps et al., 2007, 2008; Maestri et al., 2010) and also in other plant species (Marmiroli et al., 2011). Many of these studies were performed in plants grown in controlled environments, but phenotypic plasticity, as a supplementary resource for adaptation, is more evident in natural environments.

The aim of this study was to correlate differences in morphological traits with protein variations among individuals of *N. caerulescens* from MP living in four micro-environments with different geo morphology. We focused our attention on some classes of proteins such as metal transporter and chelators, plant defence proteins, protein linked to energy metabolism, proteolytic enzymes and regulatory and signal transduction proteins that seemed more affected by the micro-environmental perturbations.

2. Materials and methods

2.1. In field observations

Various sub-populations of *N. caerulescens* subsp. *caerulescens* in MP, a Natural Reserve from the Northern Appennines in province of Parma, (Italy), were identified and followed in two different years. Plant phenotypes were thoroughly assessed in 2009 (see Supplementary Information S1 and S2) and 2010. MP Natural Reserve is one of the most striking ophiolitic outcrops of the Northern Apennines, with a total surface of 230 ha, 25% of which are outcropping rocks and scree covered. Several *N. caerulescens* individuals were found both in the serpentine rocks and screes in the western side of MP and in grasslands in the lower eastern side of the outcrop. Four populations (MPxp) sampled in different sub-sites on MP were analysed: sub-site MP1, a serpentine-clayey soil, near a humid grassy land, within a sub-montane vegetation site (44.65138°N–10.08330°E); sub-site MP2, a serpentine soil, on a steep track where no other herbaceous species were present (44.65096°N–10.08369°E); sub-site MP3, a serpentine soil, near a grassy land at the beginning of the main track of MP (44.64282°N–10.07951°E); sub-site MP4, a serpentine brown soil indicative for the presence of iron oxides (44.64886°N–10.08317°E) (Fig. 1). The four populations were less than 4 km apart, but separated by natural barriers preventing inter-crossing.

2.2. Plant analyses

Five individuals per population were randomly sampled at each sub-site in 2010 at the stage of rosette and their phenotypes were monitored *in situ* every two weeks, starting from mid-March to the beginning of June. In particular, we measured morphological and reproductive traits such as: (i) the number of leaves of the rosettes; (ii) the length and width of leaves of the rosettes; (iii) the height of stalk; (iv) the number of inflorescence and (v) the size within a small sample of seeds (40 seeds per plant) with Scanning Electron Microscope (SEM) (Oxford Instrument, Oxford, UK). The same analyses were carried out in 2009, but this time only morphological parameters were measured: the length and width of leaves and the number of leaves of the rosettes (Supplementary Information S1). Since the mission of the MP Natural Reserve is the preservation of the biodiversity of this particular natural habitat, strict rules on sample collection were followed.

For plant mineral analysis five plants were randomly sampled per sub-site at the stage of rosette and carefully washed with deionised water. Roots were washed thoroughly with deionised water to eliminate any visible soil particle. Roots were separated from leaves and both were oven dried for three days at 70 °C. Dried roots and leaves were grinded with a mortar and a pestle

and 100 mg of powder was digested in 10 ml of 37% (v/v) HCl in 0.5 l glass cylinder at 160 °C for 20 min. The digestion was stopped by adding 8 ml of MilliQ water. Ni concentration was determined by flame atomic absorption spectrometry (F-AAS) (Perkin Elmer 1100B, Waltham, MA, USA). Accuracy of each measure was assessed in duplicate analyses on selected samples. Less than 10% relative variation between measures was observed.

2.3. Soil sampling and analyses

Different soil parameters were analysed to correlate the plant morphological traits to the soil micro-environment. Soil samples from the four different sub-sites (MPxs) in MP were collected. Soil samples were representative of the surface horizon corresponding to a maximum sampling depth of about 20 cm. Sampling was performed with two different strategies: (i) five soil samples per sub-site, randomly collected in an area of 10 m^2 near the plants' population; (ii) five samples of soils collected at each site from the rhizosphere of each analysed plant, for a total of 20 samples.

Ni concentration was analysed in soils collected in the four different sub-sites of MP. Aliquots of 300 mg of soil were dried overnight at 70 °C and wet-ashed, the metal concentration in the soil was determined by F-AAS (Perkin Elmer 1100B). The same procedure was applied for determining the Ni content in plant rhizosphere. To evaluate Ni extractable fraction, a diethylenetriaminepentaacetic acid (DTPA) extraction was performed in the latter samples following Lindsay and Norwell protocol (1978). Soil pH was measured with a glass electrode from a soil-deionised water suspension (20 g soil/50 ml water) with agitation for 1 h and overnight settlement. Determination of soil organic matter was performed on soils sampled at 10 and 20 cm of depth by the "weight loss on ignition method" (Heiri et al., 2001). Aliquots of 2 g of each soil were placed in ceramic crucibles for 3 h at 450 °C. The organic matter was then calculated with the equation: organic matter % = (initial weight − final weight) × 100. Water content measurement was performed on 20 g of soil samples, placed in closed thermo resistant plastic containers that were previously weighted and placed in an oven at 70 °C for 24 h. Percentage of water in the sediment calculated with the equation: water content % = (initial weight − final weight) × 100.

2.4. Protein extraction and analyses

Leaf samples from the five rosettes collected at the different sub-sites on MP that were used for mineral analyses, were utilised for protein extraction, following the method of Pirondini et al. (2006). Since there are restrictions on the sampling of biological material from the Natural Reserve, protein analysis was performed on pools of leaves rather than on leaves from a single plant in replicates. Proteins were quantified spectrophotometrically at 562 nm by using BCA Protein Assay Kit (Novagen, Merck KGaA, Darmstadt, Germany). Equal amounts (1.5 mg) of total protein extracts were separated by two dimensional liquid chromatography (2D-LC), using the ProteomeLab™ PF 2D instrument (Beckman Coulter, Fullerton, CA, USA) (Pirondini et al., 2006). To test the reproducibility of the method, three technical replicates for each biological sample were analysed. A set of proteins showing different levels of abundance among MP populations from different sub-sites was identified and the proteins were then subjected to trypsin digestion before mass spectrometry (MS) analyses as previously described (Visioli et al., 2010a). Proteins identification was carried out with a MS/MALDI-TOF Micromass LR apparatus (Micromass Waters Corporation, Milford, MA, USA).

