



The University of
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**Zinc Hyperaccumulation in *Thlaspi*
*caerulescens***

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Abstract

The total land available to farm globally is only one quarter of the land available. With the current world population currently rising, standing at over 6.6 billion people in August 2008, a need to produce larger food quantities is an ever increasing pressure to scientists and farmers. The options available to support demands are to produce crops that have higher yields grown on land we currently have available, crops with increased tolerance to abiotic stresses, such as saline toxicity and crops to reclaim land that has been damaged by human use such as heavy metal contaminated land. There are currently over 400 plant species belonging to 45 different families that can tolerate and accumulate excessive amounts of heavy metals, such as nickel, cadmium and zinc. *Thlaspi caerulescens* a member of the family Brassicaceae (which is therefore closely related to *Arabidopsis thaliana*), is a well studied model for studying heavy metal accumulation as it accumulates zinc, nickel and sometimes cadmium to high levels without showing signs of toxicity.

The primary aim of this research was to identify and confirm potential genes responsible for the hyperaccumulation of zinc, using microarray and qPCR technologies. The second aim was to functionally test any highlighted, potential candidate genes through transgenics, therefore this project aimed to develop a transformation protocol to study potential candidate genes *in planta*.

The microarray successfully identified genes that were differentially expressed in the hyperaccumulator *T. caerulescens* compared to *T. arvense*, several were confirmed by qPCR. A good candidate gene from this and other studies on *Thlaspi caerulescens* and *Arabidopsis halleri* was *HMA4* which is a member of the P_{1B}-

ATPase family. An RNAi construct was successfully made of the *HMA4* gene in an attempt to silence the gene *in planta*. Attempts were made to transform *Thlaspi caerulescens* through tissue culture and floral dip methods; however these were unsuccessful due to difficulties of *T. caerulescens* cultivation and transformation.

Future strategies would include rapid cycling of plants and heterologous expression of native *T. caerulescens* genes in *Arabidopsis thaliana*.

Abbreviations

As	arsenic
ATH1	ATH1-121501
BAC	bacterial artificial chromosome
BAP	6-benzyl amino purine: a cytokinin plant growth regulator
bp	base pair: two nucleotides on opposite complementary DNA or RNA strands
CA	carbonic anhydrase
CAX	calcium and other divalent cation exchange antiporters
Cd	cadmium
CDF	cation diffusion facilitator proteins
cDNA	complementary DNAs
CHS	chalcone synthase
Co	cobalt
cRNA	complementary RNA
Cu	copper
cv.	cultivar
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate. The units from which DNA molecules are constructed, each carrying a single nitrogenous base (adenine, guanine, cytosine or thymine).
DTPA	diethyltriaminepentaacetic acid, soil test reagent
d. wt.	dry weight

E. coli	Escherichia coli: a bacterium
e.g.	<i>exempli gratia</i> : for example
EDTA	ethylene diamine tetraacetic acid (C ₁₀ H ₁₂ N ₂ Na ₄ O ₈)
EST	expressed sequenced tag
et al.,	et alia: and others
etc.	<i>et cetera</i> : and the rest
<i>ex vitro</i>	from glass: Organisms removed from tissue culture and transplanted; generally plants to soil or a potting mixture
Fig.	figure
f. wt.	fresh weight
<i>gus</i>	β -glucuronidase reporter gene
h	hour
HCl	hydrochloric acid
HMA	heavy metal associated gene
i.e.	id est: that is
IAA	3- Indoleacetic acid: an auxin plant growth regulator
<i>in planta</i>	within plant species.
<i>in vitro</i>	in glass: plants and tissue grown under axenic conditions within a controlled environment i.e. tissue culture (L).
ka	kanamycin
kb	kilobases
kPa	kilopascals
L.	litre
LB	Luria-Bertani medium
Ltd.	limited: a private limited company by shares

M	molar: unit of concentration, or molarity, of solutions
m	metre
MBD	metal binding domain
mg	milligramme
min./mins.	minute/ minutes
ml	millilitre
mM	millimolar
MM	mis match probe on an Affymetrix GeneChip
Mn	manganese
Mol	mole: an SI unit used for an amount of a particular object based on Avogadro's number, approximately 6.02252×10^{23} .
MS	Murashige and Skoog medium: a plant growth medium
MS0	Murashige and Skoog 0: <i>In vitro</i> basal medium containing 8 g l^{-1} agar with full strength MS macro and micro salts and vitamins (4.3 g l^{-1}), 30 g l^{-1} sucrose, but lacking growth regulators, pH 5.8.
MTP1	metal transporter protein 1
NAA	α -naphthaleneacetic acid: an auxin plant growth regulator
NaCl	sodium chloride
NaOH	sodium hydroxide
NAS1	nicotianamine synthase 1
NASC	Nottingham Arabidopsis Stock Centre
NCBI	national center for biotechnology information
Ni	nickel

Abbreviations

NIH	national institute of health
NLM	national library of medicine
<i>nptII</i>	neomycin phosphotransferase type II gene
NRAMP	natural resistance-associated macrophage proteins
NR1	nitrate reductase 1
°C	degrees Celsius
OD	optical density
Pa	Pascal: an SI derived unit of pressure (1 Pa=1 kg m ⁻¹ s ⁻²).
Pb	lead
PCR	polymerase chain reaction.
pers. comm.	personal communication
pH	a measure of the activity of hydrogen ions (H ⁺) in a solution
PM	perfect match probe
qPCR	quantitative real-time PCR
RNAi	ribonucleic acid interference
s	seconds
SEM	standard error of the mean
SOD	superoxide dismutase
<i>T. arvensis</i> 'Wellesbourne'	<i>Thlaspi arvensis</i> 'Wellesbourne'
<i>T. caerulescens</i> 'Ganges'	<i>Thlaspi caerulescens</i> 'Ganges'
T ₁	first generation of a transformed plant
Taq polymerase	thermostable DNA polymerase from <i>Thermus aquaticus</i>
TDZ	thiadiazuron, N-phenyl, N-1, 2, 3-thiadiazol-5-ylurea: a cytokinin plant growth regulator

™	trademark
U.K.	United Kingdom
U.S.A.	United States of America
v/v	volume to volume ratio of a solution
W	watts: an SI unit of power, equal to one joule per second.
w/v	weight to volume ratio of a solution
ZFP	zinc-finger protein
ZIP	zinc/iron permease (ZRT, IRT-like Protein)
Zn	zinc
[Zn] _{ext}	zinc external concentration
ZNT1	zinc transporter 1
α	alpha: first letter of the Greek alphabet.
β	beta: second letter of the Greek alphabet.

1.Introduction

The world population is continuing to grow, although the rate of growth has slowed over recent years. According to the International Census data (<http://www.census.gov/>) and the Population Clock (<http://www.worldometers.info/population>) the world population was over 6.6 billion people in August 2008. This figure has doubled since the 1960s and is expected to reach 9 billion by 2042. All of these people must be fed by only 37% of the Earth's land. The FAO (Food and Agriculture Organisation of the United Nations) estimate that more than three quarters of the Earth's surface is unsuitable for agriculture due to soil type, terrain or climate. If we are to support this growing population we need to produce plants that are better suited to the soils we currently cannot access.

The UN also has reported that approximately one half of the world's population suffers from micronutrient undernourishment mainly amongst young children and mothers in developing countries. Biofortification of crops holds a key to relieve the numbers of people currently afflicted with this problem. A recent WHO report has stated that nutritional Zn deficiency affects 25% of the world's population, ranked fifth amongst the developing world's most important health and eleventh in the world overall, ranking as equally important as iron and vitamin C deficiency. Therefore Zn enriched plants such as cereals could potentially solve this problem. The Zn atom/ion has to travel a long way from soil to edible parts of the plant (e.g. cereal grains) and

therefore to be able to use crops to treat mineral deficiencies it is necessary to understand the biological processes that control the uptake and translocation of the mineral ion (Palmgren *et al.*, 2008).

This introduction will study in more detail the facts surrounding these issues and the research may be able to begin to make an impact on the road to solving these issues.

1.1 Zinc is an essential element for plant growth

1.1.1 Zn chemical and biological properties

Zinc (Zn) belongs in the group of metals known as heavy metals or trace elements. Heavy metals are defined as those metals or semimetals (metalloids) that have been known to cause contamination or toxicity. Generally these metals have a high density (greater than 6 g cm^3) and with a high atomic mass (the value varies around 20), examples of which include Copper (Cu), Zinc (Zn), Nickel (Ni), or Lead (Pb). Together they make up approximately 1% of the earth's crust. Zinc, the 23rd most abundant element found on earth (average total Zn content in the lithosphere 75 mg/kg), has an atomic number of thirty and is found in five stable isotopic forms: ^{64}Zn , ^{66}Zn , ^{67}Zn , ^{68}Zn and ^{70}Zn . In order to study Zn uptake and translocation in plants the isotope ^{65}Zn , one of thirty short lived isotopes of Zn has been used as a Zn tracer. Zn is primarily found as sulphide minerals, sphalerite (ZnS) and less commonly, smithsonite (ZnCO_3) (Alloway and Ayres, 1997; Auld, 2001; Barak and Helmke, 1993; Ross, 1994).

Zinc is an essential micronutrient required for correct plant growth and functioning. In order to function and complete their life cycles plants require at least 17 elements (Broadley *et al.*, 2001). Excluding iron, zinc is the most abundant metal to be found in living organisms, where it plays a major structural, catalytic and cocatalytic role in enzymes. Zinc is the only metal to be found in all of the six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases (<http://www.chem.qmul.ac.uk/iubmb/>). The study of Zn^{2+} , the only stable oxidation state of Zn, has shown Zn^{2+} to be required for correct protein folding. The most abundant Zn^{2+} binding protein class is the Zn finger domain containing proteins (ZFP). The ZFP are able to determine transcription rates through DNA/RNA binding. Zinc also plays a structural role in ribosomes; protein synthesis is greatly reduced in its absence due to ribosomal degradation (Auld, 2001; Broadley *et al.*, 2007; Maret, 2005). Zinc is able to bind to phospholipid and sulfhydryl groups within membranes, forming tetrahedral complexes that protect membranes from oxidative damage (Marschner, 1995). Studies on the importance of Zn in maize were reported as early as 1915, followed by barley and dwarf sunflower (Mazé, 1915; Sommer and Lipman, 1926). Earliest reports on studies of Zn deficiency in plants was carried out in the 1940s in tomato (Skoog, 1940). These early experiments looked at the symptoms of zinc deficiency in various agriculturally important crop species and confirmed a positive relationship between the presence of zinc and the levels of auxin in the plants (reviewed in Broadley *et al.*, 2007). They reported that a deficiency in Zn resulted in the retardation of growth probably due to the reduced accumulation of auxin in the plant. The issue of

zinc deficiencies in plants has not decreased in interest today and therefore is a present day research area.

1.1.2 Zn deficiency in plants

Zinc deficiency in plants has been noted as the most commonly occurring crop micronutrient deficiency, affecting 30 % of the world's soils (Fig.1.1). The total Zn concentration of soils is greatly determined by the parent rock type. Typically soils range in Zn concentration from 10-300 mg kg⁻¹, averaging around 50 mg kg⁻¹ (Alloway, 1990). Most West Australian soils, half of Indian and Turkey's cultivated land, and a third of China's agricultural land is Zn deficient. Phytoavailability of free Zn²⁺ is affected by pH, soil moisture, organic matter content and the ease of weathering of rocks. Although the average soil contains 50 mg kg⁻¹ of Zn the concentration of Zn in solution is much lower (4-270 µg l⁻¹). Zn mobility and solubility is largely affected by pH, at low pH Zn exists in the ionic form Zn²⁺. Solubility of Zn is therefore decreased as Zn forms ZnCO₃ and ZnS in the soil. In alkaline soils high pH causes Zn sorbs or precipitates in unavailable forms (Khoshgoftar *et al.*, 2004; Ross, 1994; Alloway, 1990).

Agricultural production on soils low in phytoavailable Zn can be improved through genetic improvement of crops to take up Zn or traditional agricultural practices such as the addition of foliar fertilizers containing Zn. The most commonly used and most cost effective way of altering soil concentrations are through the use of zinc salts such as zinc sulphate (ZnSO₄·7H₂O) applied on their own or mixed with fertilizers. To treat foliar parts zinc sulphate can also

be used; however calcium hydroxide is normally needed in addition to neutralise acidity. Alternatively by-products of industry may be recycled and used (Alloway, 2001). Success stories have included the treatment of little leaf in peach orchards, mottled leaf in citrus orchards, stunted growth and rosetting in pineapples and pecans respectively (Hacisalihoglu and Kochian, 2003; Graham, *et al.*, 1992; White, and Zasoski, 1999; Cakmak, 2002; 2004 respectively; reviewed in Broadley *et al.*, 2007). The limitations of application of Zn fertilisers are i) economics, ii) environmental factors associated with excessive fertiliser uses and iii) agronomic factors associated with disease interactions.

Heavy metals occur in greater quantities in igneous and metamorphic rock compared with sedimentary rock. However sedimentary rocks are the most predominant soil parent material as they overlay most igneous and metamorphic rock (Alloway and Ayres, 1997). Zn deficiency occurs in highly weathered acidic or calcareous soils (as pH increases Zn mobility decreases). Plants are said to be deficient in Zn when levels are below 15-20 ng g⁻¹ d. wt. During Zn deficiency there is often an increase in membrane permeability that results in leakage of low molecular weight solutes (Marschner, 1995). Chlorosis is a typical visible clue of Zn deficiency in plants, often called “mottle leaf” or “bronzing”, where leaves turn a reddish-brown or bronze colour. Plants often become stunted, due to a reduction in internode lengthening which is termed “rosetting”. This is seen also in auxin deficient plants. Leaf size is reduced in a disorder termed, “little leaf”; leaf laminae curl inwards in a

disorder termed “goblet leaves” and epinasty is observed. In severe cases necrosis is seen in the root apex, and is known as “die back”. (Fig.1.2).

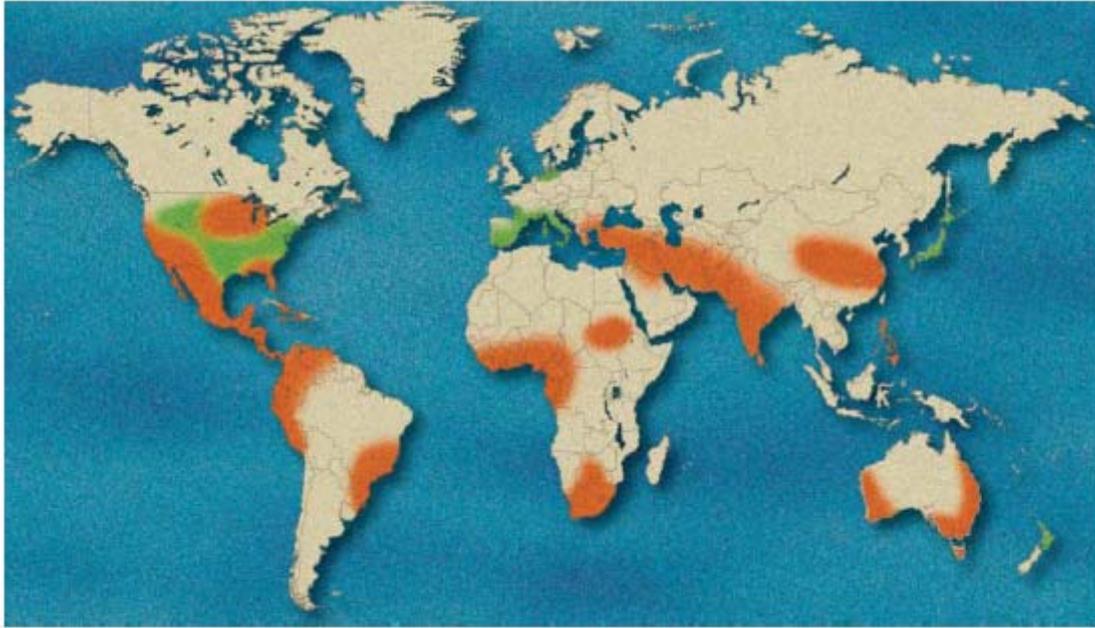


Fig.1.1 Geographic distribution of Zn deficient soil of the world. Severe areas shown in red and moderate areas shown in green (For a definition of the values of severe or critical zinc soil levels see Table 1.1). There are more zinc deficient areas not shown on the map as they are areas that are not suitable for growing crops (for example due to climatic reasons). For example the majority of Australian soils are Zn deficient however the western, eastern and southern coasts provide conditions suitable to crop growth, the majority of the rest of the land is desert (reproduced from Alloway, 2003).

Soil Extractant	Soil Levels mg Zn kg⁻¹ dry soil likely to limit crop growth
1N NH ₄ -acetate, pH 4.8	0.6
DTPA methods	0.8
0.05N HCl	1.0
EDTA methods	1.5
0.1N HCl	2.0

Table 1.1 Soil zinc levels classed as critical or severe. The levels differ depending on the extraction method that has been used. Refer to Fig.1.1 for soils that have been classed as severely zinc deficient (Alloway 2003).

