UNIVERSITA' DEGLI STUDI DI CATANIA

Dipartimento di Scienze Biologiche, Geologiche e Ambientali

PHD IN
SCIENZE GEOLOGICHE, BIOLOGICHE E AMBIENTALI
Coordinatore: Prof.ssa Agata Di Stefano

HELENA DOMENICA PAPPALARDO

Investigation on genes possibly involved in the response to heavy metals in *Cynara cardunculus* L.

Ph.D. thesis

Tutor: Prof. S.A. Raccuia

XXX cycle

AA.AA. 2014÷2017

Riassunto

Lo stress da metalli pesanti è uno stress di tipo abiotico che risulta molto pericoloso per la salute umana. Cadmio (Cd) ed arsenico (As) sono due metalli spesso presenti in terreni contaminati a causa di attività minerarie o altre attività industriali. Il Cd è uno dei più tossici tra questi, e quando assunto dall'uomo viene accumulato in fegato e reni, causando gravi danni a tali organi. L'As è un metalloide, esistente in natura in diversi stati di ossidazione: −3, 0, +3, e +5. La forma trivalente (arsenite) risulta essere più tossica della pentavalente (arsenate) Attraverso la catena alimentare, questi metalli entrano in contatto con l'uomo, causando malattie cardiovascolari e cancro. Alcune piante possono essere in grado di detossificare i suoli contaminati da sostanze tossiche, quali i metalli pesanti. Tale tecnica, nominata phytoremediation, è una metodologia economica che sfrutta la capacità di alcuni organismi vegetali di rimuovere i metalli pesanti dall'ambiante, trasformandoli in sostanze meno tossiche e meno reattive, o accumulandoli negli organi interni. È possibile, in base alla strategia adottata, classificare diversi processi: fitoestrazione, fitofiltrazione, fitostabilizzazione, fitovolatilizzazione e fitodegradazione. Nella tecnica di fitoestrazione, la pianta assorbe i metalli dal suolo, e accumula quest'ultimi negli organi interni in concentrazioni .100 - 1000 volte più elevate di una pianta non tollerante. Tali organismi vengono classificati come 'iperaccumulatori'. Il cardo (*Cynara cardunculus* L.) è una pianta pluriennale, particolarmente adattata all'ambiente mediterraneo in grado di tollerare moderate concentrazioni di NaCl e crescere in terreni inquinati. Tre taxa sono ad oggi riconosciute: *C. cardunculus* L. subsp. *scolymus* (L.) Hegi = *C. cardunculus* L. var. *scolymus* (L.) Hayek (carciofo), *C. cardunculus* L. var. *altilis* DC. (cardo domestico), and *C. cardunculus* L. var. *sylvestris* Lam. (cardo selvatico). Lo scopo di questo progetto è stato quello di indagare sui geni associati all'accumulo di metalli pesanti in *C. cardunculus* L. In particolare nell'ambito di questo lavoro l'influenza del genotipo, cardo coltivato e selvatico*,* è stata testata come percentuale di semi germinati su terreni contaminati da Cd, As e Cd + As alle concentrazioni di 0, 10. 50, 100 e 200 µM. Per lo svolgimento di questa prova, il genotipo *altilis*, e due genotipi *sylvestris* (A14SR e R14CT) sono stati considerati. Inoltre è stata valutata nelle due varietà, la capacità di crescere in terreni contaminati con metalli pesanti, misurando radici e germogli in piantine cresciute per tre settimane con Cd e As a 0, 25, 50 µM. Prendendo come modello tra i tre taxa, il cardo *altilis,* per i suoi possibili impieghi come specie che produce

biomassa da destinare alla chimica verde, abbiamo ricercato in cardo dei geni ortologhi a quelli di altre piante che risultano essere associati alla tolleranza e accumulo di metalli pesanti. Per fare ciò abbiamo disegnato i primers di clonaggio tramite il programma primer3, per amplificare e clonare l'amplificato, sequenza target nel vettore utilizzabile per il sequenziamento. Le sequenze target sono state analizzate con l'utilizzo di tools bioinformatici (Blast, CodonCode, BioEdit, Clustal omega). Con questo metodo i geni di cardo probabilmente associati al trasporto e accumulo di metalli pesanti, sono stati identificati e caratterizzati. Abbiamo saggiato tramite l'uso della real-time PCR, i livelli trascrizionali di espressione genica sui seguenti geni: Natural resistance of macrophage isoforma 1 (NRAMP1) e 3 (NRAMP3), Zinc/Iron Protein 11 (ZIP11), Heavy metal ATPase 3 (HMA3), Phosphate transporter 1 (PHT1) e ABC transporter C1 (ABCC1). L'espressione è stata saggiata in piante delle varietà *altilis* e *sylvestris (*A14SR) cresciute per due e tre settimane su ½ MS medium contaminato con Cd e As a 0, 25, 50 μM. La normalizzazione dei dati è stata effettuata con l'utilizzo dei geni di riferimento EF1-alpha e GAPDH, isolati precedentemente e valutati in funzione della loro stabilità in diverse fasi di crescita di *Cynara* (*altilis*) non trattato.

I risultati hanno mostrato che il cardo è in grado di germinare in terreni contaminati da metalli pesanti, con una percentuale media di germinazione del 65.9 %, ottenuta dalle tre maggiori sorgenti di variabilità, includendo il tipo di metallo utilizzato e la sua concentrazione e il differente genotipo della pianta. Con riferimento alla germinazione, *altilis* è risultato il genotipo che meglio tollera il Cd durante la germinazione, con una percentuale del 83 %, e il *sylvestris* A14CT il genotipo che meglio tollera l'As durante tale fase (76 %). L'analisi della crescita della pianta in terreni contaminati ha evidenziato una riduzione della crescita nel genotipo *altilis* trattato con il Cd e non con l'As, mentre, in *sylvestris* una riduzione della crescita è stata causata dalla presenza di As alla concentrazione di 50 μM. La ricerca dei geni che in cardo domestico sono associati alla risposta ai metalli pesanti, ci ha permesso di identificare ed isolare sette geni: NRAMP1, NRAMP3, ZIP11, HMA, PCS (Phytochelatin Syntase), ABCC1, PHT. Su sei di questi (PCS è stato escluso) è stata effettuata, tramite RT-PCR, l'analisi quantitative di espressione genica sulle varietà *sylvestris* ed *altilis*. I risultati hanno mostrato un significativo incremento di espressione nella varietà *sylvestris* dei geni NRAMP3 e ZIP11 trattata con entrambi i metalli, e PHT e ABCC1, trattata con As.

In conclusione, *Cynara cardunculus* L. risulta capace di germinare in terreni contaminati con As e Cd, e la risposta alla germinazione risulta fortemente influenzata non solo dalla concentrazione e dal metallo, ma anche dal genotipo. Similmente, la crescita delle plantule è risultata influenzata dalla natura e concentrazione del metallo, e dal genotipo considerato. Per la prima volta i geni: PCS, NRAMP1, NRAMP3, ZIP11, HMA3, ABCC1, e PHT sono stati identificati in *C. cardunculus* L.. Le piante trattate hanno mostrato una variazione dei livelli trascrizionali di espressione che potrebbe essere dipesa dal tipo e concentrazione metallo, sul genotipo considerato, e dall'organo della pianta analizzato, radici o steli. Con particolare riferimento a quest'ultimo fattore studiato, nelle radici e steli di *sylvestris* trattati con As, un significativo incremento dei livelli di espressione dei geni NRAMP3, ZIP11, ABCC1 e PHT è stato monitorato Da questi dati, il *sylvestris* risulta la varietà più tollerante e utilizzabile per la detossificazione dei terreni contaminati da metalli pesanti.

Future analisi saranno necessarie, per comprendere pienamente i meccanismi utilizzati dalla pianta di cardo, durante il trasporto e accumulo di metalli pesanti. La tecnica di RNAseq potrebbe mettere in un luce la presenza di altri geni strettamente coinvolti in tale meccanismo.

Abstract

In plants, heavy metals stress may ultimately lead to serious consequences for human health. Cadmium (Cd) and arsenic (As) are frequently found in soils contaminated by mining or other industrial activities. Through food chain, these metals may enter into the human body, causing cardiovascular diseases and cancer. Cardoon (*Cynara cardunculus* L.) is a perennial crop, particularly adapted to the Mediterranean environment. It is able to accumulate heavy metals from polluted soils and thus it seems to tolerate this stress. The aim of this thesis was to characterize the transcriptional modulation of six genes that may be involved in the response to heavy metals stress and accumulation in *C. cardunculus* L.. The rate of seed germination of two different *C. cardunculus* varieties, *altilis* and *sylvestris,* was scored on agar plates medium containing Cd, As and Cd + As at 0, 10. 50, 100 and 200 µM concentrations. Transcriptional levels of Natural resistance of macrophage isoforms 1 (NRAMP1) and 3 (NRAMP3), Zinc/Iron Protein 11 (ZIP11), Heavy metal ATPase 3 (HMA3), Phosphate transporter 1 (PHT1) and ABC transporter C1 (ABCC1) were assayed by real time PCR in the two cardoon varieties, *altilis* and *sylvestris,* grown for two or three weeks on solid ½ MS medium containing Cd or As at 0, 25 and 50 µM concentrations. The results showed that both cardoon varieties were able to germinate under heavy metals contamination but just in *sylvestris* a clear correlation with an increased level of expression of genes involved in Cd and As transport was observed.

Acknowledgments

I would like to begin by expressing my thanks to my tutor Dr. Raccuia for having me enable to do this wonderful experience.

I thank to my co-tutor Dr. Puglia Giuseppe for teaching me how carry out the research and for helping and supporting during these three years of PhD.

I thank to Prof. Aarts Mark and Post doc Akkers Robert for welcoming in Wageningen University to improve my research on analysis of transcriptional levels.

I thank to my colleagues/ friends for their help and their important support during these three years.

I thank to my family to their supports.

Finally, I would like to express my sincerest gratitude to my boyfriend/husband for his support and patience, being close to me in this great goal.

Index

PREFACE

This work was done in collaboration with:

- ✓ **CNR - AGROFOOD Department - Institute for Mediterranean Agriculture and Forest Systems of Catania, which funded all the thesis work.**

- ✓ **UNIVERSITY & RESEARCH, University of Wageningen. A part of this work was done with the research group of Prof. Mark Aarts, laboratory of genetic, University of Wageningen, Netherland.**

:

1 INTRODUCTION

Environmental pollution, by chemicals in particular, is one of the key factors responsible for the destruction of important biosphere components, leading to serious and irreparable damages to earth. It can be classified in three different types: air, water and soil pollution. These affect human health and ecosystems. The principal criteria used to evaluate the contamination of toxic elements are: bioaccumulation, toxicity, and persistence. Air pollution is a harmful form of environmental pollution. Most metals in the atmosphere, are associated with particles, (diameter between 0.01 and 100 μm), in a gaseous state. The presence of these particles and their increase, have been clearly associate with the occurrence of lung cancer, asthma, allergies, and various respiratory problems. Water pollution is mainly caused by industrial waste products released into lakes, rivers, and other water bodies. Trace elements, especially metals, are present as suspended colloids or are fixed by organic and mineral substances. They may originate by either natural processes or man's activities. Historically soil and water pollution have been considered separately by environmental policy makers, but of lately, they are seen as synergistic factors that can seriously threat agricultural production and human health (Rajaganapathy et al., 2011).

Soil pollution

Soil pollution is defined by the presence of toxic chemicals (pollutants or contaminants) in concentrations high enough to pose a risk to human health and/or to the ecosystem. Soil is a very specific component of the biosphere because it is involved in the transport of chemical elements and substances to the atmosphere, hydrosphere and biota. However, its most important role for human health, relates to the quality and safety of agricultural products (Wuana, et al., 2011).

Soil contaminants include metals, inorganic ions and salts (e.g. phosphates, carbonates, sulphates, nitrates), and many organic compounds (such as lipids, proteins, DNA, fatty acids, hydrocarbons, PAHs, alcohols, etc.). Soil contamination caused by metals is largely dependent on the agricultural practices carried out in crop farms. In fact, some phosphate

fertilizers contain potentially toxic elements, including As, Cd, Cr, Pd, Hg, Ni, and V (Mortvedt, 1996.) and some pesticides contain Cu and As as part of their formulation (Quinton and Catt, 2007). It is through fertilizers and pesticides that the contaminant enters within the food chain, polluting drinking water, and fodder (Rajaganapathy et al., 2011). Skin contact, ingestion, inhalation, and dermal absorption are the ways that human health can be exposed to the risk (Elliot, 2001).

Heavy metals pollution

The heavy metals (HMs) are metals with high electronegative charge and density greater than 5 g/cm^3. HMs threat the sulphide bond between HMW (high molecular weight) proteins in the living system, using the outer-shell electrons (Agarwal, 2009). This kind of pollution not only degrades the quality of the atmosphere, water bodies, and food crops, but also affects the human and animal health by the mean of food chain (Dong et al., 2011). Cadmium and arsenic are very dangerous for human health. Both are frequently found in soils contaminated by mining or other industrial activities (Govil et al., 2007), irrigated with waste-water, fertilizers, soil amendments and pesticides (fig.1).

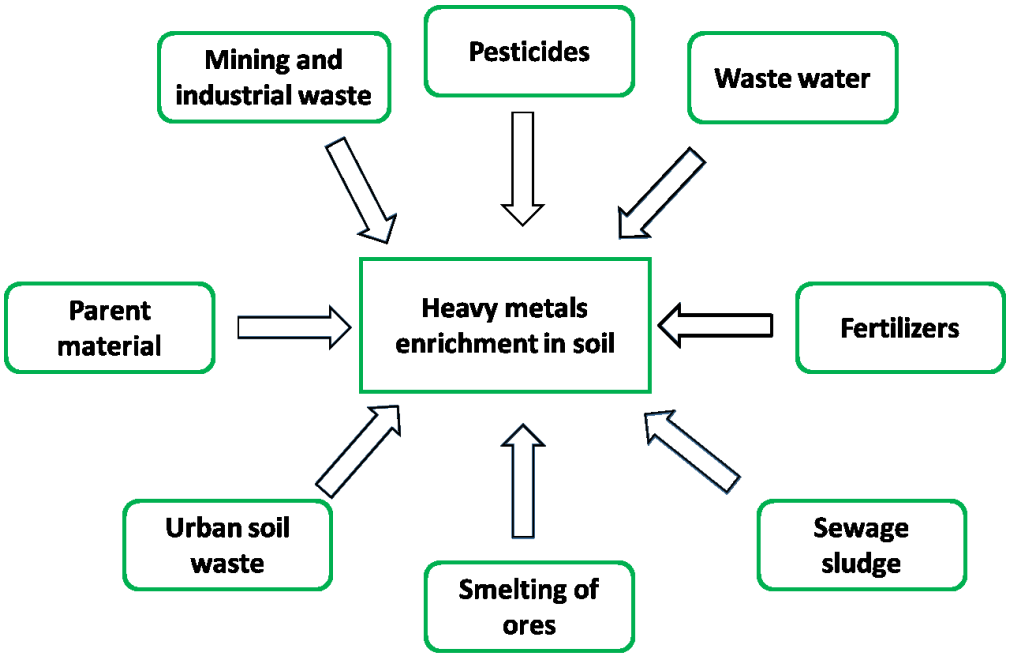


Figure 1. Scheme of possible source of heavy metals in the soil. Mahara et al., / Ecotoxicology and Environmental Safety 126 (2016) 111 – 121

The pollution level and potential ecological risk of the soils decrease in the following order: urban areas > waste disposal/treatment sites ~ industrial areas > agricultural lands ~ forest lands > water source protection areas (Hu et al., 2013). Arsenic is a metalloid of great relevance in environmental pollution, because of its toxicity and abundance (Peralta-Videa et al., 2009). It is released into the environment from smelting and mining processes, agricultural practices, fabrication and consumption of wood preservatives and food additives (Aldrich et al., 2003). About 3.5 million sites in the EU were estimated to be potentially contaminated with 0.5 million sites being highly contaminated and needing remediation. 400,000 polluted sites have been scored in European countries such as Germany, England, Denmark, Spain, Italy, Netherlands and Finland while Sweden, France, Hungary, Slovakia and Austria have 200,000 contaminated sites or less (Fig. 2) (Perez, 2012).

Figure 2. Map of the world with evidenced the sites contaminated with As http://www.bgs.ac.uk/arsenic/

Toxicity of HMs is not limited to humans or animals, but affects many other organisms, including plants. Under HMs excess, the plants show a biomass reduction, leaf chlorosis, root growth inhibition, and morphological alterations (Yadav et al., 2010; AArts et al., 2012). In plants, these types of contamination can also induce the production of reactive oxygen species (ROS), increasing lipid peroxidation and oxidative stress (Srivastava et

al., 2005). In humans, HMs are considered carcinogenic, or toxic, capable of causing damages on the central nervous system, liver, kidneys, heart, lungs, skin, and reproduction (Johnson, 1997).

Cadmium

Cadmium (atomic number 48) is a silver-grey brittle crystalline solid with atomic weight of 112.414, specific gravity 8.69, melting point 321.069 °C (at 28 atm), boiling point 767 °C, and heat of evaporation 99.87 kJ/mol. It is one of the most toxic metals that has by far a greater bioavailability than lead, arsenic, or mercury. It is a no-essential element except in marine diatoms where it can replace zinc in a specific isoform of carbonic anhydrase (Lane et al., 2005; Xu et al., 2008). In nature Cd concentration is very low and in non-contaminated soil it varies from 0.01 to 5 mg kg^{-1} of soil (Kabata-Pendias, 2004). Its bioavailability in soil depends on the concentration, pH, organic matter content, clay content, soil moisture conditions, and availability of macro- and micronutrients (Welch and Norvell, 1999). When it is in contact with humans (by the means of food chain) is accumulated in the kidney or liver. Accumulation in high levels causes stomach irritation leading to vomiting and diarrhea, and sometimes death. Cadmium impairs kidney function, reduces bone density favouring the occurrence of fractures (Fig. 3). The high amount of Cd in the human body has also been associated with breast cancer, cardiovascular diseases and obstructive pulmonary diseases (Toxicological Profile for Cadmium, 2012 ATSDR).

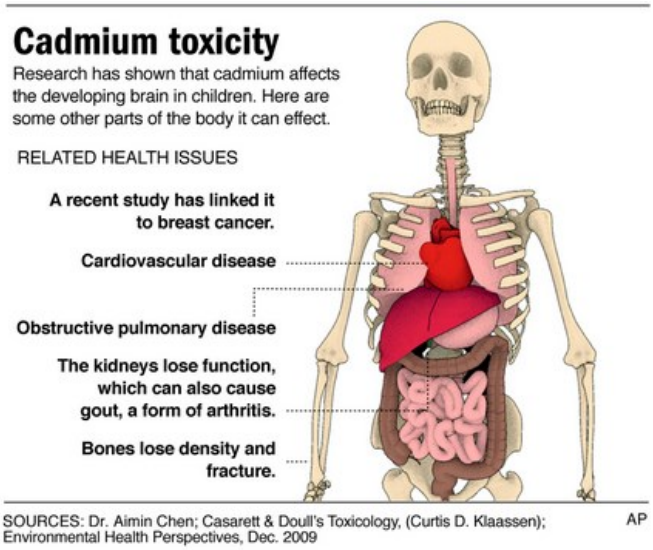


Figure 3. Health effects of Cadmium. Retrieved from: Akram (2012).

Cadmium is easily taken up by plant roots and leaf concentration greater than 5-10 mg Cd Kg^{-1} dry weight are toxic to most plants (Sanchez-Pardo et al., 2015). The uptake is achieved by those systems that operate for the absorption of essential micronutrients, mainly Zinc, iron, and calcium. High concentration of this metal in plants usually causes stunted growth, chlorosis, necrosis, leaf curling and epinasty, brown and stunted roots. It has been demonstrated that plants treated with cadmium exhibit a decrease of chlorophyll amount, with inhibition of enzymes involved in chlorophyll synthesis. This happens because the chemical structure of chlorophyll can be affected by a substitution of Mg^{2+}, with heavy-metal ions, such as Cd^{2+} (Küpper et al., 1998). A number of species have evolved Cd high-tolerance phenotype, mainly through exclusion mechanisms (Clemens, 2006; Verbruggen et al., 2009; Kupper and Kochian, 2010). There are some rare plants that display an exceptional capacity to accumulate Cd in their hypogeal biomass. These plants are recognized as Cd hyperaccumulators. *Noccaea caerulescens* (Lombi et al., 2000), *Arabidopsis halleri* (Bert et al., 2003; Zhao et al., 2006), *Noccaea praecox* (Vogel-Mikus et al., 2008), *Sedum alfredii* (Yang et al., 2004; Deng et al., 2007), *Arabis paniculata* (Tang et al., 2009), *Viola baoghanensis* (Liu et al., 2004; Wu et al., 2010), and *Potentilla griffithii* (Wang et al., 2009) can be used to clean-up the soil from this metal.

Arsenic

Arsenic (As), atomic number 33, is a silver-grey brittle crystalline solid with atomic weight of 74.9, specific gravity 5.73, melting point 817°C (at 28 atm), boiling point 613°C, and vapour pressure 1 mm Hg at 372°C (Mohan and Pittman, 2007). It is odourless and tasteless. Arsenic can combine with other elements to form inorganic and organic arsenicals (National Ground Water Association, 2001). In the environment, it is combined with oxygen, chlorine, and sulphur to form inorganic arsenic compounds. Organic arsenic compounds are used as pesticides, primarily on cotton plants (U.S. Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry, 2005). The metalloid exists in the −3, 0, +3, and +5 valence oxidation states (Mohan and Pittman, 2007), and in a variety of chemical forms in natural waters and sediments (Hasegawa et al., 2009). Inorganic species, arsenite [As^{3+}] and arsenate [As^{5+}], are the predominant species in most environments (Andrianisa et al., 2008). The pH, redox conditions, surrounding mineral composition, influence the form (inorganic or organic) and the oxidation state of As. As^{3+} is predominant in reduced redox potential conditions (Hasegawa et al., 2009). The trivalent compounds (arsenite) are more toxic than the pentavalent compounds (arsenates), which are thermodynamically more stable (Ampiah-Bonney et al., 2007; Vaclavikova et al., 2008). However, the trivalent methylated arsenic species is more toxic than inorganic arsenic because they cause DNA breakdown (Vaclavikova et al., 2008). Particularly when exposure occurs over prolonged periods, it affects the health of millions of people causing skin and nerve damages and having carcinogen effects (fig. 4). The uptake by humans mainly occurs through drinking As-contaminated water and eating plants grown in contaminated soil.

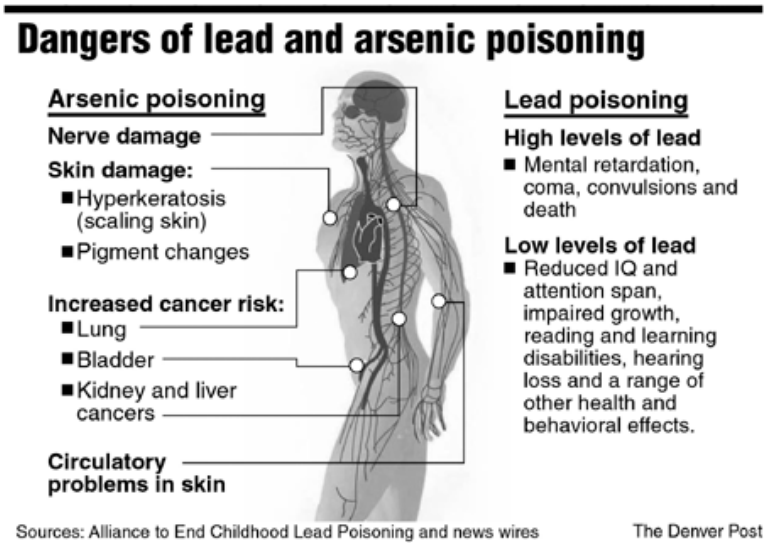


Figure 4. The effects of As in human health. Levin, et al., 2008.

As is toxic also for plants. It causes reduction of root elongation and branching, leaf chlorosis, and the shrinking and even necrosis of the plant aerial parts (Carbonell-Barrachina et al., 1998).

AsV is the primary plant-available form of As in most areas. The uptake in plants occurs via the inorganic phosphate (Pi) uptake system, because Pi transporters cannot distinguish between the similar electrochemical profiles of Pi and AsV (Sanchez-Pardo, 2015). It crosses the plasma membrane of root cells, and it is rapidly reduced to arsenite once inside the cytoplasm. Since arsenate and phosphate behave as analogues with respect to their uptake, arsenate toxicity is linked to phosphorus nutrition, and high levels of phosphate can mitigate arsenate toxicity (Esteban et al., 2003; Sanchez-Pardo, 2015). To overcome this problem, various methods have been used such as ex-situ or in-situ soil washing (Dikinya and Areola, 2010) or chemical immobilization/stabilization of heavy metals in soil (Wang et al., 2009; Houben et al., 2012). In fact, in the last decade the value of metal-accumulating plants for the environmental remediation of heavy metal polluted soil has attracted increasing interest (Hernandez-Allica et al., 2008). During the remediation process these toxic elements are extracted or stabilized by plants and metabolized in their tissues. Phytoremediation is considered an economically profitable method of exploiting plants to extract contaminants from soil (Padmavathiamma and Li, 2007).

1.1 Phytoremediation

Phytoremediation basically refers to the use of plants and/or association to microorganisms to partially or completely recover selected contaminants from soil, sludge, sediments, wastewater and ground water. It can be used for removal of organic pollutants as well as heavy metals. The term ‘‘phytoremediation’’ is a combination of two words: Greek *phyto* (meaning plant) and Latin *remedium* (meaning to correct an evil). This process is relatively cost-effective, efficient and eco-friendly compared with other remediation techniques (Wan, et al., 2016). The technique includes different processes such as phytoextraction, phytofiltration, phytostabilization, phytovolatilization and phytodegradation (Fig. 5) (Alkorta et al., 2004).

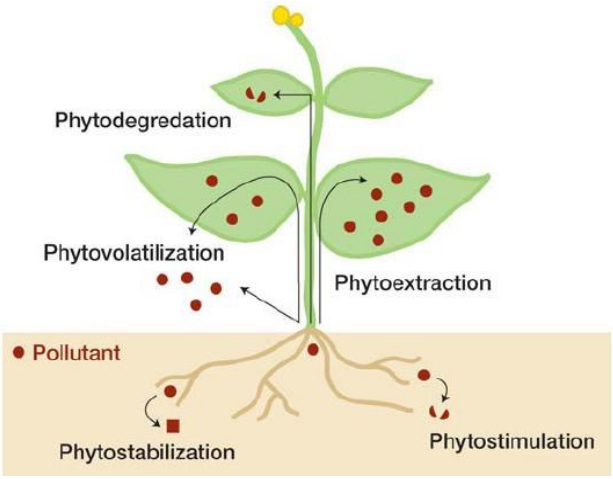


Figure 5. Different mechanisms of phytoremediation in plants. http://tinyurl.com/kolj52p

During phytoextraction, the metal is translocated from roots to shoots, through important biochemical process. The phytofiltration is another process, which includes rhizofiltration (use of plant roots), blastofiltration (use of seedlings) or caulofiltration (use of excised plant shoots) (Mesjasz-Przybylowicz et al., 2004). During phytovolatilization, the heavy metals absorbed by plants are converted into volatile forms, and released into the atmosphere. This process is limited by the fact that the metal is not completely removed but rather transferred from one medium (soil or water) to another (atmosphere), and can re-enter into soil and water. To reduce bioavailability and mobility of metals, the plants use two processes: phytostabilization or phytoimmobilization. By these processes the

plants reduce the toxic elements concentration in the soil and prevent food chain contamination (Dixit, et al., 2015). In the rhizosphere plants perform the immobilization of heavy metals by absorption through roots, precipitation and complex-formation or metal valence reduction (Poschenrieder, 2003). The plants metabolize organic pollution by the mean of enzymes such as dehalogenase and oxygenase, which are not dependent on rhizospheric microorganisms (Vishnoi and Srivastava, 2008).

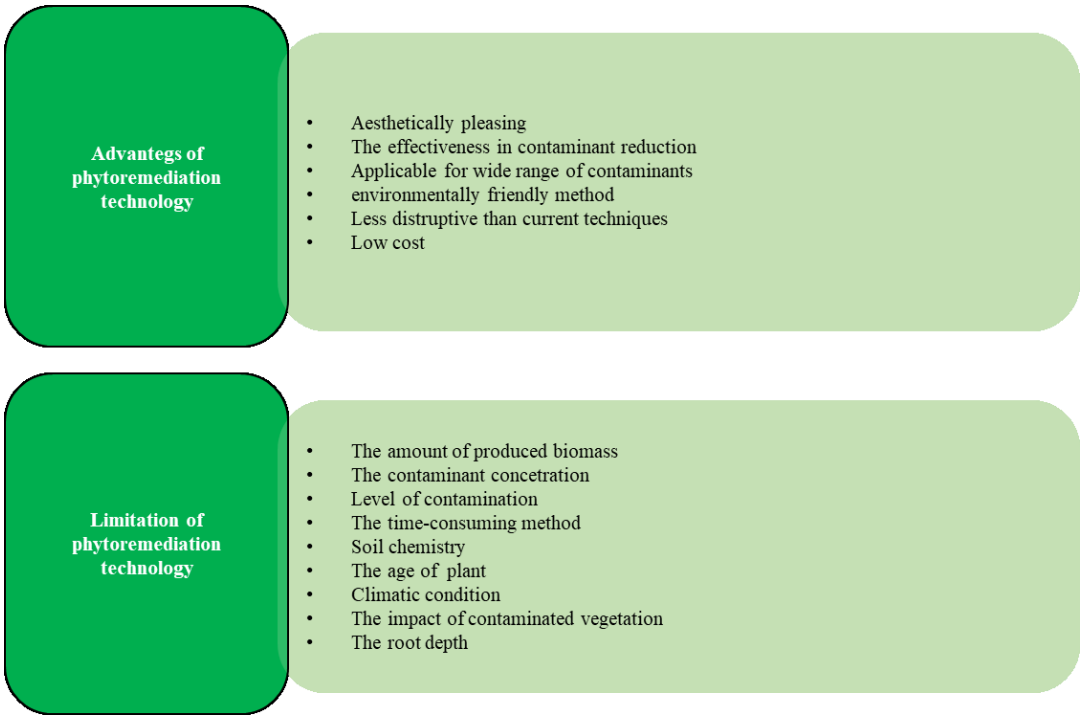


Figure 6. Scheme on the advantage and limitation of the phytoremediation. (Tangahu, et al., 2011)

During phytoextraction, specific plant species can absorb and hyperaccumulate metal contaminants and/or excess of nutrients in harvestable root and shoot in soils. This process breaks down complex organic molecules into simpler molecule contaminants (EPA, U., 2000; Prassad, et al., 2003). There are different problems on phytoremediation use. In fact, harvested plant biomass resulting from phytoextraction may be classified as a hazardous waste, hence, disposal should be properly done. Contaminants may still enter the food chain through animals/insects that eat plant material containing toxic elements (Fig. 6) (Tangahu, at al., 2011).

In fact, for phytoremediation technology, expert project designers are required to select relevant species adapted for specific metals and regions (Alkorta et al., 2004). The plants to be used should not include food crops and this satisfies green chemistry material requirements (fig.7). Green chemistry, indeed, is an area of chemistry that uses agricultural raw materials with low environmental impact to create an innovative range of bio-products (bio-plastics, bio-lubricants), home and personal care products, plant protection, additives for the rubber (www.matrica.it).