2.5. Statistical analyses

ANOVAs after log-transformation of the response variable and Tukey's Post Hoc tests were used to test for differences in plant morphology and reproductive traits, for differences in plants Ni concentration and soils Ni concentration. The response variables for Ni in plants were: total Ni in roots, total Ni in shoots and Ni translocation factor (shoot/root ratio). For soil: total Ni content, Ni extractable fraction and Ni extraction factor (EF) (concentration extracted/concentration in soil). The relationship between whole plant Ni content in plants and EF was estimated using Pearson's (r) coefficient after log-transformation of variables.

The occurrence of phenotypic differences among *N. caerulescens* populations living in the different sub-sites in MP was tested with multivariate statistical analysis. Non-metric multidimensional scaling (n-MDS) was used to test for differences in morphological characters of plants (Clarke and Warwick, 2001). n-MDS is a non-parametric ordination method which constructs a "map" of the distances between the various sub-sites. In general stress values lower than 0.1 suggest a good ordination of data. PERmutational Multivariate ANalysis Of VAriance (PERMANOVA) was then applied to test for the simultaneous response of morphological characters to the sub-sites. PERMANOVA uses permutation methods and allows for unbalanced experimental design, asymmetrical models, and also analyses without replicates (Anderson, 2001). Morphological characters used in the PERMANOVA analysis are described in Section 2.2. The dissimilarity matrix was obtained by applying the Bray-Curtis distance after $\sqrt[4]{}$ transformation of data. The software Primer 6 (Clarke and Gorley, 2006) was used for multivariate statistical analyses.

Statistical criteria to validate the differential abundance of thoroughly separated proteins were based on ANOVAs after log transformation and Tukey's Post Hoc tests. The absorbance value measured a 214 nm represent a direct detection via peptide bonds thus providing a more universal and sensitive quantification of proteins. Significant statistical value was set at the 0.01 level. Abundance values were converted in fold differences considering a cut-off limit ≥2 (obtained for each protein as a ratio between the absorbance values in the different MPxp and the lowest absorbance value found for the same protein in a particular Mpxp). Abundance values were classified: ≥2 (between 2- and 5-fold); ≥5 (between 5- and 10-fold) and ≥10 (above 10-fold).

2.6. Bioinformatic analyses

2.6.1. Protein pattern analyses

Chromatograms obtained from the protein samples extracted from leaves of rosette at different sub-sites were compared with DeltaVue software (Eprogen, Darien, IL, USA). DeltaVue compares each chromatogram peak corresponding to a protein with the same isoelectric point (p*I*) and hydrophobicity value in different samples allowing quantification by subtractive analysis.

2.6.2. Protein identification

Peptide mass fingerprinting analysis was carried out with the Mascot program (http://www.matrixscience.com). Proteins were identified by searching against Swiss-Prot database of *A. thaliana* and other plant species and against *N. caerulescens* Expressed Sequence Tags (ESTs) database downloaded from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The following parameters were used for database search: mass accuracy below 100 ppm, maximum of one missed cleavages by trypsin, carbamidomethylation of cysteine, oxidation of methionine as fixed modifications. For mass spectrometry (MS) analyses, three technical replicates for each spectrum were performed. For proteins identification, only

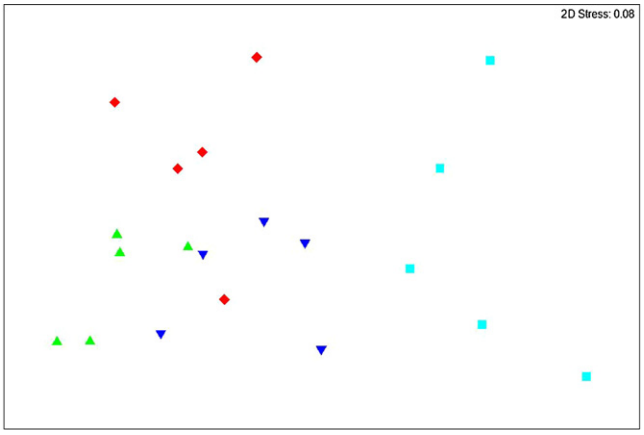

Fig. 2. Non-metric multidimensional scaling (MDS) plot obtained from the morphological data of *Noccaea caerulescens* populations of the four different sub-sites. Sub-sites are: MP1 (▲); MP2 (■); MP3 (▼); MP4 (♦). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the peptides that were common to all the resolved spectra were considered.

3. Results

3.1. Phenotypic analysis

Different phenotypic variables in the four different MPxp showed that morphological and reproductive traits differed among populations at the four sub-sites (Fig. 1; Table 1). ANOVA test of variation among sub-populations in leaf number, length and width resulted significant. Significant was also the variation observed among reproductive traits as height of flowering stalks, number of siliques and seed dimensions. The n-MDS plot groups apart the four populations (Fig. 2). The PERMANOVA test found significant differences among all populations for phenotypic traits. Pair wise tests revealed strong differences between all pairs (Table 2). Similar results were obtained with data from the year 2009 with a multivariate statistical analysis considering only morphological traits. In this case, a clearly separation was not observed among all the sub-populations (see Supplementary Information S1 and S2).

Another phenotypic parameter tested was metal accumulation in plants. The total Ni content in roots and shoots as well as the Ni translocation factor in plants of the different sub-sites are reported in Table 3. All plants showed a high accumulation of Ni in roots, from 200 to 650 $\mu g\,g^{-1}$ dry weight. Higher accumulation of Ni was observed in shoots, up to 12000 $\mu g\,g^{-1}$ dry weight, which was consistent with a hyperaccumulator phenotype. In particular the MP2p showed the highest Ni translocation factor (23), while MP3p and MP4p accumulated the highest level of total Ni per g of dry weight in different tissues. MP1p showed the lowest Ni translocation factor (8). The level of Ni in roots was as in the other MPxp.

Table 2
PERMANOVA test for the simultaneous response of phenotypic characters of *Noccaea caerulescens* to sub-sites.