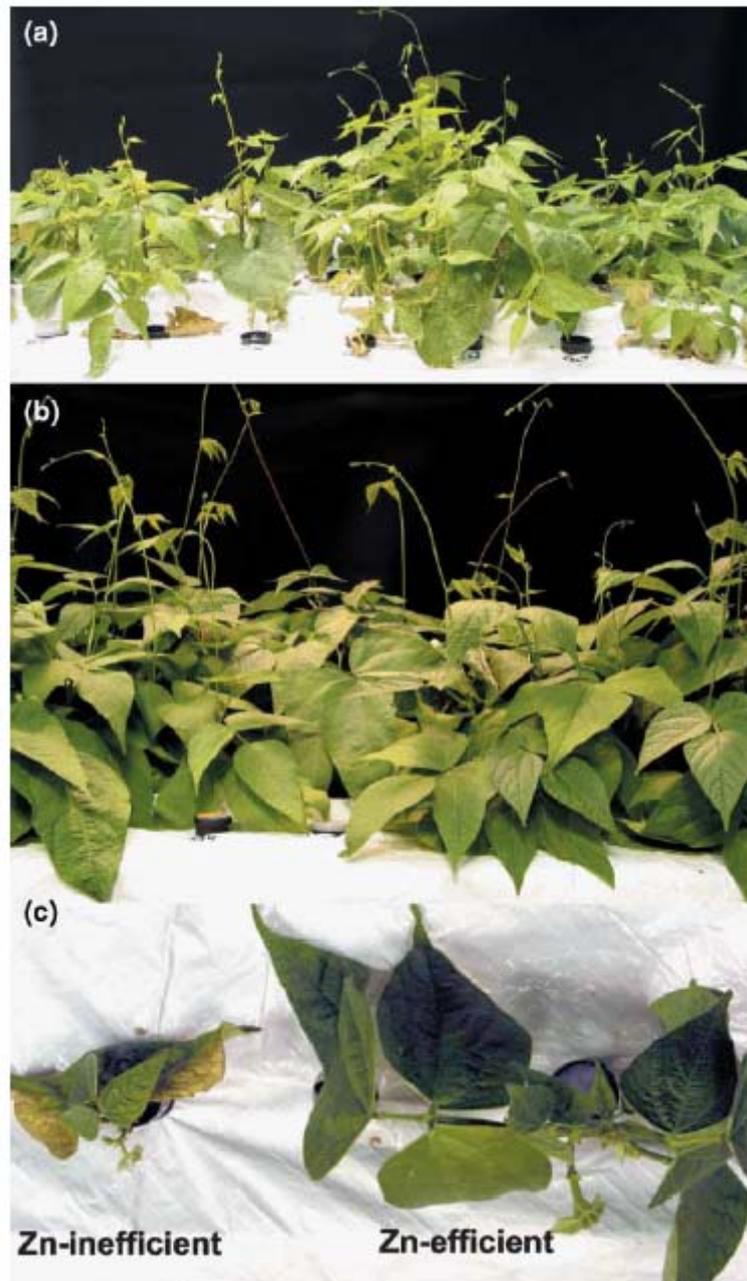


Fig.1.2 Zinc deficiency in common bean. (a) Plants grown on Zn deficient nutrient solution (b) Plants grown on solution containing 150 ppm Zn (c) Bean plants showing stunted growth, reduction in leaf size and chlorosis (Hacisalihoglu and Kochian, 2003).

1.1.3 Zn toxicity

The majority of plants find high tissue concentrations (>0.03% d. wt.) of Zn toxic. Some plant species exhibit toxicity symptoms when containing as little as 0.01% dry weight (d. wt.) or less. In nature and agriculture, Zn toxicity is a far less common occurrence than Zn deficiency. Zinc toxicity is mainly found in mining and smelting areas or on agricultural land contaminated with sewerage sludge. Toxicity may also occur in soils with a low pH enriched with anthropogenic inputs of Zn (Chaney, 1993; Marschner, 1995). Symptoms of Zn toxicity include chlorosis of leaves, inhibition of root elongation and often inhibition of photosynthesis (Marschner, 1995). High cytosolic Zn^{2+} concentrations interfere with cellular processes in most plants causing toxicity. Zn^{2+} concentrations are controlled through selective cation uptake, translocation to the shoot, and through compartmentalising Zn^{2+} within and between cells (Andrews, 2001; Gaither and Eide, 2001). Other symptoms of Zn toxicity include reduction in crop yields, stunted growth, reduction in chlorophyll synthesis and chloroplast degradation, which induces chlorosis and a disruption of potassium and magnesium uptake (Chaney, 1993).

The symptoms of toxicity that are observed in plants may be due to interactions at the cellular and molecular level. One mechanism for toxicity may be through the inhibition of enzyme activity. Two methods of enzyme inhibition are the binding of metals to the sulphhydryl group of proteins which may be either responsible for the catalytic action or structural integrity of the enzyme, or the metal may displace another essential element leading to symptoms of deficiency (Van Assche and Clijsters, 1990). Heavy metals are

known to inhibit chlorophyll biosynthesis through the binding to two key enzymes in this pathway, 5-aminolaevulinic acid (ALA) dehydratase and Protochlorophyllide reductase (Van Assche and Clijsters, 1990).

Some heavy metals such as iron and copper, due to unpaired electrons, can be responsible for catalysing oxygen reduction. Hydroxyl radicals (.OH) produced can cause biological damage and also may result in cell death. If this occurs in proximity to DNA the addition or removal of hydrogen ions to the DNA bases or DNA backbone may result (Brait and Lebrun, 1999). This same reaction involving, in particular, iron and oxygen may be responsible for lipid peroxidation. Therefore biological membranes rich in polyunsaturated fatty acids would be disposed to damage (Brait and Lebrun, 1999). Metals are capable of binding to the cell nucleus which results in mutagenesis via base changes, crosslinking of DNA and proteins, base pair mismatches and breaks in the DNA strands.

There are some plants however that can grow on heavy metal contaminated soils and not only survive but thrive there without showing any signs of toxicity. These plants are able to accumulate levels of zinc above $30 \text{ mg g}^{-1} \text{ d. wt.}$ (Hammond *et al.*, 2005; McGrath *et al.*, 2006). These plants also show the ability to actively seek out heavy metals in the soil and actively uptake them, accumulate them and traffic them to aerial parts (Clemens, 2001). The hyperaccumulation trait is an interesting one and has led to many people researching the plant to determine how this trait is controlled, why the plants actively uptake heavy metals and why the trait has evolved.

Studies which have previously looked at *Thlaspi caerulescens* and the hyperaccumulation trait have identified potential genes involved with the hyperaccumulation trait (Assunção *et al.*, 2004; Papoyan and Kochian 2004; Pence *et al.*, 2000)

The whole picture is still not clear despite extensive studies, which is why this research was carried out. One further question of interest that will be answered is how these plants can be of benefit to humans. Firstly the plants themselves and the areas they are commonly found in will be looked at.

1.1.4 Zinc tolerance vs zinc hyperaccumulation

Studies have confirmed that zinc tolerance and zinc hyperaccumulation are genetically independent characters (Macnair *et al.*, 1999). These authors describe how tolerance is a response to external Zn concentrations and is not affected by internal Zn levels within aerial plant parts. Tolerance involves the prevention of build up of toxic metals in sensitive areas within the cell (Hall, 2002). In comparison hyperaccumulation of Zn involves the translocation of Zn to the aerial parts from the roots. This conclusion was drawn following an experiment that involved a cross between the hyperaccumulating plant, *A. halleri* and a closely related non-hyperaccumulating plant, *A. petraea*. The F₂ generation that was produced was subjected to a series of tolerance experiments and it was deduced that the F₂ generation segregated for the two characteristics, tolerance and hyperaccumulation independently.

Plants have evolved ways to maintain the concentration of essential metals at a physiological level and to minimize any damage caused by non-essential metals through a series of mechanisms including; uptake, translocation and detoxification via chelators. A review of these tolerance mechanisms will be covered here.

1.1.4.1 Sequestering ions in root vacuoles

Current evidence suggests that the vacuole is the main storage site for metals in both yeast and plant cells. In yeast (*Saccharomyces cerevisiae*) Ni compartmentalization in the vacuole is achieved by pH gradient existing across the vacuolar membrane (Yang *et al.*, 2005). Studies in plants and yeast have identified several transporters involved in the sequestration of ions to the root vacuole. Included in this is the ATP-dependent, ABC transporter which has been localized to the tonoplast and is associated with Cd tolerance and was first discovered in a yeast mutant through the complementation with the *HMT1* gene (Clemens, 2000; Hall, 2002). Similarly a system involving the *HMT1* gene has been found in oat root cells where it is thought to transport Cd-phytochelatin complexes (Salt and Rauser, 1995; reviewed in Hall and Williams, 2003).

There is more evidence for the sequestration of Zn to the vacuole for example in meristematic cells of *Festuca rubra* where it was shown to have elevated vacuolation on application of external Zn. Likewise studies in barley showed that rapid vacuolar compartmentation was vital in managing the high levels of Zn (Brune *et al.*, 1995, reviewed in Hall, 2002). Further to this it was found

that despite the importance of vacuolar compartmentation to Cd and Zn, Ni was found in the cytosol not the vacuole.

Studies were carried out looking at the root tonoplast vesicles of two ecotypes of *Silene vulgaris*, one Zn tolerant, one Zn sensitive. When observed at elevated Zn levels, Zn transport into the vesicles was 2.5 fold higher in Zn tolerant ecotypes compared with Zn sensitive ecotypes. This suggests that the tonoplast plays an important role in Zn tolerance (Verkleij *et al.*, 1998; reviewed in Hall 2002). A separate study (Chardonens *et al.*, 1999; reviewed in Hall, 2002) looked at a genetic cross and concluded that the tonoplastic uptake system correlated with Zn tolerance. In more recent studies an *Arabidopsis* gene (ZAT) which shows similarities to the animal *ZnT* gene (Zn transporter gene- localized to the plasma membrane, which was isolated from rats and proposed to transport Zn out of cells), has been isolated. This gene was found to be constitutively expressed in plants however its expression was not elevated in the presence of Zn (van der Zaal *et al.*, 1999) However in transgenic plants where the gene was over expressed, Zn tolerance was elevated and accumulation was increased, therefore proposing that the Zn transporter is involved in Zn tolerance.

A further two vacuolar-located genes, *CAX1* and *CAX2* have been isolated from *Arabidopsis* and have been proposed to be H^+/Ca^{2+} exchangers (Hirschi *et al.*, 1996). Again evidence has been found for a Cd and Ca H^+ antiporter in oat root cells. No evidence has been found for a Ni/ H^+ antiporter or a

nucleotide-dependent Ni transporter which supports earlier evidence that Ni is stored elsewhere (Gries and Wagner, 1998; reviewed in Hall, 2002).

1.1.4.2 Mycorrhizas, rhizosphere bacteria

It is thought that the majority of plants have mycorrhiza when growing under natural conditions. They are beneficial in that they increase the surface area of the roots to increase the assimilation of nutrients (Khan *et al.*, 2000).

Mycorrhizal fungi including ectomycorrhizas have been known to alleviate the effects of heavy metal toxicity of plants including trees and shrubs. For example Marx and Artman (1979; reviewed in Jentschke and Godbold, 2000) showed that conifer seedling survival and growth on contaminated mine soil was greatly increased following inoculation of the roots with *Pisolithus tinctorius*. The mechanisms involved in this process are still unresolved and appear to be diverse and may be species and metal specific. For example *Pinus sylvestris* retained less Zn due to the ectomycorrhizal fungi *Paxillus involutus* containing higher levels of Zn. On the other hand the fungus *Thelephora terrestris* held less Zn and increased the Zn content of the host (Colpaert and Van Assche, 1992; reviewed in Hall, 2002). It is thought that the mechanisms undertaken by the fungi to be able to tolerate elevated heavy metal concentrations are similar to the cellular mechanisms exhibited by higher plants such as binding of the metals to extracellular materials or sequestration to the vacuole. It was noted that tolerance to Cu and Zn was achieved in *Pisolithus tinctorius* through the binding of Cu and Zn to extrahyphal slime, (Tam, 1995; reviewed in Hall, 2002). Comparatively

Paxillus involutus maintained Cd tolerance through the binding of Cd to the cell wall and compartmentalising the Cd in the vacuole (Blaudez *et al.*, 2000; reviewed in Hall, 2002).

The mycorrhizal fungi are able to assist in plant metal tolerance by restricting movement of heavy metals to plant host roots through the absorption of metals to the fungal hyphal sheath and absorption onto the external mycelium. Due to the hydrophobicity of the fungal sheath, access by the heavy metals to the apoplast is restricted (Jentschke and Godbould, 2000)

Arbuscular mycorrhizas have been linked, less frequently, to metal tolerance. For example Weissenhorn *et al.*, (1995; reviewed in Hall 2000) showed that arbuscular mycorrhizas were either responsible for reducing the heavy metal content of the plant or increased the metal uptake dependent on growth conditions.

In addition to mycorrhizal fungi, plants have associations in the soil with rhizobacteria. Links have been made that suggest rhizobacteria increase the ability of a plant to survive in the presence of elevated heavy metals in the soil (Burd *et al.*, 1998, 2000) It was found that *Kluyvera ascorbata* SUD 165, a metal-resistant bacteria, isolated from metal contaminated soils was able to affect germination and survival of plants grown in heavy metal containing soils. Plant seeds of rape (canola), Indian mustard and tomato were inoculated with the bacteria and grown on soil containing nickel, lead or zinc. The bacterium was able relieve symptoms of growth inhibition caused by the metals. Methods suggested to explain how these soil bacteriaa reduce the

effects of metal toxicity and promote plant growth include; nitrogen fixation, production of hormones such as auxin and cytokinin to enhance plant growth and the solubilization of minerals such as phosphorus.

1.1.4.3 Chelators

Chelators are responsible for the buffering of metal concentration within cells due to the lack of solubility of metals. Experiments have shown these chelators and chaperones account for the lack of “free” metal within cells, for example in yeast it was found that less than one free Cu atom was found within each yeast cell (Rae *et al.*, 1999; reviewed in Clemens, 2000). Chaperones are responsible for delivering metal ions to organelles and metal proteins. The main chelators involved in plant metal homeostasis are phytochelatins, metallothioneins, organic acids and amino acids.

1.1.4.3.1 Phytochelatins

Phytochelatins have been found in all plants that have been studied and in some fungi they have most frequently been related to cadmium (Cd). They are small metal binding peptides that are synthesized within minutes of exposure to metals or metalloids by the phytochelatin synthase enzyme. Metals which have been associated with phytochelatin complexes include, Cd, Ag, Cu and As (reviewed in Clemens, 2000). Investigations using *Arabidopsis* mutants have isolated a *CAD1* gene, the *cad1* mutant that was studied was sensitive to Cd and deficient in phytochelatins. Recent cloning has determined that the *CAD1* gene encodes a phytochelatin synthase gene. The gene has since been renamed *AtPCS1* and has been expressed in *E. coli*

which subsequently produces phytochelatins on exposure to metals (reviewed in Clemens, 2000).

Not all studies have agreed on phytochelatins' role in metal detoxification and tolerance (Hall, 2002) for example De Knecht et al., (1992; 1994, reviewed in Hall, 2002) concluded that Cd tolerance in *Silene vulgaris* was not due to a differential production of phytochelatins. They therefore may have other roles such as in sulphur metabolisms or anti-oxidants (Hall, 2002).

1.1.4.3.2 Metallothioneins

Metallothioneins are low molecular weight, cystine rich proteins which bind metal ions, namely Zn, Cu and Cd. Reports have shown Cu can induce metallothioneins and they have been suggested to play a role in metal detoxification in animals and fungi. Their role in plants is yet to be recognized (Hall, 2002).

Several studies have used *A. thaliana* genes (metallothioneins1- MT1 and MT2 expressed in *S. cerevisiae* to eliminate sensitivity to metals such as Cu. Also metallothioneins production has been induced in *Arabidopsis* on exposure to Cu but less so by Cd and Zn (Hall, 2002)

As yet their actual role is yet to be deduced, this function may vary for different metals, other suggested roles are detoxification of metals, in particular Cu, cytosolic Zn buffering, metal scavenging during leaf

senescence, secretion of metals via leaf trichomes, as antioxidants and in plasma membrane repair (Clemens, 2000; Hall, 2002).

1.1.3.4.3 Organic acids and amino acids

Other suggested ligands involved in metal detoxification are carboxylic acids and amino acids including malate, citrate and histidine. Reported experiments in this field include the induction of histidine (36-fold) on exposure to Ni in a Ni hyperaccumulator *Alyssum lesbiacum* found in xylem sap (Kramer, 1996; reviewed in Hall, 2002). Also applying histidine to non-accumulator plants increased their tolerance to Ni and increased translocation to the shoot. Conversely an experiment with *T. goesingense*, a Ni hyperaccumulator showed no link between histidine and Ni tolerance within its tissues (Persans *et al.*, 1999; reviewed in Hall, 2002).

Citric acid is thought to form complexes with Cd, Ni and Zn ions in hyperaccumulating plant species where it may be involved in metal transport through the xylem and sequestration in the vacuole. In addition malate has been suggested as a cytosolic Zn chelator in Zn hyperaccumulating plant species (Mathys, 1977; reviewed in Clemens, 2000).