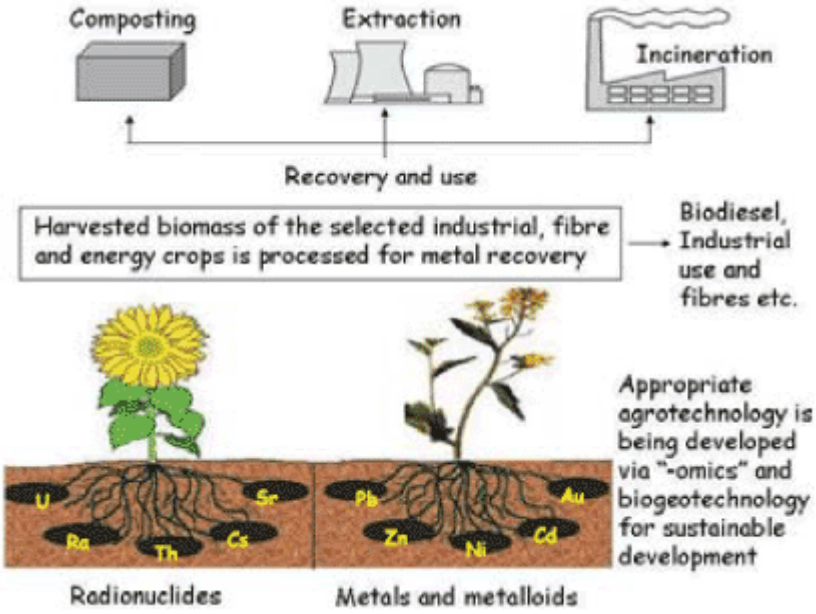


Figure 7. The approach of green technology for cleaning the soil from heavy metals. Braz. J. Plant, 2005

As regards to heavy metal tolerance and accumulation, the plants are classified into three categories:

- *excluders*: plants which have high levels of heavy metals in the roots with shoot/root quotients lower than 1 (Boularbah et al., 2006);
- *indicators*: plant which reflects metal levels in the soil (Baker, 1995);
- *accumulators*: plant species that concentrate metals in their tissues to levels far exceeding those present in soil (Mganga et al., 2011).

Plants that accumulate high concentrations of metals in their shoots are called hyperaccumulators. These can take up toxic metal ions at level of thousands of ppm and the shoot-to-root metal concentration ratio is greater than one. Hyperaccumulators can concentrate heavy metals like Cd, Zn, Co, Mn, Ni, and Pb up to 100 or 1000 times those taken up by nonaccumulator (excluder) plants. Non-accumulating plants typically have a shoot-to-root ratio considerably less than one. Multiple mechanisms are involved in the tolerance of metal toxicity. Storage in the vacuole appears to be a major one. Some plant species have been identified for soil remediation including either no food high biomass plants (Landberg and Greger, 1996) or low biomass plants with high hyper-accumulating features such as *Thlaspi* and *Arabidopsis* species.

1.2 *Cynara cardunculus* L.

Cardoon is a perennial species, belonging to Asteraceae family, particularly well adapted to the Mediterranean environments (Zohary and Basnizki, 1975; Raccuia et al., 2004a). It comprises three taxa, *C. cardunculus* L. subsp. *scolymus* (L.) Hegi = *C. cardunculus* L. var. *scolymus* (L.) Hayek (globe artichoke), *C. cardunculus* L. var. *altilis* DC. (leafy or domestic cardoon), and *C. cardunculus* L. var. *sylvestris* Lam. (wild cardoon), considered to be the wild ancestor of globe artichoke (Fig. 11) (Rottenberg and Zohary, 1996; Raccuia et al., 2004b).

a b c

Figure 11. *Cynara cardunculus* varieties. a) *C. cardunculus* L. var. *scolymus* (L.) Hayek (globe artichoke); b) *C. cardunculus* L. var. *altilis* DC. (domestic cardoon); *C. cardunculus* L. var. *sylvestris* Lam. (wild cardoon).

The aerial biomass is harvested every year at the end of the growth cycle. During that time, the plant canopy dries up and the fruits become ripe. Later on - when the climate conditions are favourable - some buds of the plant stock sprout and a leaf rosette is

gradually formed. This is the beginning of a new growth cycle. The aboveground biomass produced is harvested once a year, in summer time (Fernandez et al., 2006). Cardoon could be considered as a facultative halophyte. It can grow on slightly saline soils. When salt level rises growth is inhibited (Raccuia et al., 2004a; Benlloch-Gonzàlez et al., 2005). For this feature it represents a species especially suitable for phytoremediation.

1.2.1 Uses of *Cynara cardunculus*

Each *Cynara cardunculus* variety is used for a specific purpose.

Globe artichoke plays an important role in human nutrition, especially in the Mediterranean region. It is acknowledged for the benefits associated with its antioxidant contents (polyphenolic compounds) and for the presence of inulin which is a soluble food fibre that cannot be digested by humans and it is used as low-caloric replacement for fat (Lattanzio et al., 2009; Raccuia and Melilli, 2010). Globe Artichoke is also known as antidiabetic, choleretic, diuretic, cardiotonic agent (Kukic et al., 2008). Caffeic acid derivatives are the main phenolic compounds in artichoke heads and leaves, with a wide range of caffeoylquinic acid derivatives. Chlorogenic acid (5-*O*-caffeoylquinic acid) is the most important of these derivatives. Inulin belongs to a group of fructose-based polysaccharides called fructans, which are not digested in the small intestine because humans can't hydrolyse the fructan chain. For this reason is a low-caloric fibre that has potential for use in the production of fat-reduced foods (Frehner et al., 1984; Rapaille et al., 1995; Hellwege et al., 2000).

Leafy cardoon is an intensive growth crop with a high production of epigeal biomass, roots and grain used for green chemistry (Raccuia and Melilli, 2007, 2010). It is a very promising energy and biofuel crop. Its biomass has different uses. The lignocellulosic biomass is used for alternative energy production (solid biofuel) by combustion, pyrolysis and gasification (Gonzales, et al., 2004; Ochoa and Fandos, 2004). Biomass residues pellets combustion for domestic heating and raw material for green chemistry. With regard to the latter purpose, biomass can be used to prepare biodiesel from either the oil extracted from *C. cardunculus* L. seeds or the lignocellulosic fraction (Toscano et al., 2016).

1.2.2 Cardoon for phytoremediation

In early studies, *C. cardunculus* was shown to be able of growing in a polluted environment. Hernandez-Allica (2007) reported that cardoon is able to translocate to the shoots the metal-chelate complexes (EDTA - Pb, Cd or Zn) even at high concentration levels. Into the stele EDTA (ethylenediaminetetraacetic acid) increases the root flux through the apoplast and then increases the metal shoot/root ratio. In addition, the Pb–EDTA complex is known to be less phytotoxic than free Pb^{2+} or protonated EDTA metals (Tandy et al., 2006). Furthermore, EDTA combined with metals can reduce metal toxicity and, at the same time, efficiently enhance shoot accumulation increasing metal absorption and translocation via apoplast pathway.

Papazoglou et al., (2011) reported that under Cd treatment *C. cardunculus* grows and develops in a similar way as in the absence of cadmium showing no evident phytotoxicty symptoms. Furthermore, *C. cardunculus* has an efficient Cd translocation system from roots to shoots and growth is unaffected by the uptake activity. *C. cardunculus* can be initially considered as a Cd accumulator, because under elevated Cd soil concentrations, it shows high tolerance and accumulation in plant organs. The translocation factor from roots to shoots is higher than one. In contrast, cardoon does not tolerate elevated Ni concentrations, and could not be considered as Ni accumulators.

Llugany et al., in 2012 showed that root elongation in *C. cardunculus* plants, a reliable indicator for Cd sensitivity (Vázquez et al., 1992), is reduced of approximately 10% after 5 weeks of exposure to 5 μM Cd. The leaves of treated plants displayed the same length respect to the untreated control. The metal is translocated from roots to shoots. In contrast, in presence of arsenic, As III or As V, cardoon retained the metal in the roots and phytoextraction is not feasible. According to these results, *C. cardunculus* plants can indeed be considered as good candidates for phytoremediation, and can be used as an energy crop on As polluted soils.

Recent studies (Spagnuolo, et al., 2017), reported that *C. cardunculus* is able to accumulate Cd and Pb in its organs. In fact, the distribution of the Cd between shoots and roots is homogeneous and the plants did not show any effect on photochemistry. Pb, instead, is accumulated only in roots. Cd caused the increase of the level of Rubisco and D1, two enzymes involved in photosynthesis, a response that is useful to neutralize

chloroplast damage and enhance photosynthesis efficiency. The accumulation of Pb in root, can explain the high increase of the HSP70 level induced in this organ in Pb-treated plants.

Recently, Leonardi (2017), showed that the combination of As and Cd compounds increased the resistance of plants promoting survival. Therefore, depending on metals concentration and the presence or absence of Cd, plants could be used as excluders of As in As-contaminated sites, or accumulators in sites co-contaminated by As and Cd, respectively.

1.3 Heavy metals cellular metabolism

In general, plants with higher ability to reduce the toxicity effects are able to survive in heavy metal/metalloid contaminated sites and are promising candidates for phytoremediation purposes. Arsenic hyperaccumulation capacity seems to be confined to the Pteridaceae family of ferns. Cd hyperaccumulation is present only in some populations of *T. caerulescens, T. praecox*, and *Arabidopsis halleri*, all belonging to the *Brassicaceae* family, and *Sedum alfredii* (Crassulaceae). The use of several chelating agents, such as EDTA (ethylenediaminetetraacetic acid), EDDHA (ethylenediamine di(o-hyroxyphenylacetic acid), EGTA (ethylene glycol-O,O'-bis-[2-amino-ethyl]-N,N,N',N',-tetraacetic acid), and citric acid, has been used to enhance phytoextraction by mobilizing metals and increasing metal accumulation (Cooper et al., 1999).

To understand the mechanisms supporting phytoextraction of heavy metals, different gene expression studies with different type of metals (As, Cd, Zn, Fe etc.) and different concentrations of these (5, 10, 50…200 μM) have been carried out. A large array of genes are constitutively highly expressed in Cd hyperaccumulators compared to a non-hyperaccumulating closely related species. Until now, transport to the storage organs, chelation, efflux from the plant body, or subcellular compartmentalization are the most common mechanisms used for detoxification. The similarity between Zn and P to Cd and As respectively, causes the toxicity of the latter because they tend to replace Zn and P in cellular metabolism.

1.3.1 Genes associated with heavy metals transport

The plant use specific low-molecular-weight chelators to detoxify trace metal (-loid)s. By the mean of these proteins the contaminants are transported into the vacuoles. The uptake of Cd from the soil seems to occur mainly via Ca^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} transporters. The best-studied non-specific transporter is the ZIP IRT1, which is the major transporter responsible for high-affinity iron, zinc and consequently cadmium uptake from the soil. However, in *T. caerulescens,* model plant for Cd accumulation, there is no evidence that TcIRT1 can transport Cd from soil to roots and shoots. The As(V) is potentially toxic because can substitute for phosphate in phosphorylation reactions, including ATP synthesis, instead As(III) is mainly assimilated through members of the NIP (nodulin 26-like intrinsic protein) subfamily of aquaporins (Verbruggen, et al., 2009) (Fig. 9). Toxicity of As(III) like that of Cd is probably primarily due to high sulphydryl reactivity. Both metals causes oxidative stress, and can deplete reduced glutathione, an important cellular antioxidant, through the formation of As(III)–glutathione or Cd–glutathione complexes [As(III)–GS3 or Cd(II)– GS2] and As(III)-induced or Cd-induced phytochelatin (PC) synthesis. Stress-responsive MAP kinases seem to be involved in transcriptional responses to Cd as they are activated possibly by ROS under Cd^{2+} excess. Different genes are associated with HMs tolerance or accumulation.

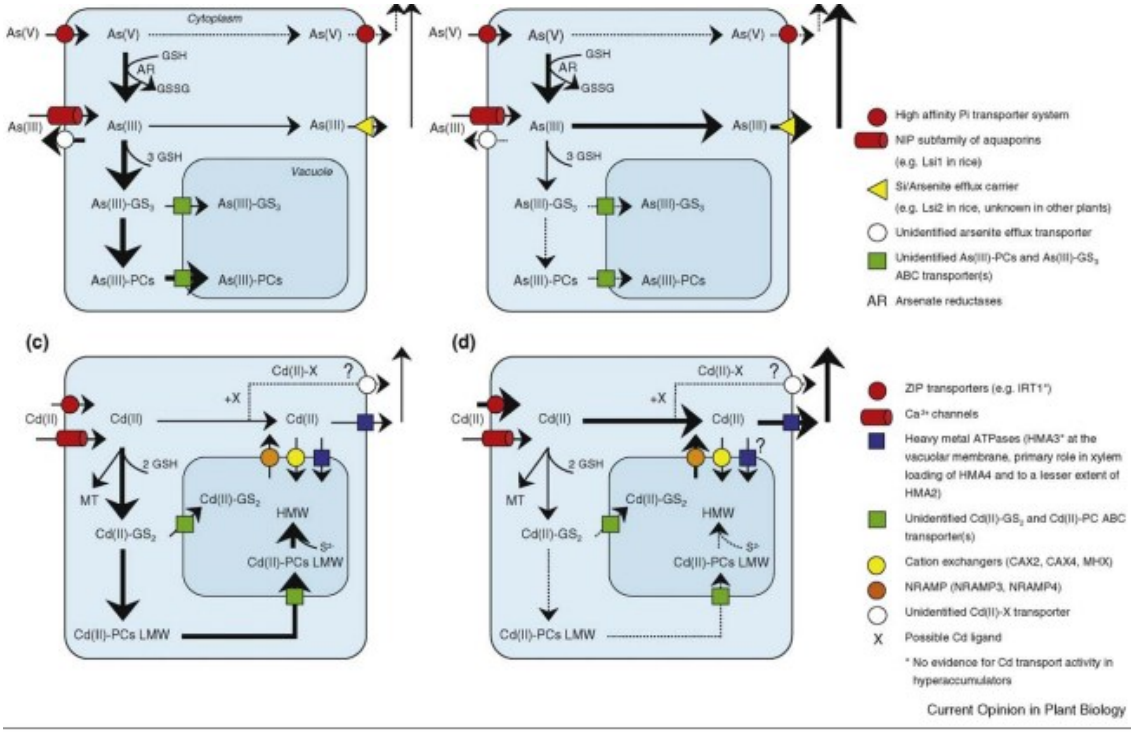


Figure 9. Mechanisms to cope with arsenic or cadmium excess in plants. Verbruggen, et al., 2008.

GLUTATHIONE

The tripeptide glutathione (Glu-Cys-Gly), GSH, is synthesized by gamma-glutamylcysteine synthetase (g-ECS) and glutathione synthetase (GS). It is involved in the control of cellular redox balance. Increasing GSH synthesis is considered a means of increasing metal (loid) binding capacity as well as a way to increase cellular defence against oxidative stress. GSH and Phytochelatins (PCs) chelate heavy metals and metalloids such as Cd, Cu, and As, facilitating their sequestration into vacuoles (Cobbett, 2000; Pilon-Smits, 2005).

PHYTOCHELATIN

Phytochelatin (PC) is a oligomers family characterised by the general structure (gGlu-Cys)n-Gly where n = 2–11. Synthesized from GSH, the reaction is catalysed by PC synthase (PCS). PCS is constitutively expressed, but to be active requires post-translational activation by metal(loid)s, as As and Cd. This protein is found in all plants, some fungi and animals. The formation of As–GS3 or Cd–GS2 thiolates, which act as high-affinity substrates for the enzyme, seems to be sufficient for its activation. PC

synthesis seems to be the main factor for basal Cd and As tolerance but not in hypertolerant plants or hyperaccumulators (Verbruggen et al., 2009). The induction of PC synthesis depends on the combination of PCSs and hazardous elements. Only As(III) can bind to thiols and activate PCS. PC synthesis under As(V) exposure may be limited by the arsenate reductase capacity, rather than by PC synthetic capacity itself.

This might explain why only combined expression of g-ECS and arsenate reductase (ArsC) substantially increased As(V) tolerance in *A. thaliana*. Heavy metal are immobilized in the endocellular compartment by complexation with organic acids, like malate, oxalate (Lutts et al., 2004), and malonate (Clemens, 2001), and PCs carries out sequestration (Gadapati and Macfie, 2006; Mishra et al., 2006), compartmentation into vacuoles (Kramer et al., 2000; Shevyakova et al., 2003) or simply blocking by epidermal cells (Solís-Domínguez et al., 2007).

ZRT-IRT-LIKE PROTEIN

ZRT-IRT-like Protein (ZIP) family were the first metal transporters to be identified in plants (Eide et al., 1996). Fifteen ZIP genes have been identified in *A. thaliana*, based on whole genome sequencing. They are transporters of divalent cations including Zn^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+} (Guerinot, 2000) and their expression is regulated by plant metal status that reflects environmental metal levels. ZIP proteins have eight putative transmembrane domains (TM) and contain a histidine repeat in the variable region that has been proposed as the metal binding and/or sensing site (Grossoehme et al., 2006).

AtIRT1 transporter is involved in Fe and Cd uptake (Gallego et al., 2012; Romè et al., 2016), and with the other ZIP proteins transfer these metals from soil to roots (Guerinot, 2000). *ZNT1* and *ZNT2* are highly expressed in the roots of *T. caerulescens* while expression is barely responsive to the Zn status in the other parts of the plants (McGrath, et al., 2003). *ZNT1* and *ZNT2* show high-affinity uptake for Zn^{2+} as well as low-affinity uptake for Cd^{2+}. *AtZIP2* and *AtZIP4* are associated with Cd uptake in Cd treated plants, but their expression decreased significantly in Cd+Ca-treated plants (Aarts, et al., 2009).

HEAVY METAL TRANSPORTING ATPase

The P1B-type ATPases are a class of proteins, also named HMAs (Heavy Metal transporting ATPases), that operate in heavy metals transport and play a role in metal homeostasis and tolerance. In *A. thaliana* there are eight P1B-type ATPase members, subdivided into two major subgroups depending to the metal transported (Baxter et al., 2003). AtHMA1 to AtHMA4 classify into the Zn/Cd/Co/Pb transporting subgroup, while AtHMA5 to AtHMA8 belong to the Cu/Ag transporting subgroup, although AtHMA1 has also been shown to transport Zn, Cu and Ca (Axelsen and Palmgren, 1998; Kim et al., 2009). The P1B-ATPase HMA3 seems to be also involved in the vacuolar storage of Cd in non-hyperaccumulators, as demonstrated in *A. thaliana* (Verbruggen, et al., 2009). TcHMA3 belongs to the P1B-type ATPase subfamily. Members of this group transport several heavy metal ions from the cytosol to organelles or out of the cell (Williams and Mills, 2005). TcHMA3, in Cd hyperaccumulating ecotypes, is expressed in leaves where it is involved in transport and accumulation into the vacuole. By contrast, in species that are not hyper-accumulator, such as *A. thaliana*, the metal is transported to cells of the shoot such as hydathodes and guard cells (only in these type of cells) and does not accumulate in other leaf cells. In roots, HMA3 is predominantly expressed in the pericycle cells (Morel et al., 2009), and before xylem transport, the metal can be stored into the vacuole (Ueno, et al., 2011). The HMA4 role is to allow Cd and Zn efflux from the root symplasm into the xylem vessels, necessary for shoot hyperaccumulation. Its expression is up-regulated when these plants are exposed to high levels of Cd and Zn, whereas it is down-regulated in non-hyperaccumulator plants. Interestingly, the increased expression of *HMA4* enhances the expression of genes belonging to the *ZIP* family, implicated in heavy metal uptake. This strongly suggests that the root-to-shoot translocation acts as a driving force of the hyperaccumulation (M. Hanikenne et al., 2008).

NATURAL RESISTANCE OF MACROPHAGES

NRAMPs, are membrane spanning proteins, characterized by nearly 12 highly hydrophobic transmembrane domains. They define a ubiquitous family of metal transporters with several homologues in fungi, animals, plants and bacteria (Cellier et al., 1995). Some Arabidopsis NRAMPs (AtNRAMP1, AtNRAMP3 and AtNRAMP4) are high affinity Fe transporters (Curie et al., 2000; Thomine et al., 2003), and AtNRAMP1, AtNRAMP3 and AtNRAMP4 are also associated with Mn transport (Cailliatte et al., 2010; Lanquar et al., 2010). In addition, several studies reported that NRAMPs also retain heavy metals transport (Ni and Cd) ability (Thomine et al., 2003; Oomen et al., 2009). In rice, OsNRAMP1 expression is induced during As stress at the same time of other stress responsive genes, transporters, heat-shock proteins, metallothioneins and sulphate-metabolizing proteins (Gautam et al., 2012). Furthermore, a few reports have revealed that As uptake in rice root is related to Fe availability in the soil and its accumulation was correlated with Fe in rice tissues (Zhao et al., 2010; Rahman et al., 2011). In transgenic rice lines over-expressing NRAMP1, significant higher accumulation of As was recorded in comparison to Wild Type.

NRAMP3 and NRAMP4 are responsible for Cd^{2+} efflux from the vacuole. Their overexpression increased Cd sensitivity in *Arabidopsis* and they are responsible for the release of vacuolar Fe^{2+}. In *T. caerulescens* these are overexpressed both in roots and shoots where their roles are still unclear. The up-regulation of AtNRAMP4 in roots and shoots upon Cd stress may also be a consequence of Cd-induced decrease in Fe availability. The decrease of essential metals under Cd stress could be caused by the inability to recycle Fe and Mn from the vacuole into the chloroplast. Mn is involved in PSII photo activation, and Cd replacing the Mn in the PSII (Faller et al., 2005). For this reason Cd inhibits photosynthesis, by inhibiting PSII it activates a continual cycle of damage and repair (Edelman and Mattoo, 2008; Mollins, 2012).

PHOSPHATE TRANSPORTER

In plants, phosphate transport is involved in Arsenate uptake from the soil to the roots (Abedin et al., 2002; Meharg and Hartley-Whitaker, 2002; Wang et al., 2002). In *Pteris*

vittata the influx of arsenate was strongly reduced by the presence of phosphate in the uptake solution. In *Arabidopsis*, overexpression of PHT1 or PHT7 causes hypersensitivity to arsenate, due to increased arsenic uptake, while arsenic resistance is enhanced through YCF1-mediated vacuolar sequestration (Smith, et al., 2013). The arsenate uptake is enhanced in P-deficient plants, as reported in barley (*Hordeum vulgare*) (Lee, 1982), and in the As non-resistant population of *H. lanatus* (Meharg et al., 2002). *P. vittata* can also hyperaccumulate As, when present as arsenite, but the uptake does not share the same transport systems for phosphate. In the absence of phosphate in the uptake solution, *P. vittata* assumed As (III) very slowly, at a rate that was about one-tenth of the arsenate influx, (Wang, et al., 2002).

ARSENATE REDUCTASE 2

Arsenate reductase (ACR2), like AtACR2 in *Arabidopsis* and OsACR2.1 and OsACR2.2 in rice, may be involved in AsV reduction (Dhankher et al., 2006; Duan et al., 2007). However, more recent evidence showed that canonical ACR2 arsenate reductase probably does not play a significant role in arsenate reduction (Liu et al., 2012; Chao et al., 2014). Instead, a novel arsenate reductase, HAC1 (High Arsenic Content1) (Chao et al., 2014), is critical for AsV reduction and AsV tolerance in *Arabidopsis*. This protein reduces AsV to AsIII in the outer cell layer of the roots, causing AsIII efflux out into the external environment (Chao et al., 2014).

ABCC1

AtABCC1 and AtABCC2, in *Arabidopsis*, mediate AsIII–PC complex transport to the vacuole. The overexpression of AtABCC1 increases As tolerance only when co-expressed with PCS, indicating the cooperation of PC synthesis and AsIII–PC complex transporters in plant As detoxification (Song et al., 2010).

The two genes are not synthesized de novo but they are constitutively present in a plant cell to rapidly respond to toxic metal (loid)s and xenobiotic stresses. In root rice, OsABCC1 is expressed in the exodermis and pericycle inducing the biosynthesis of thiol compounds that bind to As in cytoplasm (Song et al., 2014). Overexpressing transporters

for As sequestration in the shoots may lead to As accumulation in plants (Zhu and Rosen, 2009; Guo et al., 2012).

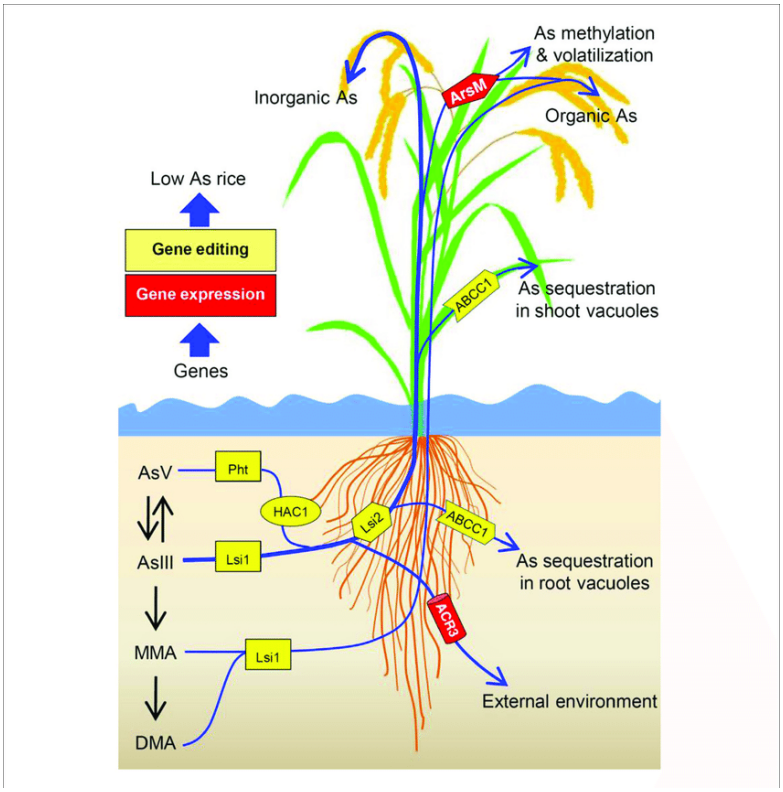


Figure 10. Mechanism of As uptake in rice plant and role of ABCC1, (Chen, et al., 2009).

However, overexpression in the roots may decrease As accumulation in the shoots, because the metals is storage only in root and the uptake can not happen (Zhu and Rosen, 2009; Ueno et al., 2010). Because complexation of AsIII by thiols is a critical step for As transport into the vacuoles, in plants simultaneously expressing the ABC transporters and PC synthase, the rate-limiting step in PC biosynthesis, may maximize As sequestration (Fig. 10) (Chen, et al., 2017). As(V) exhibits a complex metabolism in plants, and apparently accumulation of PC–As complexes in the cytosol induces mechanisms that reduce the transfer of arsenic to the shoot (Song, et al., 2010).

2 Aim of work

Phytoremediation is a technique where specific plants are used for removing toxic elements, such as heavy metals, from contaminated soils. In order to preserve the quality of soils, waters and food, the use of specific plants for contaminants removal is considered an environmentally friendly technology, safe and cheap (Cunningham et al., 1995). Insofar, few studies have investigated about the molecular mechanisms that in plants are involved in heavy metal tolerance as a part of the more general phytoremediation response (Pollard et al., 2002).

In this PhD thesis we studied the response mechanisms to heavy metals stress in *Cynara cardunculus* L., with the aim of using this plant for phytoremediation purposes.

This research was carried out analysing the following different aspects:

1. The influence of the genotype on seed germination in soil contaminated with Cd, As and a combination(s) of Cd + As;
2. The influence of these heavy metals on seedling growth;
3. The identification of cardoon orthologous of plant genes associated with heavy metals transport and accumulation;
4. The transcriptional modulation of genes involved in the response to different heavy metals stresses, in different genotypes and at different metal concentrations and the similarity of the gene expression response, activation or down regulation, between cardoon and accumulator model plants.

3 Materials and methods

During this research activity, five different trials were carried out to investigate on genes associated with heavy metals accumulation in *C. cardunculus*: germination test, seedling growth analysis, identification of genes associated with heavy metals transport, identification of genes usable as housekeeping genes, and analysis of the gene expression levels. Below are explicated the methodology used.

3.1 Plant materials

For the different trials of this study, three genotypes were assessed: two wild cardoon genotype (*sylvestris*) and one domestic cardoon variety (*altilis*). All the three genotypes belonged to the *C. cardunculus* L. genetic bank of the section of U.O.S. Catania of the ‘ Istituto sui Sistemi Agricoli e Forestali del Mediterraneo (ISAFOM) CNR (Italy).

In particular, for the germination tests, we considered all three genotypes. The wild cardoon populations were collected in two very different sites of Eastern Sicily: the first placed at 900 m above sea level in the territory of Randazzo (CT) within Nebrodi Regional Park (R14CT) and the second placed in the territory of Augusta (SR) at 4 m above sea level within the industrial area (A14SR). The domestic cardoon (*Cynara cardunculus* var. *altilis* DC.), is a selected line to produce biomass for use in Green Chemistry by CNR-ISAFOM UOS Catania. The seed of all three genotype were harvested in the year 2014.

To carry out the molecular analyses, the seeds of the line of domestic cardoon (*Cynara cardunculus* var. *altilis* DC.) collected during the summer 2015, were grown in incubator at 25/15 °C and 12h of photoperiod and germinated seeds (GS) are used to identify the presence of genes associated with heavy metals transport.

To find the housekeeping genes usable during gene expression analyses, five different stages of plant cycle of *C. cardunculus* var. *altilis,* collected in 2015, were used: water imbibed seed (IDS), germinated seeds (GS), young leaf (YL), flower open head (capitulum) (OC4), and flower closed capitulum (CC1). IDS and GS samples were grown

in incubator at 25/15 °C and 12h of photoperiod. The seeds were sown with 3 mL bidistilled water on Whatman filter paper and were harvested respectively after 24 h and 48 h since imbibition time. YL, OC4 and CC1 samples were grown in the field where YL represents young leaf stage, CC1 the flower primordial and OC4 the final stage of heads flowering.

Three biological replicates were used in this study for the different conditions. All samples after harvesting were immediately frozen in liquid nitrogen and stored at -80 °C.

For the gene expression analyses, the seeds of *altilis* genotype and wild cardoon (A14SR) were collected during the summer 2015. The seeds were surface-sterilized with 0.5% (w/v) sodium hypochlorite for 1 minute, followed by three thorough rinses with sterile water. Three biological replicates, each of which, consist of two plants, were germinated and grew in ½ MS solid Medium with 0, 25 and 50 µM of Cadmium Sulphate hydrate and Sodium Arsenate di–basic heptahydrate, in 20/25 °C of temperature and 12h light/dark cycle photoperiod.

The plants were harvested after two and three weeks, separated into shoots and roots, and immediately frozen in liquid nitrogen. The samples were grinded with sterile mortar and pestle in liquid nitrogen, and 100 mg of tissue were used for RNA extraction.

3.2 Germination tests

The experiments were carried out in agar medium contaminated with Cd and As at different concentrations (Di Salvatore et al., 2008). The solid medium consists in 9% plant agar and bidistilled water, at 5.5 - 6.0 pH. The seeds of R14CT, A14SR and "Altilis" were used for these tests.