Sub-sites	*t*	*P* (perm)	Perms
MP1–MP2	22.0013	0.027	126
MP1–MP3	4.1167	0.009	126
MP1–MP4	2.1691	0.01	126
MP2–MP3	2.7386	0.011	126
MP2–MP4	1.9846	0.018	126
MP3–MP4	3.1877	0.007	126

Table 3
Ni content in *Noccaea caerulescens* plants of the four sub-sites.

Sub-sites	Roots	Shoots	Ratio shoot/root
MP1s	574 ± 10.2a	4538 ± 73a	8 ± 0.2a
MP2s	253 ± 4.9b	5789 ± 666b	23 ± 0.1b
MP3s	656 ± 11.7c	12406 ± 156c	19 ± 0.3c
MP4s	655 ± 12.7c	12707 ± 154c	19 ± 0.1c

Values corresponded to an average of 5 plants per site that were analysed separately.
Metal content in plants is expressed in $\mu g\,g^{-1}$ of tissue d wt.
Values with the same letter (a, b, c, and d) are not significantly different (**$P < 0.01$)

Table 4
Soils parameters in the four sub-sites: total Ni content, pH values, water content, organic matter. Five replicate of soils were taken from the 4 different sub-sites of MP in around an area of 10 m^2 each.

Sub-sites	Total Ni content	pH	Water content (%)	Organic matter (%)
MP1s	1694 ± 84a	7.1 ± 0.1a	25.38 ± 0.23a	4.84 ± 0.22a
MP2s	2032 ± 101b	7.2 ± 0.2a	19.01 ± 0.2b	2.95 ± 0.14b
MP3s	1976 ± 77b	7.0 ± 0.1a	19.50 ± 0.15b	3.01 ± 0.17b
MP4s	1904 ± 99b	7.3 ± 0.1a	18.25 ± 0.2b	3.15 ± 0.11b

Metal content in soils is expressed in $\mu g\,g^{-1}$ of soil d wt.
Water content in soil is expressed as % of initial weight – final weight.
Values with the same letter (a, b, c, and d) are not significantly different (**$P < 0.01$).

3.2. Soil parameters

Ni contents in soils of the different sub-sites was analysed (Table 4); Tukey's tests revealed that MP1s showed a significant lower Ni concentration compared to the other three sub-sites. The analysis of the soil present in the rhizosphere confirmed the results (Table 5). The lowest Ni DTPA extractable fraction was observed in MP1s respect to the other three sub-sites (Table 5). The total Ni content in plants and the extractable Ni fraction (EF) were positively correlated between all the sub-sites (Pearson's $r = 0.50$, $p < 0.05$), suggesting that the accumulation capacity depends on the extractable fraction of Ni in soil. Soils pH did not differ among

Table 1
Phenotypic traits in *Noccaea caerulescens* plants in different sub-sites of Monte Prinzera.

Sub-populations	Times of measurement								
	24/3			6/4			24/4		15/5
	Leaf number	Leaf width	Leaf length	Leaf number	Leaf width	Leaf length	Height of flowering stalk	Number of siliques per flowering stalk	Seeds length
MP1p	22.16 ± 1.53a	1.28 ± 0.07a	3.57 ± 0.72a	24.16 ± 1.74a	1.36 ± 0.14a	3.16 ± 0.61a	35.58 ± 3.60a	59.66 ± 11.30a	1.83 ± 0.043a
MP2p	19.20 ± 2.68a	1.14 ± 0.18ac	3.42 ± 0.70a	26.40 ± 3.89b	1.26 ± 0.20a	2.88 ± 0.47a	23.4 ± 2.29b	51.40 ± 7.39b	1.74 ± 0.084a
MP3p	16.20 ± 4.55b	0.80 ± 0.16b	2.24 ± 0.65b	18.50 ± 4.21c	0.88 ± 0.11b	1.87 ± 0.58b	13.75 ± 3.79c	39.83 ± 15.21c	1.50 ± 0.199b
MP4p	14.40 ± 3.02b	1.06 ± 0.16c	2.96 ± 0.64b	21.20 ± 1.04d	1.18 ± 0.29ab	2.90 ± 0.72a	27.12 ± 8.84d	64.40 ± 8.84d	2.23 ± 0.094c

Leaf measures were taken in two different times and before and after the emergence of the flowering stalk. Data are means of measures of five plants ± sd. Values with the same letter (a, b, c, and d) are not significantly different (**$P < 0.01$).

Table 5
Ni content in soil of the rhizosphere of the five *Noccaea caerulescens* plants per sub-site analysed also for phenotypical parameters and protein abundance.

Sub-sites	Total Ni	DTPA Ni extraction	EF (concentration extracted/ concentration in soil)
MP1s	1282 ± 81a	30 ± 1.2a	0.023a
MP2s	1609 ± 32b	50 ± 0.8b	0.031b
MP3s	1641 ± 60b	51 ± 0.9b	0.031b
MP4s	1700 ± 42b	53 ± 0.9b	0.031b

Metal content in soil is expressed in $\mu g\, g^{-1}$ soil d wt.
Values with the same letter (a, b, c, and d) are not significantly different ($^{**}P < 0.01$).

sub-sites, but MP1s had the highest organic matter and water content (Table 4).

3.3. Proteomic analysis

To find whether the phenotypic differences reported above had any correlation with protein variation in plants of the different Mpxp, a proteomic approach was also performed, starting with a "gel free" 2D-LC protein separation. More than 300 proteins were resolved in each sample. The chromatograms of the proteins extracted from plants in the four sub-site were compared. Twenty-one proteins among the most abundant ones showed different abundance between the four MPxp and were analysed by MS. Proteins were classified according to their putative function: (i) metal transporter and chelators; (ii) plant defence proteins; (iii) protein linked to energy metabolism; (v) proteolytic enzymes; (vi) regulatory and signal transduction proteins (Table 6). Significant differences in mean abundance of specific proteins (ANOVA $p < 0.01$) were found among MPxp (Table 6; Supplementary Information S3). The population which showed the highest Ni translocation factor (MP2p), had also an higher level of proteins putatively correlated to transport and metal chelation. Regulatory proteins and proteins linked to energy metabolism were also up-regulated in MP2p whereas in MP1p we observed an up-regulation of some defence related proteins (Table 6). MP3p and MP4p did not shown any peculiar phenotypic differences but nevertheless showed lower amounts of many of the proteins identified as compared with MP1p and MP2p (Table 6).