1.2 Species that hyperaccumulate Zinc

1.2.1 Elevated Zn soil concentrations and Zn-tolerant plant species

It is thought that up to 25% of the world's soils may deter plant growth either due to natural causes or through man's interference (Macnair, 1993). Soil found around mine sites and near to corroded galvanised material such as

electricity pylons have been of interest to those studying Zn tolerance. These soils have a Zn concentration that would normally be toxic to most plant species, but many plants have been found to flourish there (Ernst *et al.*, 1992; Macnair, 1993). Plants possess a series of cellular mechanisms to detoxify Zn and thereby tolerate high tissue Zn concentrations.

Tolerant plants possess mechanisms for compartmentalising Zn, avoiding its build up in sensitive areas where toxic effects could occur. Such mechanisms of avoiding Zn stress include extracellular compartmentalising into mycorrhizas, particularly in trees and shrubs (Hall, 2002; Hutterman *et al.*, 1999; Jentschke and Goldbold, 2000; Marschner, 1995). Older leaves may be used as a way of compartmentalizing Zn to avoid its toxic effects on younger and more vulnerable parts of the plant. Other mechanisms include reducing Zn fluxes from root to shoot, increased organic acid production, vacuolar compartmentalisation and Zn efflux across the plasma membrane (Broadley *et al.*, 2007; Ernst *et al.*, 1992; Hall, 2002).

1.2.2 Hyperaccumulating plant species

Although this thesis focuses on Zn and Zn hyperaccumulation, a broader account of the hyperaccumulation trait will be considered including other plants species and other metals. This is important to be considered here as accumulation pathways of different heavy metals may be linked within a species and have implications with evolution.

There are upward of 400 reported plant species from families such as the Asteraceae (including sunflowers and daisies), Brassicaceae (includes *T. caerulescens* and the cabbage family), Caryophyllaceae (includes carnations), Poaceae (includes grasses), Violaceae (including violets and pansies) and Fabaceae (including peas and beans) that are able to tolerate high levels of heavy metals in the soil and in their shoots. Of these families the Brassicaceae family is most highly studied, containing 87 classified hyperaccumulators. The term hyperaccumulator was first termed by Brooks *et al.*, 1977 (reviewed in Milner and Kochian, 2008; Peer *et al.*, 2003) to plants found to tolerate and accumulate heavy metals approximately 100 times higher than nonaccumulator species (accumulate greater than $1000 \mu\text{g g}^{-1}$ Ni, $10\,000 \mu\text{g g}^{-1}$ Zn or Mn, $1000 \mu\text{g g}^{-1}$ Co or Cu and $100 \mu\text{g g}^{-1}$ Cd when grown in native soils). Despite this plants have been identified as possessing this trait since 1885 (see 1.2.2.3) (Peer *et al.*, 2003). Plant hyperaccumulators have been recorded hyperaccumulating Nickel (Ni), Zn, Cadmium (Cd), Lead (Pb), Copper (Cu), Arsenic (As), Cobalt (Co) and Manganese (Mn) (Baker & Brooks, 1989; Brooks, 1994; Ma *et al.*, 2001).

Within the Brassicaceae family two species have received the largest attention above all hyperaccumulator plant species; these are *Thlaspi caerulescens* and *Arabidopsis halleri*. These plant species have been studied mainly due to their ability to accumulate the metals zinc (Zn), Cadmium (Cd) and Nickel (Ni) with reported findings of levels exceeding $10,000 \text{ mg Zn kg}^{-1}$ d. wt., $100 \text{ mg Cd kg}^{-1}$ d. wt. and $1000 \text{ mg Ni kg}^{-1}$ d. wt. (Milner and Kochian, 2008).

1.2.2.1 Ni Hyperaccumulation

The majority of the 400 plant hyperaccumulators are Ni accumulators (317 species) (Reviewed in Assunção *et al.*, 2003; Reeves and Baker, 2000). Over half of all plant Ni hyperaccumulators belong to the family Brassicaceae the rest are found within 21 other families. Of these some of the most studied include *Thlaspi caerulescens*, *Alyssum bertolonii*, *Alyssum lesbiacum* and *Thlaspi goesingense* (Küpper *et al.*, 2001). A Ni hyperaccumulator has been defined, by Reeves, (1992) as a “species for which at least one wild-collected specimen has been shown to contain at least 1000 $\mu\text{g Ni g}^{-1}$ in aboveground tissues (on a dry mass basis)”.

Plants typically require only small amounts of nickel to remain healthy (1.7 nmol g^{-1} Ni or less d. wt.). Toxicity to Ni can be observed at levels between 0.19 and 0.85 $\mu\text{mol g}^{-1}$ Ni d. wt. Symptoms of toxicity include inhibition of root elongation and interveinal chlorosis, possibly due to the Ni interrupting normal chlorophyll production (Krämer *et al.*, 1997). The levels of Ni found in plant species known to hyperaccumulate Ni have been recorded to be higher than 1000 parts per million (17 pmol g^{-1}) in fresh weight and 650 pmol g^{-1} Ni in dry weight (Baker and Brooks, 1989; Reeves, 1992; reviewed in Krämer *et al.*, 1997) which compared to non-hyperaccumulating plants species is considerably higher (normal range 5 nmol g^{-1} - 1.7 pmol g^{-1}).

These notably high levels of Ni within the plant possibly protect plants from insect herbivory or fungal and bacterial pathogens (Martens and Boyd, 2002). For the plants to have this large concentration of heavy metal within the plant

suggest some detoxification of the metal within the plant system. Low molecular weight chelators, such as citrine, have been suggested to play a role in metal detoxification (reviewed in Krämer *et al.*, 1997).

Studies of Ni hyperaccumulators, namely *T. goesingense* have revealed that hypertolerance of Ni is due to the storage of Ni²⁺-organic acid complex within the vacuole. Besides vacuolar compartmentalization Ni is also accumulated outside of the vacuole (Krämer *et al.* 1997; Krämer *et al.* 2000; Küpper *et al.*, 2001; Persans *et al.*, 2001).

A more recent study that is relevant to this thesis, was the study of the hyperaccumulator of Zn and Ni, *T. caerulescens* (Vacchina *et al.*, 2003). It was reported that a Ni²⁺ complex with the metal chelate nicotianamine, which has been suggested plays an important role in detoxification of extravacuolar Ni in plant hyperaccumulators. Evidence to support this lies with the constitutive overproduction of nicotianamine and the corresponding enzyme nicotianamine synthase within *T. caerulescens* and *A. halleri* (reported by Vacchina *et al.*, 2003; Becher *et al.*, 2004; Weber *et al.*, 2004). Krämer *et al.*, (1996) report on another metal chelator, histidine, which possibly forms a complex with Ni to aid transport in the xylem. The observed links between species and between types of metal suggests that the exclusive study of uptake of one metal is not useful.

1.2.2.2 Cd Hyperaccumulation

Cadmium (Cd) is not an essential or beneficial metal to plants however it can be found in plant roots and to a lesser extent in shoots. Cadmium is taken up by the roots before being transported to the leaves and seeds, although uptake is greater at low soil pH and varies between plant species (Assunção *et al.*, 2003b). Elevated Cd levels (10-200 mg kg⁻¹, sometimes even higher) are generally found in soils containing waste materials from the mining of ZnS and other Zn ores, but may also occur in soils treated with industrial wastes or Cd-rich phosphate fertilisers. Cadmium concentrations in plants are generally <3 mg kg⁻¹ but may reach 20 mg kg⁻¹ or more in plants growing on Cd-enriched soils. Tissue concentrations >100 mg kg⁻¹ may be regarded as exceptional, even on Cd contaminated sites. *Sedum alfredii*, a known Cd hyperaccumulator, has been recorded to accumulate between 3317 and 4512 mg kg⁻¹ d. wt. in the stem and leaf respectively when grown on 400 µM Cd nutrient solution without showing any sign of phytotoxicity (Ni and Wei, 2003). This species has the benefit over *T. caerulescens* because of its characteristic fast growth, large biomass, it reproduces asexually and can grow up to 40 cm in height. If the environmental conditions are suitable it can propagate 3–4 times in a year (Yang *et al.*, 2000). There are currently only four species of Cd hyperaccumulators, all of which also accumulate Zn, which possibly suggests a common genetic basis. Several studies have highlighted a correlation between Zn accumulation variation and Cd variation among populations of *T. caerulescens* (reviewed in Verbruggen *et al.*, 2009).

Cadmium is deemed to be a major environmental pollutant that bioaccumulates in the food chain, entering through plant uptake from the soil. Therefore it is important that the genes responsible for this trait are known in order to control this.

1.2.2.3 Zn Hyperaccumulation

Possibly the first recording of a heavy metal tolerant plant accumulating exceptional high levels of Zn in its aerial parts was in the late 19th Century, in the calmine flora or “galmei” (*Thlaspi calmainare* now *Thlaspi caerulescens*), found on the border of Germany and Belgium in the Aachen region (Forchhammer, 1855; reviewed in Reeves and Baker, 2000; Assunção *et al.*, 2003a). Soon after this, *Thlaspi alpestre* L. was reported as having a shoot Zn concentration of >1% d. wt. (Sachs 1865 reviewed in Reeves and Baker, 2000). Following these discoveries, Zn hyperaccumulation was defined as the presence of >10,000 µg Zn g⁻¹ d. wt. in a plant’s aerial parts when growing in its natural environment (Baker and Brooks, 1989; reviewed in Broadley *et al.*, 2007). At present there are twelve known Zn hyperaccumulators within the Brassicaceae family, 11 of which are within the genera *Thlaspi* and one belongs to the genus *Arabidopsis* (*Arabidopsis halleri*) (Pollard *et al.*, 2002; Assunção *et al.*, 2003). Table 1.2 gives a list of Zn hyperaccumulators that have been reported in the ecological literature as containing >0.3 % Zn as d. wt. They have been discussed as a potential means of transferring the characteristic to crop species for use in phytoremediation, phytomining and crop biofortification (Baker and Brooks, 1989; reviewed in Broadley *et al.*,

2007; Chaney, 1993; Gueriot & Salt, 2001; Krämer, 2005; Macnair, 2003; Salt *et al.*, 1998).

Table 1.2 Plant species whose [Zn]_{shoot} has been observed to exceed 0.3% d. wt. (unless stated) where grown under natural conditions

Species ^a	Potential synonyms ^a	Family (Order)	Locality	Maximum [Zn] _{shoot} (% d.wt observed).	References and Comments
<i>Acer pseudoplatanus</i> L.	–	<i>Sapindaceae</i> (<i>Sapindales</i>)	UK	0.35	Johnston & Proctor (1977)
<i>Arenaria patula</i> Michx.	<i>Minuartia patula</i> (Michx.) Mattf.	<i>Caryophyllaceae</i> (<i>Caryophyllales</i>)	USA	1.31	Brooks (1998). Uncertain record (Macnair, 2003; R. D. Reeves, pers. comm.)
<i>Arabidopsis arenosa</i> (L.) Lawalrée	<i>Cardaminopsis arenosa</i> (L.) Hayek	<i>Brassicaceae</i> (<i>Brassicales</i>)	France	0.52	Reeves <i>et al.</i> , (2001)
<i>Arabidopsis halleri</i> (L.) O'Kane & Al-Shehbaz	<i>Arabis gemmifera</i> Makino, <i>Cardaminopsis halleri</i> (L.) Hayek, <i>C. ovirensis</i> (Wulf.) O. Schwarz	<i>Brassicaceae</i>	France	2.07	R. D. Reeves, pers. comm.
<i>Arabidopsis thaliana</i> (L.) Heynh.	<i>Arabis thaliana</i> L., <i>Sisymbrium thalianum</i> (L.) J. Gay & Monnard, <i>Stenophragma thalianum</i> (L.) Čelak.	<i>Brassicaceae</i>	USA	2.67	Reeves (1988)
<i>Biscutella laevigata</i> L.	<i>Biscutella alsatica</i> Jord., <i>B. austriaca</i> Jord., <i>B. longifolia</i> Vill., <i>B. lucida</i> Balb. ex	<i>Brassicaceae</i>	France	0.41	R. D. Reeves, pers. comm.

		DC., <i>B. sempervirens</i> L., <i>B. varia</i> Dumort., <i>B. variegata</i> Boiss. & Reut., <i>B. vincentina</i> (Samp.) Rothm. ex Guinea				
<i>Cochlearia pyrenaica</i> DC.	<i>Cochlearia officinalis</i> L. subsp. <i>pyrenaica</i> (DC.) Rouy & Foucaud	<i>Brassicaceae</i>	UK	0.53	Reeves (1988)	
<i>Dianthus</i> sp.	–	<i>Caryophyllaceae</i>	France	0.49	R. D. Reeves, pers. comm.	
<i>Dichapetalum gelonioides</i> (Roxb.) Engl.	<i>Chaillietia gelonioides</i> (Roxb.) J. D. Hook., <i>Dichapetalum howii</i> Merrill & Chun., <i>Moacurra gelonioides</i> Roxb.	<i>Dichapetalaceae</i> (<i>Malpighiales</i>)	Indonesia, Malaysia, Philippines	3.00	Reeves & Baker (2000)	
<i>Galium mollugo</i> L.	<i>Galium album</i> Mill., <i>G. cinereum</i> All., <i>G.</i> <i>corrudifolium</i> Vill., <i>G. elatum</i> Thuill., <i>G.</i> <i>insubricum</i> Gaudin, <i>G. kerneranum</i> Klokov, <i>G. lucidum</i> All., <i>G. neglectum</i> Le Gall ex Gren., <i>G. tyrolense</i> Willd.	<i>Rubiaceae</i> (<i>Gentianales</i>)	France	0.30	Reeves <i>et al.</i> , (2001)	
<i>Gomphrena canescens</i> R. Br.	–	<i>Amaranthaceae</i> (<i>Caryophyllales</i>)	Australia	0.90	Nicolls <i>et al.</i> , (1965)	
<i>Haumaniastrum katangense</i> (S. Moore) Duvign. & Plancke.	–	<i>Lamiaceae</i> (<i>Lamiales</i>)	D. R. of the Congo	1.98	Brooks (1998). Uncertain record (Paton & Brooks, 1996; Macnair, 2003)	
<i>Minuartia verna</i> (L.) Hiern	<i>Alsine verna</i> (L.) Wahlenb., <i>Arenaria</i> <i>verna</i> L., <i>Minuartia caespitosa</i> (Ehrh.) Degen	<i>Caryophyllaceae</i>	Yugoslavia	1.14	Various studies cited in Reeves & Baker (2000)	

<i>Noccaea boeotica</i> F. K. Mey.	–	<i>Brassicaceae</i>	Greece	0.31	R. D. Reeves, pers. comm.
<i>Noccaea eburneosa</i> F. K. Mey.	<i>Noccaea salisii</i> (Brugger) F. K. Mey, <i>Thlaspi salisii</i> Brugger	<i>Brassicaceae</i>	Switzerland	1.05	Reeves & Brooks (1983), taxonomic status uncertain (R. D. Reeves, pers. comm.)
<i>Polycarpaea synandra</i> F. Muell.	–	<i>Caryophyllaceae</i>	Australia	0.70	Reeves & Baker (2000)
<i>Rumex acetosa</i> L.	–	<i>Polygonaceae</i> (<i>Caryophyllales</i>)	UK	1.10	Johnston & Proctor (1977)
<i>Rumex acetosella</i> L.	<i>Acetosella vulgaris</i> Fourr., <i>Rumex acetoselloides</i> Balansa, <i>R. angiocarpus</i> Murb., <i>R. fasciobus</i> Klokov, <i>R. multifidus</i> L., <i>R. pyrenaicus</i> Pourr. ex Lapeyr., <i>R. salicifolius</i> auct., <i>R. tenuifolius</i> (Wallr.) Á. Löve	<i>Polygonaceae</i>	France	0.31	Reeves <i>et al.</i> , (2001)
<i>Sedum alfredii</i> Hance	–	<i>Crassulaceae</i> (<i>Saxifragales</i>)	China	0.50	Yang <i>et al.</i> , (2002)
<i>Silene vulgaris</i> (Moench) Garcke	<i>Silene angustifolia</i> Mill., <i>S. campanulata</i> Saut., <i>S. cucubalus</i> Wibel, <i>S. inflata</i> Sm., <i>S. latifolia</i> (Mill.) Britten & Rendle, non Poir., <i>S. tenoreana</i> Colla, <i>S. venosa</i> Asch., <i>S. vulgaris</i> (Moench) Garck	<i>Caryophyllaceae</i>	USA	0.47	Brooks (1998)
<i>Thlaspi apterum</i> Velen.	<i>Noccaea aptera</i> (Velen.) F. K. Mey.	<i>Brassicaceae</i>	Bulgaria	0.31	Reeves & Brooks (1983). Uncertain

					record (R. D. Reeves, pers. comm.)
<i>Thlaspi alpinum</i> Crantz	<i>Noccaea alpestris</i> (Jacq.) Kerguélen, <i>N. sylvia</i> (Gaudin) F. K. Mey., <i>T. sylvium</i> Gaudin	<i>Brassicaceae</i>	France	0.54	Reeves <i>et al.</i> , (2001)
<i>Thlaspi brachypetalum</i> Jord.	<i>Noccaea brachypetala</i> (Jord.) F. K. Mey.	<i>Brassicaceae</i>	France	1.53	Reeves & Brooks (1983)
<i>Thlaspi brevistylum</i> (DC.) Mutel.	<i>Noccaea brevistyla</i> Steud.	<i>Brassicaceae</i>	Corsica	0.31	Taylor (2004)
<i>Thlaspi bulbosum</i> Spruner ex Boiss.	<i>Raparia bulbosa</i> (Boiss.) F. K. Mey.	<i>Brassicaceae</i>	Greece	1.05	Brooks (1998)
<i>Thlaspi caerulescens</i> J. & C. Presl.	<i>Noccaea arenaria</i> (J. E. Duby) F. K. Mey., <i>N. caerulescens</i> (J. & C. Presl) F. K. Mey., <i>N. occitanica</i> (Jord.) F. K. Mey., <i>Thlaspi alpestre</i> L., <i>T. arenarium</i> Jord., <i>T. caerulescens</i> subsp. <i>caerulescens</i> , <i>T. caerulescens</i> subsp. <i>calaminare</i> (Lej.) Lej. & Court, <i>T. caerulescens</i> subsp. <i>occitanicum</i> (Jord.) M. Lánz, <i>T. caerulescens</i> subsp. <i>tatrense</i> (Zapał.) Dvořáková, <i>T. gaudinianum</i> Jord., <i>T. huteri</i> A. Kern., <i>T. mureti</i> Gremli, <i>T. occitanicum</i> Jord., <i>T. pratulorum</i> Gand., <i>T. rhaeticum</i> Jord., <i>T. salisii</i> Brugger, <i>T. suecicum</i> Jord., <i>T. sylvestre</i> Jord., <i>T.</i>	<i>Brassicaceae</i>	W. and C. Europe	4.37	Various studies (Reeves & Baker, 2000). Also a Cd hyperaccumulator