The trials were conducted using a completely randomized block design with five replications. Three treatments were investigated: Cd, As and combined Cd + As solutions. Four different metal concentrations were used per treatment (10 µM, 50 µM, 100 µM and 200 µM), no metal concentration was used for the control (CTRL). To remove the issues associated with using variable form of metal salts as a source of heavy metals we used a pure single metal element solution (ICP standard solution, Sigma Aldrich) which has certified guaranteed purity (99.99 %) (Bae et al., 2014).

Seeds were surface-sterilized with 5% (w/v) sodium hypochlorite for 15 minutes, followed by three thorough rinses with sterile water. Then they were sown on Petri dishes, thirty per plate, containing 25 mL of agar medium supplemented, after autoclave sterilization, with heavy metals alone and combined at different concentration as described above and placed in a growth chamber under a 12/12 h light/dark cycle photoperiod at 25/15 °C thermoperiod. Germination was determined at 24 h intervals until no further germinated seeds were observed for three consecutive days. The seeds were considered germinated when there was radicle protrusion through the seed coat. Germination percentage was calculated by the ratio between the total number of germinated seeds and total number of seeds germinated at 0 µM (this value was considered 100 %).

3.3 Seedlings growth analysis

The experiment was carry out on *altilis* and *sylvestris* seedlings, growth in ½ MS medium under 0 µM, 25 µM and 50 µM of Cd and As, at 20/25 °C and 12h photoperiod. The seedlings were harvested after 3 weeks, separated into shoot and root, and the length was measured. Shoot height was measured from culms base to the tip of the longest leaf and

root length was measured from the root-shoot junction to the tip of the longest root. These plant materials were used for the gene expression analysis.

3.4 Identification of cardoon genes likely associated with heavy metals transport and accumulation

To asses in cardoon the presence of the genes that usually are involved in heavy metals transport, different methodologies were carried out.

3.4.1 RNA extractions

The RNA extraction, was performed on Germinated seeds (GS) without metal. 100 mg of seedling was grinded with mortar and pestle in liquid nitrogen. Total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN, Germany), whose technology allows to capture RNA on a silica membrane spin filter. During the reaction, the DNase treatment was made to remove the gDNA residual. The extract was analysed with QIAxcell instrument by the mean of capillary electrophoresis, that measures the pick of 18S and 28S rRNA (the most abundant). The RNA purity and integrity were asses considering RIS number (RNA integrity score)., Reverse transcription reactions were performed by using ImProm-II™ Reverse Transcription System (Promega, Madison USA) according to the manufacturer's instructions. 1 µg of total RNA was used for the cDNA synthesis.

3.4.2 Primer designing

To design the cloning primers for the genes of interest, the complete cds of other plants, such as *Arabidopsis* or *N. caerulescens*, were blasted, with BLASTx alghorithm available at the NCBI website (http://www.ncbi.nlm.nih.gov), against *Cynara* database (taxid: 59895). GI number obtained for each gene, was searched inside the artichoke genome sequences (Scaglione et al., 2016). Then we aligned the genomic cardoon sequences against the complete cds of the model plants using Clustal Ω program (Sieveres et al., 2011). In the conserved domain, with high identity, we designed the cloning primers, using primer3 website (Rozen et al., 1999). We tried to amplify the entire sequence, using

two pairs of primers, one on the first part of the sequence, one on the last part, with an overlap region.

3.4.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction was used to understand if the genes are expressed in cardoon, and to obtain the RNA sequences in *C. cardunculus*. The cDNA was used as template to amplify the selected genes using cloning primers and PCR products were analysed on a 1.5% agarose gel.
The PCR was performed with PerfectTaq DNA polymerase (5 PRIME, Hilden, Germania), according to the manufacturer's instructions.

For the reaction 700 ng of cDNA were used. Different annealing temperatures were considered for the different genes amplified. The PCR products were check by the mean of Electrophorese on agarose gel at 1.5 %, and Gel Red is used as intercalates.

3.4.4 Cloning and miniprep

The PCR products that showed a single band in agarose gel, were cloned into the pJET vector (CloneJET PCR Cloning Kit, Thermo Scientific). This vector contains a lethal gene eco47IR enables positive selection of recombinant plasmid, which is disrupted by ligation of a DNA insert into the cloning site (Fig.12).

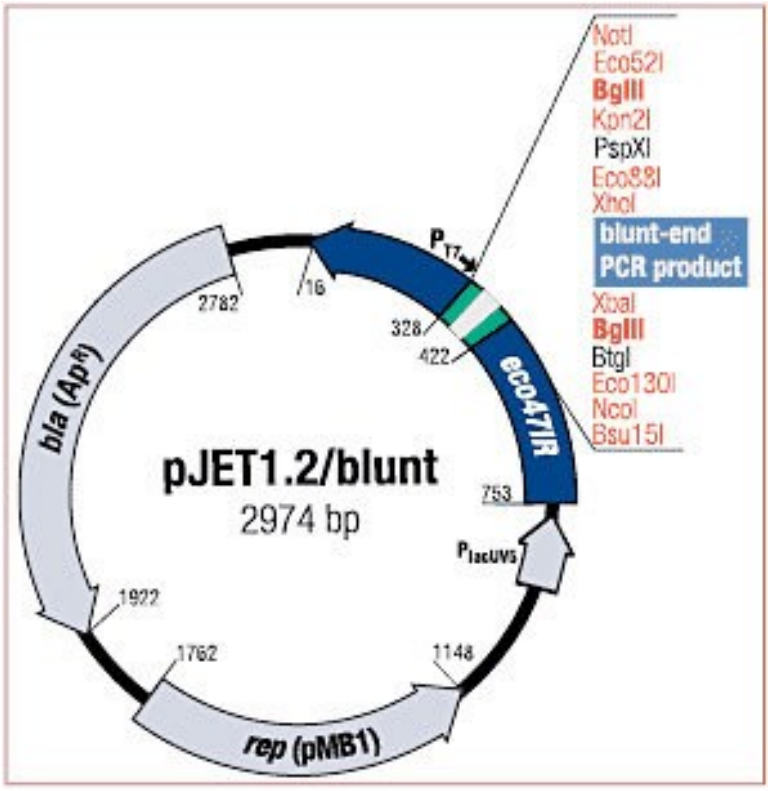


Figure 12. Map of pJET1.2/blunt vector, with the restriction sites evidenced. Eco47IR is the lethal gene, bla is the gene that caused the Ampicillin resistance.

Only the cells with recombinant plasmids were able to propagate. The growth medium was LB - ampicillin (50 mg/L) plates, pH 7.0. After 12h at 37 °C the colonies were picked, put in 1 mL sterile water and a part stored in a LB - ampicillin plates for the future analyses, and a part vortexed for 10 sec. 10 µL of the latter, were used as template for the screening of the recombinant clones during colony PCR. The amplification was carried out with Taq My Taq at Ta of 60 °C. The vector primers used were: pJET1.2 forward sequencing primer 5'- CGACTCACTATAGGGAGAGCGGC- 3' and pJET1.2 reverse sequencing primer 5'- AAGAACATCGATTTTCCATGGCAG- 3' that are at the positions 310 and 428 of the vector respectively. The PCR products were verified on agarose gel at 1.5 %. Positive clones were incubated for 16 h at 37 °C in LB Lennox liquid broth with ampicillin. Plasmid DNA was extracted by the means of QIAprep Miniprep Kits (QIAGEN, Germany), that uses silica membrane technology (Fig. 13) and amplified using vector primers and Taq My Taq. Quantitative and qualitative analyses of extracted plasmid DNA were performed by using spectrophotometer.

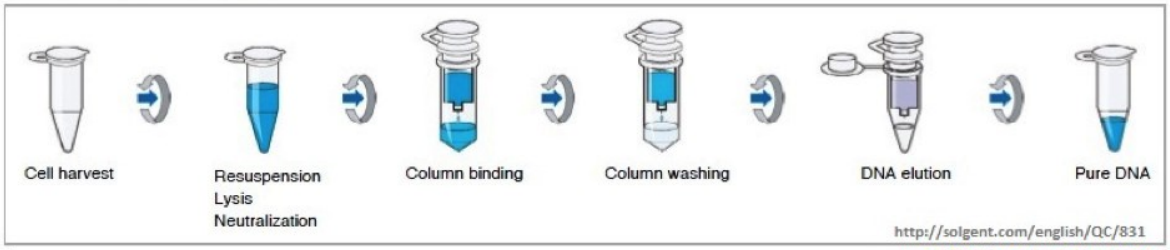


Figure 13. Scheme of miniprep protocol, with the resuspension, column binding, column washing, DNA elution phases showed.

The sequences were analysed using Sanger method, with vector primers and the results were investigated with bioinformatics tools.

3.4.5 Bioinformatics tools

The 5' and 3' sequences of the genes obtained from sequencing were used to make the contigs using CodonCode software. Then the constructs were evaluated by comparing them with the nucleotide sequences deposited in National Center for Biotechnology Information (NCBI) databases using the BLASTn algorithm. The sequences were blasted against all DNA and protein databases, and against *Cynara cardunculus* var. *scolymus* (taxid: 59895) using the BLASTx algorithm available at the NCBI website (http://www.ncbi.nlm.nih.gov). The contigs were translate with Expasy program (Artimo et al., 2012) and the amino acidic sequences were blasted against all database and against *Cynara cardunculus* var. *scolymus* (taxid: 59895) with BLASTp algorithm. The sequences were aligned with the nucleotide and amino acidic sequences with the higher score during blast analyses, by using of Clustal Ω program (Sieveres et al., 2011) and BioEdit program. (Hall, 1999).

3.5 Housekeeping gene isolation

Seven genes were selected to obtained reference genes for further quantitative analysis in cardoon plant. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), β-TUBULIN, ACTIN, ELONGATION FACTOR, 18S, ANAPHASE PROMOTING COMPLEX and TRASDUCIN/WD40 were investigated. To obtain the sequence of the genes in cardoon plant, the sequences of DNA from *Arabidopsis* were selected and used as reference (Dekkers et al., 2012). The protocols used for the RNA extraction, cloning primer

designing, cloning and sequence analysis were the same of that described above, in paragraph 3.4.1. –.3.4.5. GS samples were used as template for PCR analyses.

3.5.1 Primer designing for qPCR

To design the primers for the qPCR, Primer3 website (Rozen et al., 1999) was used. The parameters used for GAPDH, β-TUBULIN, ACTIN, ELONGATION FACTOR, 18S, ANAPHASE PROMOTING COMPLEX, WD40 (reference genes) were 70-150 bp the length of amplicones and 60-64 °C the melting temperature. The program used was Primer3 (Rozen et al., 1999).

3.5.2 qPCR

To analyse the stability of the reference genes Real time PCR was carried out. 100 ng of water imbibed seed (IDS), germinated seeds (GS), young leaf (YL), flower open head (capitulum) (OC4), and flower closed capitulum (CC1) cDNA and QuantiNova™ SYBR® Green PCR Kit (QIAGEN, Germany) were used. Three technical replicates were made for each sample. The RT–qPCRs were run on a Rotor Gene-6000 (QIAGEN, Germany) with the following condition: first step at 95 °C for 2 min and afterwards 40 cycles alternating between 5 s at 95 °C and 10 s at 63 °C. Each 20 µL reaction mixture consisted of: 10 µLof 2x QuantiNova SYBR Green PCR Master Mix, 1.25 µL each of forward and reverse primer (10 µM), and 1 µL of cDNA (100 ng). The Ct value was inserted manually, about at lower 1/3 or 1/2 of the linear phase of amplification.

3.5.3 Data analysis

The expression data of the seven reference genes were used for analyses with two Microsoft Excel-based statistical algorithms: geNorm (v 3) and NormFinder (v 0.953). The two software packages were used according to the manufacturer's instructions. The M value measured represents the "average expression stability". The RT–qPCR data were normalized per reference gene. The rate between the Ct at different conditions and the average of Ct of each gene were considered to compare the stability of HK genes, with reference to the geNorm and NormFinder user manuals.

3.6 Gene expression analysis

To assess the gene expression level of the gene associated with heavy metal tolerance, the seedlings of *altilis* and *sylvestris* varieties, grew for two and three weeks in ½ MS medium, contaminated with 0, 25, 50 µM of Cd and As, according to the methodology described in paragraph 3.1, were used.

3.6.1 RNA extraction

Shoots and roots RNA extractions were based on Chang et al., 1993, modified for cardoon. To lysate the tissue, 600 µL of extraction buffer (CTAB 2 %, PVP 2 %, Tris-HCl 100mM (pH 8), EDTA 25 mM, NaCl 2 M), warmed at 65 °C plus 2 % β-mercaptoethanol were added to 100 mg of the sample, mix completely by inverting and incubate at 65 °C with vigorous shaking every 3 min. To separate the two phases (organic and aqueous phases), 500 µL of Chloroform:IAA (24:1) were added, than vortex and centrifuged at 13000 rpm for 10 min at Room Temperature (RT). The upper layer was transferred in a new tube, and an equal volume of Chloroform:IAA (24:1) was added, than vortexed and centrifuged at 13000 rpm for 10 min at RT. The upper layer was transferred in a new tube, and an equal volume of cold isopropanol was added. To precipitate the RNA, LiCl at final concentration of 2 M was added to the sample, and incubated at 4 °C overnight. The next day the samples were centrifuged at 13000 rpm for 30 min at RT. The supernatant was discard, and the pellet was resuspended in 1 mL of DEPC water, 250 µL of LiCl 10 M and incubated for 3 hours on ice. Than the samples were centrifuged at 13000 rpm for 10 min at RT, and the supernatant was discarded. The pellet was washed in 250 µL of DEPC water, 25 µL of Sodium Acetate 3M and 1 mL of absolute ethanol. Than the RNA was precipitated for 3 hours at -20 °C. Than the samples were centrifuged at 13000 rpm for 10 min at RT, and the pellet resuspended in 50 µL of DEPC water. The RNA purity was tested with Nanodrop, and only the sample with the ratio 260/280 ≥ 2.00 and the ratio 260/230 ≥ 2.00 are used for the gene expression analysis. gDNA residue was eliminated with RQ1 RNase-Free DNase (Promega™, USA) treatment. The same protocol above described, in paragraph 3.4.1- 3.4.5. were used for the reverse transcription reaction, cloning and sequence analyses.

3.6.2 Primer designing

To design the primers for qPCR, the sequences obtained from the sequencing were used. After verification of these, with bioinformatics tools, the primers for NRAMP1, NRAMP3, ZIP11, HMA3, ABCC1, PHT (genes associated with heavy metals tolerance) were designed using primer3 website (Rozen et al., 1999), setting these parameters: 60 °C Ta, size of product 100 – 150 bp, 50 % GC, and when possible, the 3' – end of the primers, had to end with G or C, because more stables.

3.6.3 qPCR

For the reaction 600 ng of cDNA were added to master mix, including Syber green (promega), primers, and samples were puts on plates (Biorad) and in the qPCR machine (Biorad). The program of qPCR was denaturation 95 °C, 72 °C extension, and 60 °C annealing temperatures. The melt curves were asses for the different primers, and the quality check of the primers was made before use, with scalar dilutions with a range 5 (5, 25, 125, 625 fold). The expression levels were compared with the two genes housekeeping (EF1 alpha and GAPDH). The threshold was set at 1000 for each reaction. Ct values of each samples were used to analyse the data.

3.7 Data analysis

All data were submitted to the Barlett's test for the homogeneity of variance and then analysed using analysis of variance (ANOVA) with CoSTAT program. Angular transformation of the germination data, was carry out. Means were separated on the basis of the least significant different (LSD), when the 'F' test of ANOVA for treatments was significant at least at 0.05 probability level.

4 Results

4.1 Germination tests

Seeds germination was influenced by the genotypes, heavy metals, their concentrations, and the combination of these three parameters (Fig. 14). Response resulted strongly affected by the genotype (the line of domestic cardoon and two population of wild cardoon). The major differences were found between the two populations of *sylvestris* collected in very different sites of Eastern Sicily: the first grow at 900 m above sea level in the territory of Randazzo (CT) within Nebrodi Regional Park (R14CT) and the second grow in the territory of Augusta (SR) at 4 m above sea level within the industrial area (A14SR).

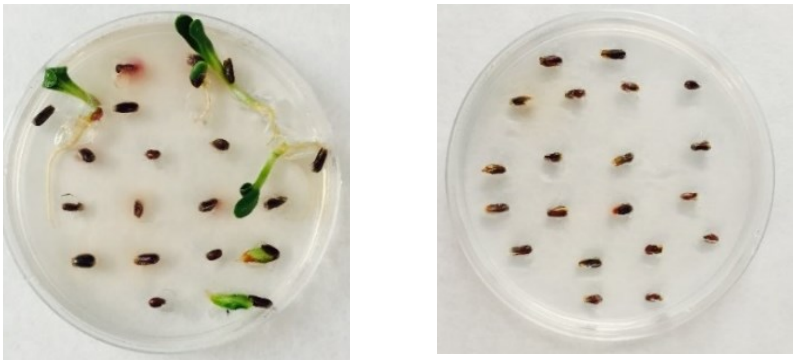


Figure 14. Seeds of Cardoon germinated in agar medium supplemented with Cd 50 um (left) and As 100 um (right) after 10 day from sowing.

As predicted, the ANOVA analyses showed that heavy metals concentration was the main cause of variation contributing for 64 % of the total (Table 1). In particular 'altilis', a selected line of cardoon, showed more tolerance to Cd and Cd + As treatments compared to wild cardoon R14CT, which belong to plants grown in uncontaminated soils. A14SR, grown in contaminated soils, resulted more tolerant to As, compared to the other genotypes. For this reason, the variety of *sylvestris* corresponding to the A14SR genotype was used in the transcriptional studies reported below. R14CT, the genotype harvested in

uncontaminated soil, showed the lower germination percentage compared to other genotypes, independently of the metal used.

Figure 15 shows the rate of germination at different concentrations of heavy metal. Data can be explained by the analysis of variance reported in table 1.

Table 1. Analysis of variance of final germination percentage of cardoon with heavy metals and partition of the treatment of squares into main effect and interaction.

Source of Variation	Mean squares	
	Absolute value	% of total
Genotype (G)	0.31 ***	12.50
Metal (M)	0.38 ***	15.47
Concentration (C)	1.58 ***	64.02
G * M	0.08 ***	3.11
G * C	0.03 ***	1.15
M * C	0.04 ***	1.52
G * M * C	0.05 ***	2.22

Germination, averaging the contribution of all the three sources of variation, was 65.9 %. Compared to this value, the A14SR genotype with 73.0 %, resulted the genotype with the most capacity to germinate in contaminated soil, and wild cardoon R14CT, with 54.07 %, the less tolerant.

Regarding the heavy metal used as contaminant in the soil, Cd was the less effective with a germination percentage of 77.0 % and a 17.23 % higher value than average. The germination under Cd + As, showed the largest decrease, 14.24 %, compared to the average value.

Compared to the interaction of the different factors, genotype x metal, with 3.11 %, results the main cause of variation. In particular, under Cd treatment, *altilis* genotype showed the highest germination percentage (83.0 %). Moreover, A14SR genotype showed the highest germination percentage (76.0 %) under As treatment. Instead. the genotype R14CT showed a lowest germination percentage under both treatments, 69.4 % and 45.6 %, respectively (Fig. 15).

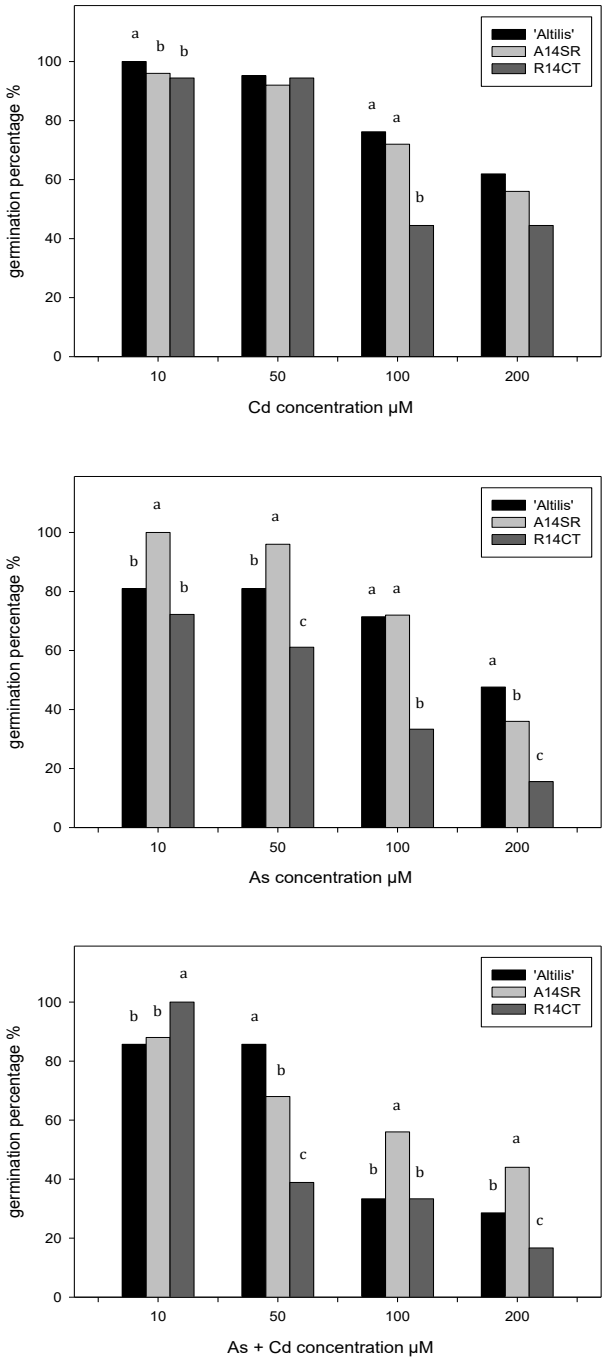


Figure 15. Germination percentage of 'altilis', A14SR and R14CT at different treatments and concentrations. Different letters indicate significant differences at $P \leq 0.05$ among genotypes within the same concentration followig Student-Newman-Keuls test.

4.2 Seedling growth analysis

Seedlings length, measured after three weeks of treatments with 0, 25 and 50 μM concentrations of either As or Cd, was differentially affected in the two varieties (fig.17). *Altilis* genotype showed growth similar to the control (CTRL, 0 μM), under As treatment while under Cd treatment, a significant reduction in roots and shoots length was observed showing a lower tolerance to the metal than that observed in the previous germination phase. However, shoots, in contrast to roots, remain vitals with no evidence of chlorosis.

In *sylvestris* under both metals, roots and shoots length decreases with the increase of concentrations but at 25 μM this is more evident with Cd than As.

In particular, the roots and shoots length, averaging the contribution of all the three sources of variation, including the different genotypes, was 2.99 cm and 2.97 cm respectively, with a ratio shoot / root ~1.

Compared to this value, the genotype *altilis*, with a ratio 1.2, resulted the variety with the highest epigeal part, while *sylvestris* with a ratio 0.65, resulted the genotype with the shortest aerial part.

As shown in table 3, the differences scored for both metals were significant.

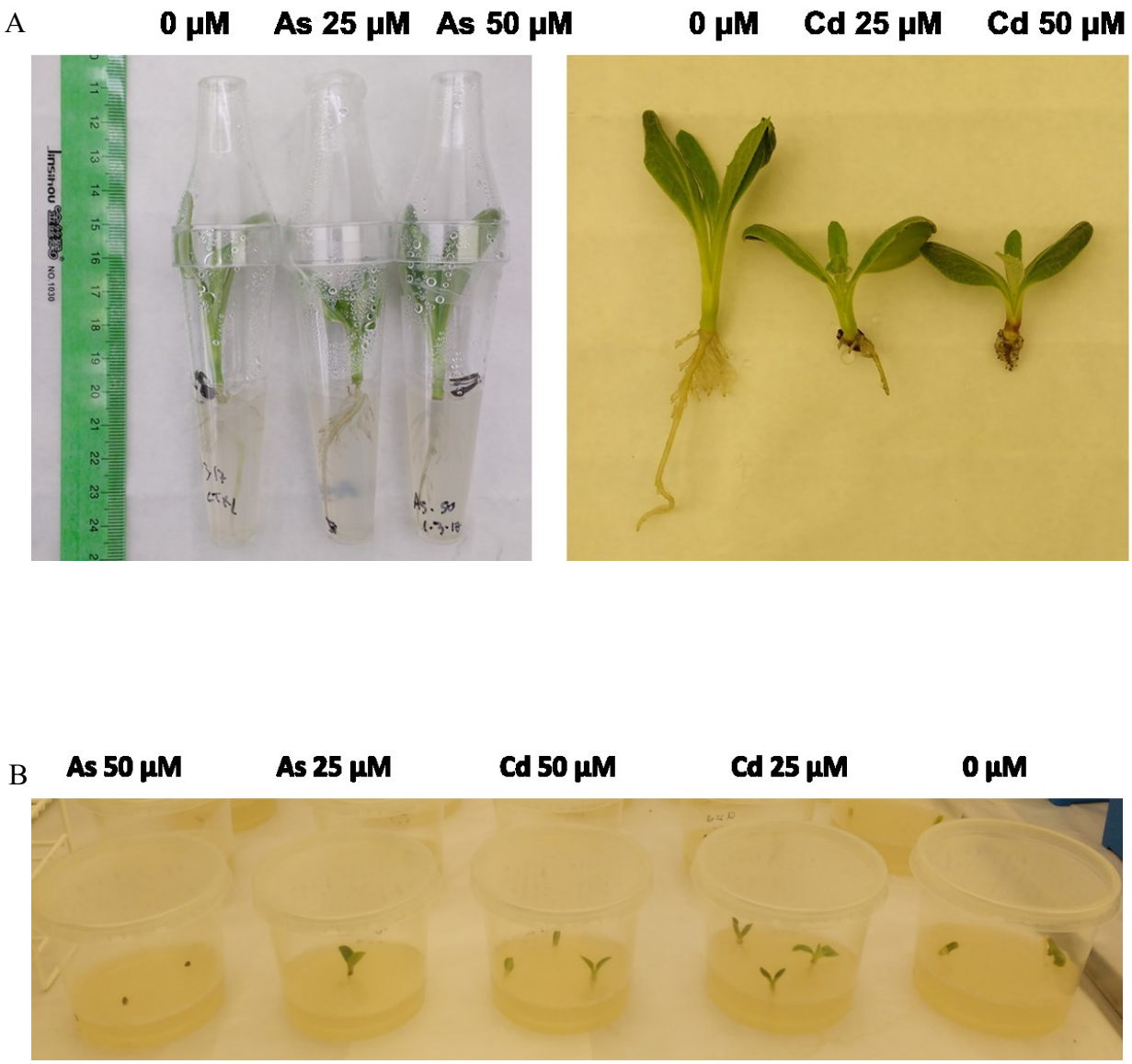


Figure 17. Seedling of *altilis* (A) and *sylvestris* (B) 3 weeks old, growth in ½ MS solid medium under different concentration of metals.

In the variety *sylvestris* plants length decreased with Cd treatment. Root length resulted more than 2 folds lower compared to untreated controls (Fig. 18). Under As treatment, the effects of metal concentration were evident at 50 µM. In fact, at 25 µM, the seedlings grow, and the ratio shoot/roots is more than 1, in contrast to the control. At 50 µM, the seeds germinated, but didn't grow, and the browning of the roots was evident.

The variance analysis showed a different behaviour in the two genotypes considered.

Table 3. Analysis of variance of plant length, separated in shoot and root, measured after 3 weeks of treatment with As and Cd. Partition of the treatment sum of squares into main effect and interaction.

Source of Variation	sylvestris Mean squares			altilis Mean squares		
	df	Absolute value	% of total	df	Absolute value	% of total
Organ (O)	1	5.84 *	13.94	1	5.14 **	6.83
Metal (M)	1	1.17 ns	2.80	1	30.25 ***	40.24
concentration (C)	2	15.13 ***	36.11	2	29.40 ***	39.11
O * M	1	0.06 ns	0.15	1	1.14 ns	1.51
O * C	2	16.97 ***	40.48	2	1.33 ns	1.77
M * C	2	2.59 ns	6.18	2	7.62 ***	10.13
O * M * C	2	0.15 ns	0.35	2	0.31 ns	0.41

As shown in table 3 in *sylvestris* variety, organ x concentration, with 16.97 %, results the main cause of variation with respect to all the factors considered.

On the contrary, in *altilis* variety, metal x concentration results the main and solely significant cause of variation.

In particular, with Cd, the increase of concentrations caused a reduction of seedling length that was 31% and 51% in *sylvestris* and 59% and 70% in *altilis*, at 25 µM and 50 µM compared to the control. With As treatment, the decrease was 21% and 100% in *sylvestris* and 14% and 28% in *altilis,* at 25 µM and 50 µM compared to the control.

The ratio root length/ seedling length was also influenced by the metal and concentration used. In particular, with Cd, the ratio was 80%, 42% and 47% at 0, 25 and 50 µM respectively in *sylvestris,* while it was 43%, 39% and 33% in *altilis* at the same concentrations. With As treatment, the ratio was 80%, 35% at 0 and 25 µM in *sylvestris* (at 50 µM the plants germinate but didn't grown), while it was 43%, 49% and 52% in *altilis* at 0, 25 and 50 µM, showing this latter no changes compared to control. The change of ratio shoot/roots is very strong in *sylvestris*, compared to both *altilis* and the control

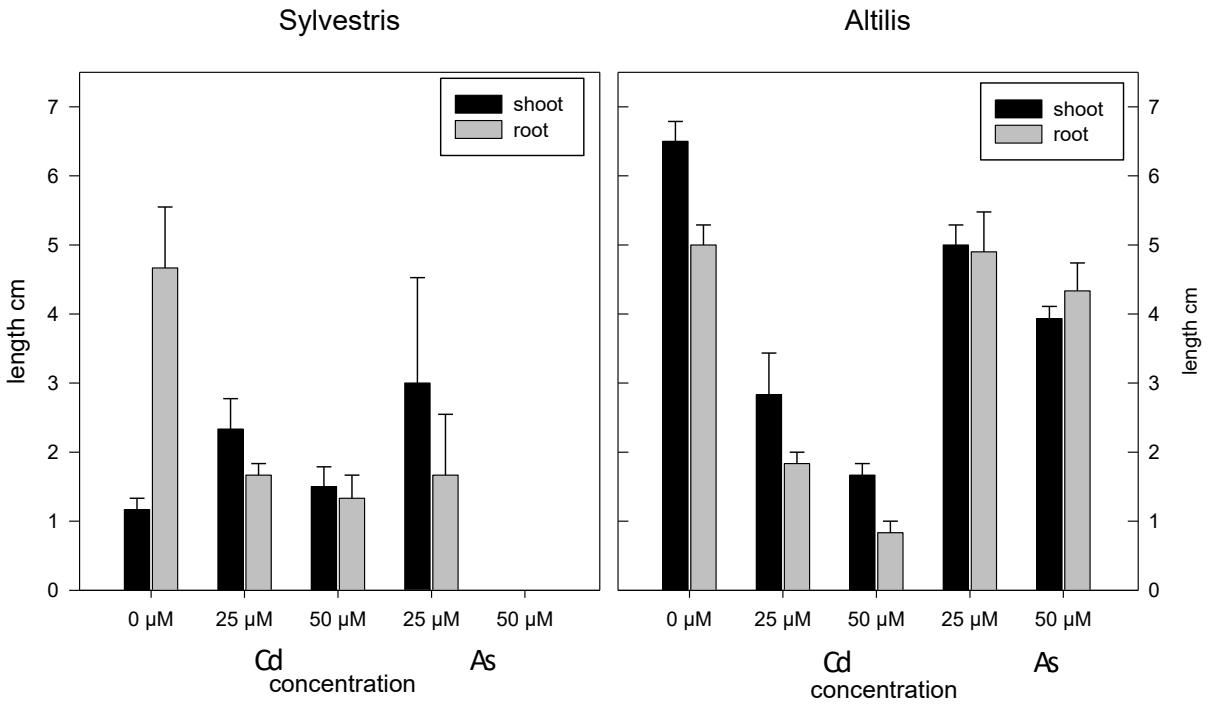


Figure 18. Root and shoot lengths of 'altilis', and 'sylvestris' harvested after 3 weeks of treatment under different concentrations of As and Cd. The error bars indicate the Standard Deviation of three biological replicates.