4. Discussion

4.1. Plant and soil relationship

The significant variation on morphological and reproductive traits observed among *N. caerulescens* individuals from the different sub-sites on MP could be the result of both the genetic and phenotypic plasticity developed within this population. The first data observed in 2009 (Supplementary Information S1 and S2) and the data of 2010 on additional reproductive traits (Figs. 1 and 2, Table 2) has prompted in deep discrimination between Mpxp. The soil micro-environment, the presence of heavy metals and other chemical factors in soils as well as differences in Ca status, iron availability and sulphur supply could have influenced the emergence of particular plant phenotypes in each sub-site. Other environmental conditions such as moisture/humidity, temperature and shadow sensitivity could have contributed to differentiate among phenotypes (Baker et al., 2010).

Differences in Ni accumulation were also observed among individuals at the different sub-sites, in particular in MP1p respect to the others MPxp (Tables 3 and 4). In literature, phenotypic variations were previously evidenced among different *N. caerulescens* accessions in hydroponic media. In the work of Assunção et al. (2003a) differences in chlorosis symptoms and metal concentrations were found in four *N. caerulescens* populations growing in hydroponics under different metal concentrations (Zn, Cd, Ni); The Ni hyperaccumulor *N. caerulescens* MP and the non-metal adapted *N. caerulescens* from Czech Republic exposed to different Ni concentrations showed differences both in growth and morphology traits as well as in Ni accumulation capacity (Visioli et al., 2010a). Unfortunately the data available in the literature consider accessions with great differences in metal accumulation and metal tolerance and therefore a true comparison cannot be done with the data reported here.

Correlations were also found between metal bioavailability and metal accumulation in hyperaccumulators. Analyses carried out *in situ* showed that the accumulation capacity is positively correlated with the soil Ni extractable fraction in the hyperaccumulator *A. bertolonii* also present in MP (Lombini et al., 1998). Other factors, such as bacterial rhizosphere community and root exudates, could play a role in the metal accumulation process (Alford et al., 2010). By investigating the relationship between morphological and reproductive traits and metal soil composition Dechamps et al. (2007) demonstrated that in the non-metalliferous *N. caerulescens* populations from Luxemburg there was a negative influence on reproductive traits in condition of Zn supply whereas metal concentration in soil did not affect vegetative growth. The trade-off between cost and gain in plants growing on metalliferous soils is not universal among *N. caerulescens*. No cost for a higher tolerance to Zn was paid in phenotypic and reproductive traits in a metalliferous populations from Prayon and Angleur in Belgium. A similar observation was reported by Jiménez-Ambriz et al. (2006) after a comparison of morphological and phenological traits in *N. caerulescens* between populations at contaminated and non-contaminated sites in southern France. In our work, although using the less accumulating sub-site (MP1p) showed a greater vegetative growth, there was no clear relationship between phenotypic variability for Ni accumulation and morphological and reproductive traits (Fig. 1; Table 1).

Correlation between phenotype and genetic variation, in *N. caerulescens* populations were also assessed in different European *N. caerulescens* populations. Besnard et al. (2009) investigating the genetic variation in metalliferous and non-metalliferous *N. caerulescens* populations from Switzerland by the use of cleaved amplified polymorphic sites (CAPs) and microsatellites found that heavy metal concentrations in soils was correlated with the variation at target loci for genes encoding for metal transporters. Similar results were found investigating single nucleotide polymorphisms (SNPs) in target and non-target genes in different *N. caerulescens* populations growing in different type of soils (Basic et al., 2006). These results suggested that natural selection may limit gene flow between individuals of metalliferous and non-metalliferous locations at least for some genetic loci more directly involved in determining fitness. Similar evidence was also found in *Populus*, comparing clones which showed different capacity to accumulate Cd with evidence of a positive correlation between SNPs at some target genes and selection (Marmiroli et al., 2011). On the contrary, in the four *N. caerulescens* MP sub-populations considered in this work, a high genetic similarity within individuals was suggested by the existence of a very low genetic divergence within and among individuals in target genes involved in uptake, chelation and sequestration of metals (Pirondini PhD thesis).

4.2. Proteins as functional molecular markers

For the purpose of correlating differences observed in phenotypic traits with proteome variations, we considered those proteins that seemed more affected by the micro-environmental perturbations. The correlation between proteome and phenotypic traits that we found involved the protein species described below related

Table 6
MALDI-TOF/MS identification of proteins from *Noccaea caerulescens* shoots of plants from the different Monte Prinzera sub-sites.