	<i>tatrense</i> Zapal., <i>T. virgatum</i> Gren. & Godr., <i>T. villarsianum</i> Jord., <i>T. vogesiacum</i> Jord., <i>T. vulcanorum</i> Lamotte				
<i>Thlaspi cepaeifolium</i> (Wulfen) W. D. J. Koch	<i>Noccaea cepaeifolia</i> (Wulfen) Rchb., <i>N. limosellifolia</i> (Burnat) F. K. Mey., <i>N. rotundifolia</i> (L.) Moench, <i>Thlaspi cepaeifolium</i> subsp. <i>rotundifolium</i> (L.) Greuter & Burdet, <i>T. limosellifolium</i> Reut. ex Rouy & Fouc., <i>T. rotundifolium</i> (L.) Gaudin subsp. <i>cepaefolium</i> (Wulfen) Rouy & Fouc.	<i>Brassicaceae</i>	Italy	2.10	Various studies (Reeves & Baker, 2000)
<i>Thlaspi epirotum</i> Hal.	<i>Noccaea epirota</i> (Hal.) F. K. Mey.	<i>Brassicaceae</i>	Greece	<0.30	Reeves & Brooks (1983)
<i>Thlaspi goesingense</i> Hal.	<i>Noccaea goesingensis</i> (Hal.) F. K. Mey., <i>Thlaspi tymphaeum</i> Hausskn., <i>T. umbrosum</i> Waisb.	<i>Brassicaceae</i>	Austria	0.38	Reeves & Baker (1984)
<i>Thlaspi graecum</i> Jord.	<i>Noccaea graeca</i> (Jord.) F. K. Mey., <i>Thlaspi taygeteum</i> Boiss.	<i>Brassicaceae</i>	Greece	<0.30	Reeves & Brooks (1983)
<i>Thlaspi kovatsii</i> Heuff.	<i>Noccaea kovatsii</i> (Heuffel) F. K. Mey., <i>Thlaspi affine</i> Schott & Kotschy, <i>T. avalanum</i> Panč., <i>T. jankae</i> Kern., <i>T. trojagense</i> Zapal.	<i>Brassicaceae</i>	Bulgaria	0.49	Reeves & Brooks (1983)
<i>Thlaspi magellanicum</i> Pers.	<i>Noccaea magellanica</i> (Pers.) J. Holub	<i>Brassicaceae</i>	Argentina	0.39	Reeves (1988)

<i>Thlaspi montanum</i> L.	<i>Noccaea alpestris</i> (Jacq.) Kerguélen subsp. <i>sylvium</i> (Gaudin), <i>N. montana</i> (L.) F. K. Mey., <i>Thlaspi lotharingum</i> Jord.	<i>Brassicaceae</i>	USA	0.43	Hobbs & Streit (1986)
<i>Thlaspi ochroleucum</i> Boiss. & Heldr.	<i>Noccaea lutescens</i> (Velen.) F. K. Mey., <i>N. ochroleuca</i> (Boiss. & Heldr.) F. K. Mey., <i>N. phrygia</i> (Bornm.) F. K. Mey., <i>N. rhodopensis</i> F. K. Mey., <i>N. versicolor</i> (Stoj. & Kitanov) F. K. Mey., <i>Thlaspi balcanicum</i> Janka, <i>T. heterochroum</i> Boiss., <i>T. lutescens</i> Velen., <i>T. phrygium</i> Bornm.	<i>Brassicaceae</i>	Turkey	0.63	Reeves & Brooks (1983), Reeves (1988)
<i>Thlaspi parviflorum</i> A. Nels.	<i>Noccaea parviflora</i> (A. Nels.) Holub	<i>Brassicaceae</i>	USA	0.31	Reeves <i>et al.</i> , (1983)
<i>Thlaspi pindicum</i> Hausskn.	<i>Noccaea pindica</i> (Hausskn.) J. Holub, <i>N. tymphaea</i> (Hausskn.) F. K. Mey., <i>Thlaspi tymphaeum</i> Hausskn.	<i>Brassicaceae</i>	Greece	<0.10	Taylor & Macnair, 2006. Note, plants collected from serpentine soils with low [Zn] _{soil} . [Zn] _{shoot} >1.00% d. wt. observed under laboratory conditions.
<i>Thlaspi praecox</i> Wulf.	<i>Noccaea praecox</i> (Wulf.) F. K. Mey, <i>Thlaspi affine</i> Schott & Kotschy ex Bioss.	<i>Brassicaceae</i>	Bulgaria	2.10	Brooks (1998). Cd hyperaccumulator (Vogel-Mikuš <i>et al.</i> , 2005)
<i>Thlaspi stenopterum</i> Boiss. & Reut.	<i>Noccaea stenoptera</i> (Boiss. & Reut.) F. K. Mey.	<i>Brassicaceae</i>	Spain	1.60	Brooks (1998)

<i>Thlaspi viridisepalum</i> (Podp.) Greuter & Burdet	<i>Noccaea viridisepala</i> (Podp.) F. K. Mey.	<i>Brassicaceae</i>	Bulgaria	0.63	Reeves & Brooks (1983)
<i>Viola calaminaria</i> (Gingins) Lej.	<i>Viola tricolor</i> L.	<i>Violaceae</i> (<i>Malpighiales</i>)	Germany	1.00	Brooks (1998). Note, uncertain record (Macnair, 2003; R. D. Reeves, pers. comm.)

^a Nomenclature and potential synonyms compiled from (i) original data sources, (ii) Flora Europaea (digital online edition; <http://rbg-web2.rbge.org.uk/FE/fe.html>), (iii) <http://www.diversityoflife.org/>, (iv) USDA, ARS, National Genetic Resources Program. *Germplasm Resources Information Network - (GRIN)* [Online Database]. National Germplasm Resources Laboratory, Beltsville, Maryland, <http://www.ars-grin.gov/cgi-bin/npgs/html/taxgenform.pl>, (v) Muséum national d'Histoire naturelle [Ed]. 2003-2006 . Inventaire national du Patrimoine naturel, <http://inpn.mnhn.fr.>, (vi) CWRIS PGR Forum Crop Wild Relative Information System, <http://www.pgrforum.org/cwriscwrisc.asp?fact=426656>.

Thlaspi caerulescens J. & C. Presl is by far the most studied Zn hyperaccumulator to date (Milner and Kochian 2008). *T. caerulescens* is a short-lived, non-mycorrhizal perennial plant that can be found on calamine (containing high Zn, Pb and sometimes Cd concentrations), serpentine (containing high Co, Cr, Fe, Mg and Ni concentrations) and non-mineral soils. *T. caerulescens* is highly tolerant of Zn and can accumulate up to 30 mg Zn g⁻¹ shoot dry weight without showing toxicity symptoms when grown experimentally under hydroponic systems (Broadley *et al.*, 2007).

1.2.3 Hyperaccumulators; environmental and sociological impacts

Hyperaccumulator plant species make an interesting topic for study due to their potential involvement in phytoremediation of soils. Contaminated soils are increasingly becoming a health risk to humans and also large areas are deemed useless agronomically through excessive Zinc in this ever-increasing period of farmable land shortage. Hyperaccumulator plants or their genes may offer an economical and bio-friendly method for cleaning contaminated land when a better understanding of the plants physiological and molecular genetics has been gained (Assunção *et al.*, 2003a; Krämer, 2005; Salt *et al.*, 1998; Whiting *et al.*, 2003). There are at least two strategies for phytoremediation of soils: phytostabilisation and phytoextraction. The former employs tolerant vegetation to cover contaminated land to stabilise the soil from erosion by wind and rain. Plants that would be employed must develop a good rooting system, give good soil cover and preferably contain contaminants within the rhizosphere. Phytoextraction is more favoured, but is also more technical. These plants have to concentrate the heavy metal in their aerial parts so they can be harvested, dried, or incinerated. The waste must be disposed of

safely or smelted down. The cultivated crop must be able to accumulate large amounts of Zn in comparison to the soil concentrations in order for it to be economically feasible. This is where transgenic approaches are favourable to transfer the trait to plants with a high biomass, such as trees. However the transformation of such species is less advanced due to time scales in their growth. (Krämer, 2005). Further analysis of genes responsible for Zn accumulation will enable genetic engineering of fast growing crop species for phytoremediation purposes. Phytoremediation technologies have been successfully used in the removal of arsenic from soils. Naturally selected ferns that hyperaccumulate arsenic have been employed and accumulate arsenic at very high levels in their upper tissues (Gonzaga *et al.*, 2006; Kertulis-Tartar *et al.*, 2006; Ma *et al.*, 2001).

More recently, hyperaccumulators have been highlighted as a potential source to increase the nutritional value of food for those people living on a predominately vegetarian diet, this is known as biofortification. The human diet requires at least 22 elements to fulfil its metabolic role (White and Broadley, 2009). Anything below the required amount can lead to poor health and sickness, particularly in children where a balanced diet is essential for growth and development. It is recommended that male adults take in 15 mg Zn a day and adult females 12 mg (FAQ/WHO, 2000; reviewed in Welch and Graham, 2004). The preferred delivery of these micronutrients is directly from agricultural products. However, in developing nations agricultural systems cannot always support this. It is estimated that over 1.5 billion people world wide currently suffer from zinc deficiency. Indeed, deficiencies in Zn, Fe (Iron) and vitamin A account for almost two thirds of childhood deaths throughout the

world (Welch and Graham, 2004; White and Broadley, 2005; White and Broadley, 2009).

The development of micronutrient-enriched crop plants can be achieved in one of two ways, either by traditional plant breeding or through plant genetic manipulation. The latter technique, by far harbours the most powerful means of introducing micronutrients to edible plants and therefore could greatly benefit many susceptible people (Assunção *et al.*, 2003a; Welch and Graham, 2004; White and Broadley, 2005; White and Broadley, 2009). Examples of biofortified crops include “golden rice” (rice grains that are engineered to contain higher than average levels of β -carotene, a precursor to vitamin A) (Ye *et al.*, 2000) and high ferritin-Fe rice grains (Lucca *et al.*, 2001). By genetically altering crop plants with genes responsible for Zn uptake, plants may be created that accumulate higher levels of Zn, which may be used to “treat” people affected by Zn deficiency due to consuming a predominantly vegetative diet (Welch and Graham, 2004; White and Broadley, 2005; White and Broadley, 2009).

1.3 The selective advantages of the hyperaccumulation trait

The selective advantage of the plant heavy metal hyperaccumulator trait is yet unknown however it is thought that Zn hyperaccumulation has evolved probably only three times in angiosperms (Macnair, 2003; Broadley *et al.*, 2007). Taxonomic studies of the genus *Thlaspi* L. have determined that it is likely that its background is polyphyletic (derived from more than one ancestral type), and through analysis of seed coat and sequence data has divided the genus into several genera including *Thlaspi* s.s., *Vania* and a clade containing *Thlaspiceras*, *Noccaea*, *Raparia*,

Microthlaspi and *Neurotropis*. The hyperaccumulation trait is most commonly a feature of *Noccaea* and *Raparia* (Taylor, 2004; reviewed in Broadley 2007). The *Thlaspiceras* genus does however contain hypertolerant species (e.g. *Thlaspiceras oxyceras*), the more distantly related genera *Microthlaspi* and *Neurotropis* contain only non-Zn hypertolerant species. This therefore suggests that the trait most likely evolved at the base of the *Noccaea/Raparia* clade or less likely at the base of the *Noccaea/Raparia/Thlaspiceras* clade which would involve a reversion to low $[Zn]_{shoot}$ trait in *Thlaspiceras*. It has been suggested that Zn hyperaccumulation may actually be a modification to the Ni hyperaccumulation trait as this has also been shown to have evolved at the base of the *Noccaea/Raparia* clade. As there are more Ni hyperaccumulators than Zn, this and the previously discussed occurrences suggest that Zn hyperaccumulation has evolved more recently at two evolutionary events within *Brassicaceae* and isolated events elsewhere amongst the angiosperms (possibly in *Sedum* L.) (Macnair, 2003; Taylor, 2004; reviewed in Broadley 2007).

Five hypotheses have been summarized for the potential reasons behind the selective advantages of the trait (Boyd; 2007; Boyd and Martens, 1992) which include (1) a method of increasing metal tolerance, (2) metal based allelopathy, (3) drought resistance, (4) cation uptake which has been termed “inadvertent uptake” and (5) resistance to pathogens and herbivory. (reviewed in Pollard *et al.*, 2002).

It has been proposed that metal hyperaccumulation evolved as an adaptive trait allowing plants to develop on niche areas. The current opinion is that the trait evolved independently in different species and genera. The most plausible reason for evolution of the trait to date was suggested by Boyd and Martens, (1992)

(reviewed by Assunção *et al.*, 2003a) who hypothesized that the trait evolved as a means to reduce damage caused by insect herbivory and parasitism. Many studies into this theory have been carried out and produced data that supports the hypothesis (Assunção *et al.*, 2003a; Jiang *et al.*, 2005; Pollard and Baker, 1997).

There is more supporting evidence for the hypothesis that the selective advantage for this trait is that of defence against herbivory and pathogens, termed the “defence hypothesis” (Boyd 2007) maybe because more studies have been carried out in this area. Experiments have concentrated on the feeding habits of herbivores of various species on metal hyperaccumulators. The aims of the experiments have been to determine if heavy metal concentrations at toxic levels could provide a deterrent to pathogens and herbivores (Boyd and Martens, 1992; Pollard and Baker, 1997). It was found that herbivores such as locusts, slugs and caterpillars may have a preference for *Thlaspi caerulescens* leaves low in Zn. However for *Arabidosis halleri* snails showed no preference for leaves of varying Zn concentrations. Therefore the hypothesis of the evolution of hyperaccumulation as a deterrent against herbivory may well be different depending on the metal accumulator species (Huitson and Macnair, 2003; Pollard and Baker, 1997). Boyd (2007) reports on the 34 studies carried out over a period of 13 years and includes defence against herbivory and pathogens shown by hyperaccumulated As, Cd, Ni, Se and Zn. The plant taxa studied have been limited along with the natural enemies included in the studies. Half of these studies looked at Ni accumulation and used indicators such as survival and growth as measures of amount of insect herbivory. The effect of Zn on plants’ defence against insects was covered by 8 studies and of these three tests showed that levels of Zn above the threshold for hyperaccumulation ($10,000 \text{ mg kg}^{-1}$)

defended the test plant and three failed to defend the plant against folivores. Two of the three positive studies report that the plant tested was *T. caerulescens* and focused on leaf chewing/scraping insects. Studies involved in the accumulation and hyperaccumulation of Se showed defence effects in 12 out of 14 tests. Three tests confirmed Cd defence against herbivory and arsenic has only one reported study which confirmed it contributed to defence against herbivory.

In order to understand the basis of the evolution of this trait it may be of interest to study variation within populations as it is at this level that natural selection may act to evolve the hyperaccumulation trait; if for example, plants that are able to accumulate higher levels of metal are more reproductively successful. However little research has been done in this area; focus has been given to sib families, families that share at least one parent. This can be achieved by collecting seeds from individual maternal plants and has been used to show significant differences between sib families of *T. caerulescens* on zinc concentrations in two independent studies suggesting genetic variation in ability to hyperaccumulate Zn (Meerts and Van Isacker, 1997; reviewed in Pollard *et al.*, 2002; Pollard and Baker, 1996). Similarly variation has been observed for both Zn and Cd hyperaccumulation and Zn hyperaccumulation in *A. halleri* (Escarré *et al.*, 2000; Macnair, 2002; reviewed in Pollard *et al.*, 2002). Heavy metal accumulation in *Thlaspi* spp. has been associated with limestone and serpentine soils, which are generally found to be low in nutrients with the exception of calcium and magnesium in limestone and serpentine soils respectively. Therefore this has led to the hypothesis that hyperaccumulation has resulted more from “inadvertent uptake” on nutrient poor soil instead of a specific selection of the zinc (Macnair, 2003).