4.3 Identification of cardoon genes likely associated with heavy metals transport and accumulation

Oligonucleotide primers designed on the annotated sequences of *Arabidopsis* or *Thlaspi* with the use of the primer3 website program were used for the isolation and characterization by PCR of orthologous cardoon genes putatively associated with heavy metals transport and accumulation (Supplementary Table 1). After sequencing, the genes identified in Cynara were NRAMP1, NRAMP3, ZIP11, HMA, ABCC1, PHT and PCS. Contigs obtained with the use of the BLASTn and BLASTp platforms are shown in Supplementary Tables 2 and 3. The BLASTn of the sequences against all database, resulted with a percentage of identities that was always > than 72 % (ST.2).The identities of the BLASTp against all database resulted > 76 %, and against *Cynara scolymus* scored > 91 % for all genes. These results, and the score obtained by BLAST, confirmed the identity and reliability of our sequences, not previously recognized in cardoon. In fact, the nucleotide sequences present in cardoon database are just predicted from amino acid sequences.

The query cover is a percent of the query sequence that overlaps the subject sequence. It resulted over 90 % in NRAMP3, PHT, and PCS but was very low in ABCC1. The Expect value (E) is a parameter that describes the number of hits that one ‘expects’ to see by chance when searching a database of a given size. It decreases exponentially as the Score (S) of the match increases. The E value was always low in our studies.

4.3.1 Alignment of sequences

To proceed with the identification of genes associated with heavy metals transport, different alignments of corresponding orthologous *A.Thaliana* proteins against the *Cynara* protein database were carried out. Table 4 shows BLASTx of the different isoforms known in *Arabidopsis* compared to *Cynara*. Different *A. thaliana* isoforms could not always find a corresponding accession protein in Cynara.

Table 4. BLASTX of Arabidopsis gene isoforms against *Cynara* database.

BLASTX OF *ARABIDOPSIS* AGAINST *CYNARA*						
PROTEIN	Max score	Total score	Query cover	E value	Ident	Accession
NRAMP1	672	672	63%	0.0	70%	KVI06072.1
NRAMP2	793	793	77%	0.0	81%	KVH98279.1
NRAMP3	747	747	75%	0.0	77%	KVH98279.1
NRAMP4	712	712	61%	0.0	71%	KVH98279.1
NRAMP5	669	669	74%	0.0	68%	KVH98279.1
NRAMP6	723	723	49%	0.0	71%	KVI06072.1
PHT1.1	818	818	77%	0.0	79%	KVH91481.1
PHT1.4	805	805	76%	0.0	84%	KVH91495.1
PHT1.5	794	794	86%	0.0	76%	KVH91481.1
PHT1.9	588	828	82%	0.0	55%	KVI05143.1
PHT1.8	537	783	80%	0.0	55%	KVI05143.1
PHT1.2	814	814	83%	0.0	80%	KVH91481.1
PHT1.7	878	878	76%	0.0	85%	KVH91495.1
PHT1.3	843	843	99%	0.0	79%	KVH91481.1
	843	843	99%	0.0	79%	KVH91495.1
PHT1.6	705	705	99%	0.0	68%	KVH91495.1
PHT2.1	676	676	70%	0.0	70%	KVI05770.1
PHT3.1	547	547	53%	0.0	87%	KVI09253.1
PHT3.2	512	512	70%	0.0	69%	KVI09253.1
PHT3.3	430	430	53%	6.00E-149	67%	KVI08722.1
PHT4.1	721	721	59%	0.0	83%	KVI03657.1
PHT4.6	656	656	57%	0.0	78%	KVH97383.1
PHT4.2	499	499	61%	3.00E-171	58%	KVH94681.1
PHT4.3	635	635	55%	0.0	75%	KVH94681.1
PHT4.5	282	282	34%	3.00E-90	63%	KVH93765.1
PCS1	404	404	74%	2.00E-135	53%	KVI12347.1
PCS2	404	404	83%	2.00E-136	49%	KVI12347.1
HMA1	963	963	73%	0.0	70%	KVH92487.1
HMA2	845	845	63%	0.0	64%	KVI01438.1
HMA3	631	828	71%	0.0	64%	KVH87487.1
	579	765	79%	0.0	59%	KVI01438.1
HMA4	732	732	48%	0.0	64%	KVI01438.1
HMA5	1422	1422	93%	0.0	73%	KVH90063.1
HMA6	955	955	66%	0.0	68%	KVI01812.1
GAPDH	185	185	42%	8.00E-58	79%	KVH97848.1
EF1A	175	175	11%	2.00E-51	72%	ACC99594.1
ZIP1	298	298	60%	5.00E-98	60%	KVI02328.1
ZIP2	383	383	62%	3.00E-131	66%	KVI11955.1
ZIP3	275	275	58%	2.00E-88	53%	KVH89209.1
ZIP4	346	346	66%	1.00E-114	65%	KVH52325.1
ZIIP5	285	285	64%	3.00E-92	54%	KVH89209.1
ZIP11	395	395	64%	3.00E-136	65%	KVI10407.1
ABCC1	2090	2090	85%	0.0	66%	KVH87904.1

Alignments of the nucleotide sequences (contigs resulted from cloning) against other plant species that showed a high score using BLASTn, can be found in the Appendix. Each nucleotide is identifiable by a different colour allowing an easy recognition of the nucleotide matches. These results showed a high similarity of the contigs resulted from the cloning with the sequences present in the annotated cds of the database, but the nucleotide sequence size was lower than that of the annotated cds

The amino acidic (aa) sequences of our contigs translate with Expasy program, were aligned with the database aa sequences by the mean of BLASTp algorithm. The results showed that in the different species, phylogenetically similar to *Cynara cardunculus* L.*,* as *Helianthus annuus* L.*,* the alignment has a high match number and high identity among the aa sequences.

4.4 Housekeeping genes analysis

GAPDH, β-TUBULIN, ACTIN, ELONGATION FACTOR, 18S, ANAPHASE PROMOTING COMPLEX and TRASDUCIN/WD40 housekeeping genes were assayed in different times of growth (water imbibed seed (IDS), germinated seeds (GS), young leaf (YL), flower open head (capitulum) (OC4), and flower closed capitulum (CC1)) to select which one was better suitable to act as reference in gene expression analyses. Cloning primers for such genes, used in PCR, are shown in Supplementary Table 4. The results of the contigs blasted with BLASTn and BLASTp are shown in Supplementary Tables 5 and 6. The identities of the BLASTp against all database were > 83%, and against *Cynara scolymus* > 88 % for all the genes assayed, except for GAPDH that was 61 %. The BLASTn of the sequences against all database, resulted with a percentage of identities always > than 85 % (ST. 6).

The dissociation curves during qPCR, showed the specificity and efficiency of the primers for real time PCR. As showed in figure 19, only a single peak was observed, for any of the tested housekeeping genes with the exception of β-TUBULIN that showed two peaks and was therefore discarded.

Ct values showed that the ribosomal 18S gene is the most expressed reference genes in the different developmental phases assayed (Fig. 18).

After geNorm analysis, all the selected housekeeping genes showed M value < 0.5, confirmatory of the stability of expression of the six selected genes for reference. Among them, the gene encoding for the ribosomal 18S subunit showed the lower stability value while WD40 and APC resulted the more stables (Fig. 20).

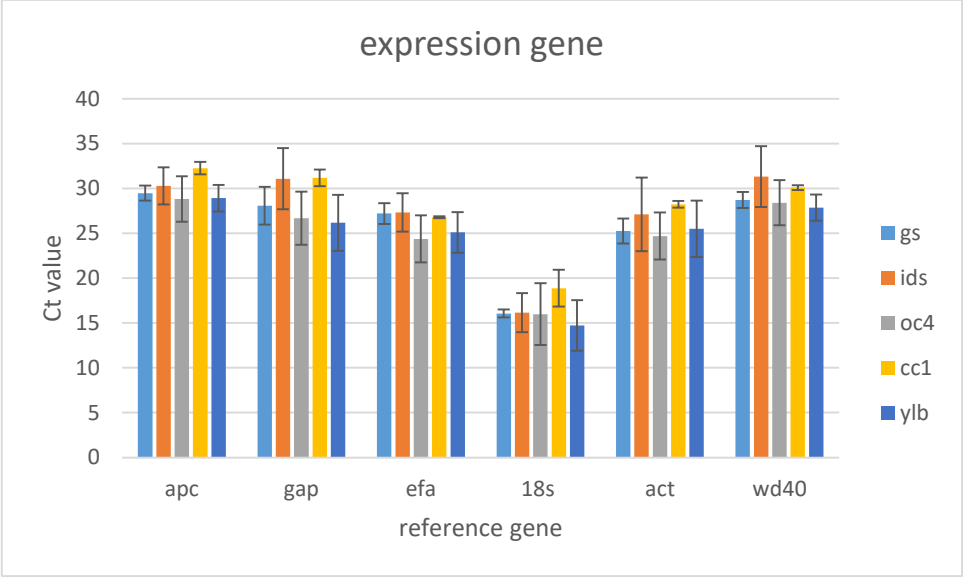


Figure 18. The gene expression patterrn of selected housekeeping genes for reference. Three biological replicates are shown for each time of growth (GS, IDS, OC4, CC1 and YLB). Error bars show the standard deviation (SD) resulting from three replicates during qRT-PCR analysis. Under different times of growth the genes are stably expressed, 18s is the gene that shows the major variability ;

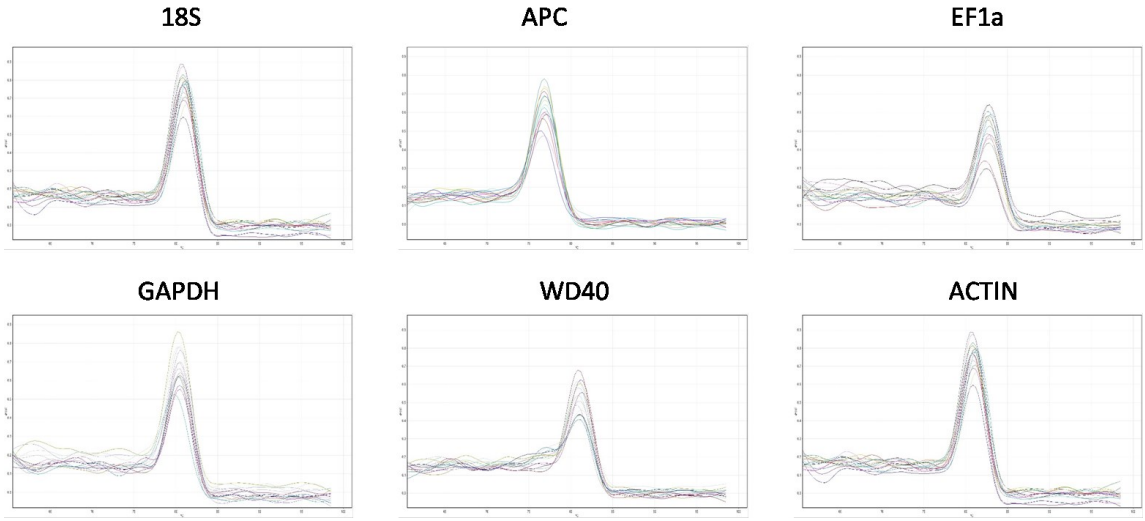


Figure 19. The Dissociation curves with single peaks of the six reference genes analysed generated for all amplicons from three replicates for five times of growth.

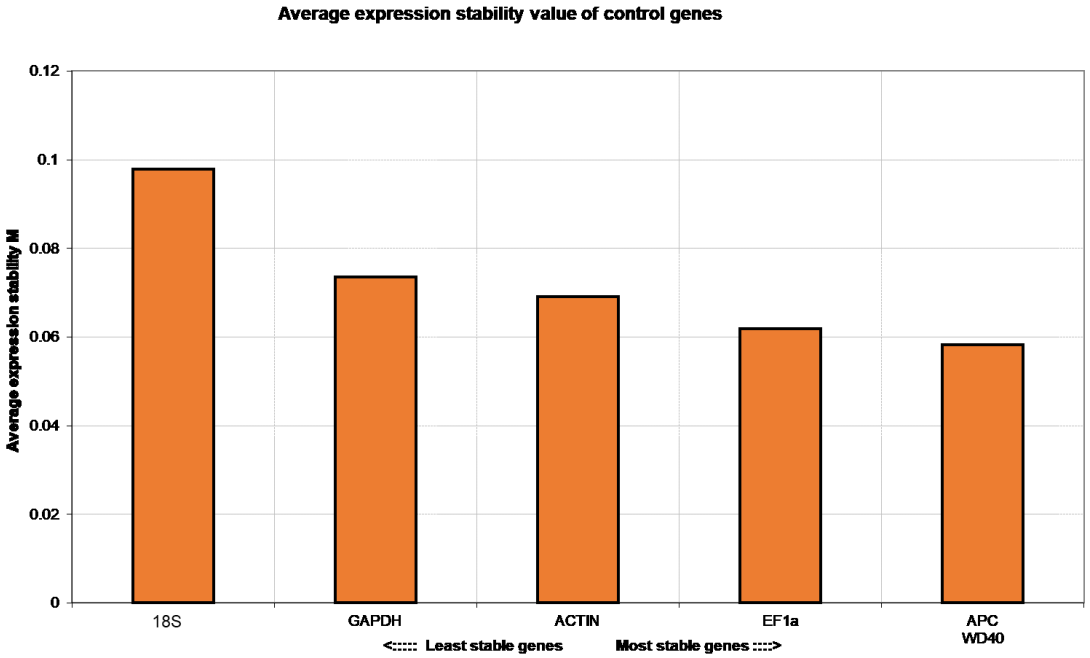
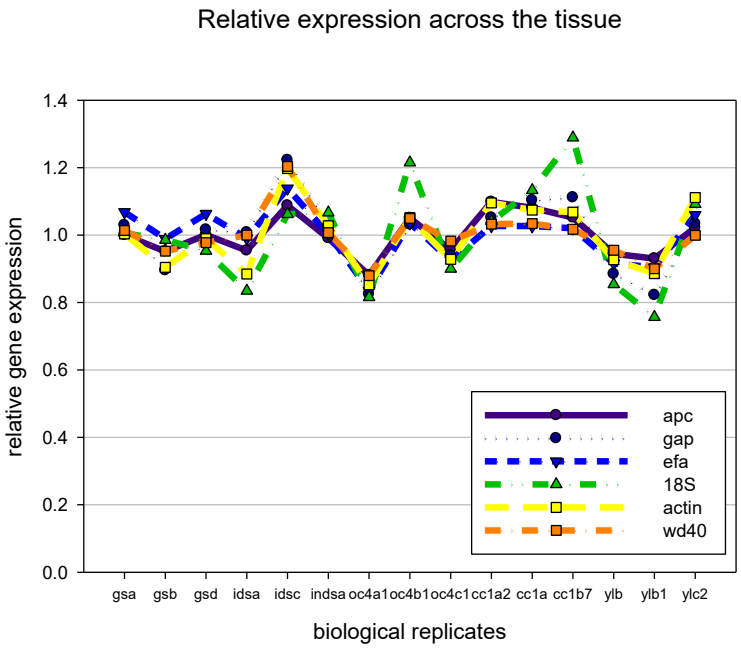


Figure 20. Relative expression of the HKGs analysed, known in Arabidopsis references on 3 biological replicates for five times of growth and their average. The dates are normalized with the average of Ct value. The ratio between the effective Ct for each sample and the average of Ct of all samples is showed. 18S is the gene with more variability. Apc and Wd40 are more stably expressed genes.

The ratio between the effective Ct for each sample and the average of Ct of all samples was considered for each gene (Fig. 21). The results showed that for all genes, this ratio is near to 1, but in 18 S gene, the trend is different, with a ratio of 1.2 in CC1 samples.



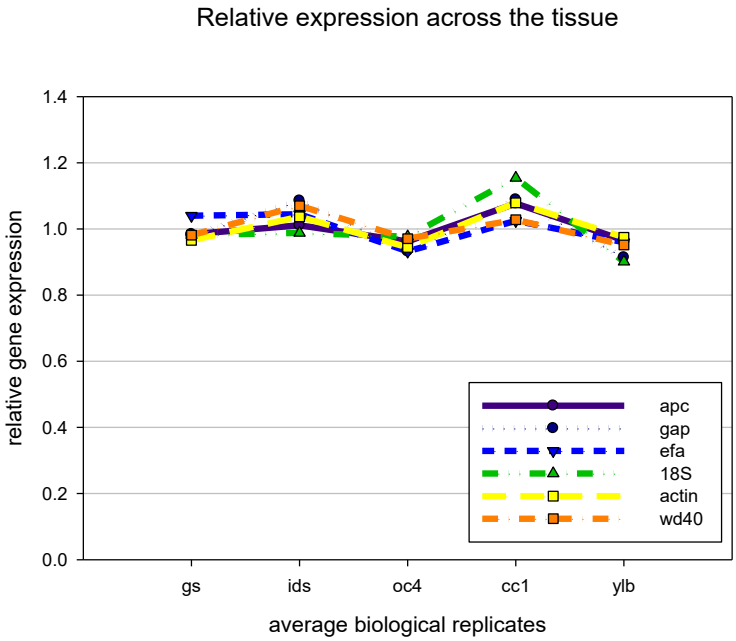


Figure 21. Relative expression of traditional HKGs, known Arabidopsis references on 3 biological replicates for each tissue, and their average. The dates are normalized with the average of Ct value. The ratio between the effective Ct for each sample and the average of Ct of all samples is showed. 18S is the gene with more variability. Apc and Wd40 are the most stably genes

4.5 qRT-PCR analyses

The six genes identified in cardoon for the response to heavy metals (NRAMP1, NRAMP3, ZIP11, HMA, PHT and ABCC1) were suitable for the transcriptional analyses performed by qPCR. PCS was discarded because its dissociation curve showed two peaks. Overall, the qPCR results obtained from the six selected genes showed a different transcriptional response in-the different varieties of metal, time and concentration used. All data were normalized against GAPDH and EF1 alpha used as reference genes. The fold change represent how many time the gene is expressed compared to the untreated control. In both genotypes, *altilis* and *sylvestris,* transcriptional levels can be influenced by the concentration of metals in the medium, type of metals, and time of growth.

In **NRAMP1,** transcriptional levels resulted not influenced by the treatments used (type of metal, its concentration and time of growth) as showed in tab.5 and tab.6, with exception of root under As treatment, where genotype and interaction genotype x concentration resulted statistically significant. In shoots, *altilis* showed expression levels lower than control, while expression in *sylvestris* results highest at 25 μM and then decreased at 50 μM, with exception of As 2 weeks (fig.22). In roots, but not in shoots, the expression levels resulted similar to control in *altilis*, while it is down regulated in *sylvestris*. In particular, in *sylvestris* roots with As treatment, at 2 weeks, the expression decreases near to 0, but at 3 weeks is near to control. This result could be caused by different responses occurring during the exposition time. In particular, highly significant values, contributed by 2 of the 3 sources of variation (genotype, time and concentration of metal) were observed only in roots treated with As, as inferable by comparing the data reported in table 5 and 6.

Table 5. Analysis of variance of fold change of NRAMP1 gene expression respect to the CTRL in shoot and root of cardoon treated with Cd and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	1.01	*	29.63	1	0.02	ns	1.38
time (T)	1	0.00	ns	0.03	1	0.03	ns	2.22
concentration (C)	2	0.98	*	28.52	2	0.41	ns	33.04
G * T	1	0.64	ns	18.70	1	0.47	ns	37.37
G * C	2	0.61	ns	17.69	2	0.07	ns	5.31
T * C	2	0.00	ns	0.01	2	0.10	ns	8.14
G * T * C	2	0.19	ns	5.43	2	0.16	ns	12.54

Table 6. Analysis of variance of fold change of NRAMP1 gene expression respect to the CTRL in shoot and root of cardoon treated with As and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	0.06	ns	0.90	1	4.44	***	46.92
time (T)	1	0.47	*	7.35	1	0.13	ns	1.35
concentration (C)	2	1.56	ns	24.59	2	1.25	**	13.24
G * T	1	1.35	ns	21.31	1	1.55	**	16.39
G * C	2	0.69	ns	10.89	2	1.14	***	12.02
T * C	2	1.76	ns	27.87	2	0.56	ns	5.96
G * T * C	2	0.45	ns	7.08	2	0.39	**	4.12

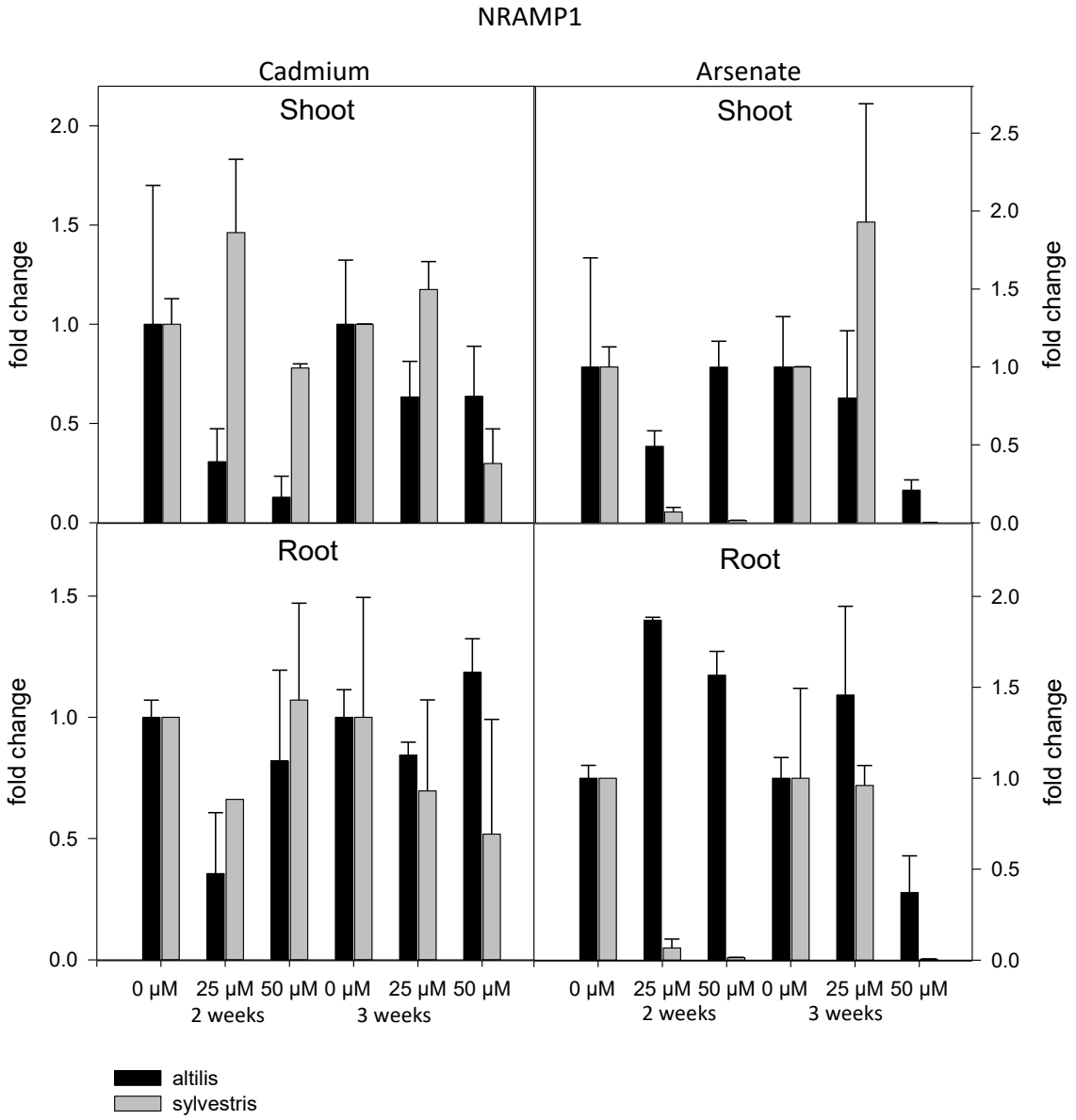


Figure 22.The level of gene expression of NRAMP1 in seedlings of *altilis* and *sylvestris* after 2 and 3 weeks of growth under Cd and As at different concentration. The fold change is the Ct value respect to the untreated control, that is made equal to 1. The error bars represents the error mean of three replicates.

The expression level of **NRAMP3** or 4 (the two proteins have the same accession number) resulted strongly influenced by the genotype and the time of growth. In shoots and roots, made the average of all the variable factors, transcriptional levels are over expressed in *sylvestris* and down regulated in *altilis*. In particular, after 3 weeks, it is observed an increase of expression levels in *sylvestris* that is at least 2 times than control at 25 µM and 4 times than control at 50 µM with both metals. In roots, which are closer

to contaminated medium, the increase of transcriptional levels is marked compared to shoots in both genotypes, with the highest value in *sylvestris* scored at 3 weeks. The type of metal, Cd or As, did not influence the relative expression of NRAMP3, because with both stresses, the transcriptional levels increased after 3 weeks compared to control in wild cardoon. However, with As, transcriptional level in *sylvestris* is 5.22 times more than *altilis*, with the highest value found in roots at 50 μM after 3 weeks (fig.23). In particular, highly significant values, contributed by the 3 sources of variation (genotype, time and concentration of metal) were observed in root treated with As, as inferable by comparing the data reported in table 7 and 8.

Table 7. Analysis of variance of fold change of NRAMP3 gene expression respect to the CTRL in shoot and root of cardoon treated with Cd and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	7.00	**	28.90	1	12.84	*	16.59
time (T)	1	1.42	ns	5.84	1	7.99	ns	10.32
concentration (C)	2	1.61	ns	6.63	2	14.51	*	18.75
G * T	1	8.38	**	34.57	1	16.23	*	20.98
G * C	2	2.91	ns	11.99	2	11.58	*	14.97
T * C	2	0.73	ns	3.02	2	3.57	ns	4.61
G * T * C	2	2.19	ns	9.05	2	10.66	*	13.78

Table 8. Analysis of variance of fold change of NRAMP3 gene expression respect to the CTRL in shoot and root of cardoon treated with As and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	29.47	***	19.26	1	256.34	***	19.11
time (T)	1	41.18	***	26.91	1	245.44	***	18.29
concentration (C)	2	8.88	***	5.80	2	118.24	***	8.81
G * T	1	34.37	***	22.46	1	391.02	***	29.14
G * C	2	11.59	***	7.58	2	99.62	***	7.42
T * C	2	12.89	***	8.43	2	102.21	***	7.62
G * T * C	2	14.63	***	9.56	2	128.85	***	9.60

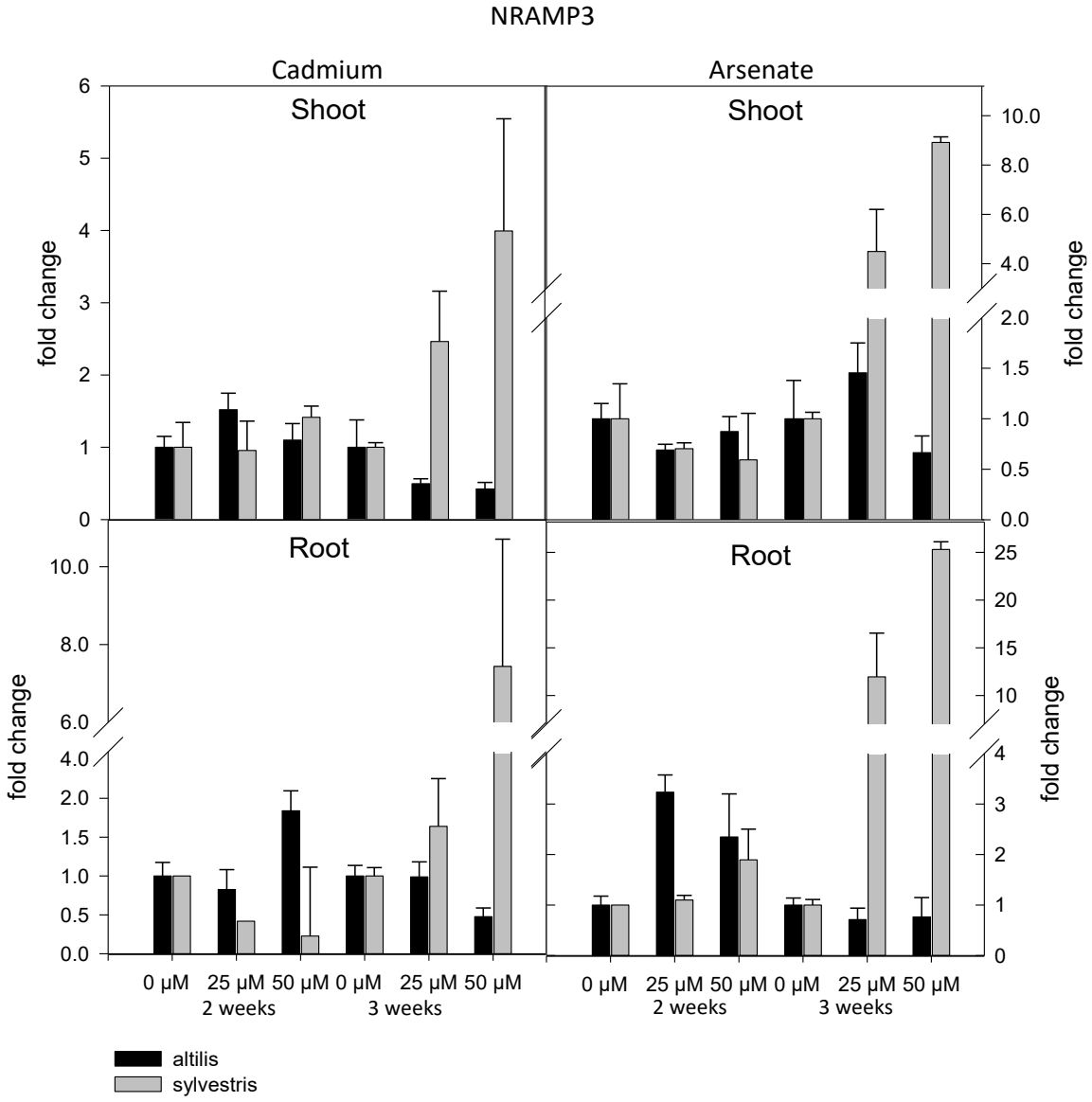


Figure 23. The level of gene expression of NRAMP3 in seedlings of *altilis* and *sylvestris* growth 2 and 3 weeks under Cd and As at different concentration. The fold change is the Ct value respect to the control, that is considered 1. The error bars represents the error mean of three replicates.

Similarly to what observed with NRAMP3/4 the expression/transcriptional level of **ZIP11** is influenced by the genotype and time of growth. In both genotypes, the type of metal did not influence the transcriptional levels. In roots of *sylvestris* after 3 weeks, the level of ZIP11 mRNAs significantly increases in presence of Cd and As, while in *altilis* the transcriptional levels decrease with both metals.