Protein class	Protein name[a]	Accession number[a]	MW (kDa)/p*I*[b]	Mascot score[c]	Coverage (%)[c]	EST *Thlaspi*[d]	MP1[e]	MP2[e]	MP3[e]	MP4[e]
Metal transport and chelation	Metallothionein-like protein 4C	MT4C_ORYSJ	8193/6.02	17	34	gb|DN925349.1|		>2		
	ABC transporter G family member 27	AB27G_ARATH	82232/9.12	28	6	gb|DN924916.1|		>10		
Defence proteins	Pectinesterase/pectinesterase inhibitor	PME_MEDSA	49558/7.55	33	12	gb|DN925135.1|	>10	>5	>10	
	Putative defensin-like protein 40	DEF40_ARATH	9965/8.94	14	25	gb|DN927710.1|	>10	>10	>10	
	Putative defensin-like protein 235	DF235_ARATH	10034/8.53	29	41	gb|DN926171.1|	>5			
	Antifungal protein (Fragment)	AFP_CULCO	2368/4.54	22	85	/	>10	>10	>5	
	Defensin-like protein 74	DEF74_ARATH	9216/6.07	15	17	gb|DN924158.1|	>10	>10	>10	
	Disease resistance protein RPM1	RPM1_ARATH	107556/8.58	13	3	gb|DN924454.1|	>5			
Energy metabolism	Acyl-coenzyme A oxidase, peroxisomal	ACOX2_CUCMA	77841/8.76	28	7	gb|DN926205.1|		>2	>5	
	NAD(P)H-quinone oxidoreductase subunit I, chloroplastic	NDHI_COFAR	19956/5.91	44	20	gb|DN926022.1|				
	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, cytoplasm	GAPN_ARATH	53710/6.23	30	10	gb|DN927432.1|	>5	>5		
	Oxygen-evolving enhancer protein 1, chloroplastic	PSBO_BRARA	3473/6.34	17	0	gb|DN927183.1|		>2		
	NAD(P)H-quinone oxidoreductase subunit J, chloroplastic	NDHJ_DAUCA	18677/6.58	28	20	gb|DN925536.1|	>10	>10		
	Probable complex I intermediate-associated protein 30, mitochondrion	CIA30_ARATH	25404/5.97	44	29	gb|DN923776.1|		>10		
Proteolysis	Probable thylakoidal processing peptidase 2	TPP2_ARATH	40581/6.54	20	8	gb|DN927173.1|				
Regularory proteins	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin	SM3L1_ARATH	99352/8.9	34	7	gb|DN924888.1|	>10	>10		
	Glycine-rich protein 2	GRP2_NICSY	20076/5.64	28	19	gb|DN926340.1|	>5	>5	>5	
	Zinc finger protein CONSTANS-LIKE 9	COL9_ARATH	41868/5.89	36	10	gb|DN927296.1|		>5		
	Heat stress transcription factor B-1	HSFB1_ORYSJ	33121/9.35	37	18	gb|DN927520.1|		>3	>2	> 2
	Serine/threonine-protein phosphatase PP1	PP1_ORYSJ	36827/5.07	39	14	gb|DN925611.1|	>10	>10	>10	
	Serine/threonine-protein kinase SRK2H	SRK2H_ARATH	41984/5.19	34	14	gb|DN927651.1|	>2	>10	>2	
	F-box protein DOR	DOR_ARATH	45254/9.39	36	10	gb|DN926000.1|				
Others	Uncharacterised 2.5 kDa protein in tRNA-Arg-tRNA-Asn intergenic region	YCX1_MAIZE	2534/4.14	19	65	/				

[a] Putative protein identification and accession number of the closest match in the SWISS PROT database.
[b] Predicted MW of the closest match in the SWISS PROT and experimentasl p*I* values estimated from the 2D-LC.
[c] Score and percentage of coverage of the peptide sequence tags matched.
[d] *Noccaea caerulescens* ESTs clones identified by tBLASTn search.
[e] Fold variation in peak abundance between proteins in the four different *Noccaea caerulescens* MPxp: ≥2 (between 2- and 5-fold); ≥5 (between 5- and 10-fold) and ≥10 (above 10-fold). No value in the table indicate that, for the corresponding peak was not possible to identify a change that exceeds a fold variation ≥2. ANOVAs test was performed on the absorbance values at 214 nm and data are reported in Supplementary Information S3.

to different classes: (i) metal transporter and chelators; (ii) plant defence proteins; (iii) protein linked to energy metabolism; (v) proteolytic enzymes; (vi) regulatory and signal transduction proteins (Table 6).

4.2.1. Metal chelation and transport

MP2p, the most efficient in translocation of Ni in the shoot, showed a significant higher level of abundance of a putative metallothionein protein (MT4C) and the ABC27 transporter (Table 6). Both are needed for an efficient translocation and sequestration of metal ions in the shoot for an efficient system of xylem loading (Clemens, 2006; Xing et al., 2008) and for sequestration in vacuoles of root cells (Xing et al., 2008).

Chelation of trace elements by organic acids, aminoacids, peptides and proteins is relevant to plant's detoxification. Although a correlation between levels of MTs and metal concentration in plant could not be established, higher levels of these proteins were evident in hyperaccumulators. For instance MT 1B showed an increase in the metallicolous MP *N. caerulescens* growing in the laboratory in presence of Ni whereas a similar increase was not observed in the non-metallicolous population from Czech Republic (Visioli et al., 2010a). Besides *N. caerulescens*, other MTs have a constitutively higher expression in different hyperaccumulators: *Arabidopsis halleri*, *Silene paradoxa* and *Silene vulgaris* (Roosens et al., 2005; Chiang et al., 2006; van de Mortel et al., 2006; van Hoof et al., 2001; Mengoni et al., 2003). Yeast complementation studies with the *Arabidopsis* type-4 MTs (MT4a and MT4b) genes conferred greater Zn tolerance and a higher accumulation of Zn than other MTs (Guo et al., 2008).

MP2p also showed a significant higher level of abundance of a member of the ABC transporter proteins. ABC transporters are involved in sequestration of metals in vacuoles and in other subcellular compartments or outside the cell wall (Sanchez-Fernandez et al., 2001; Martinoia et al., 2002). AtMRP10 homolog was differentially expressed in shoots of two *N. caerulescens* populations with contrasting Zn tolerance and accumulation (Hassinen et al., 2007) and ATH13 was more expressed in the shoot as compared with *A. thaliana* (van de Mortel et al., 2008). Recently a role in tolerance to Cu, Zn, Cd and in response to oxidative stress of ABC transporters found in micorrhyzae has been postulated (González-Guerrero et al., 2010).

4.2.2. Defence proteins

Plant's defensins were abundant in the four MPxp. They also play a role in metal stress response: in the metal hyperaccumulator *A. halleri* defensin pool is Zn-responsive, both at the mRNA and protein level (Mirouze et al., 2007). Our proteomic analysis showed that in *N. caerulescens* the levels of these proteins change significantly among sub-sites. In particular the MP1p showed the higher level of defensin like proteins. Defensins' level can be instrumental to the trade-off between metal hyperaccumulation and organic defence in *N. caerulescens,* confirming previous proteomic data in which a higher level of metal accumulation in *N. caerulescens* and *A. halleri* leads to a down regulation of the defence signalling (Visioli et al., 2010a; Farinati et al., 2009).