1.4 Mechanisms of hyperaccumulation

The molecular mechanisms that are responsible for the traits involved in hyperaccumulation are largely unknown. However it is known that hyperaccumulators of Zn exhibit several adaptations that contribute to this trait. *Thlaspi caerulescens* has been noted to express three features of Zn homeostasis that have been linked to its ability to hyperaccumulate Zn, (i) an unusually high active mechanism of Zn uptake and translocation, (ii) the synthesis of organic acids that may help in detoxification and (iii) the compartmentalising of Zn to epidermal cell vacuoles and cell walls of shoot tissues (Broadley *et al.*, 2007).

1.4.1 Zincophilic root foraging

Hyperaccumulators show zincophilic root foraging. When grown in a natural environment, hyperaccumulators show root proliferation in areas of higher zinc concentrations (Haines 2002; Whiting *et al.*, 2000). Whiting *et al.*, (2000) concluded that *T. caerulescens* did exhibit root responses to localised Zn enrichment i.e. a high root to shoot mass ratio and long root hairs. However it was not possible to confirm that the roots were foraging for Zn. *T. caerulescens* has been reported to exhibit such root foraging when grown in industrially contaminated, heterogeneous soils containing patches of high Zn and/or Cd concentrations (Schwartz *et al.*, 1999). Similar studies carried out in other species have shown that active root foraging occurs for the nutrients phosphorus and nitrogen by way of increased root growth and root branching in high nutrient patches of soil (Hodge, 2004). Of particular interest has been the isolation of a gene (*ANR1*) thought to be responsible for increased lateral root proliferation in localised patches of nitrate (NO_3^-) (Zhang and Forde, 1998). Not all accessions of *T. caerulescens* possess the root foraging trait

and the possession of this trait did not correlate with total Zn accumulation (Haines, 2002). Indeed, the accession containing highest tissue Zn concentrations did not possess the Zn root foraging trait. Thus, it is clear that zincophilic root foraging is not a prerequisite for Zn hyperaccumulation. Further studies on metal tolerant plants have suggested that root foraging may be associated with tolerance rather than accumulation, since the most tolerant species possess the trait (Haines, 2002; Whiting *et al.*, 2000). At present a clear explanation as to why the plants exhibit this mechanism is unavailable (Assunção *et al.*, 2003a; Haines, 2002).

1.4.2 Zn uptake, efflux and translocation

Zinc enters the plant as the Zn^{2+} ion, entering the root cell wall free space by diffusion. The movement across the plasma membrane is via transport proteins, an active process. At present, there is evidence that members of least six families of transport proteins can transport Zn. These include ZIPs (Zrt (Zinc regulated protein) and Irt (Iron regulated protein) related proteins, CDFs (Cation Diffusion Facilitator proteins), P type ATPases (metal transporting ATPases), NRAMPs (natural resistance-associated macrophage proteins) and CAXs (calcium and other divalent cation exchange antiporters) (Colangelo and Guerinot, 2006). (Fig.1.3).

Hyperaccumulators of Zn express a higher rate of uptake of Zn than closely related non-hyperaccumulator species, possibly due to a higher expression of Zn-transporting genes in root and shoot cells. Lasat *et al.*, (1996) compared *Thlaspi arvense* (a non-hyperaccumulator) with *Thlaspi caerulescens* (a hyperaccumulator) using a radiotracer flux technique to quantify the rate of Zn^{2+} influx into the root. They showed that Zn uptake followed a Michaelis-Menten relationship with increasing

external Zn concentrations in both species. It was shown that although the K_m values (the external Zn concentrations at which Zn^{2+} uptake was half the maximum) for both species were similar the V_{max} , (the maximum rate of Zn^{2+} uptake) was considerably higher in *T. caerulescens* than in *T. arvense*. This is consistent with a greater expression of genes encoding transport proteins facilitating Zn uptake in *T. caerulescens* compared to *T. arvense* (Lasat *et al.*, 1996; 1998; 2000).

ZIP proteins are known to be involved in Zn homeostasis through their function as cation transporters into the cytoplasm. They consist of eight transmembrane domains and a histidine rich variable loop between the transmembrane domains III and IV. To date over a 100 ZIP proteins have been characterised and functionally analysed in plants, animals, bacteria and yeast.

Lasat *et al.*, (2000) isolated a Zn transporter gene (*ZNT1*) from *T. caerulescens*, which showed homology to transporter genes in *Arabidopsis thaliana* (ZIP family). Functional complementation of the yeast *zhy3* mutant, which is defective in Zn uptake, with the *ZNT1* gene restored its capacity to take up Zn. Both *ZNT1* and related genes were found to be expressed in non-hyperaccumulators (*T. arvense*) only during Zn deficiency, whereas they were constitutively expressed in *T. caerulescens*, the hyperaccumulator (Lasat *et al.*, 2000). This feature allows the hyperaccumulator to prevent toxic build up of Zn accumulating in the roots enabling high Zn concentrations to be tolerated in the soil.

There is strong evidence suggesting a correlation between the expression of genes encoding heavy metal transporters, such as *TcZNT1*, *TcZNT2* and *TcZTP1* and the

hyperaccumulation trait. Their expression has been found to be constitutively higher in both roots and shoots of *T. caerulescens* compared with *T. arvense* (Assunção *et al.*, 2001; Pence *et al.*, 2000). *TcZNT1* and *TcZNT2* share sequence and structural similarities with members of the *ZIP* transporter family, which are thought to be responsible for translocating essential metals within the cell and the detoxification of unwanted toxic metals (Pence *et al.*, 2000).

P-type ATPases translocate cations across biological membranes facilitated by the energy derived from ATP hydrolysis. In one subfamily, P_{1B}-ATPases, the heavy metal transporters consist of eight transmembrane spanning domains, a CPx motif responsible for translocation and a putative metal-binding domain. In *Arabidopsis* the eight P-type ATPases have been designated *HMA1-8*. Studies in yeast, of *HMA2* and *HMA4* have given evidence of enzymatic functions associated with Zn uptake and translocation. Expression of *HMA4* within the heterologous systems of wild type and heavy metal sensitive yeast strains has indicated *HMA4* transports Zn, Cd and Pb. Reverse genetics experiments in plants have yielded corresponding results (Williams *et al.*, 2000).

The CDF family of metal transporters consist of six transmembrane domains and function as proton antiporters that efflux heavy metals out of the cytoplasm. The first CDF protein to be described was discovered in *A. thaliana*, named *ZAT1* or Zinc transporter gene, later renamed Metal Tolerance Protein 1 (MTP1). Overexpression and knockouts of this gene have confirmed its role in Zn tolerance. Also localisation studies of the MTP1 protein with GFP expression has shown its expression is

localised in the vacuolar membranes of root and leaf cells which suggests it may function in Zn sequestration in the vacuole (Dräger *et al.*, 2004; Kobae *et al.*, 2004). Long distance transport of Zn^{2+} ions occurs via the xylem stream. Concentration of Zn in the xylem sap has been shown to vary from 2 to nearly 100 μM . Zn transport through the phloem is not thought to occur at significant volumes (Hacisalihoglu and Kochian, 2003).

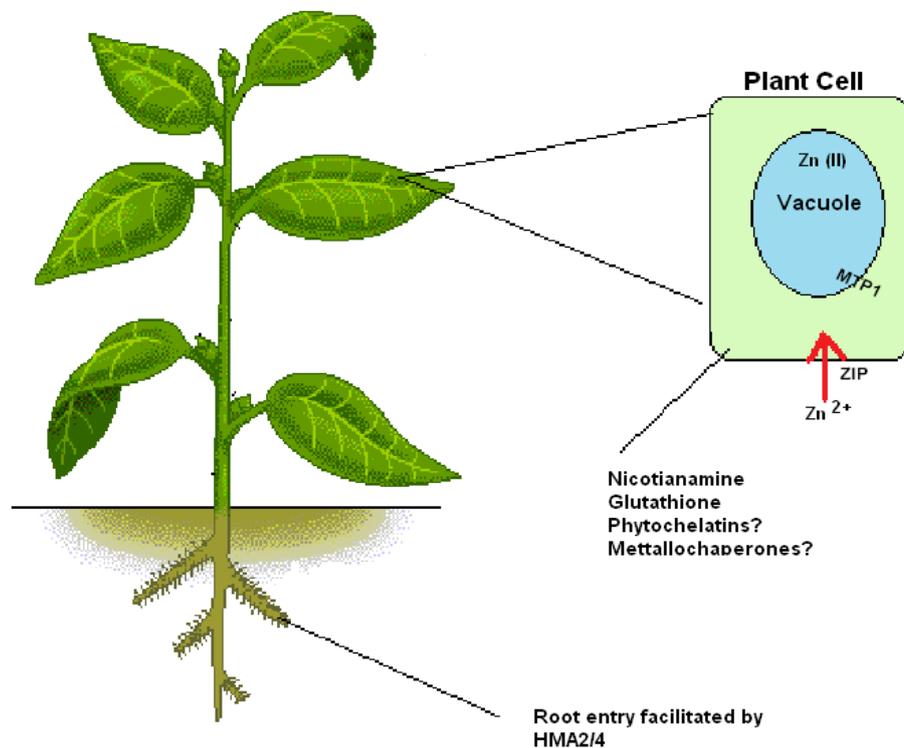


Fig.1.3 A summary of the genes involved in zinc uptake and transport in plants. Zinc must pass through many membranes in the root before reaching the xylem where it can be delivered to the growing tissues. In the root two P_{1B} -ATPases, *HMA2* and *HMA4* are responsible for translocating zinc from root to aerial tissues. Zinc homeostasis is shown in the plant cell. ZIP transporters are known to assist entry into the cell. Nicotianamine and glutathione are low molecular weight ligands for zinc. Possibly Metallochaperones are also involved in zinc binding. *HMA2* and *HMA4* may also be involved to export zinc to the apoplast (not shown). Finally detoxification and compartmentalisation may occur by transporting zinc to the vacuole by MTP1. Here zinc may be chelated by organic acid anions or other unknown binding mechanisms.

1.4.3 Metal detoxification

Detoxification methods are required by some plants to manage the high levels of heavy metals accumulated within the plant. Metals chelators, including organic acids, phytochelators and metallothioneins all play roles in detoxification. (Assunção *et al.*, 2003a). Zn Hyperaccumulators typically display high levels of several organic acids, usually citrate or malate. In *T. caerulescens* it has been shown that genes regulating malate and citrate levels in the leaves are constitutively expressed. However in the roots their expression increases upon exposure to heavy metals (Assunção *et al.*, 2003a; Salt *et al.*, 1999).

The amino acid, histidine, has been shown to play a role in Zn homeostasis. However it plays a greater role in Ni (nickel) hyperaccumulation. Zn-histidine complexes are found to be very stable within the cytoplasm and this suggests a similar role as with Ni hyperaccumulation.

Phytochelators and their role in *T. caerulescens* have been studied in some detail in response to Cadmium (Cd) accumulation. Levels of phytochelators have been found to rise in response to increasing levels of Cd. Metallothioneins' (MT) role in hyperaccumulator and metal tolerant species is as yet undecided however their transcript abundance in plant tissues is reportedly very high, close to 3% of all the transcribed genes studied. For example amongst 700 *T. caerulescens* ESTs around 10 MT like genes were present. A suggested explanation for their presence has been a role in Cd homeostasis (Assunção *et al.*, 2003a).

1.4.4 Zn compartmentalisation

Finally it is known that hyperaccumulators show the ability to translocate Zn to specific shoot cell types for storage. Elemental distribution maps are able to show specifically the distribution of essential elements within the plant. Frey (2000) showed that Zn is primarily located within the epidermal cells of plants, where it can exist in an ionic form at concentrations exceeding $60 \text{ mg Zn g}^{-1} \text{ d. wt.}$ This compartmentalising of Zn prevents toxic levels accumulating in the cytoplasm, Zn is rarely found in the mesophyll or stomatal complexes of hyperaccumulator species. Fig.1.4 shows first the black and white image along side the false colour distribution map for Zn. The epidermal and subsidiary cell can be seen, clearly showing the high concentrations of Zn within the epidermal cell.

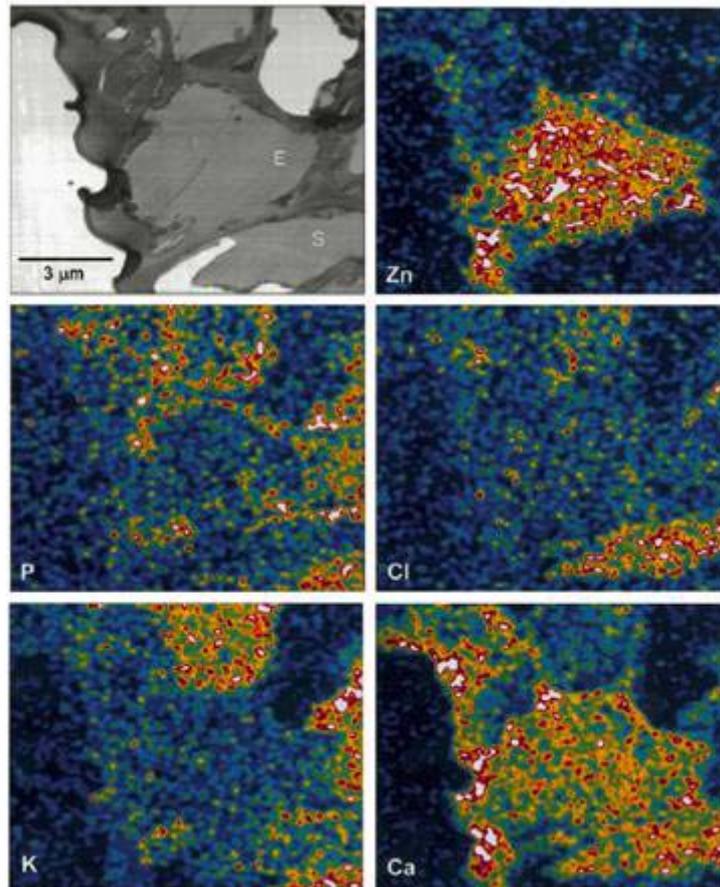


Fig.1.4 Elemental distribution maps. The first image is the black and white microscopic view showing epidermal (E) cell and subsidiary (S) cell. Along side is the elemental map for Zn. Other elemental maps can be seen below. The map shows Zn concentration is highest within the epidermal cell (white and red shows the highest concentration with blue being the lowest (Frey *et al.*, 2000).

1.5 Strategies to determine the genes involved in hyperaccumulation

Many techniques and approaches have been used to study hyperaccumulators to try to determine the genes responsible for the trait discussed. Here are some of the approaches to date, some of which will be employed in this thesis.

1.5.1 Yeast Expression Libraries

Initial identification of candidate genes involved in Zn hyperaccumulation came via the screening of cDNA expression libraries in yeast. Yeast is easily manipulated and studied due to its relatively small genome size. Having been widely studied, protocols for its transformation and gene cloning are available along with its widely available libraries and mutants making it an excellent tool to aid the identification of putative genes in other species. A yeast mutant defective in Zn uptake (*zhy3*) was used to carry out a functional complementation test to isolate a Zn transporter gene from *T. caerulescens* (Pence *et al.* 2000). A *T. caerulescens* cDNA library was constructed within a yeast expression vector which was used to transform the *zhy3* mutant. The transformed yeast strains that grew (20 initial colonies) on a Zn-limited medium highlighted cDNAs of interest. Of the 20 colonies 7 were found capable of restoration of growth on low Zn-medium. Sequence analysis of the cDNA clones showed that of the seven clones, five represented the same gene, which was subsequently, called *ZNT1*. Insertion of the *ZNT1* gene into the *zhy3* mutant restored the yeast's ability to uptake Zn. Sequence analysis further confirmed that this gene showed homology with the *IRT1* gene, a putative Fe transporter isolated from *Arabidopsis thaliana* (Zhao & Eide, 1996), *ZRT1*, a high affinity Zn transporter discovered in yeast (Zhao & Eide, 1996) and *ZIP4*, one of four Zn transporters isolated from *Arabidopsis* (Grotz *et al.*, 1998; Lasat *et al.*, 2000).

1.5.2 Mutants

Due to the number of available centres stocking knockout mutant lines (for example, The National Arabidopsis stock centre (NASC) situated within The University of Nottingham) this method of studying gene function is widely used. Any putative genes may be investigated by studying the “knockout” mutant (a mutant with a particular gene switched off) within the gene of interest. This has been facilitated by whole genome sequencing of agronomically important crops and model plant species such as *Arabidopsis thaliana*, the first plant to have its genome sequenced (Bouche and Bouchez, 2001). Previously with molecular genetics, as with this study to some degree, genes were identified that controlled a biological function. More recently with the production of mutant libraries, in particular through T-DNA insertions, biological functions are being assigned to genes using reverse genomics or functional genomics as it is termed (Bouche and Bouchez, 2001).