In particular, highly significant values, contributed by the 3 sources of variation (genotype, time and concentration of metal) were observed in shoot treated with As, as inferable by comparing the data reported in table 9 and 10.

In shoot and roots, after 3 weeks transcriptional levels were up- regulated in *sylvestris* compared to control, while in *altilis*, the expression was down-regulated, allowing for the contribution of all the three sources of variation. In fact, in *sylvestris,* the transcriptional level resulted at 3 weeks 51.5 fold more than that in *altilis*. Although type of metal not influences the transcriptional levels, in *sylvestris*, As increase transcriptional levels 4 time more than Cd.

Table 9. Analysis of variance of fold change of ZIP11 gene expression respect to the CTRL in shoot and root of cardoon treated with Cd and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				Root Mean squares			
	df	Absolute value		%t of total	df	Absolute value		% of total
genotype (G)	1	435.92	*	13.11	1	256.34	*	26.08
time (T)	1	503.78	*	15.15	1	245.90	*	25.02
concentration (C)	2	459.72	**	13.82	2	44.46	ns	4.52
G * T	1	477.43	*	14.36	1	248.37	*	25.27
G * C	2	473.41	**	14.24	2	64.11	ns	6.52
T * C	2	529.26	**	15.92	2	61.64	ns	6.27
G * T * C	2	445.84	**	13.41	2	62.11	ns	6.32

Table 10. Analysis of variance of fold change of ZIP11 gene expression respect to the CTRL in shoot and root of cardoon treated with As and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				Root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	15554.67	**	22.01	1	314.07	*	24.25
time (T)	1	15861.47	**	22.44	1	286.82	*	22.15
concentration (C)	2	5961.16	*	8.44	2	91.99	ns	7.10
G * T	1	15461.28	**	21.88	1	320.44	*	24.75
G * C	2	5955.58	*	8.43	2	100.98	ns	7.80
T * C	2	6036.21	*	8.54	2	87.83	ns	6.78
G * T * C	2	5840.03	*	8.26	2	92.80	ns	7.17

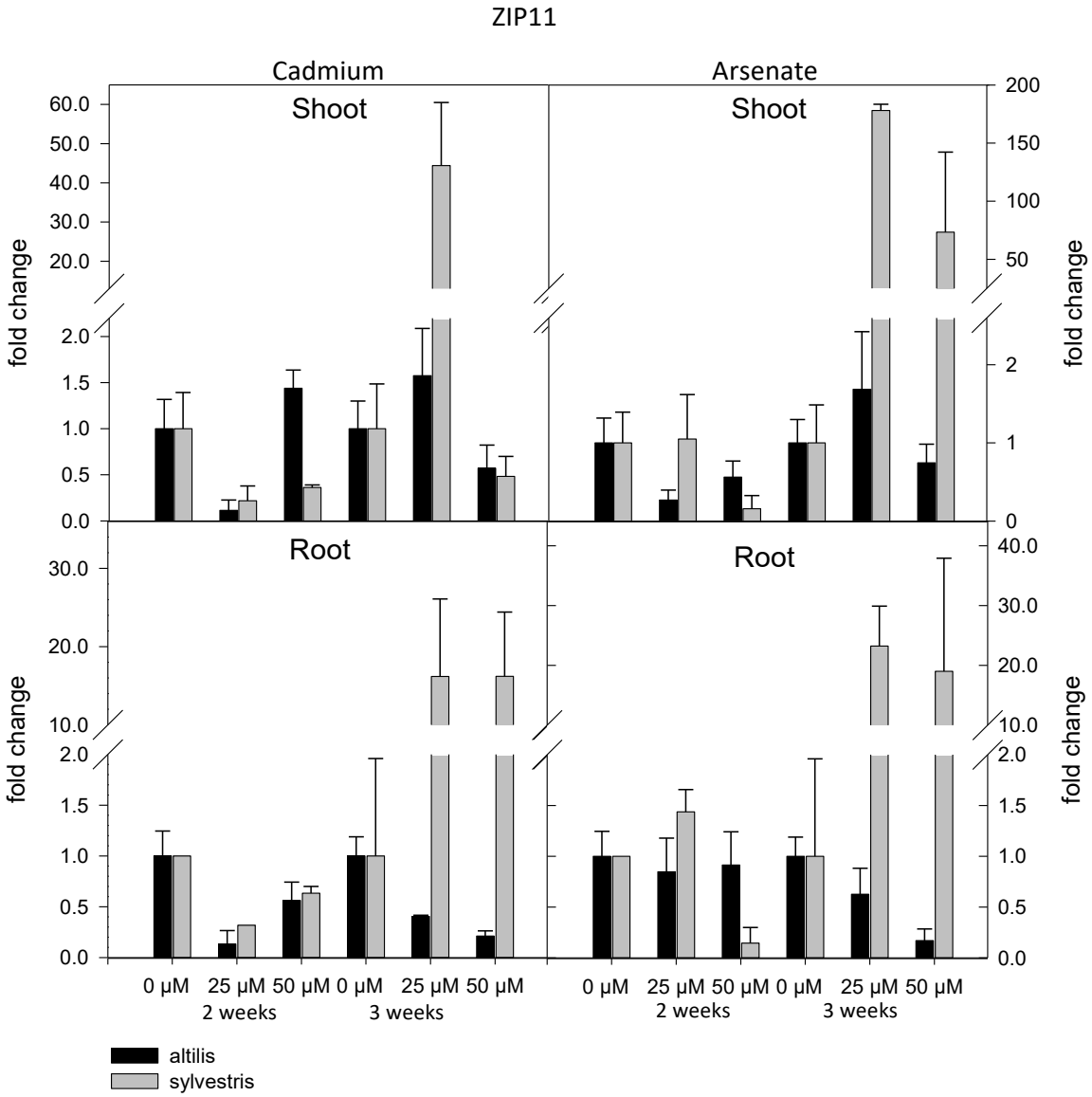


Figure 24. The level of gene expression of ZIP11 in seedlings of *altilis* and *sylvestris* growth 2 and 3 weeks under Cd and As at different concentrations. The fold change is the Ct value respect to the control, that is considered 1. The error bars represents the error mean of three replicates.

The expression level of **HMA** resulted influenced by concentration and genotype.

In particular, highly significant values, contributed by 2 of the 3 sources of variation (genotype, time and concentration of metal) were observed in root treated with Cd, as inferable by comparing the data reported in table 11 and 12.

Shoots and roots show similar response to stresses, averaging the contribution of all the three sources of variation, but transcriptional level at 3 weeks in shoots of *altilis* with As resulted 3 times more than *sylvestris* (fig.25). From these data, *altilis*, resulted over-express 1.36 times HMA transcript compared to *sylvestris* with As treatment.

Table 11. Analysis of variance of fold change of HMA gene expression respect to the CTRL in shoot and root of cardoon treated with Cd and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	0.23	ns	0.72	1	6.34	**	25.18
time (T)	1	11.80	**	37.16	1	0.29	ns	1.17
concentration (C)	2	1.02	ns	3.20	2	4.91	***	19.49
G * T	1	1.34	ns	4.23	1	2.53	*	10.04
G * C	2	6.20	*	19.51	2	7.02	***	27.91
T * C	2	4.23	ns	13.32	2	1.48	ns	5.88
G * T * C	2	6.95	*	21.87	2	2.60	*	10.33

Table 12. Analysis of variance of fold change of HMA gene expression respect to the CTRL in shoot and root of cardoon treated with As and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	13.47	ns	14.27	1	2.70	ns	4.23
time (T)	1	35.94	**	38.08	1	7.59	ns	11.88
concentration (C)	2	4.53	ns	4.81	2	12.07	**	18.89
G * T	1	18.79	*	19.91	1	6.65	ns	10.40
G * C	2	4.56	ns	4.84	2	8.92	*	13.96
T * C	2	11.08	ns	11.74	2	17.11	***	26.77
G * T * C	2	5.99	ns	6.35	2	8.86	*	13.87

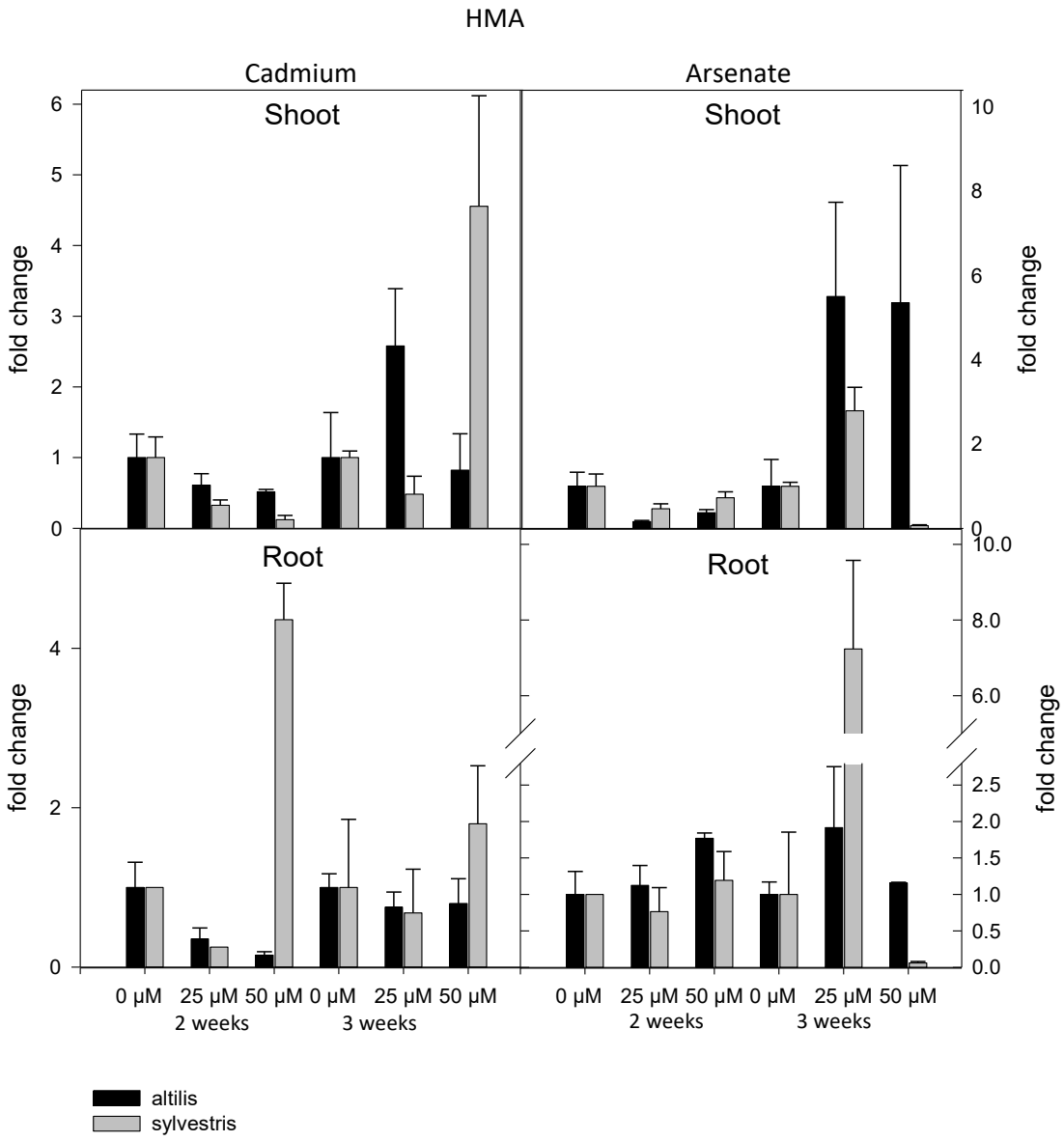


Figure 25. The level of gene expression of HMA in seedlings of *altilis* and *sylvestris* growth 2 and 3 weeks under Cd and As at different concentrations. The fold change is the Ct value respect to the control, that is considered 1. The error bars represents the error mean of three replicates.

In **ABCC1,** transcriptional levels resulted influenced by the treatments used (type of metal and its concentration). In particular, highly significant values, contributed by the 3

sources of variation (genotype, time and concentration of metal) were observed in root treated with As, as inferable by comparing the data reported in table 13 and 14.

On average, transcriptional levels, resulted not affected by the organs (shoots and roots), but influenced by the type of metal. In fact, with As, and not with Cd, the transcriptional levels increase 2 times in *sylvestris* compared to *altilis* (fig. 26). In particular, in wild cardoon, in shoots at 3 weeks, and in roots with As at 2 and 3 weeks, the ABCC1 mRNA is up-regulated, showing a clear engagement of this transcript in As response.

Table 13. Analysis of variance of fold change of ABC gene expression respect to the CTRL in shoot and root of cardoon treated with Cd and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		Percent of total	df	Absolute value		Percent of total
genotype (G)	1	0.10	ns	2.54	1	3.97	***	17.82
time (T)	1	1.36	*	36.22	1	3.42	ns	15.33
concentration (C)	2	0.03	ns	0.73	2	2.53	**	11.37
G * T	1	0.67	ns	17.82	1	2.23	**	10.02
G * C	2	0.49	ns	12.99	2	5.13	***	23.00
T * C	2	0.39	ns	10.48	2	2.44	ns	10.95
G * T * C	2	0.72	ns	19.22	2	2.57	**	11.51

Table 14. Analysis of variance of fold change of ABC expression gene respect to the CTRL in shoot and root of cardoon treated with As and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	3.88	**	15.13	1	9.79	***	37.33
time (T)	1	8.75	***	34.13	1	0.90	ns	3.44
concentration (C)	2	3.61	**	14.08	2	3.17	**	12.08
G * T	1	2.25	*	8.77	1	4.15	**	15.83
G * C	2	0.99	ns	3.87	2	4.50	***	17.16
T * C	2	5.36	***	20.93	2	1.16	ns	4.41
G * T * C	2	0.79	ns	3.09	2	2.56	**	9.75

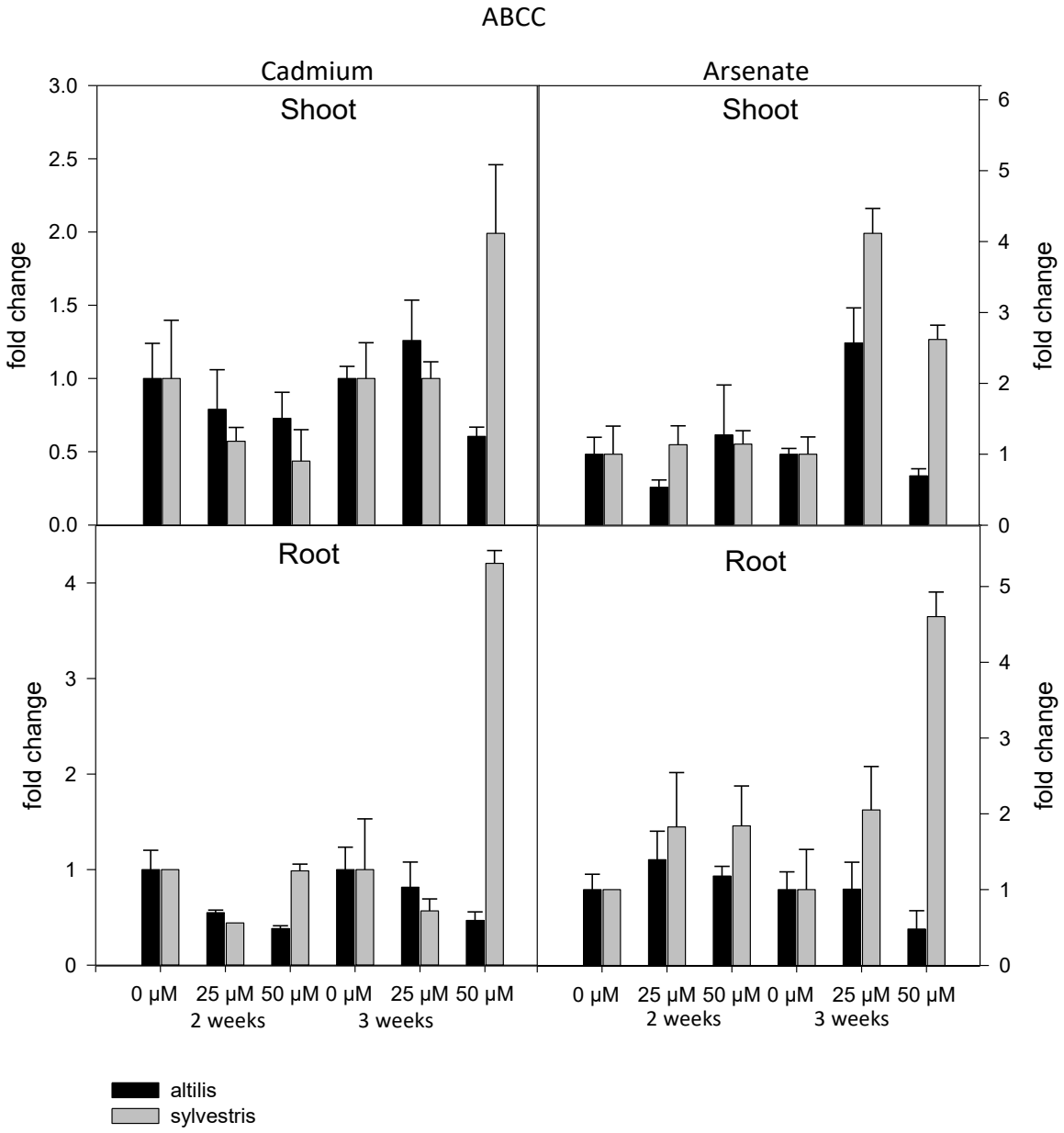


Figure 26. The level of gene expression of ABCC in seedlings of *altilis* and *sylvestris* growth 2 and 3 weeks under Cd and As at different concentrations. The fold change is the Ct value respect to the control, that is considered 1. The error bars represents the error mean of three replicates.

The transcriptional level of **PHT** was strongly influenced by the metal and genotype. In particular, highly significant values, contributed by the 3 sources of variation (genotype, time and concentration of metal) were observed in shoot and root treated with As, as inferable by comparing the data reported in table 15 and 16.

In roots, but not in shoots, the expression of the PHT transcript, averaging the contribution of all the three sources of variation, increases compared to control, and this is more evident in *sylvestris* than *altilis*. The type of metal had influenced the expression of PHT. In fact, with As, the transcriptional level resulted 2 times more than Cd. In particular, in the *sylvestris* roots, the expression increased linearly with the concentration of metal. After 2 weeks of treatment, the increase of transcriptional level was from 0 to 25 and from 25 µM to 50 µM 3 and 5 folds respectively (Fig. 27). Under As treatment the genotype, that is *sylvestris*, was the factor that more pronouncedly contributed to transcriptional increase in both roots and shoots after 3 weeks of treatment. (tab. 16).

Table 15. Analysis of variance of fold change of PHT gene expression respect to the CTRL in shoot and root of cardoon treated with Cd and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	0.04	ns	1.00	1	6.15	***	36.65
time (T)	1	1.31	*	37.16	1	0.37	ns	2.19
concentration (C)	2	0.68	ns	19.43	2	0.48	*	2.84
G * T	1	0.68	ns	19.37	1	1.52	**	9.03
G * C	2	0.07	ns	1.89	2	1.76	***	10.46
T * C	2	0.46	ns	13.08	2	2.46	***	14.62
G * T * C	2	0.28	ns	8.07	2	4.07	***	24.22

Table 16. Analysis of variance of fold change of PHT gene expression respect to the CTRL in shoot and root of cardoon treated with As and partition of the treatment sum of squares into main effect and interaction.

Source of Variation	shoot Mean squares				root Mean squares			
	df	Absolute value		% of total	df	Absolute value		% of total
genotype (G)	1	4.58	***	14.54	1	24.53	***	49.01
time (T)	1	9.85	***	31.25	1	6.11	*	12.20
concentration (C)	2	3.31	***	10.50	2	9.71	***	19.39
G * T	1	3.63	**	11.52	1	0.04	ns	0.08
G * C	2	1.79	**	5.69	2	6.72	**	13.42
T * C	2	7.13	***	22.62	2	2.24	ns	4.48
G * T * C	2	1.22	*	3.88	2	0.71	ns	1.41

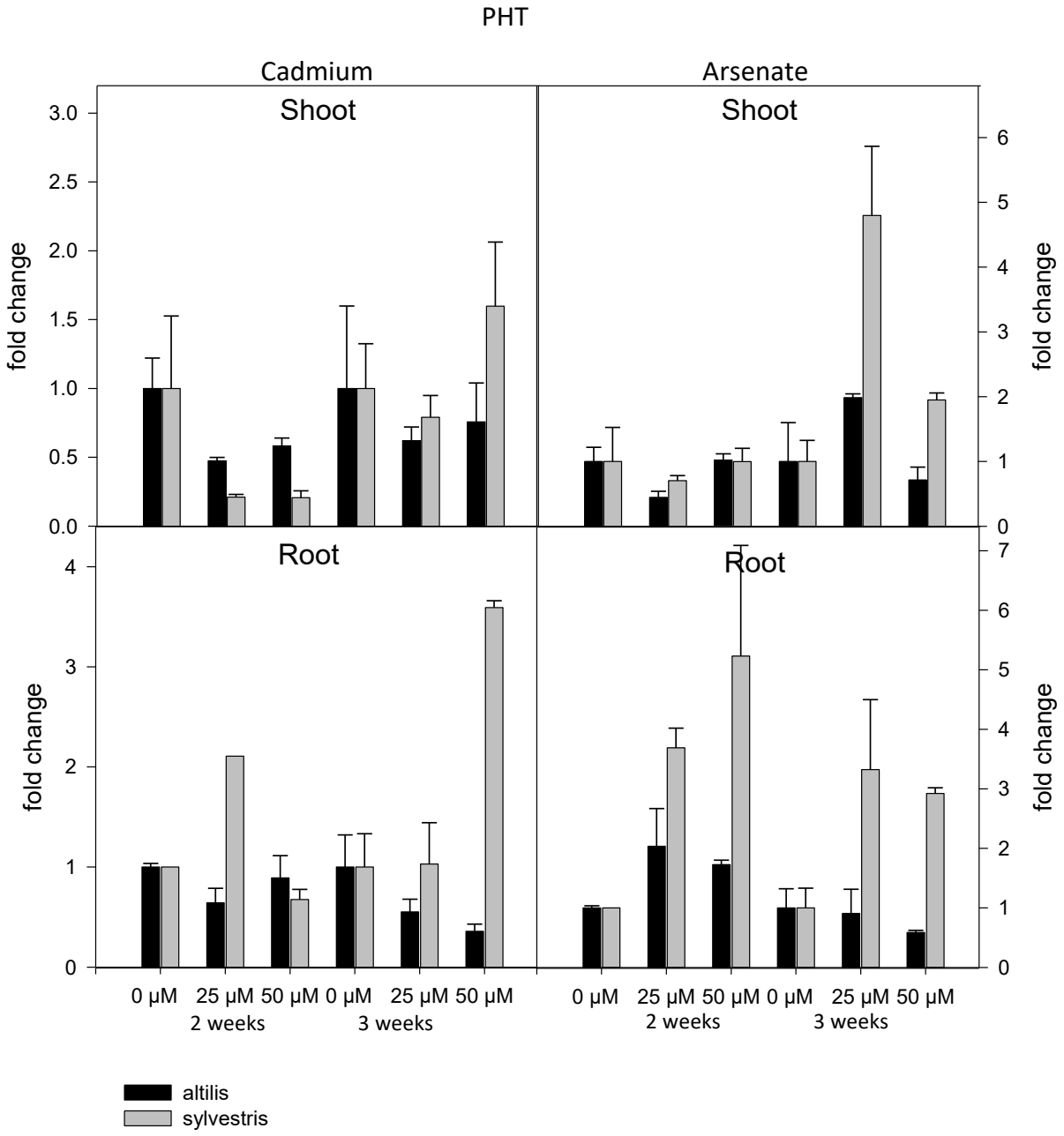


Figure 27. The level of gene expression of PHT in seedlings of *altilis* and *sylvestris* growth 2 and 3 weeks under Cd and As at different concentrations. The fold change is the Ct value respect to the control that is considered 1. The error bars represents the error mean of three replicates.

5 Discussion

In the present study, seed germination, seedlings growth and transcriptional levels of gene expression of six genes, identified in domestic and wild cardoon and putatively involved in phytoremediation, were investigated after treatments with heavy metals, Cadmium and Arsenic.

Concentrations of these metals in not contaminated soils are Cd < 1 mg Kg^{-1} and As < 5 mg Kg^{-1}. Concentration limited decreed by D.Lgs 152/2006 are for Cd 2 mg Kg^{-1} and for As 20 mg Kg^{-1}. In our studied we used concentrations of 2.81 mg Kg^{-1} and 5.6 mg Kg^{-1} for Cd, 1.87 mg Kg^{-1} and 3.7 mg Kg^{-1} for As (corresponding to 25 µM and 50 µM). These value are low if compared to the actual situation out in the field but, as already reported (Aarts et al., 2012; Llugany at al., 2014). it was of importance that in our studies the plants could survive at the treatments (Aarts et al., 2012; Llugany at al., 2014).

With regard to germination measurements, although highly variable depending on the different contribution of the three main factors considered in this study, genotype, heavy metal type and its concentration, it is reported that cardoon is tolerant to Cd and As. In particular, the varieties *altilis* and *sylvestris* (A14SR) show similar percentage of germination with Cd, while under As stress, wild cardoon (A14SR) shows the best tolerance.

In fact, under 50 µM Cd concentration, cardoon germination percentage, was similar to the untreated control in all genotype (> 95%). These data, are in in good association with transcriptional levels of the NRAMP3 and ZIP11 genes, which resulted up-regulated in *sylvestris* genotype, but in contrast to transcriptional levels on *altilis*. Furthermore, in response to Cd stress, these data are in contrast with the result obtained from Peralta and Li (2001) in *Medicago sativa* L. where at 5 ppm (44 µM) of Cd, seed germination was reduced of 50%. Probably cardoon, that is able to growth close to the sea, resulted more tolerant to abiotic stress, than this plant. The same authors also showed an inhibition of germination up to 50 % in *Arabidopsis* at 10 mM Cd, after 12 h treatment of the embryos (Li, et al., 2005).

Under As treatment, germination percentage of cardoon seeds was heavily dependent on the genotype. In fact, the *sylvestris* genotype A14SR showed a significantly higher

germination rate than that observed in the other genotypes (*altilis* and the *sylvestris* R14CT), and for this reason it was used as *sylvestris* variety in studies on transcriptional regulation. The experiments performed by Li et al., in 2007 showed that wheat (*Triticum aestivum* L.) seeds germination as well as root and shoot growth were stimulated at low concentrations of As (0–1 mg/kg). Such responses gradually decreased at higher As concentrations (5–20 mg/kg). In our results, at low As concentrations, the germination percentage of A14SR was the same as the untreated control, while at high concentrations, the decrease was inversely linear to increasing As amount. At 100 and 200 µM of HMs, although germinated, plants eventually died for the extensive necrosis of the tissues.

With regard to seedlings growth, the two genotypes show different response to metals. In *sylvestris* root length decreased of 64% at 25 µM Cd and 71% at 50 µM Cd compared to 0 µM. In *altilis* the increase of concentrations caused a reduction of seedling length of 59% and 70% with Cd at 25 and 50 µM, while with As the reduction was lower than 30% with highest concentration. In agreement to order considered by Li et al. in 2007 on the influence of HMs (root length > root mass > shoot length > total mass (root plus shoot) > shoot mass > germination), the roots length in *sylvestris,* resulted more affected than total seedling length.

Compared to control, the inversion of ratio shoot/roots is present in *sylvestris*, but not in *altilis*. In fact with both metals, the ratio decreases in *sylvestris*, in particular with As the incident of roots decreases from 80% (0 µM) to 35% (25 µM), while not changes in *altilis*, compared to control. This results could be explain by transcriptional levels of the genes studied. In *altilis* up- regulation of the genes under As treatment is not observed and the seedling plants could be not uptake metals, and for this reason named ‘resistant’ for As treatment.

In fact in the order above describe, shoot length and germination percentage are affected by heavy metals lower than root length. Compared to cardoon, roots length and shoots height were more inhibited by comparative lower levels of arsenic in wheat seedlings (Li et al., 2007).

Preliminary to the transcriptional studies performed on cardoon genes putatively involved in HM response, six genes of reference for gene expression studies, were tested and then used in cardoon with relation to different development stages, because normalization is a

key step to obtain reliable gene expression data by RT–qPCR. About these reference genes, we selected from literature, two 'classical' housekeeping genes such as ACTIN and 18S Ribosomal DNA genes, and other reference genes as GAPDH, WD40/TRASDUCIN, EF1 alpha and APC (anaphase promoting complex). The results showed that the 'classic' reference genes, ACTIN and 18s rDNA, resulted generally among the least stable genes in all developmental stages. However they can be used as reference genes, because they showed an acceptable value of "average expression stability". Our results are in accordance with Dekkers et al., 2012.

Seven genes, NRAMP1 and NRAMP3, ZIP11, HMA, ABCC1, PHT and PCS were identified in cardoon that could be possibly associated to heavy metals transport and accumulation response.

Transcriptional expression levels of six out of seven of the identified genes possibly involved in heavy metals stress response were investigated. PCS was also identified in cardoon, but it showed two peaks in the dissociation curve obtained, and was therefore discarded for qRT-PCR analysis. Ours results showed that in cardoon var. *sylvestris*, **NRAMP3** expression is up-regulated in roots by both As and Cd treatments. This result agrees with Fallen et al., (2005), where it was shown NRAMP3 and NRAMP4 are responsible for Cd^{2+} efflux from the vacuole. Their overexpression increases Cd sensitivity in *Arabidopsis* and they are responsible for the release of vacuolar Fe^{2+}. Instead, no data exists in literature on NRAMP3 expression with reference to As.

NRAMP1 in var. *altilis* resulted up-regulated in roots under As treatment. This is agreement to Tiwara et al., 2016, that showed in *Oryza sativa,* the induction of NRAMP1 after As(III) exposure. Earlier studies reported that expression of OsNRAMP1 was induced during As stress in rice in addition to defence and stress responsive genes, transporters, heat-shock proteins, metallothioneins, sulphate-metabolizing proteins (Norton et al., 2008). However the data obtained in our studies, show not existence of clear response to the stress in both varieties, and we can considered this gene not involved in response to heavy metals in cardoon plant. Considering the results obtained with NRAMP3 and arsenic, we can hypothesize that NRAMP isoform implicated in response to this metal in *sylvestris* is not 1, like in rice (Tiwara et al., 2016), but it is the isoform 3 as we found it.

ZIP proteins are generally responsible for the metal-ion homeostasis through the uptake of cations into the cytosol (Colangelo and Guerinot, 2006). Usually ZIP transporters are involved in the uptake and accumulation of Fe and Zn, but may also be responsible for Cd or other heavy metals transport (Guerinot, 2000). In *Solanum torvum* roots, *IRT2* and *ZIP11*, are associated to Zn transport (Xu et al., 2012). In our study, transcriptional expression of the ZIP11 transporter of wild cardoon, was increased in shoot and roots by Cd treatment. Similarly, ZIP11 mRNA was found to increase after 3 weeks of exposure of the seedlings to As. The result obtained under As, is not in agreement with the literature of ZIP genes.