4.2.3. Energy metabolism

In recent proteomic analyses on plant hyperaccumulators exposed to metals, a consistent up-regulation of proteins involved in energy metabolism was observed (Tuomainen et al., 2006, 2010; Farinati et al., 2009; Walliwalagedara et al., 2010; Zeng et al., 2011). The common interpretation is that the enhancing energy demand of the entire cellular metabolism was instrumental to obtain the best performances for metal hyperaccumulation. The level of these enzymes varies in the different Mpxp revealing a slight increase in the MP2p, with the highest translocation factor. The GAPDH involved in carbon metabolism instead, did not show significant differences in abundance between individuals.

4.2.4. Proteolysis

The role of processing peptidases is to contribute to the correct folding of nascent proteins in the chloroplast. The high level of a thylakoidal processing peptidase 2 in our work could be correlated with an increase in energy metabolism and in the production of active proteins in thylacoid membranes, where this protein is localised (Table 6 and Tuomainen et al., 2006). The same enzyme was found to increase in abundance in a *Populus nigra* clone showing high performance in Cd uptake and translocation (Visioli et al., 2010b). A similar relative pattern was seen in the *N. caerulescens* accession La Calamine protein disulfide isomerase and in the FKBP-type peptidyl-prolyl-cis-trans-isomerase, involved in protein folding (Tuomainen et al., 2006)

4.2.5. Regulatory proteins

Two important regulatory proteins, the heat shock-transcription factor (HSF) B-1 and a serine/threonine-protein kinase SRK2H were more abundant in MP2p. Signalling and regulation play a fundamental role in metal accumulation and tolerance. Heat shock (HS) and metal stress were both associated with enhanced production of ROS and activation of an oxidative burst (Vacca et al., 2004). HSFs might function as direct sensors of hydrogen peroxide in plants (Miller and Mittler, 2006), as shown in roots of wheat and rice where Cd stress caused increase in HSFA4a expression, together with the MT genes, thus conferring Cd tolerance (Shim et al., 2009). In *N. caerulescens* MP2p the highest translocation of Ni in the shoots was coupled with a higher level of abundance of a putative MT and the HSF B-1, indicating a possible cross-talk of these two proteins in determining the Ni translocation capacity. HSF could be a general transducer for abiotic stress response mechanisms when different stresses are combined as: metals, light, heat and drought. Protein phoshorylation is another important aspect of the response to environmental stimuli such as the presence of metals in soils. The importance of kinases like SRK2 in the induction of several distinct transduction pathways during metal stress was evidenced also for MAPK kinases in condition of excess of Cd, Cu, Zn, Pb and Hg in *Medicago sativa* and *Oryza sativa* (Lin et al., 2005; Yeh et al., 2003, 2007; Huang and Huang, 2008; Jonak et al., 2004).

5. Conclusions

In this work a correlation was reported between phenotypic and proteomic variation in the Ni hyperaccumulator *N. caerulescens* population of MP, in its natural environment. This population, which on the basis of a molecular marker analysis has a low level of genetic diversity (Pirondini PhD thesis), was clearly separated into four sub-populations according to phenotypic differences, thus suggesting phenotypic plasticity in response to differences in micro-environments. Proteome gives an important contribution to the phenotype. Specific protein variation may be interpreted as phenotypic plasticity because proteins transduce information from environmental factors, such as biotic and abiotic stresses, to genes and metabolites. In addition, proteins may have a role in epigenetic variation, which is not simply predictable from the gene sequence, but play a significant role in determining a specific phenotype (Richards, 2011).

The occurrence and strength of phenotypic plasticity is a relevant topic of investigation in modern evolutionary and ecological research, since its role for faster adaptations to new or changing environments with respect to changes at genotypic level. Thus, proteomic studies in the natural environments like the one we have reported for *N. caerulescens* of MP in Italy shall become instrumental

to understand how the relationship between genes and environment and between cellular protein functions and environment are relevant in determining fitness and plasticity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2011.11.016.