Mutants, whether naturally occurring or laboratory induced have been used to determine gene function on many previous occasions e.g. for determining flowering control in plants (Samach and Coupland, 2000). Laboratory induced mutants may be induced by physical means e.g. gamma rays or by chemical means e.g. ethyl methane sulphonate (EMS).

Naturally occurring mutants can be identified by screening populations under stress conditions either at low or high zinc concentrations to pick out a mutant deficient in Zn uptake among a population of hyperaccumulators or a hyperaccumulator among a population of non-accumulators respectively (Cobbett, 2003).

1.5.3 QTL

Forward genetics approaches have traditionally been the starting point to many genetic studies in plants in the past. The ability of the plant to self-pollinate and with a limited out-crossing rate allows the scientist to investigate the genetic variation of metal accumulation in two different populations of, for example, *T. caerulescens*. (Assunção *et al.*, 2006; 2003b; Deniau *et al.*, 2001; Pollard and Baker, 1996). QTL (Quantitative Trait Locus) mapping has been employed as a tool for gene discovery for over two decades. This method allows phenotypes to be linked to known loci. In terms of plant breeding this allows phenotypes for yield or quality to be mapped and advantageous alleles to be combined together. One successful use of this approach was used on the hyperaccumulator *Arabidopsis halleri*. A cross was carried out between *A. halleri* and its close relative *A. petraea*. This allowed the location and number of genomic regions associated with Zn hyperaccumulation to be determined. Twenty five markers were identified on all eight chromosomes following the cross of the two plants being grown on high (100 μM) and low (10 μM) Zn concentrations (Filatov *et al.*, 2007). In more recent years other genetic tools have been developed to assist the work of QTL mapping so it is no longer used alone. Additional techniques include microarrays, genome sequencing and knockout lines (Borevitz and Chory, 2004; Macnair *et al.*, 2003).

1.5.4 Transcriptomics

Whole-genome transcriptomic profiling is becoming a valuable tool in revealing the functions of genes. Microarrays allow the comparison of two whole transcriptomes either from different species or of the same species but subjected to different conditions. One reported example is the comparison of *T. caerulescens* with *T.*

arvense. This allows the identification of genes that are up regulated in the hyperaccumulator but that are not transcribed in the non-hyperaccumulator. In this case approximately 5000 genes were highlighted as differentially expressed in the hyperaccumulator compared with the non-hyperaccumulator. From this several of the putative heavy metal accumulator genes were analysed by quantitative real time PCR to confirm the initial findings (Hammond *et al.*, 2006) (Chapter 3 of this thesis).

Similarly other known hyperaccumulators were compared to closely related non-hyperaccumulators in this way, namely, Becher *et al.*, (2004) and Weber *et al.*, (2004) who made comparisons between *Arabidopsis halleri*, a plant hyperaccumulator and *Arabidopsis thaliana*, a closely related non-hyperaccumulator. Filatov *et al.*, (2006) compared *A. halleri* with its relative and non-hyperaccumulator, *Arabidopsis petraea*. A review of these studies can be found in chapter 3.1.

1.5.5 Genetic manipulation approaches

The development of genetic manipulation as a research tool and not just a means of improving an agronomically important tool, has benefited the progress of functional gene analysis. Putative genes can be studied *in planta* or in heterologous species either using knockout, overexpression or promoter fusion constructs (Assunção *et al.*, 2003a).

Antisense methods were originally the easiest and most commonly used method of silencing or knocking out genes using DNA or RNA of the complementary sequence to the gene you wish to silence. These two sequences subsequently annealed to each other effectively halting or reducing translation. Issues with this method include

incomplete efficiency and artefacts (Hammond *et al.*, 2001). More recently the discovery of the RNAi technique has improved efficiency considerably. Originally used in the model worm, *Caenorhabditis elegans*, the presence of a few molecules of dsRNA per cell initiates gene silencing. With *C. elegans* it is necessary only to soak the worms in dsRNA or to feed them *E. coli* cells that are expressing the dsRNA. This method of gene silencing differs in that its effect is post transcription (Hammond *et al.*, 2001).

Mutant lines produced through T-DNA, previously discussed, are produced by genetic manipulation. Random insertions of T-DNA of *Agrobacterium tumefaciens* are inserted to produce plant lines that have insertions that collectively almost cover the whole genome. PCR can then be used to clone genes using primers for the known insertion sequence (Bouché and Bouchez, 2001).

Other methods of creating gene knockouts include specific gene knockouts for example using, antisense constructs or RNAi (RNA interference) constructs. This will be discussed in more detail in chapter 4 of this thesis as this method was employed to study the *HMA4* gene.

Of further interest in studying gene function is the use of promoter fusions. The most commonly used reporters are GUS (β -glucuronidase) and GFP (Green Fluorescent Protein, taken from the jelly fish *Aequorea victoria*). Constructs are designed to include the gene of interest's promoter fused to the reporter gene and inserted into the plant. Whenever the gene of interest would normally be transcribed the reporter gene will also be transcribed. This allows studies to be made into what conditions

stimulate the gene of interest. This has been successfully used in many higher plants including tobacco (Chiu *et al.*, 1996).

1.5.6 Localisation studies

As shown in Fig.1.4, elemental distribution maps allow the localisation of the heavy metal to be determined. This allows investigation at the tissue or organelle level to determine gene expression. Such methods include the use of immunology, for example Kobae *et al.*, (2004) created antibodies against the AtMTP1 and immunoblots of microsomal fractions were analysed to determine the localisation in different organelles and also its change in expression in response to altering levels of Zn concentration.

1.6 Aims of this thesis

The aim of this thesis was to determine the genetic control of zinc hyperaccumulation in *Thlaspi caerulescens*, the model heavy metal accumulator plant. Previous work had identified *Thlaspi caerulescens* as a potential model plant due to its genetic similarities to the model plant species, *Arabidopsis thaliana*, for which there are considerable resources available. The aim of the work was to highlight potential candidate genes that could be studied through transformation methods such as gene knockouts. Prior to the commencement of this project progress had been made with a microarray using *Thlaspi caerulescens* cDNA and an *Arabidopsis thaliana* chip, a novel approach made possible through the genetic homology between the two species. This study gave a basis to narrow down the search for a potential candidate gene. The long term aim was to transfer the heavy

metal hyperaccumulating trait to agricultural important crop species for biofortification purposes or phytoremediation.

The starting point for this thesis was to study the hyperaccumulator species, *Thlaspi caerulescens*, physiologically and compare it with its close relative, non-hyperaccumulator, *Thlaspi arvense*. The physiological studies aimed to confirm previous reported work that the plant indeed could tolerate and accumulate excessive amounts of heavy metals, in this case the focus being zinc. All documented reports of hyperaccumulation in *Thlaspi caerulescens* had previously been carried out hydroponically or in soil. This thesis differs as it studied *Thlaspi caerulescens* grown *in vitro*. The physiological studies would evaluate the amounts of zinc that were taken up by the plants and also how this affected growth. This was achieved using radio labelled zinc, again a novel method.

The microarray experiment, carried out prior to the commencement of this project, highlighted many genes that were differentially expressed, either upregulated or downregulated in the presence of zinc. In this study a short-list of genes, of potential interest as candidate genes responsible for hyperaccumulation, was created. These genes were picked as previous work had been carried out looking at their role in zinc uptake in yeast and *Arabidopsis*. A qPCR experiment was then used to determine if in fact the microarray was correct at identifying that these genes were up regulated in the presence of zinc. Therefore following the microarray and the qPCR the gene *HMA4* (Heavy metal accumulating gene 4), part of a family of heavy metal transporting gene was identified a potential candidate gene to be studied further.

Having identified potential genes that were differentially expressed in the microarray experiment, narrowing down the genes to be further confirmed by the qPCR and finally highlighting a candidate gene, *HMA4*, the next step was to analyse its function *in planta*. This was to be done by transformation of *Thlaspi caerulescens* using a knockout construct. There is no efficient protocol available for transformation of *Thlaspi* species. Therefore part of this study focussed on developing a transformation protocol. Both tissue culture and floral dip methods were explored.

Ultimately, transformation of *Thlaspi caerulescens* was not possible however a *HMA4* RNAi construct was produced to analyse the effects of knocking out the *HMA4* gene, through transgenic approaches on the hyperaccumulation trait.

The following figure gives an outline plan of the thesis detailing how each part links in.

THESIS PLAN

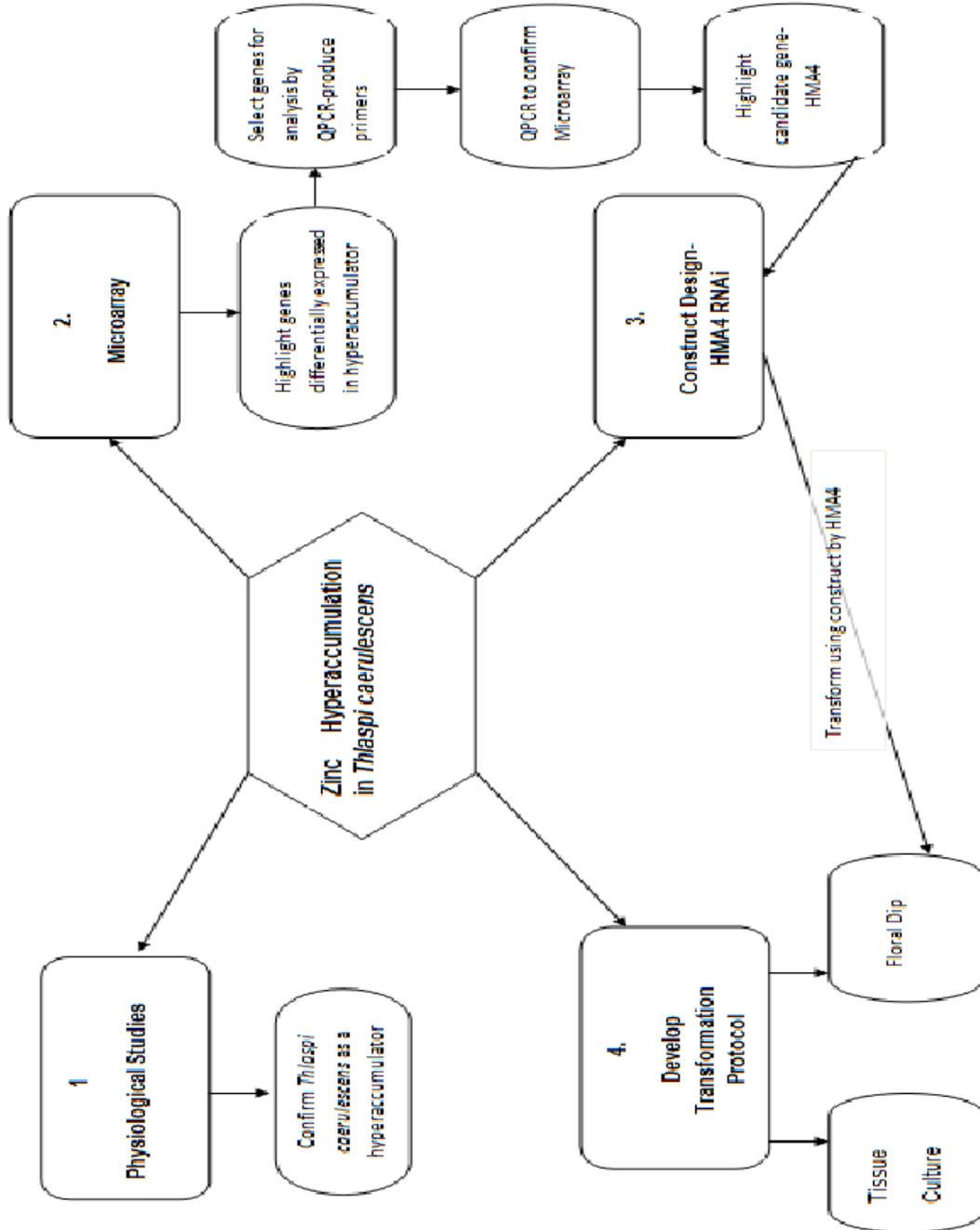


Fig.1.6 Summary of the Thesis

2. Comparing the accumulation and tolerance of zinc in *T. caerulescens* and *T. arvense*

The aim of this chapter was to compare the responses to increasing external Zn concentrations of the model hyperaccumulating plant, *Thlaspi caerulescens* and its close relative *T. arvense* which does not possess the hyperaccumulation trait. Basic physiological tests were carried out to determine the levels of zinc (Zn) the plants can accumulate and to determine the plants' level of tolerance to increasing Zn concentrations. These experiments are background investigations to confirm hyperaccumulation status in *T. caerulescens*.

2.1 Introduction

Thlaspi caerulescens is a well studied model for heavy metal hyperaccumulation. In this particular study *Thlaspi caerulescens* accession Ganges (France) (Fig.2.1), was chosen as the hyperaccumulator with comparisons being made with the non-hyperaccumulator *Thlaspi arvense* accession Wellesbourne (Warwick, UK) (Fig.2.2). There has been much interest in studying hyperaccumulating plant species for a varying number of reasons. As population size increases so does the demand for food however the land available for farming has not changed. In order to keep up with demand changes must be made either to farmable land or to the tolerance of plants to abiotic stresses such as metal or salt tolerance. Secondly the interest in this area stems from their possible use as phytoremediators of contaminated land. Figures collected in 1998 stated that 1,400,000 sites in Western Europe were contaminated with heavy metals including Zn, Cd, Pb and Cu, 50,000-100,000 of

these sites are located in the UK alone. This has potential health hazards as it may easily contaminate water courses and find its way into food chains (Collins, 1999; Mulligan *et al.*, 2001). Hyperaccumulators have the potential to clean up soils through absorption, accumulation and detoxification. Current remediation technologies, which are not suitable on the large scale, render soil biologically inactive (McGrath *et al.*, 2001). Lastly the hyperaccumulators have increased peoples' interest in them as potential means of biofortification. Presently the half the world's population is suffering from lack of micronutrients in their diet, according to UN data (Broadley *et al.*, 2007).

2.1.1 Plant Hyperaccumulators

It has been well documented that many plant species possess the ability to accumulate and tolerate high levels of heavy metals with no signs of toxicity. To date there has been noted over 400 taxa holding this trait. These plants have been found all over the world but notably have been found on metalliferous soils (Reeves and Baker, 2000). The uptake and hyperaccumulation of these metals is an active trait that has evolved possibly as a defence mechanism against predators and disease. Alongside the ability to accumulate and tolerate these usually deadly levels of heavy metals the plants also possess the ability to scavenge and seek out the metals (Haines, 2002).

Studies in the 1970s on plants that accumulated Ni first led to the term hyperaccumulators and referred to plants that contained nickel at levels above 1000 $\mu\text{g g}^{-1}$ dry weight (Brooks *et al.*, 1977). Later studies defined the thresholds for other levels of heavy metals such as 100 $\mu\text{g g}^{-1}$ dry weight of cadmium, 100 $\mu\text{g g}^{-1}$ dry

weights for copper, cobalt and lead and 10,000 $\mu\text{g g}^{-1}$ dry weight for zinc and manganese (Baker and Brooks, 1989).

The majority of the 400 plus hyperaccumulator species are nickel hyperaccumulators, found within 45 different families, the highest occurrence being within the Brassicaceae family (Reeves and Baker, 2000). Zinc hyperaccumulators make up the second largest group (described in Baker *et al.*, 1992; Bert *et al.*, 2002; Brooks, 1994; Meerts and Van Isacker 1997; Schat *et al.*, 2000).

The first reported evidence of Zn hyperaccumulation was in *Viola calaminaria* (Ging.) Lej in the Aachen region on the border of Belgium and Germany when >1 % Zn was reported in the ash of the plants aerial parts in 1855. Shortly after this in 1865 >1 % $[\text{Zn}]_{\text{shoot}}$ d. wt. was reported in *Thlaspi caerulescens* (previously *Thlaspi alpestre* L.) (Reeves and Baker, 2000). Reports more recently have described levels up to 3 % d. wt. in the plant's aerial parts (Kochian, 2002).

2.2. Materials and methods

2.2.1 Plant Materials, culture media and culture conditions

Seeds of *T. caerulescens* cv. Ganges (France) were surface sterilized with 70 % (v/v) ethanol for 10 min followed by a wash in reverse osmosis purified water. The seeds were then immersed in 50 % (v/v) "Domestos™" bleach solution (NaOCl Sodium hypochlorite 1 % active chlorine) (Diversey Levre, Northampton, UK) for 20 min with a final 6 washes in reverse osmosis purified water. This procedure was carried out in a Bassaire class II cabinet (Bassaire LTD, Southhampton, UK) to

ensure sterile working conditions. The seeds were refrigerated at 5 °C for 6 days to induce germination.

The culture medium used to germinate the seeds was composed of MS basal salts (Murashige and Skoog, 1962) 4.3 g L⁻¹ inorganic salts (Appendix 8.1), 10 g L⁻¹ sucrose and 8 g L⁻¹ agar adjusted to a pH of 5.6 with 0.1 M NaOH. Zinc sulphate was used to produce agar at the following zinc concentrations 3, 30, 150, 300, 600, 1200, 1800 µM. The medium was autoclaved at 121 °C at 104 kPa for 20 min. After autoclaving the agar was spiked with activity concentration of 2.1 KBq ⁶⁵Zn µmol Zn to trace the amount of Zn uptake.