In cardoon **ABCC1** transcriptional levels, measured under As treatment, in roots of var. *sylvestris*, resulted up-regulated compared to untreated control. The increase of the expression was influenced by the time of exposure, with the highest level at 50 μM after 3 weeks. A similar response was observed in shoots after 3 weeks of As treatment. In *altilis,* where seedling growth analysis showed low reduction length in shoot and root with As compared to untreated plants, significant differences was not observed in the transcriptional levels of the genes investigate compared to control with As treatment. May be the As is not uptake by *altilis*, such as not accumulator/resistant plants. In *sylvestris* seedlings, As treatment caused biomass reduction, because As uptake and storage at high toxic concentrations in the vacuole eventually lead to cell death. These results are in accordance to Song et al., (2010), who showed that *Arabidopsis* isoforms AtABCC1 and AtABCC2 mediate AsIII–PC complex transport to the vacuole, and overexpression of AtABCC1 increases As tolerance only when co-expressed with PCS. In rice, a similar ABC transporter, OsABCC1, is critical for the vacuolar AsIII–PC sequestration and As detoxification, thus reducing As accumulation in rice grains. For this reason, knockout of OsABCC1 leads to the increase of As sensitivity (Song et al., 2014).

The uptake of As(V) in plants occurs via inorganic phosphate (Pi) system, because Pi transporters cannot distinguish between the similar electrochemical profiles of Pi and AsV (Sanchez-Pardo, 2015). In our experiments, the **Phosphate transporter** resulted up-regulated in roots under As treatment, in *sylvestris* genotype, where the increase of expression level was strongly influenced by the concentrations of metal. In *altilis*, the expression levels of ABCC1 and PHT resulted elevated also in control, for this reason, the variability on the gene expression levels was not observed.

These results are in according to Di Tusa et al., (2016) that showed in *Pteris vittata,* an increase of As accumulation when the plans express PvPht1;3. In *Arabidopsis*, the expression pattern of PHT1;1 in the presence of As(V) decreased significantly as compared to limiting Pi condition in the natural variants, while the expression of PHT1;4 was higher in presence of AsV to limiting Pi condition (Shukla et al., 2015).

6 Conclusion

The response mechanisms of *Cynara cardunculus* L. at heavy metals stress were investigated. Two different varieties were used in this thesis: *C. cardunculus* L. var. *altilis* D.C. (domestic cardoon) vs *C. cardunculus* L. var. *sylvestris* Lam. (wild cardoon).

Seed germination resulted primarily influenced by the genotype, regardless metal type and concentration used. Wild cardoon A14SR resulted more able to germinate and grow in soil contaminated with Arsenate. In soil contaminated by Cadmium wild cardoon A14SR and domestic cardoon were more able to germinate and grow than var. *sylvestris* R14CT.

Seedlings length was influenced by the combination of genotype and heavy metals concentration. The two genotypes, *altilis* and *sylvestris*, responded differently to stress. In *altilis* the ratio shoot/roots remains constant compared to control, and the seedling length was more affected by Cd than As (the latter is probably not absorbed by the plants) while in *sylvestris*, the ratio changes compared to control, and the seedling length at 25 µM resulted affected less than 50 %, by both Cd and As as confirmed by the transcriptional analysis.

The *C. cardunculus* genes PCS, NRAMP1, NRAMP3, ZIP11, HMA3, ABCC1, and PHT, orthologous to genes shown to be involved in HM response in other plants, were identified and used as target sequences for transcriptional studies, with the exception of PCS.

Cardoon plants showed differential transcript levels that could be influenced by the genotype, metal used, concentration, and length of treatment/exposure. Compared to domestic cardoon, wild cardoon significantly increases the expression of genes involved in the Cadmium and Arsenate uptake (NRAMP3, ZIP11, ABCC and PHT) after 3 weeks of exposure. In leafy cardoon, expression patterns are less influenced by the concentration of the metal, and more influenced by the organ considered.

The gene expression levels of NRAMP3, ZIP11, ABCC and PHT that usually are activated in accumulator model plants under Cd or As stress, were activated also in wild cardoon: NRAMP3 and ZIP11 by both stresses, and ABCC1 and PHT just by As.

From this preliminary study we can conclude that *sylvestris* A14SR variety could be used for detoxification of soils polluted with heavy metals, especially if As is present. They should be considered good candidates for phytoremediation.

Until now, no information is available in literature, on the mechanisms that in *Cynara cardunculus* L. are involved in the uptake and accumulation of heavy metals.

For this reason further experiments will have to be performed to sort out which genes, including those of this study, are actually associated to the mechanisms activated by the plant during phytoremediation. RNAseq technique may be potentially useful to understand what are the genes associated with heavy metals accumulation, tolerance and transport in plants grown in contaminated soil.

A better knowledge of the molecular aspects of this process in *Cynara cardunculus* will help in the breeding and selection of new cardoon lines with improved features specifically suitable for phytoremediation purposes.

7 Supplementary Tables

Supplementary Table 1. PCR primers designed with primer3 website. Two pairs of primers were designed for each gene.

GENE	CLONING PRIMER CODE	FORWARD PRIMER SEQUENCE	REVERSE PRIMER SEQUENCE	Ta
Natural Resistence of Macrophage 3	NR3_05	GATTACGCCAAGCTTTGGGCGGGGAAGTTGTTGTGGATCA	GATTACGCCAAGCTTTGGAACCACGAGACCAACAAGAAGCTCT	60 °C
	NR3_06	TTGATGCTACAATCAACCGA	GGGCAGAGTAGTACCATCAC	57 °C
	NR3_07	ACTTCTCGTAATCAAGGCCC	TTCCTCTTTCACTCACTCGG	<50 °C
Zinc Iron Protein 1	ZP1_06	TCAAAGGTGCATCTATTAACACA	AAGATGCATCCTCCAAGACC	50 °C
	ZP1_07	TGTCTGTTGGTGCTTCTGAA	GCAAGAAGCGACATACAACCA	54 °C
Zinc Iron Protein 2	ZP2_06	CCCAAATCATGACAACTGCGA	CGGAAAAGGAGGTCATCATGG	55 °C
	ZP2_07	ACCAACAGCAATACCCTCGA	ACTCAGCTCCCATGGCTATC	<50 °C
Zinc Iron Protein 6	ZP6_06	ACTTTGTGTGCCGCAGA	GGCCCATCCCTTCGAAG	<48 °C
	ZP6_07	AACGATGGGGATGTCACAG	ACTTCAAGCCCAAAGAGCA	52 °C
Zinc Iron Protein 9	ZP9_06	GACACCACTTGAAGCCTAACA	CCGGAAAAGAGAGCAACGTT	63 °C
	ZP9_07	CCGTGGGAGTGAGAATGCAT	TCTGAATCCATGTCGACGGT	53 °C
Zinc Iron Protein 11	ZP11_06	ATCCCAGATCATAACAACAGCA	GATTGGAATCGCGGACACC	54 °C
	ZP11_07	GAGATTGTCCATAGAGCTTTCCA	CCATGCCTCGTTTCCTTTTCT	48 °C
Heavy Metal ATPase 4	HM4_06	CATTGATGAACATAGCTCCTCAG	TGGAACTGATAACACCCTGCT	54 °C
	HM4_07	CGCACAGTCTCGTTCAGTTG	GGTTCCCACGTCCGCAAG	50 °C
Heat Shock Protein 23	HSP_06	CTCTACTTGAATCTGCCTCACT	TCATGGGACGCAAATGAAAAC	48 °C
	HSP_07	GCTTGTATACATCCATCGGC	CGATAGTCATCTACCACCGG	51°C
Phytochelatin syntase 2	PS2_06	ACGGGATAGATAATTGACGGAAA	CTAACGGGTTTTGCTGTGGG	63 °C
	PS2_07	TCACCAATGCCATTCGTCAC	GTTCTTCCATCTCCTCCTGCT	48 °C
Phosphate Transporter	PHT_06	GCATCTTCCTCATTCTCGCC	GTCATGGCCACCCTTTGTTT	48 °C
	PHT_07	GGTTTCCGGCATCTTCATCC	GCAACTCCAAGTGCTTAACG	48 °C

Supplementary Table 2. BLASTn of the contigs obtained from the sequencing against all database. .

ESM_4. The results of sequence alignment of sequenced clones and selected squences in NCBI					
Gene	**Accession num ber**	**Results of sequence alignment (blastn)**			
		Query cover	**Expect**	**Identities**	**Gaps**
NRAMP1	XM_010260353.2	74%	0.0	79%	1%
NRAMP3/4	XM_013744626.1	99%	2.00E-81	74%	1%
ZIP11	XM_004291513.2	99%	2.00E-52	78%	0%
HMA	XM_010550291.1	87%	3.00E-75	72%	2%
ABCC1	XM_017379379.1	44%	0.0	81%	1%
PHT	KC812501.1	99%	0.0	82%	0%
PCS	GQ372840.1	100%	0.0	93%	0%

Supplementary Table 3. BLASTp of the contigs obtained from the sequencing against all database and against *cynara scolymus*.

ESM_1. Comparison of product of candidate reference genes in *Cynara cardunculus* with orthologs sequences												
Gene	***sequence name***	***ortholog sequence***	***ortholog species name***	**The results of BLASTP**				***cynara* accession number**	**The results of BLASTP**			
				Total Score	**Expect**	**Identities**	**Positives**		**Total Score**	**Expect**	**Identities**	**Positives**
NRAMP1	cyn00008	OTG21116.1	*Helianthus annuus*	2044	0.0	82%	85%	KVI06072.1	2414	0.0	94%	94%
NRAMP3/4	cyn00009	OTG06734.1	*Helianthus annuus*	1046	7.00E-139	91%	95%	KVH92457.1	1088	3.00E-148	96%	96%
ZIP11	cyn00010	OTG07698.1	*Helianthus annuus*	931	2.00E-121	77%	83%	KVI10407.1	1110	4.00E-152	91%	91%
HMA	cyn00011	OTG15082.1	*Helianthus annuus*	1781	0.0	85%	91%	KVI01438.1	1863	0.0	92%	91%
ABCC1	cyn00012	OTG28642.1	*Helianthus annuus*	6476	0.0	76%	81%	KVH87904.1	8321	0.0	95%	95%
PHT	cyn00013	AGK29560.1	*Chrysanthemum x morifolium*	1900	0.0	87%	90%	KVH91481.1	2024	0.0	93%	93%
PCS	cyn00014	ACU44656.1	*Sonchus arvensis*	928	1.00E-121	91%	93%	KVI12347.1	661	8.00E-85	96%	95%

Supplementary Table 4. Primer sequences designed with primer3 website for the reference genes. Two couples of primers are created for the cloning, and a couple for the qPCR

GENE	CLONING PRIMER CODE	FORWARD PRIMER SEQUENCE	REVERSE PRIMER SEQUENCE	Ta	REAL TIME FORWARD PRIMER SEQUENCE	REAL TIME REVERSE PRIMER SEQUENCE	Tm	HOMOLOGOUS TO ARABIDOPSIS GENE
GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE	GAP_01	TGARTCHACYGGTGTCTTCA	TCRAYVACACGRGARCTGTA	53 °C	AGTACGACAGTGTTCATGGCC	CTGAAGCCGAAAACAGCGAC	63 °C	AT1G13440
	GAP_02	TGARTCHACYGGTGTCTTCA	TCRAYVACACGRGARCTGTA	50° C			63 °C	
β-TUBULIN	TUB_01	ATCCTGACCTTCTTCTTCCTCT	TCTTAACCACTTGATTTCCGCC	53 °C	TATTACAACGAGGCCAGCGG	CAGGCCTGAAGATCTGTCCG	63 °C	AT5G44340
	TUB_02	GAGCAAACCCCACCATGAAG	CAGATCGGTGCCAAGTTCTG	53 °C			63 °C	
ACTIN	ATA_01	ACCGAAGATATTCAGCCCCT	CTTAGGATTCAAAGGTGCCTCC	48 °C	ACATGTTCACCACCACTGCC	GCTACTCTTTGCGGTTTCAAGC	63 °C	AT3G18780
	ATA_02	CTTCGTGTTGCTCCTGAGGA	TGTTGGAAGGTGCTGAGTGA	48 °C			63 °C	
ELONGATION FACTOR	EFA_01	TCCTTCTTGTCCACGCTCTT	AGTTGGCCGTGTTGAAACTG	53 °C	TGACCCCAGTTTCAACACGG	AAGAGGCCATCAGACAAGCC	63 °C	AT5G63390
	EFA_02	CAACATTGTCACCCGGCAA	GACTACTACCGGGCACTTGA	48 °C			63 °C	
18S	18S_06	GGTTGATCCTGCCAGTAGTC	GCGGAGTCCTAWAAGCAACA	65 °C	TGCGGCCCAGAACATCTAAG	CGAGACCTCAGCCTGCTAAC	63 °C	AT2G01010.1
	18S_07	AAAAGCTCGTAGTTGRACYTTGG	GTTCACCTACGGAAACCTTGT	48 °C			63 °C	
ANAPHASE PROMOTING COMPLEX	APC_01	TGAGCTAATTGATTTATGGTGGC	TGACAAGCAAGCATGGATTGA	48 °C	GCTCCAATGTGCGTATTTCAGT	TGAATATCGTGTTATGCTGGCTG	63 °C	AT2G04663
	APC_02	GATACCGGCTGCCCTTCA	GCTCCTGCCATTGAAGACTT	53 °C			63 °C	
WD40	WD40_01	GGTTGAGAGGGATGGAAAACA	GTGGGATATGCGTCAAAGGG	48 °C	CATGGTTCTGAAAGGGCACAAG	CATCCCATGCCCTCAGTGTC	63 °C	AT2G43770
	WD4_02	TCCTAAGATCCCACACTTTGACA	TGCAAGCAGGTGAAGGTAC	48 °C			63 °C	

Supplementary Table 5. BLASTn of the sequence against all database

ESM_4. The results of sequence alignment of sequenced clones and selected squences in NCBI					
Gene	**Accession number**	**Results of sequence alignment (blastn)**			
		Query cover	**Expect**	**Identities**	**Gaps**
EF1-α	NM_001247106.2	99%	0.0	87%	0%
ACTIN	KJ634809.1	100%	0.0	96%	0%
GAPDH	KF563904.1	99%	0.0	95%	0%
WD40	XM_002277595.4	97%	5.00E-150	84%	0%
APC	XM_016039563.1	95%	9.00E-96	86%	0%
TUB	KP752084.1	85%	0.0	88%	0%
18S	KT179688.1	100%	3.00E-170	100%	0%

Supplementary Table 6. BLASTp of the contigs obtained from the sequencing against all database and against *cynara scolymu*

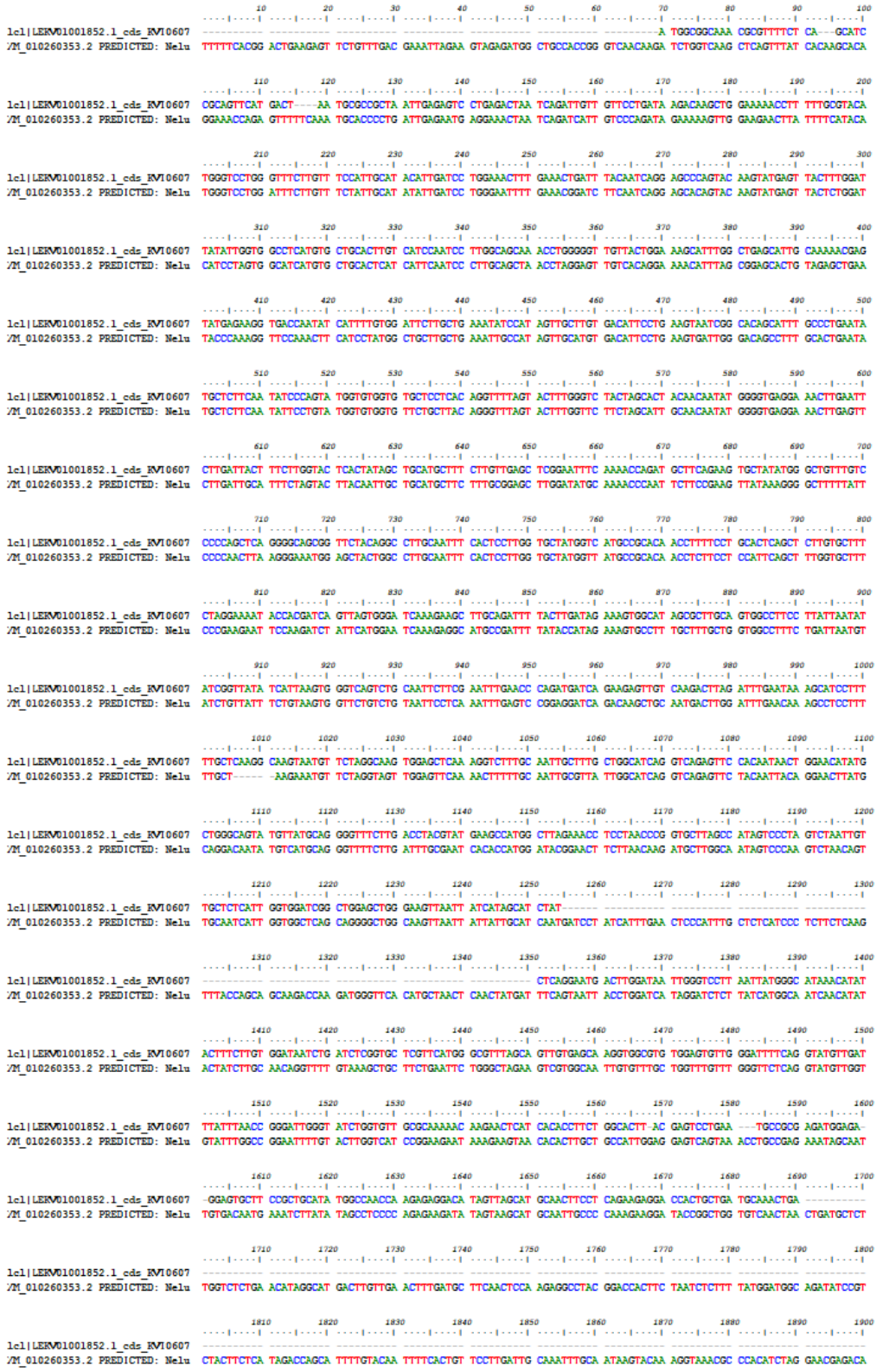
ESM_1. Comparison of product of candidate reference genes in *Cynara cardunculus* with orthologs sequences												
Gene	***sequence name***	***ortholog sequence***	***ortholog species name***	**The results of BLASTP**				***cynara* accession number**	**The results of BLASTP**			
				Total Score	**Expect**	**Identities**	**Positives**		**Total Score**	**Expect**	**Identities**	**Positives**
EF1-α	cyn00001	XP_015058086.1	*Solanum pennellii*	1862	0.0	92%	93%	KVI09543.1	1889	0.0	94%	94%
ACTIN	cyn00002	OTF93595.1	*Helianthus annuus*	1629	0.0	92%	92%	KVI08516.1	1630	0.0	92%	92%
GAPDH	cyn00003	AGX26868.1	*Saussurea involucrata*	617	2.00E-78	83%	86%	KVI10262.1	391	5.00E-45	61%	72%
WD40	cyn00004	OTG35852.1	*Helianthus annuus*	873	2.00E-115	95%	96%	KVH91003.1	905	1.00E-123	91%	90%
APC	cyn00005	OTG04417.1	*Helianthus annuus*	362	5.00E-37	87%	90%	KVH95643.1	375	2.00E-43	92%	93%
TUB	cyn00006	OTG08113.1	*Helianthus annuus*	1285	6.00E-175	92%	92%	KVH94866.1	1176	2.00E-161	88%	89%
18S(*)	cyn00007	KT179661.1	*Cirsium undulatum*	1164	0.0	100%		no available				

*Blastn was applaied

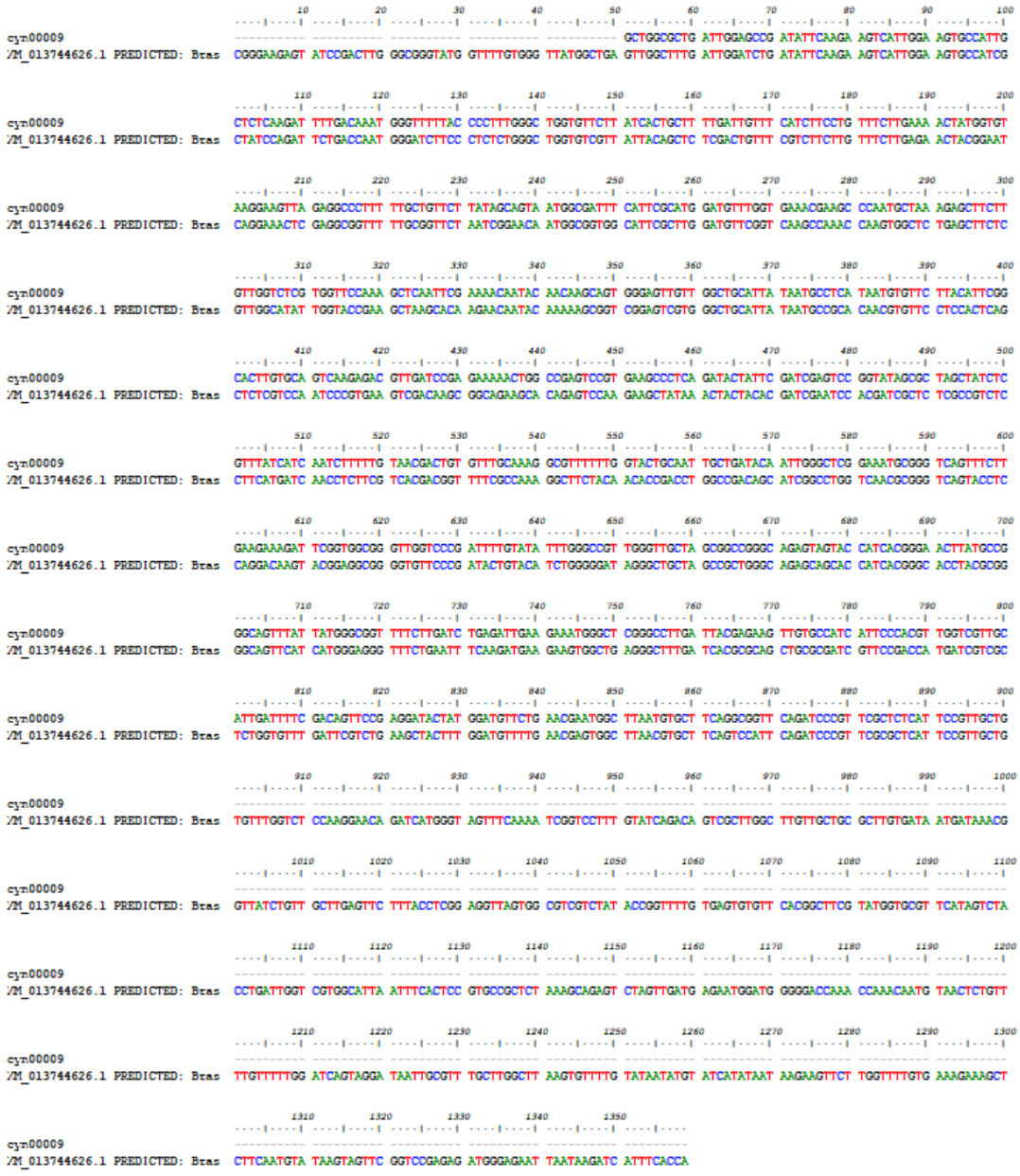
Supplementary Table 7. qPCR primers designed with primer3 website.

GENE	PRIMER CODE	REAL TIME FORWARD PRIMER SEQUENCE	REAL TIME REVERSE PRIMER SEQUENCE	Tm
NRAMP1	NR1_01	GCAAGTGGAGCTCAAAGGTC	GGTCAAGAAACCCCTGCATA	60 °C
NRAMP3/4	NR3_02	GGTGTAAGGAAGTTAGAGGCCC	AGCTTTGGAACCACGAGACC	60 °C
ZIP11	ZIP11_06	TGCCTCGTTTCCTTTTCTTC	CTCGGGTGCTTCGTCGT	60 °C
HMA	HMA_07	CGGGCACGATTACTAGAGGA	CTAGCCTTGCTCTCGATGCT	60 °C
ABCC1	ABC_01	TAAGTCTTTCGCGTGCATTG	TTCTTCGCGAATGGTCTTTT	60 °C
PHT	PHT_02	AAGATTTCAGCAGGGACGAC	ACGACAACCGTCTTGGATTC	60 °C
PCS	PCS_02	AGCTTCAACCTTTGCTCCAG	CCAATCCACAATAGGCAGGT	60 °C

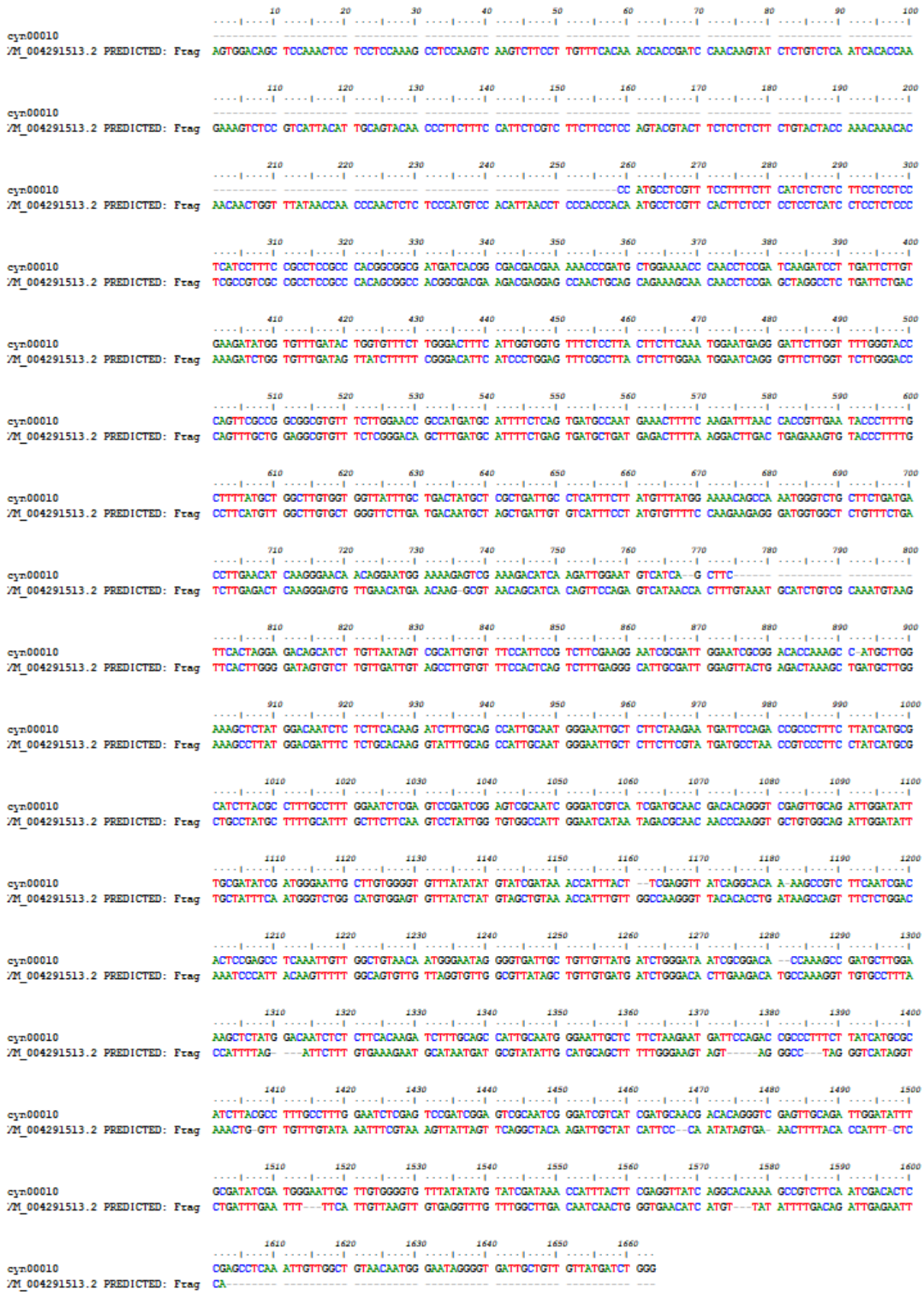
8 Appendix



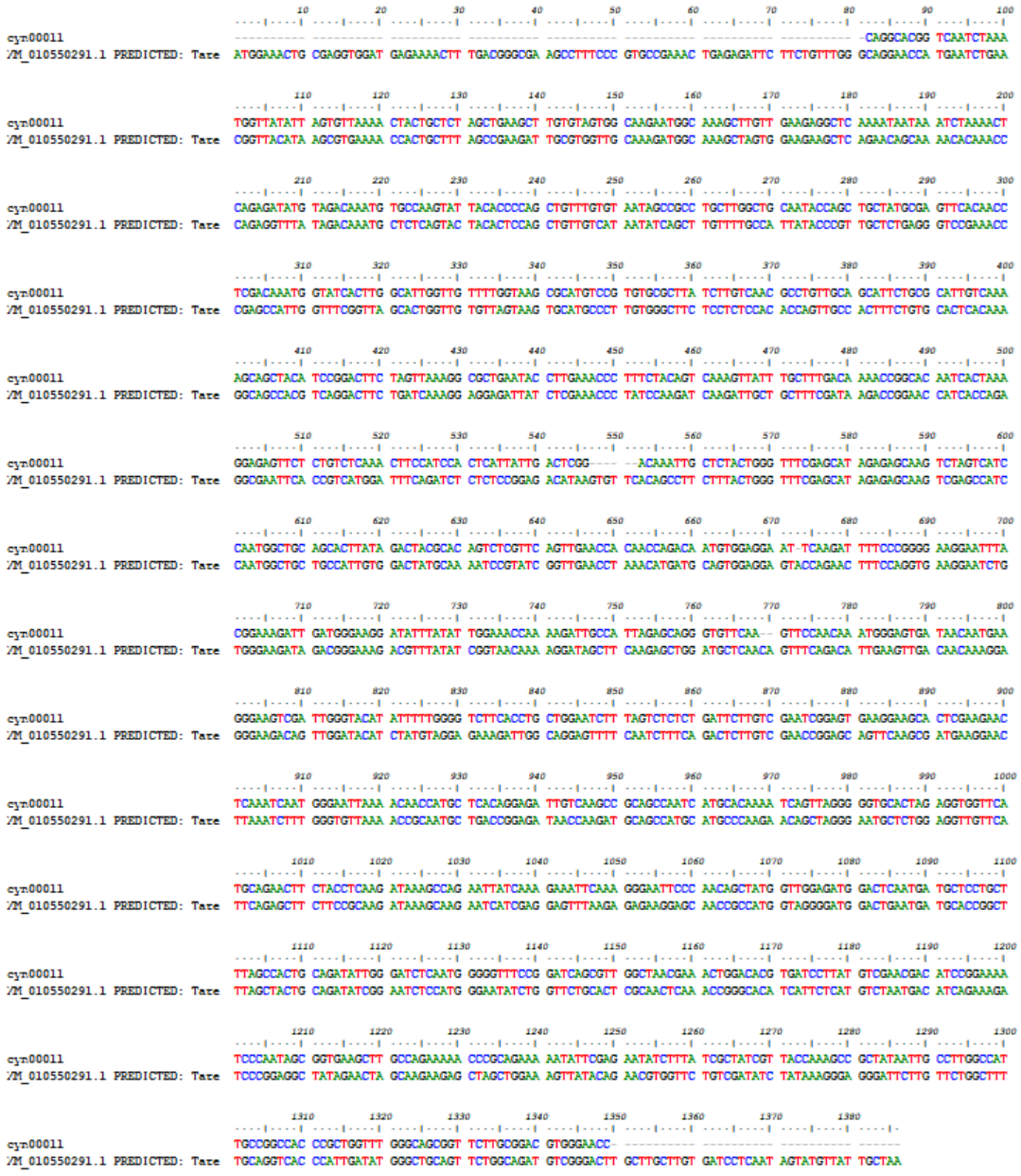
Alignment of *Cynara scolymus* sequences NRAMP1 against *Nelumbo nucifera* metal transporter with BioEdit program. Different colours represent the different nucleotides.