References

Alford, E.R., Pilon-Smits, E.A.H., Paschke, M.W., 2010. Metallophytes – a view from the rhizosphere. Plant Soil 337, 33–50.
Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. Austral. Ecology 26, 32–46.
Assunção, A.G.L., Da Costa Martins, P., De Folter, S., Vooijs, R., Schat, H., Aarts, M.G.M., 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ. 24, 217–226.
Assunção, A.G.L., Schat, H., Aarts, M.G.M., 2003a. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytol. 159, 411–419.
Assunção, A.G.L., Ten Bookum, W.M., Nelissen, H.J.M., Vooijs, R., Schat, H., Ernst, W.H.O., 2003b. A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. New Phytol. 159, 383–390.
Baker, A.J.M., Ernst, W.H.O., 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, L.C., Hallberg, K.B. (Eds.), Ecology of Industrial Pollution. Cambridge University Press, Cambridge, pp. 7–40.
Baker, A.J.M., McGrath, S.P., Reeves, D.R., Smith, J.A.C., 2000. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry, N., Banuelos, G. (Eds.), Pytoremediation of Contaminated Soils and Water. CRC Press LLC, Boca Raton (FL), pp. 171–188.
Basic, N., Besnard, G., 2006. Gene polymorphisms for elucidating the genetic structure of the heavy-metal hyperaccumulating trait in *Thlaspi caerulescens* and their cross-genera amplification in Brassicaceae. J. Plant Res. 119, 479–487.
Basic, N., Salamin, N., Keller, C., Galland, N., Besnard, G., 2006. Cadmium hyperaccumulation and genetic differentiation of *Thlaspi caerulescens* populations. Biochem. Syst. Ecol. 34, 667–677.
Besnard, G., Basic, N., Christin, P.-A., Savona-Bianchi, D., Gallard, N., 2009. Thlapsi caerulescen (Brassicaceae) population geneticsin western Switzerland: is the genetic structure affected by natural variation of soil heavy metal concentrations? New Phytol. 181, 974–984.
Chiang, H.C., Lo, J.C., Yeh, K.C., 2006. Genes associated with heavy metal tolerance and accumulation in Zn/Cd hyperaccumulator *Arabidopsis halleri*: a genomic survey with cDNA microarray. Environ. Sci. Technol. 40, 6792–6798.
Clarke, K.R., Gorley, R.N., 2006. Primer v6: User Manual/Tutorial. PRIMER-E, Plymouth, UK, p. 192.
Clarke, K.R., Warwick, R.M., 2001. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation, 2nd ed. PRIMER-E, Plymouth, p. 172.
Clemens, S., 2006. Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88, 1707–1719.
Coleman, R.G., 1977. Ophiolites. Springer-Verlag, New York, p. 227.
Coleman, R.G., Jove, C., 1992. Geological origin of serpentinites. In: Baker, A.J.M., Proctor, J., Reeves, R.D. (Eds.), The Vegetation of Ultramafic (Serpentine) Soils. Intercept, Andover, pp. 1–17.
Dechamps, C., Lefèbvre, C., Noret, N., Meerts, P., 2007. Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. New Phytol. 173, 191–198.
Dechamps, C., Noret, N., Mozek, R., Escarré, J., Lefèbvre, C., Gruber, W., Meerts, P., 2008. Cost of adaptation to a metalliferous environment for *Thlaspi caerulescens*: a field reciprocal transplantation approach. New Phytol. 177, 167–177.
Dickinson, N.M., Baker, A.J.M., Doronila, A., Laidlaw, S., Reeves, R., 2009. Phytoremediation of inorganics: realism and synergies. Int. J. Phytorem. 11, 97–114.
Escarrè, J., Lefebvre, C., Gruber, W., Leblanc, M., Lepart, J., Riviere, Y., Delay, B., 2000. Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implication for phytoremediation. New Phytol. 145, 429–437.
Farinati, S., DalCorso, G., Bona, E., Corbella, M., Lampis, S., Cecconi, D., Polati, R., Berta, G., Vallini, G., Furini, A., 2009. A proteomic analysis of *Arabidopsis halleri* shoots in response to the heavy metals cadmium and zinc and rhizosphere microorganisms. Proteomics 9, 4837–4850.
González-Guerrero, M., Benabdellah, K., Valderas, A., Azcón-Aguilar, C., Ferrol, N., 2010. GintABC1 encodes a putative ABC transporter of the MRP subfamily induced by Cu, Cd, and oxidative stress in Glomus intraradices. Mycorrhiza 20, 137–146.
Gregorius, H.R., Kleinschmit, J.R.G., 1999. The environmental dichotomy of adaptation and the role of genetic diversity. Silvae Genet. 48, 193–199.
Guo, W.-J., Meetam, M., Goldsbrough, P.B., 2008. Examining the specific contributions of individual Arabidopsis metallothioneins to copper distribution and metal tolerance. Plant Physiol. 146, 1697–1706.
Hassinen, V.H., Tervahauta, A.I., Halimaa, P., Plessl, M., Peräniemi, S., Schat, H., Aarts, M.G.M., Servomaa, K., ·Kärenlampi, S.O., 2007. Isolation of Zn-responsive genes from two accessions of the hyperaccumulator plant *Thlaspi caerulescens*. Planta 225, 977–989.
Heiri, O., Lotter, A.F., Lemcke, G., 2001. Loss on ignition as a method for estimating organic and carbonate content in sediments: reproducibility and comparability of results. J. Paleolimn. 25, 101–110.
Hoffmann, A.A., Parsons, P.A., 1991. Evolutionary Genetics and Environmental Stress. Oxford University Press Inc., New York.
Huang, T., Huang, H., 2008. ROS and CDPK-like kinase-mediated activation of MAP kinase in rice roots exposed to lead. Chemosphere 71, 1377–1385.
Jiménez-Ambriz, G., Petit, C., Bourrié, I., Dubois, S., Olivieri, I., Ronce, O., 2006. Life history variation in the heavy metal tolerant plant *Thlapsi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. New Phytol. 173, 199–215.
Jonak, C., Nakagami, H., Hirt, H., 2004. Heavy metal stress activation of distinct mitogen-activated protein kinase pathways by copper and cadmium. Plant Physiol. 136, 3276–3283.
Krämer, U., 2010. Metal hyperaccumulation in plants. Annu. Rev. Plant Biol. 61, 517–534.
Lin, C.W., Chang, H.B., Huang, H.J., 2005. Zinc induces mitogen activated protein kinase activation mediated by reactive oxygen species in rice roots. Plant Physiol. Biochem. 43, 963–968.
Lindsay, W.L., Norwel, W.A., 1978. Development of a DTPA soil test for zinc, iron, manganese, and copper. Sci. Soil Soc. Am. J. 42, 421–428.
Lombi, E., Tearall, K.L., Howarth, J.R., Zhao, F.J., Hawkesford, M.J., McGrath, S.P., 2002. Influence of iron status on cadmium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens*. Plant Physiol. 128, 1359–1367.
Lombini, A., Dinelli, E., Ferrari, C., Simoni, A., 1998. Plant-soil relationships in the serpentinite screes of Mt. Prinzera (Northern Appennines, Italy). J. Geochem. Explor. 64, 19–23.
Maestri, E., Marmiroli, M., Visioli, G., Marmiroli, N., 2010. Metal tolerance and hyperaccumulation: costs and trade-offs between traits and environment. Environ. Exp. Bot. 68, 1–13.
Marmiroli, M., Visioli, G., Maestri, E., Marmiroli, N., 2011. Correlating SNP genotype with the phenotypic response to exposure to cadmium in *Populus* spp. Environ. Sci. Technol. 45, 4497–4505.
Martinoia, E., Klein, M., Geisler, M., Bovet, L., Forestier, C., Kolukisaoglu, U., Müller-Röber, B., Schulz, B., 2002. Multifunctionality of plant ABC transporters—more than just detoxifiers. Planta 214, 345–355.
Mengoni, A., Gonnellii, C., Hakvoort, H.W.J., Galardi, F., Bazzicalupo, M., Gabbrielli, R., Schat, H., 2003. Evolution of copper-tolerance and increased expression of a 2b-type metallothionein gene in *Silene paradoxa* L. populations. Plant Soil 257, 451–457.
Miller, G., Mittler, R., 2006. Could heat shock transcription factors function as hydrogen peroxide sensors in plants? Ann. Bot. 89, 279–288.
Milner, M.J., Kochian, L.V., 2008. Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. Ann. Bot. 102, 3–13.
Mirouze, M., Sels, J., Richard, O., Czernic, P., Loubet, S., Jacquier, A., François, I.E.J.A., Cammue, B.P.A., Lebrun, M., Berthomieu, P., Laurence Marque', L., 2007. A putative novel role for plant defensins: a defensin from the zinc hyper-accumulating plant, *Arabidopsis halleri*, confers zinc tolerance. Planta 225, 977–989.
Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D., Moczek, A.P., 2010. Phenotypic plasticity's impacts on diversification and speciation. Trends Ecol. Evol. 25, 459–467.
Pirondini, A., 2006. Analysis of genetic variation in the Monte Prinzera population of *Thlaspi caerulescens*. In: Genomic and Proteomic Analysis of Natural Variation for Metal Hyperaccumulation in Model Plants and its Relevance for Phytoremediation. PhD Thesis, University of Parma, Ialy.
Pirondini, A., Visioli, G., Malcevschi, A., Marmiroli, N., 2006. A 2-D liquid-phase chromatography analysis in plant tissues. J. Chromatogr. B 833, 91–100.
Pollard, A.J., Powell, K.D., Harper, F.A., Smith, J.A.C., 2002. The genetic basis of metal hyperaccumulation in plants. Crit. Rev. Plant Sci. 21, 539–566.
Richards, E.J., 2011. Natural epigenetic variation in plant species: a view from the field. Curr. Opin. Plant Biol. 14, 204–209.