As the MS basal salts contained zinc the zinc concentrations had to be made up to take this into account. The 1/10 basal salt mixture used contained 3 µM of zinc. The table 2.1 summarises how the agar was prepared to the final zinc concentrations needed.

Zn Concentration Required (μM)	Amount of 1 M ZnSO_4 needed in 1 L agar (μl)
3	0
30	27
60	57
150	147
300	297
600	597
1200	1197
1800	1797

Table 2.1 The concentration on ZnSO_4 required to make agar to test the accumulation and tolerance of the two *Thlaspi* species.

Plants were sown into sterile polycarbonate boxes (10x11x9.5 cm) with 75 ml of the appropriate agar. The boxes were marked to show *T. arvense* seeds on one side and *T. caerulescens* on the other side. Six of each species were sown per box; the boxes were sealed with Nescofilm™ (Bando Chemical Co., Kombe, Japan) to eliminate fungal and bacterial contamination. Three replicates of each concentration were prepared for comparison. The plants were cultured *in vitro* at 24 ± 1 °C in a 16 h photoperiod at a light intensity of $50\text{--}80 \mu\text{mol m}^{-2} \text{s}^{-1}$ under 58 W of white halophosphate fluorescent tube lighting (Crompton Lighting, Germany) and 8 h darkness.

2.2.2 Plant harvesting

Four randomly selected plants, per species per box were harvested at 18 days post sowing by excising the shoots from the agar, separating from the roots, and placing in separate pre weighed 5 ml tubes. Root and shoot fresh weight (f.wt.) was recorded. Samples were dried in an 80 °C oven for three days and reweighed to give root and shoot dry weight (d. wt.). The samples were then measured in a gamma counter (Wallac 1480 Wizard 3" Automatic Gamma Counter, Perkin Elmer Life sciences, Turku, Finland) to determine the amount of radioactivity in the sample and therefore calculate the total amount of Zn uptake by the plant.

2.2.3 Determining the concentration of Zn using radiolabeled ^{65}Zn

Tissue Zn concentration was determined using radiolabeled ^{65}Zn . The ^{65}Zn γ -emissions were counted for 600 seconds per sample using an automated well-type gamma counter (Wallac 1480 Wizard, Perkin-Elmer Life Sciences, Turku, Finland) designed for counting high-energy samples (up to 2000 keV). The detector consists of a thallium (TI) activated NaI crystal of the end well design. The NaI crystal has dimensions 80 mm in height and diameter of 75 mm. The principal mechanism of gamma-ray detection in the crystal is through a photoelectric effect, where photoelectrons of varying energies are emitted from the atomic electron shells of the NaI following absorption of gamma-rays (photons). The photoelectrons are amplified, and converted subsequently to a digital output via an electrical pulse. The gamma counter was normalized to adjust the parameters for detection of ^{65}Zn according to the manufacturer's guidelines. After normalization, the background activity of ^{65}Zn was determined when an empty tube was placed in the detection well. This

background reading was carried out before measurement of plant samples each time the counter was used. The background ^{65}Zn activity ranged from 6.2 to 16.2 counts per minute (cpm) with a mean value of 9 cpm. These background values were subsequently subtracted automatically from the counts per minute measured from the plant samples.

Each agar sample prepared contained an activity concentration of 2.1 kBq ^{65}Zn μmol Zn. For each experiment agar samples were taken to be analysed at the same time as analysing harvested plants. The ^{65}Zn γ -emissions were counted for 600 seconds per agar sample using an automated well-type gamma counter (Wallac 1480 Wizard, Perkin-Elmer Life Sciences, Turku, Finland), at the same time as the plant tissue samples were analysed- giving a reading in counts per minute (cpm). Since a known concentration of stable Zn was added to the agar sample (e.g. 3 μM = 3 μmoles per litre, or 3/1000 μmoles per mL), the cpm of ^{65}Zn can be related to the number of moles of Zn. By knowing the weight of plant, and the cpm, it is possible to calculate the number of moles of Zn per gram of plant material. It is possible to directly relate cpm to moles as there is no discrimination between stable zinc and radioactive zinc during plant uptake (S. Donnelly pers.com.).

Worked example of sample of 3 μM agar-emitting 60cpm

3 μM = 3 μmoles in 1L

In one ml agar sample = 3/1000 μmoles in 1L

=0.003 μmoles Zn

1 mole Zn = 65.39 g Zn

1 μmole Zn = 65.39 μg Zn

0.003 μmoles Zn = 0.19617 μg Zn

Therefore 0.19617 μg Zn gives 60 cpm

Therefore 1cpm = 0.003265 μg Zn

2.2.4 Statistical analysis

The statistical program Genstat (VSN International, Hemel Hempstead, UK) was used to analyse the data collected from root and shoot fresh and dry weight and the quantity of zinc accumulated in the sample. Analysis of Variance (ANOVA) was used to test for significance between zinc concentrations and between species.



Fig.2.1 *Thlaspi caerulescens* Ganges the model hyperaccumulator



Fig.2.2 *Thlaspi arvense* Wellesbourne the non-hyperaccumulating relative of *T. caerulescens* used for comparison.

2.3 Results

The aim of this chapter was to evaluate the differences between *Thlaspi caerulescens* and *T. arvense* in terms of Zn accumulation and tolerance. The two plant species were grown on agar supplemented with increasing concentrations of Zn, which was radiolabelled with ^{65}Zn . The differences in growth were assessed by measuring the root and shoot fresh and dry weights and the accumulation of Zn quantified by assessing the uptake of ^{65}Zn measured using an automatic gamma counter.

2.3.1 Plant growth of *Thlaspi caerulescens* and *Thlaspi arvense*

T. caerulescens and *T. arvense* were grown at a range of external Zn concentrations. Fresh weight and shoot zinc concentrations were recorded; *Thlaspi caerulescens* showed positive growth at all Zn concentrations with the exception of the highest (1800 μM), which is almost 34 times greater than the amount of Zn provided by the basal salts used to make the agar; that is 34 times greater than a plant requires for normal growth. The growth of *T. caerulescens* increased up until 300 μM then above this level the growth decreased. However some growth is maintained even at the highest Zn concentrations. When making comparisons with *T. arvense* it can be seen that root and shoot fresh weights were greatest in the 3 and 30 μM Zn concentrations. Growth above these levels decreased sharply and at levels above 600 μM no growth was observed (Fig.2.3).

In all replications *T. arvense* plants were visibly larger in terms of height and leaf size than *T. caerulescens* in the 3 and 30 μM treatments due to *T. arvense* having a faster growth rate. At levels above 150 μM in *T. arvense* the shoot and root size was greatly reduced, making harvesting and measurement more difficult. Plants remained in the cotyledon stage and were visibly chlorotic.

At levels of 600 μM only 12 plants of *T. arvense* successfully germinated; above this level no seeds germinated. Comparatively *T. caerulescens* were able to grow to the very highest concentrations despite being notably smaller.

Statistical evaluation of the data showed a significant difference in growth for both species at increasing Zn concentrations ($P < 0.001$) *T. arvense* shoot and root

amounts were higher than *T. caerulescens* for 3, 30 and 60 μM concentrations. *T. arvense* root fresh weight reached the highest quantity at 30 μM (0.0477 g) and shoot fresh weight at 3 μM (0.1701 g). Comparatively *T. caerulescens* root and shoot fresh weights were greatest at 300 μM [Zn] (0.0211 g and 0.0658 g respectively). Shoot and root fresh weight for *T. caerulescens* was lowest at the highest concentration of 1800 μM (0.0064 g and 0.0141 g respectively.)

There was a significant difference between Zn concentrations in the root fresh weight ($P=0.005$) but not between the two *Thlaspi* species ($P=0.059$) (Fig.2.3 a). There was however a significant difference between species and between Zn concentrations for shoot fresh weights (Fig.2.3b).

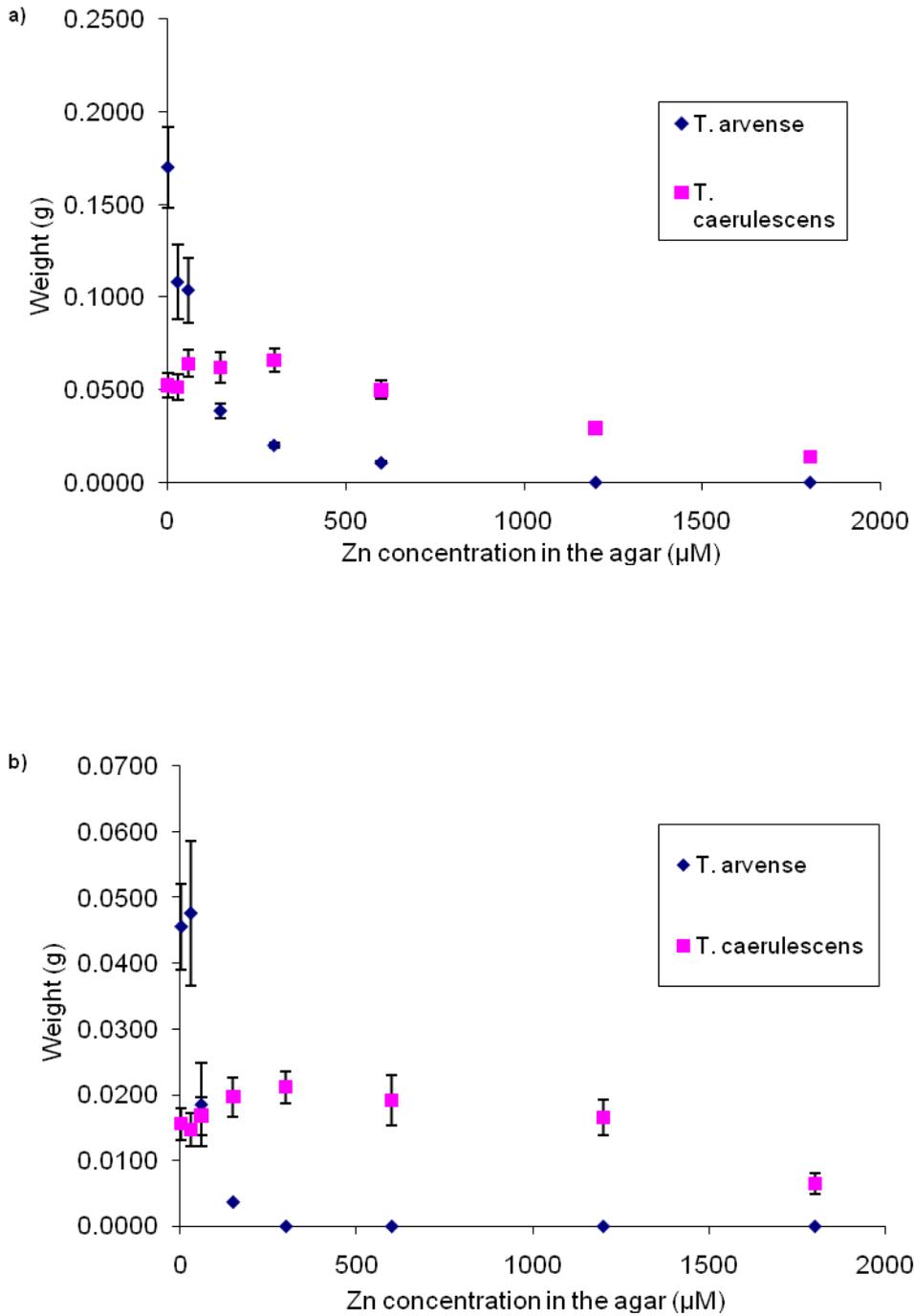


Fig.2.3 Effect of increasing Zn concentrations on the mean fresh weight of a) shoots and b) roots of *Thlaspi caerulescens* and *Thlaspi arvense*. Those not germinating were plotted as 0 g. The error bars represent two times the standard error of the mean.

The data for shoot and root dry weights were similar to the fresh weights for the increasing range of Zn concentrations (Fig.2.4 a and b). Root dry weight was greatest for *T. arvense* in the 30 μM Zn concentration (0.0031 g), and the greatest shoot weight was in the 3 μM concentration of Zn (0.0149 g). Again, *T. caerulescens* shoot and root dry weights were highest at 300 μM concentration of zinc (0.0021 g and 0.0081 g respectively). Root and shoot dry weights were greater in *T. arvense* than *T. caerulescens* for 3 and 30 μM treatments, shoot dry weight was also found to be greater than *T. caerulescens* at 60 μM Zn concentrations. At levels above this it was found that *T. caerulescens* greatly exceeded the dry weights of *T. arvense*. Similarly to the fresh weight data there was a significant difference between Zn concentrations between species with regards to shoot and root dry weights ($P < 0.001$) and a significant difference between species in terms of shoot and root dry weights ($P = 0.001$). In contrast to the data described for shoot and root fresh weight, there was a significant difference between root dry weights between species ($P = 0.010$), however there was no significant difference between shoot dry weight between species ($P = 0.116$) (Fig.2.4).

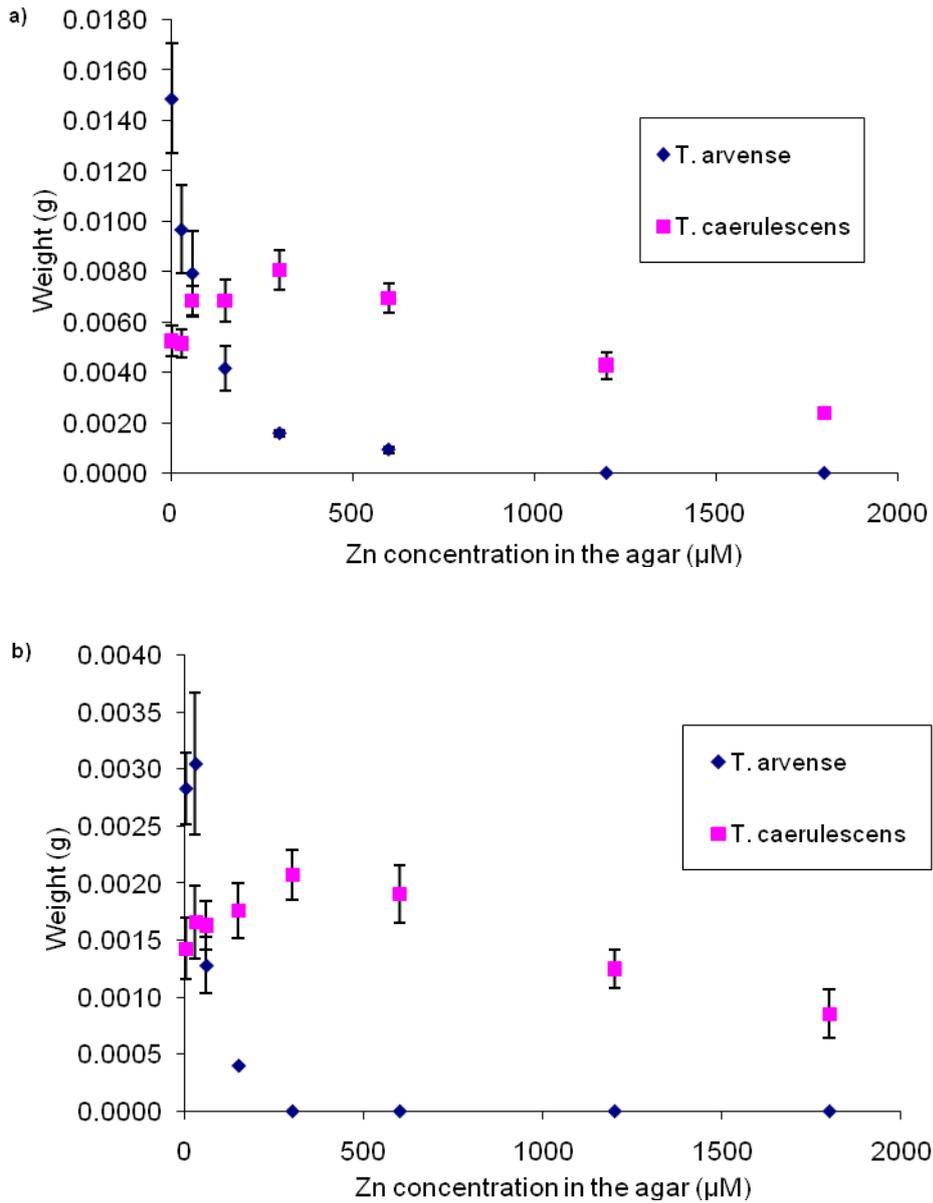


Fig.2.4 Effect of increasing Zn concentrations on the mean dry weights of a) shoots and b) roots of *Thlaspi caerulescens* and *Thlaspi arvense*. Those not germinating were plotted as 0 g. The error bars represent two times the standard error of the mean.

2.3.2 Zinc accumulation in *Thlaspi caerulescens* and *Thlaspi arvense*

The levels of Zn accumulation in the plant were measured by the presence of ^{65}Zn by a gamma counter. Root and shoot accumulation data was recorded for both *Thlaspi caerulescens* and *T. arvense*. This data was collected from the dry mass of the plants.