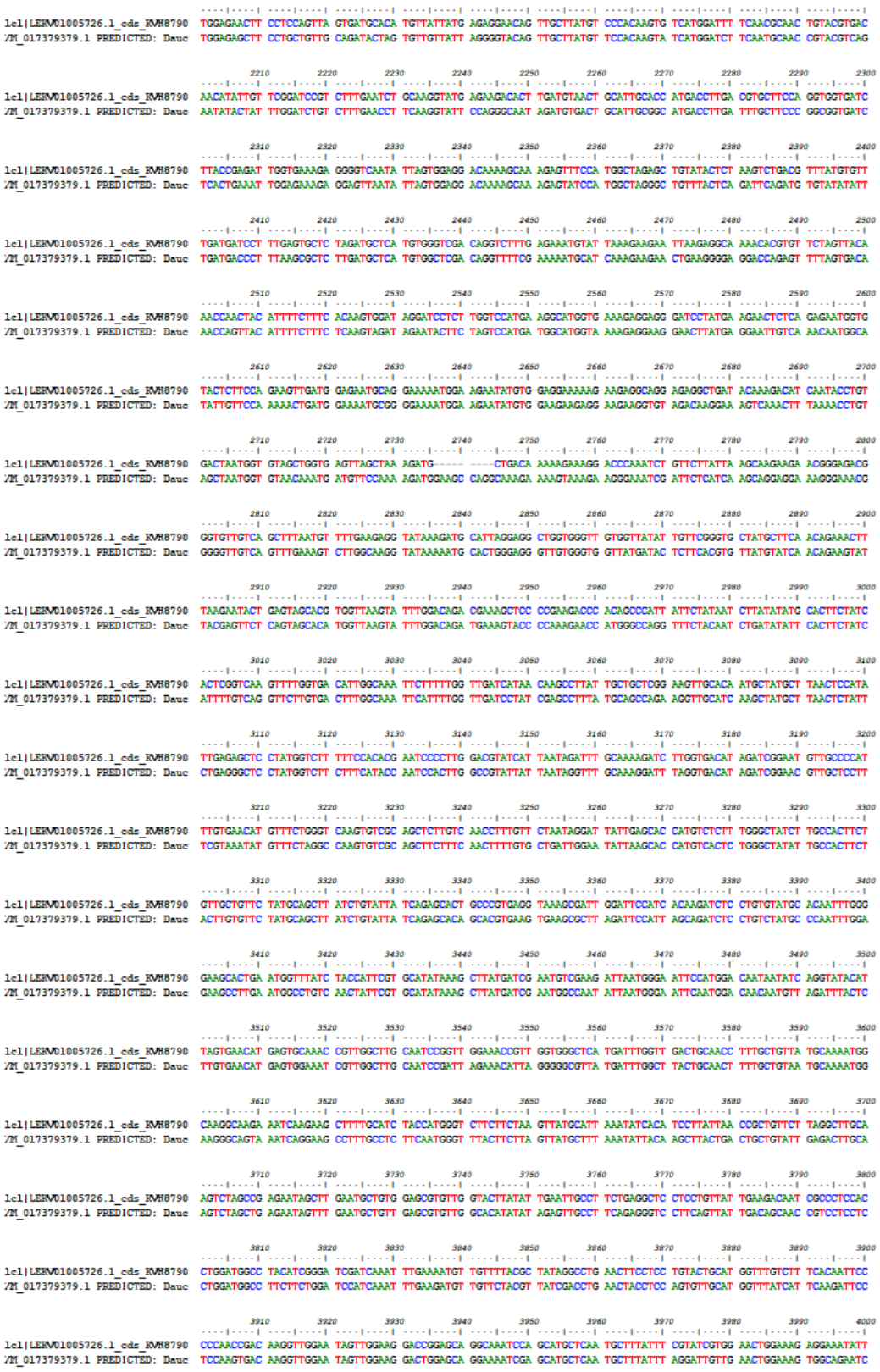
Alignment of NRAMP3 contigs against *Brassica oleracea* metal transporter Nramp3-like with BioEdit program. Different colours represent the different nucleotides.



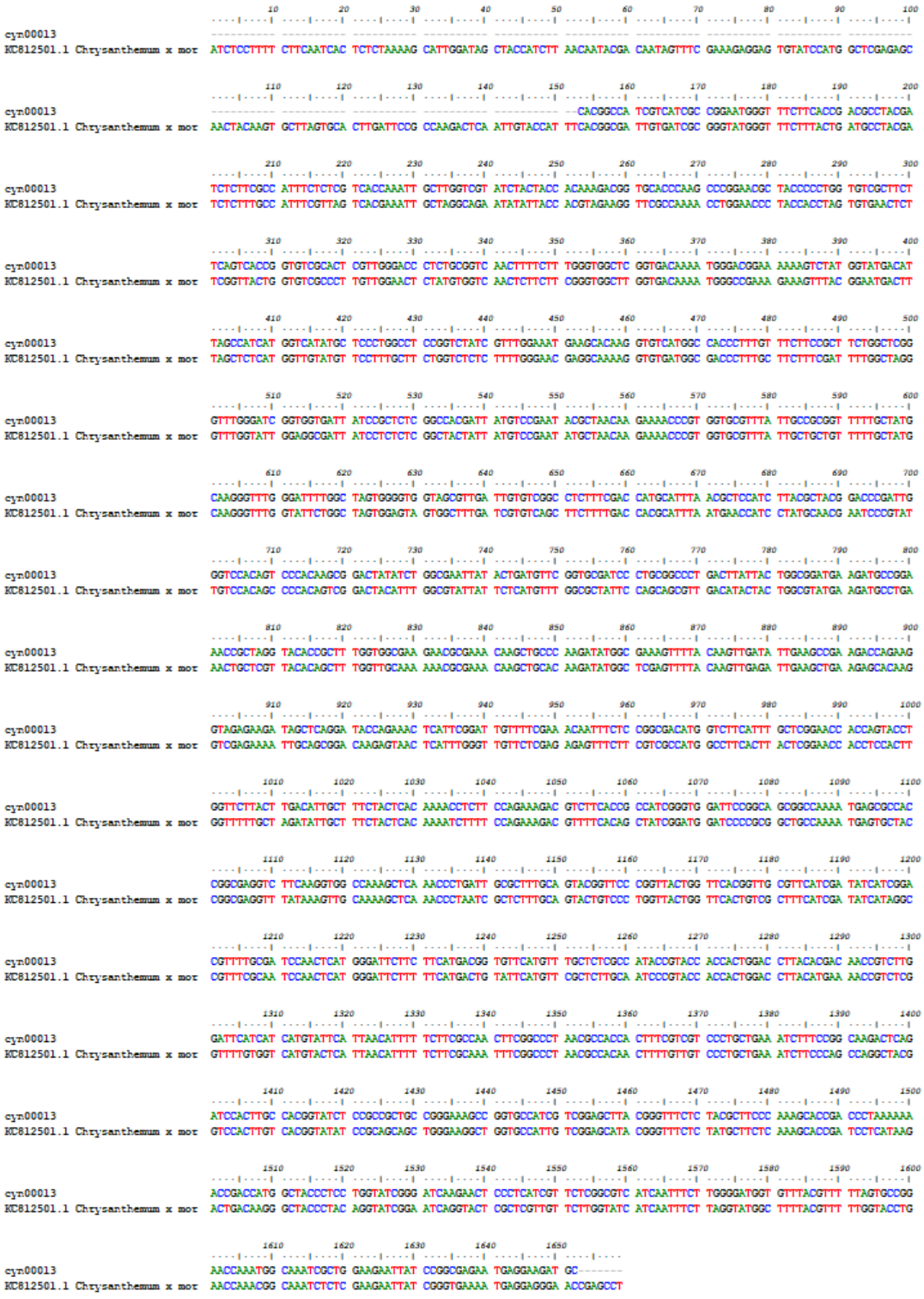
Alignment of ZIP11 contigs against *Fragaria vesca* zinc transporter 11-like with BioEdit program. Different colours represent the different nucleotides.



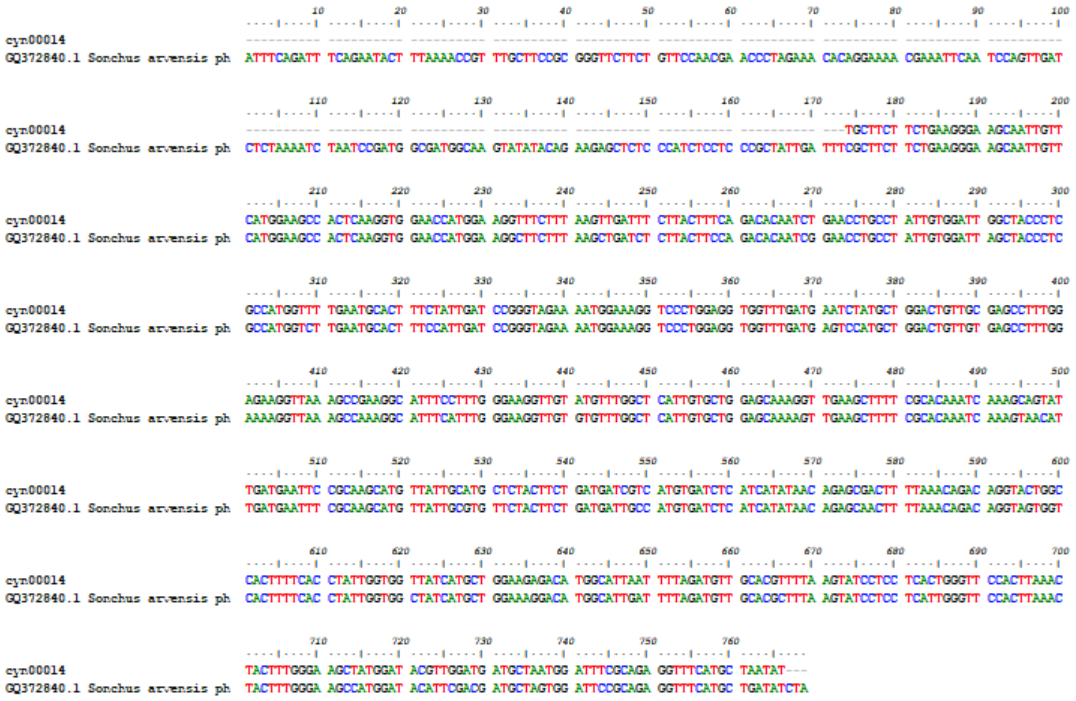
Alignment of HMA contigs against *Tarenaya hassleriana* cadmium/zinc-transporting ATPase HMA3 with BioEdit program. Different colours represent the different nucleotides.



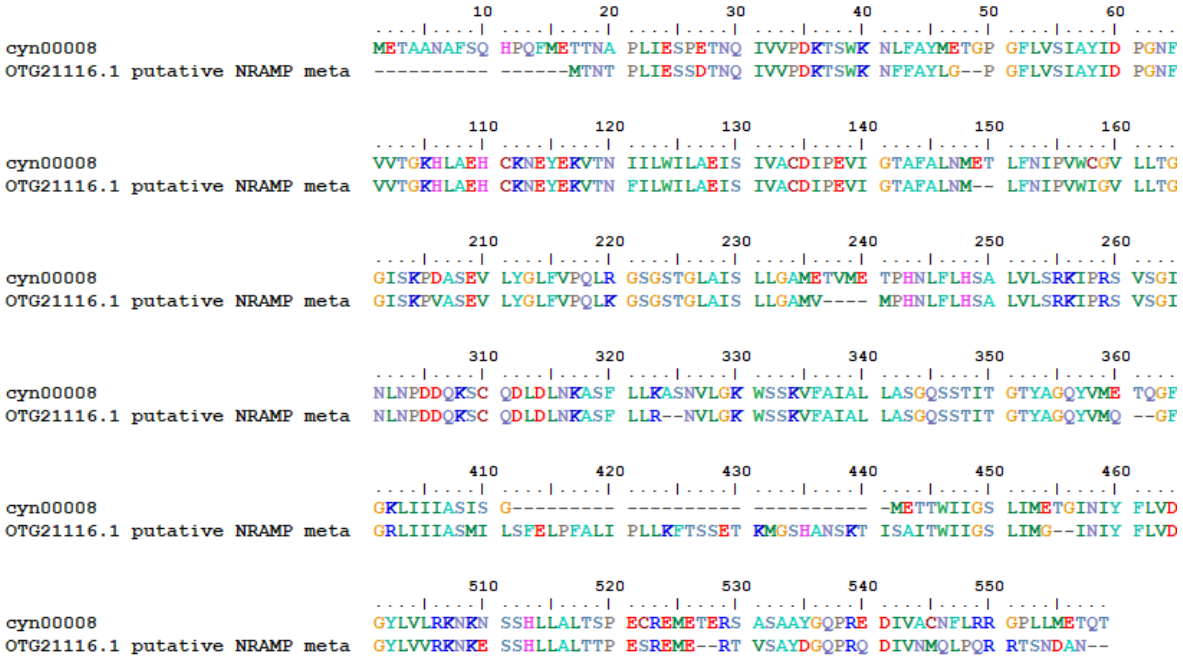
Alignment of ABCC1 contigs against *Daucus carota subsp. sativus* ABC transporter C family member 2-like with BioEdit program. Different colours represent the different nucleotides.



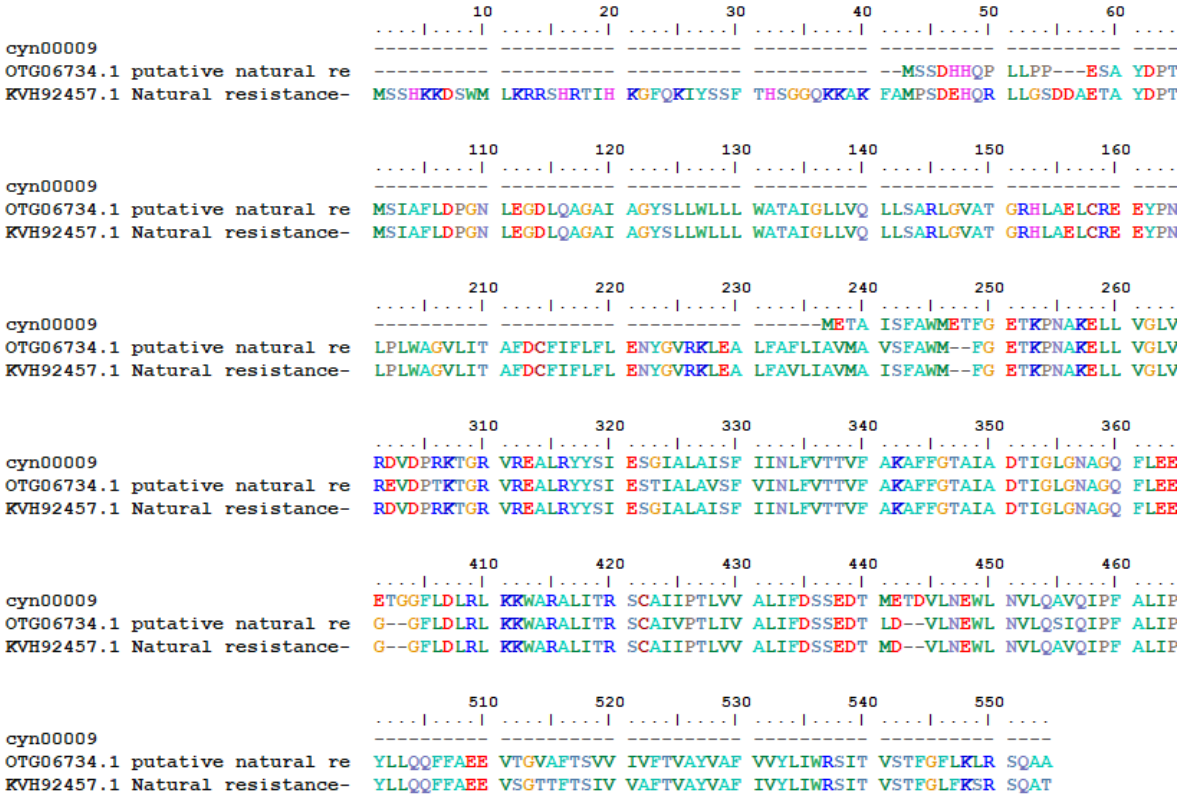
Alignment of PHT contigs against *Chrysanthemum x morifolium* phosphate transporter 1 with BioEdit program. Different colours represent the different nucleotides.



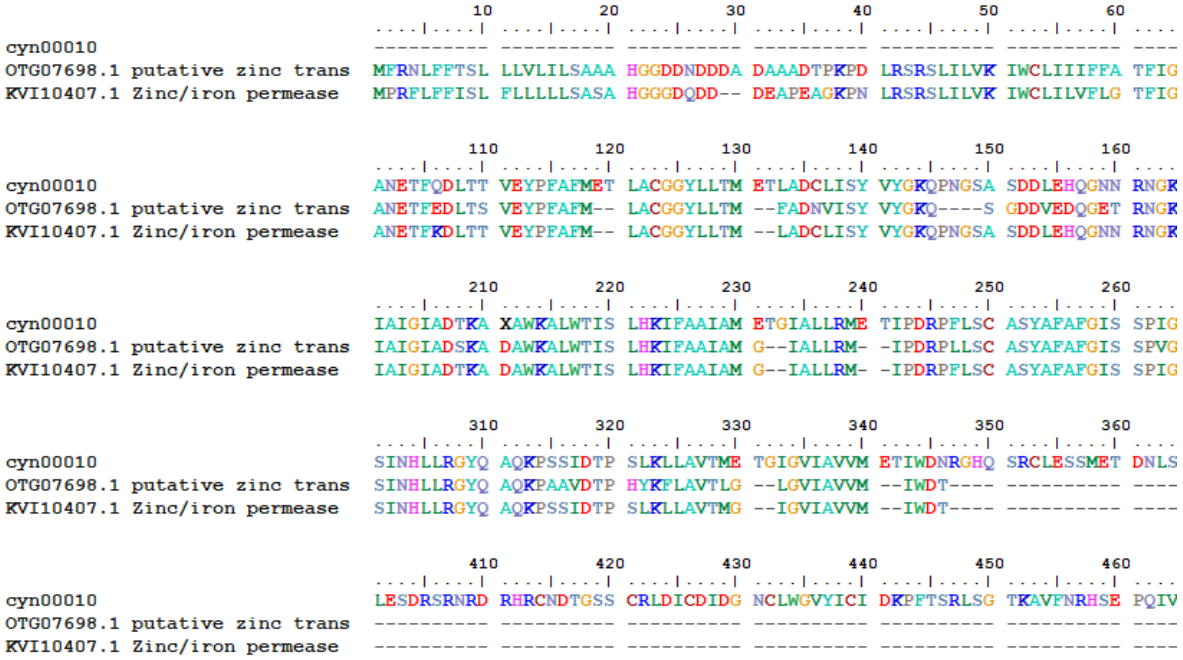
Alignment of PCS contigs against *Sonchus arvensis* phytochelatin synthase (PCS1) with BioEdit program. Different colours represent the different nucleotides.



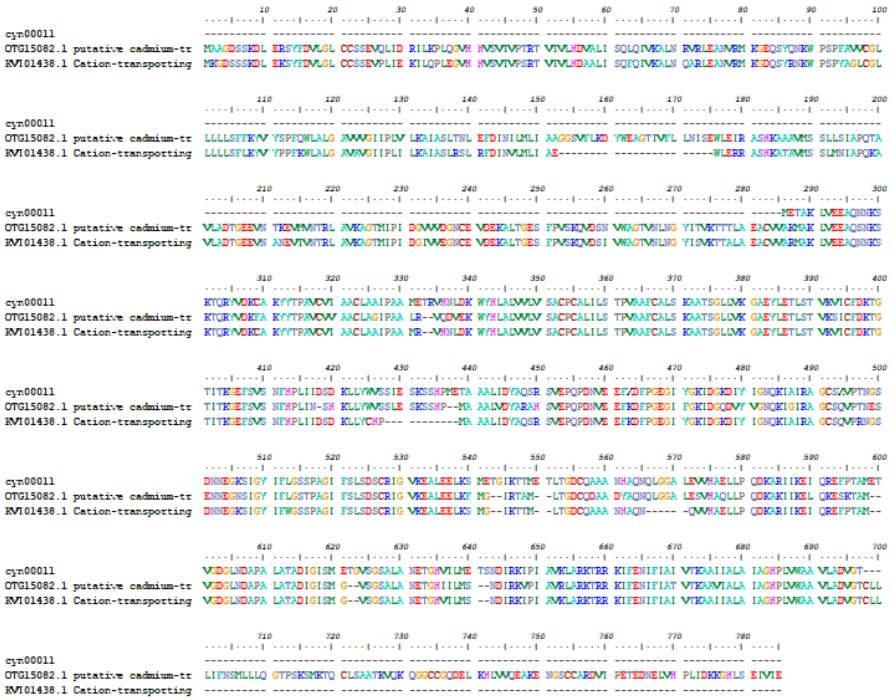
Alignment of NRAMP1 translate sequences against *Helianthus annuus* NRAMP metal ion transporter 6. Different colours represent the different nucleotides.



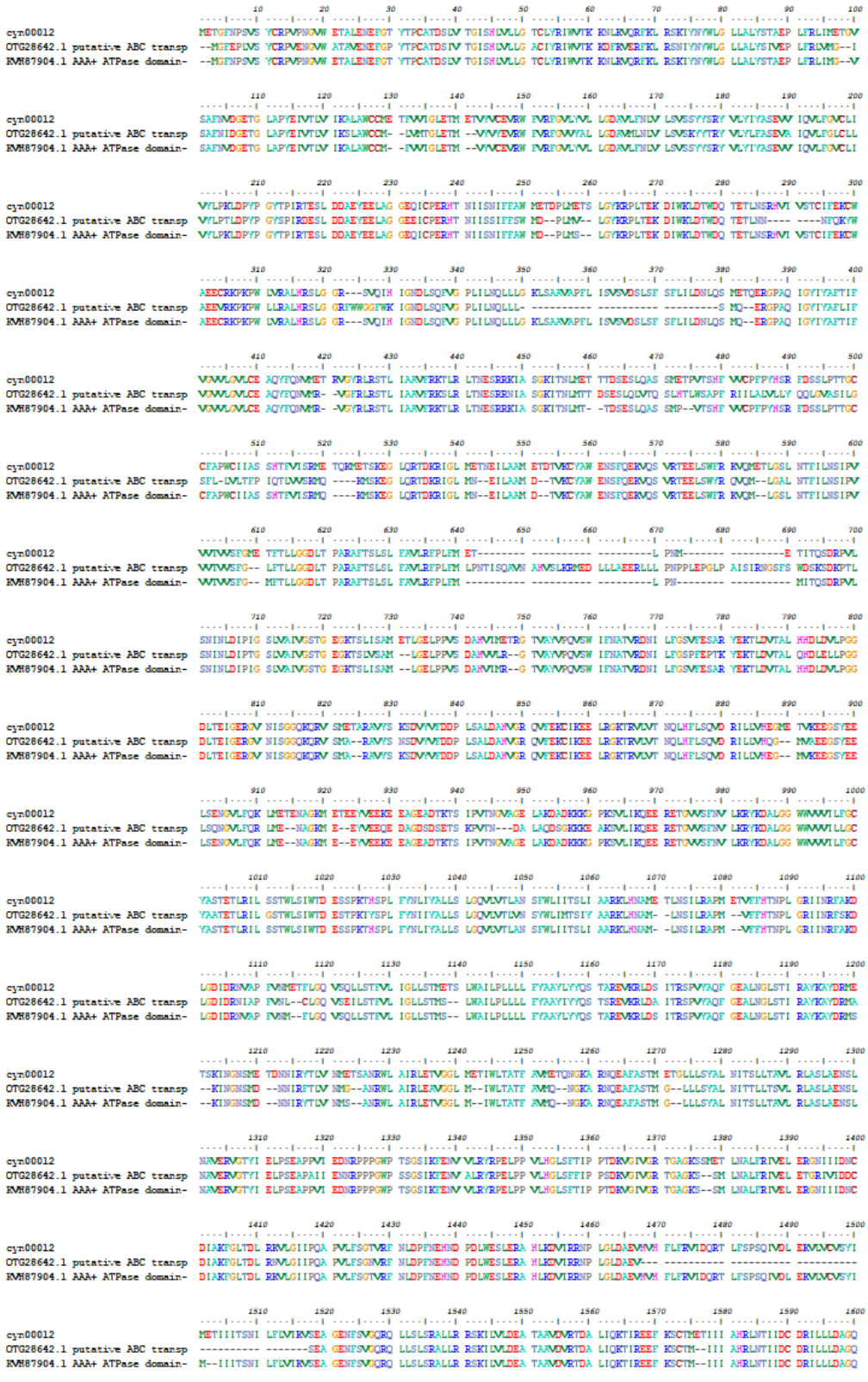
Alignment of NRAMP3 translate sequences against *Helianthus annuus* putative natural resistance associated macrophage protein 4. Different colours represent the different nucleotides.



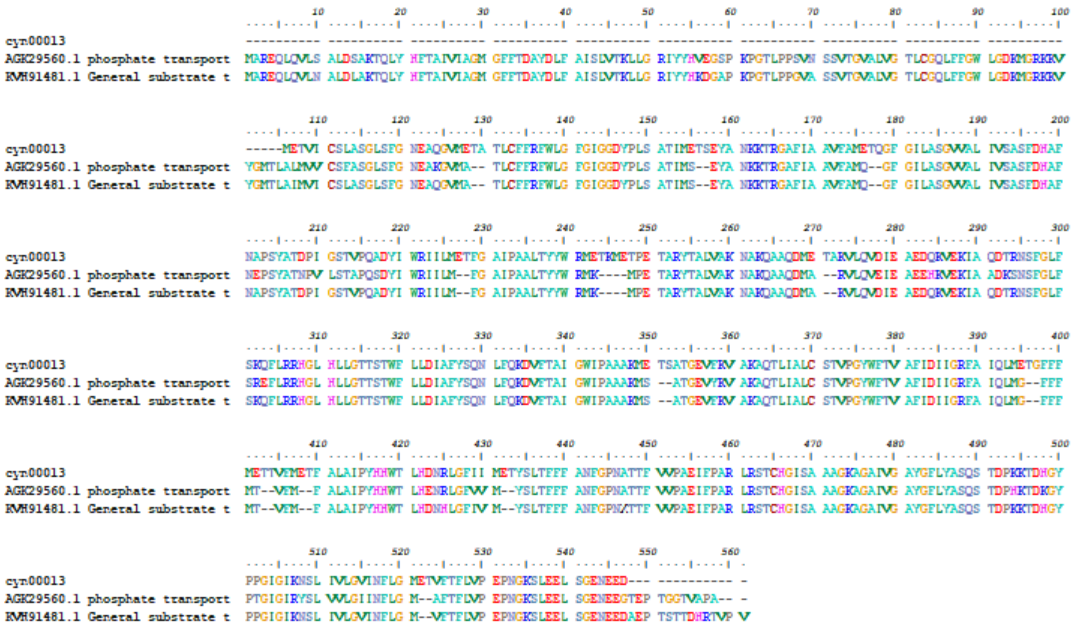
Alignment of ZIP11 translate sequences against *Helianthus annuus* putative zinc transporter 11 precursor. Different colours represent the different nucleotides.



Alignment of HMA translate sequences against *Helianthus annuus* putative cadmium-transporting ATPase. Different colours represent the different nucleotides.



Alignment of ABCC1 translate sequences against *Helianthus annuus* putative ABC transporter. Different colours represent the different nucleotides.



Alignment of PHT translate sequences against *Chrysanthemum x morifolium* phosphate transporter 1.

9 Reference

Abedin, M. J., Cotter-Howells, J., & Meharg, A. A. (2002). Arsenic uptake and accumulation in rice (*Oryza sativa* L.) irrigated with contaminated water. *Plant and Soil*, *240*(2), 311-319.

Agarwal, S. K. (2009). Heavy Metal Pollution. A P H publishing corporation

Aldrich, M. V., Gardea-Torresdey, J. L., Peralta-Videa, J. R., & Parsons, J. G. (2003). Uptake and Reduction of Cr(VI) to Cr(III) by Mesquite (Prosopisspp.): Chromate−Plant Interaction in Hydroponics and Solid Media Studied Using XAS. Environmental Science & Technology, 37(9), 1859–1864. doi:10.1021/es0208916

Alkorta, I., Hernández-Allica, J., Becerril, J. M., Amezaga, I., Albizu, I., & Garbisu, C. (2004). Recent findings on the phytoremediation of soils contaminated with environmentally toxic heavy metals and metalloids such as zinc, cadmium, lead, and arsenic. *Reviews in Environmental Science and Biotechnology*, *3*(1), 71-90.

Alley, W. M. (2001). Ground water and climate. *Ground Water*, *39*(2), 161-161.

Ampiah-Bonney, R. J., Tyson, J. F., & Lanza, G. R. (2007). Phytoextraction of arsenic from soil by *Leersia oryzoides*. *International journal of phytoremediation*, *9*(1), 31-40.

Andrianisa, H. A., Ito, A., Sasaki, A., Aizawa, J., & Umita, T. (2008). Biotransformation of arsenic species by activated sludge and removal of bio-oxidised arsenate from wastewater by coagulation with ferric chloride *Water research*, *42*(19), 4809-4817.

Arena, C., Figlioli, F., Sorrentino, M. C., Izzo, L. G., Capozzi, F., Giordano, S., & Spagnuolo, V. (2017). Ultrastructural, protein and photosynthetic alterations induced by Pb and Cd in *Cynara cardunculus* L., and its potential for phytoremediation. *Ecotoxicology and Environmental Safety*, 145, 83–89. doi:10.1016/j.ecoenv.2017.07.015

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., De Castro, E., ... & Grosdidier, A. (2012). ExPASy: SIB bioinformatics resource portal. *Nucleic acids research*, *40*(W1), W597-W603.

Axelsen, K. B., & Palmgren, M. G. (1998). Evolution of substrate specificities in the P-type ATPase superfamily. *Journal of molecular evolution*, *46*(1), 84-101.

Bae, J., Mercier, G., Watson, A. K., & Benoit, D. L. (2014). Seed germination test for heavy metal phytotoxicity assessment. *Canadian Journal of Plant Science*, *94*(8), 1519-1521.

Baxter, I., Tchieu, J., Sussman, M. R., Boutry, M., Palmgren, M. G., Gribskov, M., ... & Axelsen, K. B. (2003). Genomic comparison of P-type ATPase ion pumps in *Arabidopsis* and rice. *Plant physiology*, *132*(2), 618-628.

Benlloch-González, M., Fournier, J. M., Ramos, J., & Benlloch, M. (2005). Strategies underlying salt tolerance in halophytes are present in *Cynara cardunculus. Plant Science*, *168*(3), 653-659.

Bert, V., Meerts, P., Saumitou-Laprade, P., Salis, P., Gruber, W., & Verbruggen, N. (2003). Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and soil*, *249*(1), 9-18.