Roosens, N.H., Leplae, R., Bernard, C., Verbruggen, N., 2005. Variations in plant metallothioneins: the heavy metal hyperaccumulator *Thlaspi caerulescens* as a case study. Planta 222, 716–729.

Sanchez-Fernandez, R., Davies, T.G., Coleman, J.O., Rea, P.A., 2001. The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. J. Biol. Chem. 276, 30231–30244.

Schat, H., Llugany, M., Bernhard, R., 2000. Metal-specific patterns of tolerance, uptake and transport of heavy metals in hyperaccumulating and nonhyperaccumulating metallophytes. In: Terry, N. (Ed.), Phytoremediation of Contaminated Soils and Water. CRC Press, pp. 171–188.

Shim, D., Hwang, J.-U., Lee, J., Lee, S., Choi, Y., An, G., Martinoia, E., Lee, Y., 2009. Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. Plant Cell 21, 4031–4043.

Sultan, S.E., 2000. Phenotypic plasticity for plant development, function and life history. Trends Plant Sci. 5, 537–542.

Tuomainen, M., Tervahauta, A., Hassinen, V., Schat, H., Koistinen, K.M., Lehesranta, S., Rantalainen, K., Häyrinen, J., Auriola, S., Anttonen, M., Kärenlampi, S., 2010. Proteomics of *Thlapsi caerulescens* accessions and an interaccession cross segregating for zinc accumulation. J. Exp. Bot. 61, 1075–1087.

Tuomainen, M.H., Nunan, N., Lehesranta, S.J., Tervahauta, A.I., Hassinen, V.H., Schat, H., Koistinen, K.M., Auriola, S., McNicol, J., Karenlampi, S., 2006. Multivariate analysis of protein profiles of metal hyperaccumulator *Thlaspi caerulescens* accessions. Proteomics 6, 3696–3706.

Vacca, R.A., de Pinto, M.C., Valenti, D., Passarella, S., Marra, E., De Gara, L., 2004. Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco bright-yellow 2 cells. Plant Physiol. 134, 1100–1112.

van de Mortel, J.E., Almar Villanueva, L., Schat, H., Kwekkeboom, J., Coughlan, S., Perry, D., Moerland, P.D., van Themaat, E.V.L., Koornneef, M., Aarts, M.G.M., 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 Physiol. 142, 1127–1147.

van de Mortel, J.E., Schat, H., Moerland, P.D., Van Themaat, E.V.L., Van der Ent, S., Blankestijn, H., Ghandilyan, A., Tsiatsiani, S., Aarts, M.G.M., 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 Environ. 31, 301–324.

van Hoof, N.A., Hassinen, V.H., Hakvoort, H.W., Ballintijn, K.F., Schat, H., Verkleij, J.A., Ernst, W.H., Karenlampi, S.O., Tervahauta, A.I., 2001. Enhanced copper tolerance in *Silene vulgaris* (Moench) Garcke populations from copper mines is associated with increased transcript levels of a 2b-type metallothionein gene. Plant Physiol. 126, 1519–1526.

Visioli, G., Pirondini, A., Malcevschi, A., Marmiroli, N., 2010a. Comparison of proteomic variation in *Thlaspi caerulescens* populations from metalliferous and nonmetalliferous soils. Int. J. Phytorem. 12, 805–819.

Visioli, G., Marmiroli, M., Marmiroli, N., 2010b. Two dimensional liquid chromatography (2D-LC) technique coupled with mass spectrometry analysis to compare the proteomic response to Cd stress in plants. J. Biomed. Biotechnol., doi:10.1155/2010/567510.

Walliwalagedara, C., Atkinson, I., van Keulen, H., Cutright, T., Wei, R., 2010. Differential expression of proteins induced by lead in the Dwarf Sunflower *Helianthus annuus*. Phytochemistry 71, 1460–1465.

Xing, J.P., Jang, R.F., Ueno, D., Ma, J.F., Schat, H., McGrath, S.P., Zhao, F.J., 2008. Variation in root-to-shoot translocation of cadmium and zinc among different accessions of the hyperaccumulators *Thlaspi caerulescens* and *Thlaspi praecox*. New Phytol. 178, 315–325.

Yeh, C.M., Chien, P.S., Huang, H.J., 2007. Distinct signaling pathways for induction of MAP kinase activities by cadmium and copper in rice roots. J. Exp. Bot. 58, 659–671.

Yeh, C.M., Hung, W.C., Huang, H.J., 2003. Copper treatment activates mitogen-activated protein kinase signaling in rice. Physiol. Plant. 119, 392–399.

Zeng, X.-W., Qiu, R.-L., Ying, R.-R., Tang, Y.-T., Tang, L., Fang, X.-H., 2011. The differentially-expressed proteome in Zn/Cd hyperaccumulator *Arabis paniculata* Franch. in response to Zn and Cd. Chemosphere 82, 321–328.