Boularbah, A., Schwartz, C., Bitton, G., Aboudrar, W., Ouhammou, A., & Morel, J. L. (2006). Heavy metal contamination from mining sites in South Morocco: 2. Assessment of metal accumulation and toxicity in plants. *Chemosphere*, *63*(5), 811-817.

Brown, S. L., Angle, J. S., Chaney, R. L., & Baker, A. J. M. (1995). Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. *Soil Science Society of America Journal*, *59*(1), 125-133.

Cailliatte, R., Schikora, A., Briat, J. F., Mari, S., & Curie, C. (2010). High-affinity manganese uptake by the metal transporter NRAMP1 is essential for *Arabidopsis* growth in low manganese conditions. *The Plant Cell*, *22*(3), 904-917.

Carbonell-Barrachina, A. A., Aarabi, M. A., DeLaune, R. D., Gambrell, R. P., & Patrick, W. H. (1998). The influence of arsenic chemical form and concentration on *Spartina patens* and *Spartina alterniflora* growth and tissue arsenic concentration. *Plant and Soil*, *198*(1), 33-43.

Cellier, M., Prive, G., Belouchi, A., Kwan, T., Rodrigues, V., Chia, W., & Gros, P. (1995). Nramp defines a family of membrane proteins. *Proceedings of the National Academy of Sciences*, *92*(22), 10089-10093.

Chen, Y., Han, Y. H., Cao, Y., Zhu, Y. G., Rathinasabapathi, B., & Ma, L. Q. (2017). Arsenic transport in rice and biological solutions to reduce arsenic risk from rice. *Frontiers in plant science*, *8*.

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

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

CO2: Editorial Board. (2016). Ecotoxicology and Environmental Safety, 126, IFC. doi:10.1016/s0147-6513(16)00003-8

Cobbett, C. S. (2000). Phytochelatins and their roles in heavy metal detoxification. *Plant physiology*, *123*(3), 825-832.

Colangelo, E. P., & Guerinot, M. L. (2006). Put the metal to the petal: metal uptake and transport throughout plants. *Current opinion in plant biology*, *9*(3), 322-330.

Cooper, E. M., Sims, J. T., Cunningham, S. D., Huang, J. W., & Berti, W. R. (1999). Chelate-assisted phytoextraction of lead from contaminated soils. *Journal of environmental quality*, *28*(6), 1709-1719.

Cunningham, S. D., Berti, W. R., & Huang, J. W. (1995). Phytoremediation of contaminated soils. *Trends in biotechnology*, *13*(9), 393-397.

CURIE, C., ALONSO, J. M., Marie, L. E., ECKER, J. R., & BRIAT, J. F. (2000). Involvement of Nramp1 from *Arabidopsis thaliana* in iron transport. *Biochemical Journal*, *347*(3), 749-755.

Dekkers, B. J., Willems, L., Bassel, G. W., van Bolderen-Veldkamp, R. P., Ligterink, W., Hilhorst, H. W., & Bentsink, L. (2012). Identification of reference genes for RT–qPCR expression analysis in *Arabidopsis* and tomato seeds. *Plant and Cell Physiology*, *53*(1), 28-37.

Deng, D. M., Shu, W. S., Zhang, J., Zou, H. L., Lin, Z., Ye, Z. H., & Wong, M. H. (2007). Zinc and cadmium accumulation and tolerance in populations of *Sedum alfredii*. *Environmental Pollution*, *147*(2), 381-386.

Dhankher, O. P., Rosen, B. P., McKinney, E. C., & Meagher, R. B. (2006). Hyperaccumulation of arsenic in the shoots of *Arabidopsis* silenced for arsenate reductase (ACR2). *Proceedings of the National Academy of Sciences*, *103*(14), 5413-5418.

Dikinya, O., & Areola, O. (2010). Comparative analysis of heavy metal concentration in secondary treated wastewater irrigated soils cultivated by different crops. *International Journal of Environmental Science & Technology*, *7*(2), 337-346.

DiTusa, S. F., Fontenot, E. B., Wallace, R. W., Silvers, M. A., Steele, T. N., Elnagar, A. H., ... & Smith, A. P. (2016). A member of the Phosphate transporter 1 (Pht1) family from the arsenic-hyperaccumulating fern *Pteris vittata* is a high-affinity arsenate transporter. *New Phytologist*, *209*(2), 762-772.

Dixit, R., Malaviya, D., Pandiyan, K., Singh, U. B., Sahu, A., Shukla, R., ... & Paul, D. (2015). Bioremediation of heavy metals from soil and aquatic environment: an overview of principles and criteria of fundamental processes. *Sustainability*, *7*(2), 2189-2212.

Dong, J., Yang, Q. W., Sun, L. N., Zeng, Q., Liu, S. J., Pan, J., & Liu, X. L. (2011). Assessing the concentration and potential dietary risk of heavy metals in vegetables at a Pb/Zn mine site, China. *Environmental Earth Sciences*, *64*(5), 1317-1321.

Duan, G. L., Zhou, Y., Tong, Y. P., Mukhopadhyay, R., Rosen, B. P., & Zhu, Y. G. (2007). A CDC25 homologue from rice functions as an arsenate reductase. *New Phytologist*, *174*(2), 311-321.

Edelman, M., & Mattoo, A. K. (2008). D1-protein dynamics in photosystem II: the lingering enigma. *Photosynthesis Research*, *98*(1-3), 609-620.

Eide, D., Broderius, M., Fett, J., & Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. *Proceedings of the National Academy of Sciences*, *93*(11), 5624-5628.

Elliott, P. (2001). Risk of adverse birth outcomes in populations living near landfill sites. BMJ, 323(7309), 363–368. doi:10.1136/bmj.323.7309.363

EPA, U. (2000). *Introduction to phytoremediation*. EPA/600/R-99/107.

Esteban, E., Carpena, R. O., & Meharg, A. A. (2003). High-affinity phosphate/arsenate transport in white lupin (*Lupinus albus*) is relatively insensitive to phosphate status. *New Phytologist*, *158*(1), 165-173.

Faller, P., Kienzler, K., & Krieger-Liszkay, A. (2005). Mechanism of Cd 2+ toxicity: Cd 2+ inhibits photoactivation of Photosystem II by competitive binding to the essential Ca 2+ site. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1706*(1), 158-164.

Fernández, J., Curt, M. D., & Aguado, P. L. (2006). Industrial applications of *Cynara cardunculus* L. for energy and other uses. *Industrial crops and Products*, *24*(3), 222-229.

Frehner, M., Keller, F., & Wiemken, A. (1984). Localization of fructan metabolism in the vacuoles isolated from protoplasts of Jerusalem artichoke tubers (*Helianthus tuberosus* L.). *Journal of plant physiology*, *116*(3), 197-208.

Gadapati, W. R., & Macfie, S. M. (2006). Phytochelatins are only partially correlated with Cd-stress in two species of Brassica. *Plant science*, *170*(3), 471-480.

González, J., Pérez, F., Fernández, J., Lezaun, J. A., Rodriguez, D., & Perea, F. (2004). Study of *Cynara Cardunculus* L. lignocelullosic biomass production in dry conditions. Acta Horticulturae, (660), 221–227. doi:10.17660/actahortic.2004.660.29

Grossoehme, N. E., Akilesh, S., Guerinot, M. L., & Wilcox, D. E. (2006). Metal-Binding Thermodynamics of the Histidine-Rich Sequence from the Metal-Transport Protein IRT1 of *Arabidopsis thaliana*. *Inorganic Chemistry*, *45*(21), 8500-8508.

Guerinot, M. L. (2000). The ZIP family of metal transporters. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1465*(1), 190-198.

Guo, J., Xu, W., & Ma, M. (2012). The assembly of metals chelation by thiols and vacuolar compartmentalization conferred increased tolerance to and accumulation of cadmium and arsenic in transgenic *Arabidopsis thaliana*. *Journal of hazardous materials*, *199*, 309-313.

Hall, T. (1998). BioEdit. Biological sequence alignment editor for Windows. *North Carolina, USA: Carolina State University*.

Hanikenne, M., Talke, I. N., Haydon, M. J., Lanz, C., Nolte, A., Motte, P., ... & Krämer, U. (2008). Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. *Nature*, *453*, 391-395.

Hasegawa, H., Rahman, M. A., Matsuda, T., Kitahara, T., Maki, T., & Ueda, K. (2009). Effect of eutrophication on the distribution of arsenic species in eutrophic and mesotrophic lakes. *Science of the Total Environment*, *407*(4), 1418-1425.

Hellwege, E. M., Czapla, S., Jahnke, A., Willmitzer, L., & Heyer, A. G. (2000). Transgenic potato (*Solanum tuberosum*) tubers synthesize the full spectrum of inulin molecules naturally occurring in globe artichoke (*Cynara scolymus*) roots. *Proceedings of the National Academy of Sciences*, *97*(15), 8699-8704.

Hernández-Allica, J., Becerril, J. M., & Garbisu, C. (2008). Assessment of the phytoextraction potential of high biomass crop plants. *Environmental Pollution*, *152*(1), 32-40.

Hernández-Allica, J., Garbisu, C., Barrutia, O., & Becerril, J. M. (2007). EDTA-induced heavy metal accumulation and phytotoxicity in cardoon plants. *Environmental and Experimental Botany*, *60*(1), 26-32.

Houben, D., Pircar, J., & Sonnet, P. (2012). Heavy metal immobilization by cost-effective amendments in a contaminated soil: effects on metal leaching and phytoavailability. *Journal of Geochemical Exploration*, *123*, 87-94.

Hu, Y., Liu, X., Bai, J., Shih, K., Zeng, E. Y., & Cheng, H. (2013). Assessing heavy metal pollution in the surface soils of a region that had undergone three decades of intense industrialization and urbanization. *Environmental Science and Pollution Research*, *20*(9), 6150-6159.

Johnson, B. L. (1997). Hazardous Waste: Human Health Effects. Toxicology and Industrial Health, 13(2-3), 121–143. doi:10.1177/074823379701300203

Kabata-Pendias, A. (2004). Soil–plant transfer of trace elements—an environmental issue. Geoderma, 122(2-4), 143–149. doi:10.1016/j.geoderma.2004.01.004

Kim, Y. Y., Choi, H., Segami, S., Cho, H. T., Martinoia, E., Maeshima, M., & Lee, Y. (2009). AtHMA1 contributes to the detoxification of excess Zn (II) in *Arabidopsis*. *The Plant Journal*, *58*(5), 737-753.

Krämer, U. (2000). Cadmium for all meals–plants with an unusual appetite. *The New Phytologist*, *145*(1), 1-5.

Krishna, A. K., & Govil, P. K. (2007). Assessment of heavy metal contamination in soils around Manali industrial area, Chennai, Southern India. *Environmental Geology,* 54(7), 1465–1472. doi:10.1007/s00254-007-0927-z

Kukić, J., Popović, V., Petrović, S., Mucaji, P., Ćirić, A., Stojković, D., & Soković, M. (2008). Antioxidant and antimicrobial activity of *Cynara cardunculus* extracts. *Food Chemistry*, *107*(2), 861-868.

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

Küpper, H., Küpper, F., & Spiller, M. (1998). In situ detection of heavy metal substituted chlorophylls in water plants. *Photosynthesis Research*, *58*(2), 123-133.

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

Landberg, T., & Greger, M. (1996). Differences in uptake and tolerance to heavy metals in *Salix* from unpolluted and polluted areas. *Applied Geochemistry*, *11*(1), 175-180.

Lane, T. W., Saito, M. A., George, G. N., Pickering, I. J., Prince, R. C., & Morel, F. M. M. (2005). Biochemistry: A cadmium enzyme from a marine diatom. *Nature*, 435(7038), 42–42. doi:10.1038/435042a

Lanquar, V., Ramos, M. S., Lelièvre, F., Barbier-Brygoo, H., Krieger-Liszkay, A., Krämer, U., & Thomine, S. (2010). Export of vacuolar manganese by AtNRAMP3 and AtNRAMP4 is required for optimal photosynthesis and growth under manganese deficiency. *Plant physiology*, *152*(4), 1986-1999.

Lattanzio, V., Kroon, P. A., Linsalata, V., & Cardinali, A. (2009). Globe artichoke: a functional food and source of nutraceutical ingredients. *Journal of Functional Foods*, *1*(2), 131-144.

LeBlanc, M. S., McKinney, E. C., Meagher, R. B., & Smith, A. P. (2013). Hijacking membrane transporters for arsenic phytoextraction. *Journal of Biotechnology*, 163(1), 1–9. doi:10.1016/j.jbiotec.2012.10.013

Lee, R. B. (1982). Selectivity and kinetics of ion uptake by barley plants following nutrient deficiency. *Annals of Botany*, *50*(4), 429-449.

Leonardi C., (2017). Innovative technologies of phytoremediation for contaminated soils. PhD thesis, University of Catania.

Levin, R., Brown, M. J., Kashtock, M. E., Jacobs, D. E., Whelan, E. A., Rodman, J., ... & Sinks, T. (2008). Lead exposures in US children, 2008: implications for prevention. *Environmental Health Perspectives*, *116*(10), 1285.

LI, C. X., FENG, S. L., Yun, S., JIANG, L. N., LU, X. Y., & HOU, X. L. (2007). Effects of arsenic on seed germination and physiological activities of wheat seedlings. *Journal of Environmental Sciences*, *19*(6), 725-732.

Li, W., Khan, M. A., Yamaguchi, S., & Kamiya, Y. (2005). Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana*. *Plant growth regulation*, *46*(1), 45-50.

Lin, Y.-F., & Aarts, M. G. M. (2012). The molecular mechanism of zinc and cadmium stress response in plants. *Cellular and Molecular Life Sciences*, 69(19), 3187–3206. doi:10.1007/s00018-012-1089-z

Liu, W., Shu, W., & Lan, C. (2004). *Viola baoshanensis*, a plant that hyperaccumulates cadmium. *Chinese Science Bulletin*, *49*(1), 29-32.

Llugany, M., Miralles, R., Corrales, I., Barceló, J., & Poschenrieder, C. (2012). *Cynara cardunculus* a potentially useful plant for remediation of soils polluted with cadmium or arsenic. *Journal of Geochemical Exploration*, 123, 122–127. doi:10.1016/j.gexplo.2012.06.016

Lombi, E., Zhao, F. J., Dunham, S. J., & McGrath, S. P. (2001). Phytoremediation of heavy metal–contaminated soils. *Journal of Environmental Quality*, *30*(6), 1919-1926.

Lone, M. I., He, Z. L., Stoffella, P. J., & Yang, X. E. (2008). Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. *Journal of Zhejiang University Science B*, *9*(3), 210-220.

Lutts, S., Lefevre, I., Delpérée, C., Kivits, S., Dechamps, C., Robledo, A., & Correal, E. (2004). Heavy metal accumulation by the halophyte species Mediterranean saltbush. *Journal of Environmental Quality*, *33*(4), 1271-1279.

Ma, J. F., Yamaji, N., Mitani, N., Xu, X. Y., Su, Y. H., McGrath, S. P., & Zhao, F. J. (2008). Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences*, *105*(29), 9931-9935.

McGrath, S. P., & Zhao, F. J. (2003). Phytoextraction of metals and metalloids from contaminated soils. *Current Opinion in Biotechnology*, *14*(3), 277-282.

Meharg, A. A., & Hartley-Whitaker, J. (2002). Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. *New Phytologist*, *154*(1), 29-43.

Mesjasz-Przybyłowicz, J., Nakonieczny, M., Migula, P. A. W. E. Ł., Augustyniak, M., Tarnawska, M., Reimold, W., ... & Głowacka, E. (2004). Uptake of cadmium, lead nickel and zinc from soil and water solutions by the nickel hyperaccumulator *Berkheya coddii*. *Acta Biol Cracov Ser Bot*, *46*, 75-85.

Mganga, N., Manoko, M. L. K., & Rulangaranga, Z. K. (2011). Classification of plants according to their heavy metal content around North Mara gold mine, Tanzania: implication for phytoremediation. *Tanzania Journal of Science*, *37*(1).

Mishra, S., Srivastava, S., Tripathi, R. D., Govindarajan, R., Kuriakose, S. V., & Prasad, M. N. V. (2006). Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L. *Plant Physiology and Biochemistry*, *44*(1), 25-37.

Mohan, D., & Pittman, C. U. (2007). Arsenic removal from water/wastewater using adsorbents—a critical review. *Journal of hazardous materials*, *142*(1), 1-53.

Molins, H., Michelet, L., Lanquar, V., Agorio, A., Giraudat, J., Roach, T., … Thomine, S. (2012). Mutants impaired in vacuolar metal mobilization identify chloroplasts as a target for cadmium hypersensitivity in *Arabidopsis thaliana*. *Plant, Cell & Environment*, 36(4), 804–817. doi:10.1111/pce.12016

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., & Richaud, P. (2009). AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in *Arabidopsis*. *Plant physiology*, *149*(2), 894-904.

Mortvedt, J. J. (1996). Heavy metal contaminants in inorganic and organic fertilizers. Fertilizers and Environment, 5–11. doi:10.1007/978-94-009-1586-2_2

Nowack, B., Ranville, J. F., Diamond, S., Gallego-Urrea, J. A., Metcalfe, C., Rose, J., ... & Klaine, S. J. (2012). Potential scenarios for nanomaterial release and subsequent alteration in the environment. *Environmental Toxicology and Chemistry*, *31*(1), 50-59.

Ochoa, M. J., & Fandos, A. (2004). Evaluation of vegetable cardoon (*Cynara cardunculus* L.) populations for biomass production under rainfed conditions. *Acta Horticulturae*, (660), 235–239. doi:10.17660/actahortic.2004.660.31

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

Padmavathiamma, P. K., & Li, L. Y. (2007). Phytoremediation technology: hyper-accumulation metals in plants. *Water, Air, and Soil Pollution*, *184*(1-4), 105-126.

Papazoglou, E. G. (2011). Responses of *Cynara cardunculus* L. to single and combined cadmium and nickel treatment conditions. *Ecotoxicology and environmental safety*, *74*(2), 195-202.

Peralta, J. R., Gardea-Torresdey, J. L., Tiemann, K. J., Gomez, E., Arteaga, S., Rascon, E., & Parsons, J. G. (2001). Uptake and effects of five heavy metals on seed germination and plant growth in alfalfa (*Medicago sativa* L.). *Bulletin of Environmental Contamination and toxicology*, *66*(6), 727-734.

Peralta-Videa, J. R., Lopez, M. L., Narayan, M., Saupe, G., & Gardea-Torresdey, J. (2009). The biochemistry of environmental heavy metal uptake by plants: Implications for the food chain. *The International Journal of Biochemistry & Cell Biology,* 41(8-9), 1665–1677. doi:10.1016/j.biocel.2009.03.005

Pérez-Sanz, A., Millán, R., Sierra, M. J., Alarcón, R., García, P., Gil-Díaz, M., … Lobo, M. C. (2012). Mercury uptake by *Silene vulgaris* grown on contaminated spiked soils. *Journal of Environmental Management*, 95, S233–S237. doi:10.1016/j.jenvman.2010.07.018

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

PLANT, J. N. W. T. (2005). Public Health Service Agency for Toxic Substances and Disease Registry Division of Health Assessment and Consultation Atlanta, Georgia 30333.

Pollard, A. J., Powell, K. D., Harper, F. A., & Smith, J. A. C. (2002). The genetic basis of metal hyperaccumulation in plants. *Critical reviews in plant sciences*, *21*(6), 539-566.

Poschenrieder, C., & i Coll, J. B. (2003). Phytoremediation: principles and perspectives. *Contributions to science*, 333-344.

Prasad, M. N. V. (2003). Metal hyperaccumulation in plants-Biodiversity prospecting for phytoremediation technology. *Electron. J. Biotechnol.*, *6*, 110-146.

Quinton, J. N., & Catt, J. A. (2007). Enrichment of Heavy Metals in Sediment Resulting from Soil Erosion on Agricultural Fields. *Environmental Science & Technology*, 41(10), 3495–3500. doi:10.1021/es062147h

Raccuia, S. A., & Melilli, M. G. (2007). Biomass and grain oil yields in *Cynara cardunculus* L. genotypes grown in a Mediterranean environment. *Field Crops Research*, *101*(2), 187-197.

Raccuia, S. A., & Melilli, M. G. (2010). Seasonal dynamics of biomass, inulin, and water-soluble sugars in roots of *Cynara cardunculus* L. *Field crops research*, *116*(1), 147-153.

Raccuia, S. A., Cavallaro, V., & Melilli, M. G. (2004a). Intraspecific variability in *Cynara cardunculus* L. var. *sylvestris* Lam. Sicilian populations: seed germination under salt and moisture stresses. *Journal of Arid Environments*, *56*(1), 107-116.

Raccuia, S. A., Mainolfi, A., Mandolino, G., & Melilli, M. G. (2004b). Genetic diversity in *Cynara cardunculus* revealed by AFLP markers: comparison between cultivars and wild types from Sicily. *Plant Breeding*, *123*(3), 280-284.

Rahman, M. A., Rahman, M. M., Kadohashi, K., Maki, T., & Hasegawa, H. (2011). Effect of external iron and arsenic species on chelant-enhanced iron bioavailability and arsenic uptake in rice (*Oryza sativa* L.). *Chemosphere*, *84*(4), 439-445.

Rajaganapa, V., Xavier, F., Sreekumar, D., & Mandal, P. K. (2011). Heavy Metal Contamination in Soil, Water and Fodder and their Presence in Livestock and Products : A Review. *Journal of Environmental Science and Technology*, 4(3), 234–249. doi:10.3923/jest.2011.234.249

Rapaille, A., Gonze, M., & Van Der Schueren, F. (1995). Formulating sugar-free chocolate products with maltitol. *Food technology*, *49*(7), 51-54.

Romè, C., Huang, X. Y., Danku, J., Salt, D. E., & Sebastiani, L. (2016). Expression of specific genes involved in Cd uptake, translocation, vacuolar compartmentalisation and recycling in *Populus alba* Villafranca clone. *Journal of plant physiology*, *202*, 83-91.

Rottenberg, A., & Zohary, D. (1996). The wild ancestry of the cultivated artichoke. *Genetic Resources and Crop Evolution*, *43*(1), 53-58.

Rozen, S., & Skaletsky, H. (1999). Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics methods and protocols*, 365-386.

Sánchez-Pardo, B., Cantero, C., & Zornoza, P. (2015). Alleviation of arsenic stress in cardoon plants via the supply of a low cadmium concentration. *Environmental and experimental botany*, *109*, 229-234. doi:10.1016/j.envexpbot.2014.07.004

Scaglione, D., Reyes-Chin-Wo, S., Acquadro, A., Froenicke, L., Portis, E., Beitel, C., ... & Faccioli, P. (2016). The genome sequence of the outbreeding globe artichoke constructed de novo incorporating a phase-aware low-pass sequencing strategy of F1 progeny. *Scientific reports*, *6*, 19427.

Schmid, M., Davison, T. S., Henz, S. R., Pape, U. J., Demar, M., Vingron, M., ... & Lohmann, J. U. (2005). A gene expression map of *Arabidopsis thaliana* development. *Nature genetics*, *37*(5), 501.

Shevyakova, N. I., Netronina, I. A., Aronova, E. E., & Kuznetsov, V. V. (2003). Compartmentation of cadmium and iron in *Mesembryanthemum crystallinum* plants during the adaptation to cadmium stress. *Russian journal of plant physiology*, *50*(5), 678-685.

Shukla, T., Kumar, S., Khare, R., Tripathi, R. D., & Trivedi, P. K. (2015). Natural variations in expression of regulatory and detoxification related genes under limiting phosphate and arsenate stress in *Arabidopsis thaliana*. *Frontiers in Plant Science*, *6*, 898. http://doi.org/10.3389/fpls.2015.00898

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., ... & Thompson, J. D. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology*, *7*(1), 539.

Solís-Domínguez, F. A., González-Chávez, M. C., Carrillo-González, R., & Rodríguez-Vázquez, R. (2007). Accumulation and localization of cadmium in *Echinochloa polystachya* grown within a hydroponic system. *Journal of hazardous materials*, *141*(3), 630-636.

Song, W. Y., Park, J., Mendoza-Cózatl, D. G., Suter-Grotemeyer, M., Shim, D., Hörtensteiner, S., ... & Schroeder, J. I. (2010). Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National Academy of Sciences*, *107*(49), 21187-21192.

Song, W. Y., Yamaki, T., Yamaji, N., Ko, D., Jung, K. H., Fujii-Kashino, M., ... & Ma, J. F. (2014). A rice ABC transporter, OsABCC1, reduces arsenic accumulation in the grain. *Proceedings of the National Academy of Sciences*, *111*(44), 15699-15704.

Tandy, S., Schulin, R., & Nowack, B. (2006). The influence of EDDS on the uptake of heavy metals in hydroponically grown sunflowers. *Chemosphere*, *62*(9), 1454-1463.

Tang, Y. T., Qiu, R. L., Zeng, X. W., Ying, R. R., Yu, F. M., & Zhou, X. Y. (2009). Lead, zinc, cadmium hyperaccumulation and growth stimulation in *Arabis paniculata Franch*. *Environmental and Experimental Botany*, *66*(1), 126-134.

Tangahu, B. V., Sheikh Abdullah, S. R., Basri, H., Idris, M., Anuar, N., & Mukhlisin, M. (2011). A Review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. *International Journal of Chemical Engineering*, *2011*.

Thomine, S., Lelièvre, F., Debarbieux, E., Schroeder, J. I., & Barbier-Brygoo, H. (2003). AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *The Plant Journal*, *34*(5), 685-695.

Toscano, V., Sollima, L., Genovese, C., Melilli, M. G., & Raccuia, S. A. (2016). Pilot plant system for biodiesel and pellet production from cardoon: technical and economic feasibility. *Acta Horticulturae*, (1147), 429–442. doi:10.17660/actahortic.2016.1147.60

Toxicological Profile for Cadmium. (2002). ATSDR's *Toxicological Profiles*. doi:10.1201/9781420061888_ch48

Ueno, D., Milner, M. J., Yamaji, N., Yokosho, K., Koyama, E., Clemencia Zambrano, M., ... & Ma, J. F. (2011). Elevated expression of TcHMA3 plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. *The Plant Journal*, *66*(5), 852-862.

Ueno, D., Yamaji, N., Kono, I., Huang, C. F., Ando, T., Yano, M., & Ma, J. F. (2010). Gene limiting cadmium accumulation in rice. *Proceedings of the national academy of sciences*, *107*(38), 16500-16505.

Vaclavikova, M., Gallios, G. P., Hredzak, S., & Jakabsky, S. (2008). Removal of arsenic from water streams: an overview of available techniques. *Clean Technologies and Environmental Policy*, *10*(1), 89-95.

Vázquez, M. D., Barceló, J., Poschenrieder, C. H., Madico, J., Hatton, P., Baker, A. J. M., & Cope, G. H. (1992). Localization of zinc and cadmium in *Thlaspi caerulescens* (Brassicaceae), a metallophyte that can hyperaccumulate both metals. *Journal of Plant Physiology*, *140*(3), 350-355.

Verbruggen, N., Hermans, C., & Schat, H. (2009). Mechanisms to cope with arsenic or cadmium excess in plants. *Current opinion in plant biology*, *12*(3), 364-372.

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

Vishnoi, A., Srivastava, A., Roy, R., & Bhattacharya, A. (2008). MGDD: *Mycobacterium tuberculosis* genome divergence database. *BMC genomics*, *9*(1), 373.

Vogel-Mikuš, K.., Simčič, J., Pelicon, P., Budnar, M., Kump, P., Nečemer, M., ... & Regvar, M. (2008). Comparison of essential and non-essential element distribution in leaves of the Cd/Zn hyperaccumulator *Thlaspi praecox* as revealed by micro-PIXE. *Plant, cell & environment*, *31*(10), 1484-1496.

Wan, X., Lei, M., & Chen, T. (2016). Cost–benefit calculation of phytoremediation technology for heavy-metal-contaminated soil. *Science of the Total Environment*, *563*, 796-802.

Wang, J., Zhao, F. J., Meharg, A. A., Raab, A., Feldmann, J., & McGrath, S. P. (2002). Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant physiology*, *130*(3), 1552-1561 doi:10.1104/pp.008185

Wang, S. L., Liao, W. B., Yu, F. Q., Liao, B., & Shu, W. S. (2009). Hyperaccumulation of lead, zinc, and cadmium in plants growing on a lead/zinc outcrop in Yunnan Province, China. *Environmental geology*, *58*(3), 471.

Welch, R. M., & Norvell, W. A. (1999). Mechanisms of Cadmium Uptake, Translocation and Deposition in Plants. Cadmium in Soils and Plants, 125–150. doi:10.1007/978-94-011-4473-5_6

Williams, L. E., & Mills, R. F. (2005). P 1B-ATPases–an ancient family of transition metal pumps with diverse functions in plants. *Trends in plant science*, *10*(10), 491-502.

Wu, J., Zhao, F. J., Ghandilyan, A., Logoteta, B., Guzman, M. O., Schat, H., ... & Aarts, M. G. (2009). Identification and functional analysis of two ZIP metal transporters of the hyperaccumulator *Thlaspi caerulescens*. *Plant and soil*, *325*(1-2), 79.

Wu, F., Yang, W., Zhang, J., & Zhou, L. (2010). Cadmium accumulation and growth responses of a poplar (*Populus deltoids* × *Populus nigra*) in cadmium contaminated purple soil and alluvial soil. *Journal of Hazardous Materials*, *177*(1), 268-273.

Wu, D., Yamaji, N., Yamane, M., Kashino-Fujii, M., Sato, K., & Feng Ma, J. (2016). The HvNramp5 Transporter Mediates Uptake of Cadmium and Manganese, But Not Iron. *Plant Physiology*, *172*(3), 1899–1910. http://doi.org/10.1104/pp.16.01189

Wuana, R. A., & Okieimen, F. E. (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *Isrn Ecology*, *2011*.

Xu, J., Sun, J., Du, L., & Liu, X. (2012). Comparative transcriptome analysis of cadmium responses in *Solanum nigrum* and *Solanum torvum. New Phytologist*, *196*(1), 110-124.

Xu, Y., Feng, L., Jeffrey, P. D., Shi, Y., & Morel, F. M. M. (2008). Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. *Nature*, 452(7183), 56–61. doi:10.1038/nature06636

Yadav, S. K. (2010). Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. *South African Journal of Botany*, 76(2), 167–179. doi:10.1016/j.sajb.2009.10.007

Yang, X. E., Long, X. X., Ye, H. B., He, Z. L., Calvert, D. V., & Stoffella, P. J. (2004). Cadmium tolerance and hyperaccumulation in a new Zn-hyperaccumulating plant species (*Sedum alfredii Hance*). *Plant and Soil*, *259*(1), 181-189.

Zhao, F. J., Jiang, R. F., Dunham, S. J., & McGrath, S. P. (2006). Cadmium uptake, translocation and tolerance in the hyperaccumulator *Arabidopsis halleri*. *New Phytologist*, *172*(4), 646-654.

Zhu, Y. G., & Rosen, B. P. (2009). Perspectives for genetic engineering for the phytoremediation of arsenic-contaminated environments: from imagination to reality *Current Opinion in Biotechnology*, *20*(2), 220-224.

Zohary, D. J., Basnizki, 1975. The cultivated artichoke *Cynara scolymus*. Its probable wild ancestors. *Econ. Bot*, *29*, 233-235.

Website:

http://envirocivil.com/health-and-wellness/health-hazardous-cadmium /

http://tinyurl.com/kolj52p

http://www.bgs.ac.uk/arsenic/

www.matrica.it