There was a significant difference ($P < 0.001$) in zinc accumulation concentrations ($\mu\text{mol Zn g}^{-1}$ dry weight) between the two species in the roots. *Thlaspi caerulescens* accumulated higher concentrations of Zn at all Zn external treatments with the exception of 30 and 60 μM concentrations. Accumulated levels of Zn were higher in *T. arvense* in the roots at concentrations between 60 and 600 μM external Zn concentrations, above and below which *T. caerulescens* accumulated Zn to a higher degree. Overall however the difference in Zn accumulation in the shoots between the two species was not significantly different ($P = 0.095$). The maximum accumulated concentrations of Zn in the roots and shoots of *T. caerulescens* were $1061.4 \mu\text{mol g}^{-1}$ at concentration 1200 μM and $331.3 \mu\text{mol g}^{-1}$ at substrate concentration 1800 μM , respectively. The root concentration of Zn was approximately three times greater than was found in the shoots of *T. caerulescens*. The maximum Zn concentration reached by the shoots of *T. arvense* was $206 \mu\text{mol g}^{-1}$ at a substrate concentration of 300 μM . The corresponding value for roots was $62 \mu\text{mol g}^{-1}$ which was attained at 150 μM . The lowest Zn concentrations for both species was found at treatment 3 μM . The *Thlaspi caerulescens* shoot concentrations increased gradually as the Zn external concentration increased. This allowed a greater accumulation to be achieved in comparison to *T. arvense*. The Zn concentrations in the shoots of *T.*

arvense increased at a greater rate than in *T. caerulescens* until external Zn concentration reached 300 μM after this point accumulating levels dropped. There was however no data after 600 μM due to the seeds not being able to germinate at high Zn concentrations. In contrast the roots of *T. caerulescens* accumulated Zn at a similar level to in the shoots however between external Zn concentrations of 1200 μM and 1800 μM the accumulation levels appear to begin to decline. As for *T. arvense* the accumulation of Zn in the roots remained low at all external Zn concentrations. (Fig.2.5).

There was a significant difference between the accumulated Zn levels at increasing external Zn concentrations ($P=0.015$; Fig.2.5b). Accumulated Zn concentrations in the shoots was significantly different at increasing external Zn concentrations in both species ($P=0.03$; Fig.2.5a).

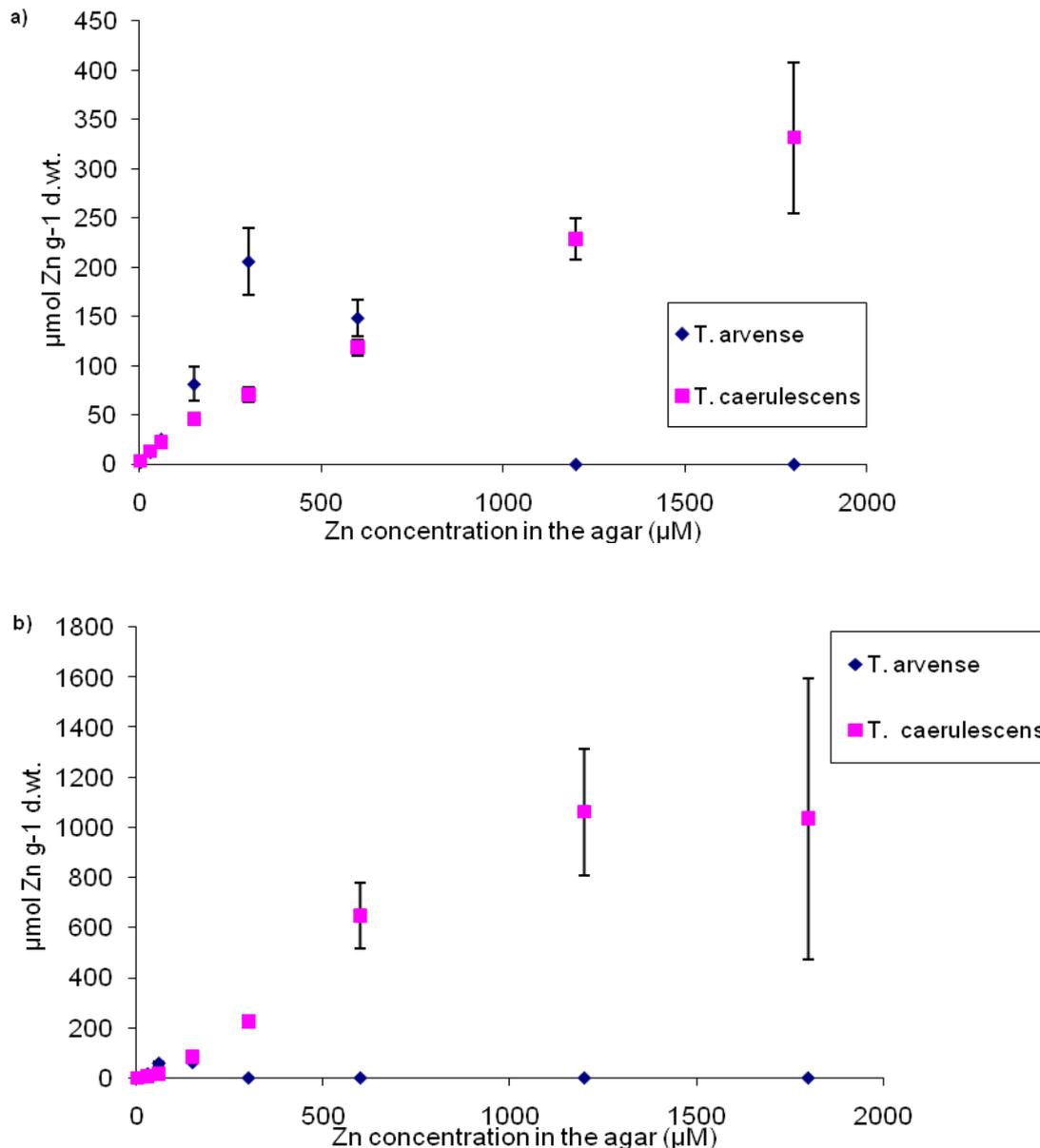


Fig.2.5 Effect of increasing [Zn] in the agar on the Zn content ($\mu\text{mol g}^{-1}$ dry weight) of a) shoots and b) roots of *Thlaspi caerulescens* and *T. arvense*. Those not germinating were plotted as 0 g. The error bars represent two times the standard error of the mean.

Data expressed in terms of $\mu\text{g g}^{-1}$ dry weight showed a similar pattern as data expressed in terms of $\mu\text{mol g}^{-1}$ dry weight, this is as expected because molarity is a function of the mass of Zn per unit tissue volume (Fig.2.6). Therefore the highest and

lowest Zn concentrations in the shoots and roots of both plant species occurred at the same external Zn concentrations.

The highest Zn concentration in the roots of *T. arvense* was 4060 $\mu\text{g g}^{-1}$; the corresponding value for the shoot was 13,474 $\mu\text{g g}^{-1}$. These values were surpassed by *T. caerulescens*, in which the highest concentrations in the roots and shoots were respectively 67,713 and 21,671 $\mu\text{g g}^{-1}$. The maximum values for both species are much higher than the typical values of 3-300 $\mu\text{g g}^{-1}$ reported by Marschner (1995). *T. caerulescens* accumulated 226 times of the maximum value cited by Marschner in its roots, whilst *T. arvense* achieved a maximum concentration in its shoots almost 44 times greater than this value. However, although *T. arvense* was capable of attaining concentrations much higher than in many plant species, this only occurred up to a point as germination was inhibited at substrate zinc concentrations exceeding 600 μM ; by contrast, *T. caerulescens* continued to germinate even at a substrate concentration of 1800 μM Zn and accumulated extremely high concentrations of zinc.

Zinc concentrations in the roots differed significantly between species ($P < 0.001$; Fig. 2.7b) and substrate Zn concentrations ($P = 0.015$). A significant species*substrate Zn concentration interaction was detected ($P < 0.001$). Zinc concentration in the shoot also varied significantly depending on substrate zinc concentration ($P = 0.003$; Fig. 2.7a), but did not differ significantly between species ($P = 0.095$).

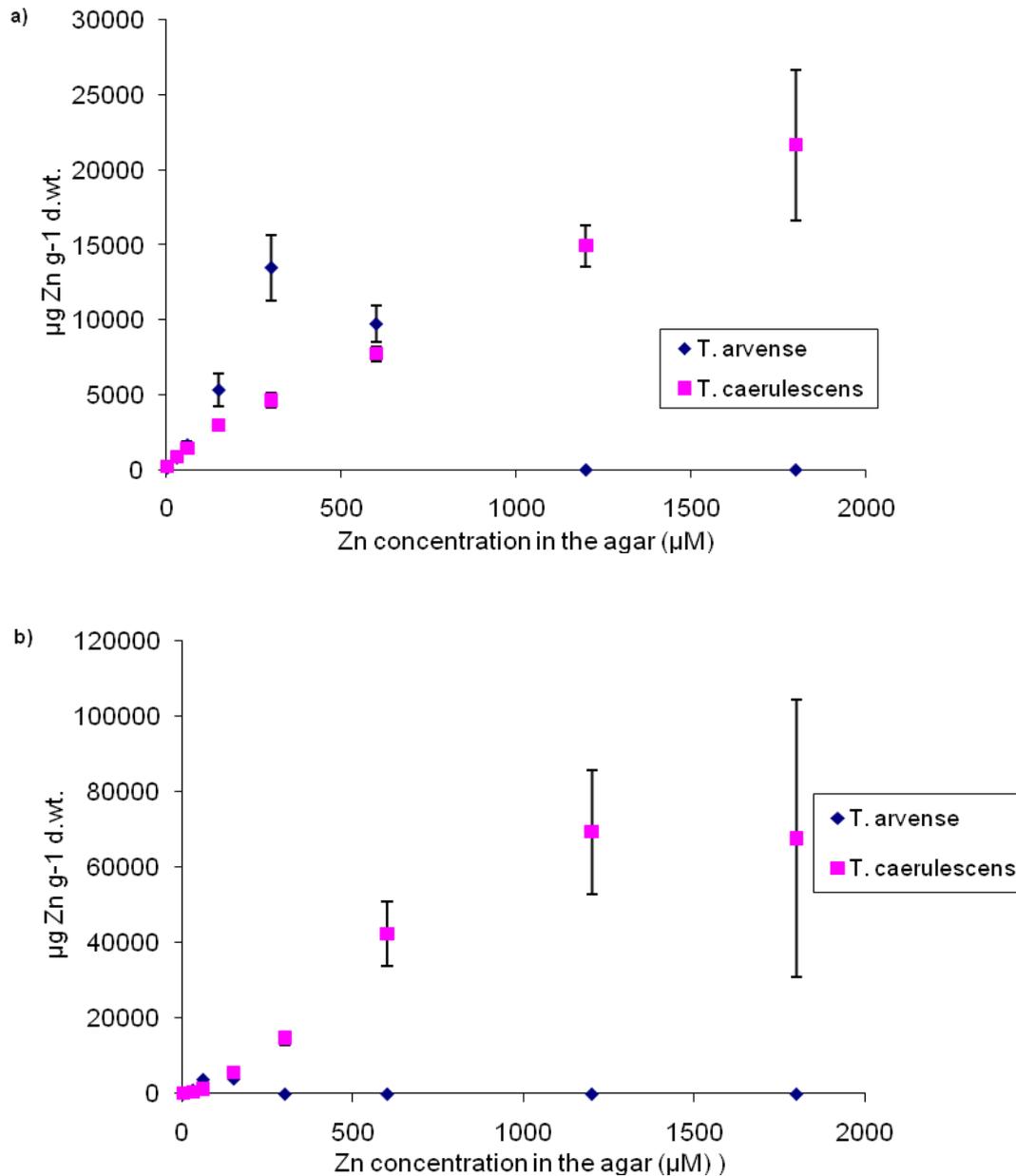


Fig.2.6 Effect of increasing [Zn] in the agar on the Zn content ($\mu\text{g g}^{-1}$ dry weight) of a) shoots and b) roots of *Thlaspi caerulescens* (Ganges) and *T. arvense*. Those not germinating were plotted as 0 g. The error bars represent two times the standard error of the mean.

2.4 Summary

The work carried out in this chapter aimed to compare the growth of two plant species on different zinc agar concentrations, one a known metal hyperaccumulator,

Thlaspi caerulescens Ganges and the second a non-hyperaccumulator, *Thlaspi arvense* Wellesborne. The study intended to quantify the amount of zinc taken up by the plants. Other similar studies have been carried out on the plants, *T. arvense* and *T. caerulescens* and these experiments were carried out to compare actual findings with published data (Brown *et al.*, 1995; Reeves and Baker, 2002). Secondly it was of interest to see if the presence of high zinc concentrations actually stimulated growth of the hyperaccumulator and whether this could be linked to growth of such plants on contaminated soils.

The method used to determine Zn concentration employed the use of radiolabelled tracers. The use of radioactive ^{65}Zn as a tracer has been used in *Thlaspi caerulescens* previously which made employing this method to the project feasible. Lasat *et al.*, (1996, 2000) used ^{65}Zn to measure Zn transport into the shoot and translocation into the shoot. Similar studies have since employed this method, for example Li *et al.*, (2007) using ^{65}Zn to study Zn adsorption and desorption in root cells of *Sedum alfredii* Hance- a hyperaccumulator.

The results confirmed that there was differential growth between *Thlaspi caerulescens* and *T. arvense* at increasing zinc concentrations. *Thlaspi caerulescens* grew well at all concentrations of zinc except the highest concentrations (1800 μM), which is 34 times higher than the basal salts provide for normal plant growth *in vitro*. In comparison to *T. arvense* which managed growth at 3 and 30 μM Zn however all levels above this growth decreased sharply. At levels above 600 μM no seeds were able to germinate.

In terms of accumulation of Zn in the roots and shoots of *T. caerulescens* there was an overall difference in comparison with *T. arvense*. In most cases, *T. caerulescens* accumulated higher levels of Zn in its roots compared with *T. arvense* with the exception of results at 30 and 60 μM Zn. This is not as reported in other studies, where all cases have reported higher concentrations of Zn in the shoot compared with the root in *Thlaspi* species and other known hyperaccumulators (Lasat *et al.*, 1998; Xing *et al.*, 2008.) This may be because in this study plants were grown in very humid atmospheres within the polycarbonate boxes. This would have limited transpiration and therefore may have affected or limited the amount of Zn that was transported to the roots from the shoots. In the shoots Zn concentrations were higher in *T. arvense* at concentrations between 60 and 600 μM Zn. The concentration of Zn in the shoots was higher in *T. caerulescens* at levels above and below those stated above. Concentrations of Zn in *T. caerulescens* reached 1061.4 $\mu\text{mol Zn g}^{-1}$ at a substrate concentration of 1200 μM . Concentrations of Zn in *T. arvense* reached 62.1 $\mu\text{mol Zn g}^{-1}$ at a substrate concentration of 150 μM .

3. Transcriptomic analysis of *Thlaspi* species

This chapter aims to outline the experiments that led to the identification of candidate genes involved in the hyperaccumulation of Zn in *Thlaspi caerulescens*. The methods that are detailed in this chapter include microarrays and qPCR (Quantitative polymerase chain reaction). These techniques aimed to highlight genes that were differentially expressed at increased zinc concentrations and also compared differential expression between the hyperaccumulator *Thlaspi caerulescens* and *Thlaspi arvense*, its non-hyperaccumulating relative. The microarray experiment was carried out prior to the start of this project and aimed to identify potential candidate genes involved in the hyperaccumulation trait. The qPCR, carried out at the start of this PhD experiment followed the microarray experiment to confirm findings in the microarray experiment. The microarray and qPCR were done as a collaborative project between University of Nottingham and Warwick HRI.

3.1 Introduction

Studying the products of gene expression gives an indication of how a trait may be controlled. Confirming the existence of a gene within a genome, and knowing its sequence does not give any indication of a part, if any it plays in the working of an organism. It is only the first step in knowing its function. Gene expression is a process where gene sequences are transcribed to produce a gene product that has a function within the organism. Regulation of gene expression is a complex interaction of genes with the environment,

resulting in the production of RNAs and proteins that are responsible for changes in growth, development, behavior and regulation of homeostasis within an organism. Therefore by comparing the expression of genes between species exhibiting different traits, for example, it is possible to link genes to playing a potential role (Schena, 1996).

Whole genome transcriptome profiling techniques using microarrays have greatly improved our knowledge of biological systems, particularly how they are regulated at the transcriptome level. Typically these studies have been restricted to several “model species” or the species with commercial interest for which the microarrays have been developed. In plant research such microarray platforms are currently commercially available for *Arabidopsis thaliana* (L.) Heynh., barley (*Hordeum vulgare*), rice (*Oryza sativa*), maize (*Zea mays*), tomato (*Lycopersicon esculentum*), soybean (*Glycine max*), sugar cane (*Saccharum officinarum*), grape (*Vitis vinifera*) and wheat (*Triticum aestivum*) (Reviewed in Aharoni and Vorst, 2001 and Donson *et al.*, 2002).

To begin studying other plant species in this way would require sequencing and production of custom arrays, which is too costly and laborious. However a method has been developed to profile and compare the transcriptomics of two non-model plant species, *Thlaspi caerulescens* J & C Presl., a zinc hyperaccumulator and *T. arvense* L., a nonhyperaccumulator using an Affymetrix *A. thaliana* ATH1-121501 (ATH1) GeneChip® array (Affymetrix, Santa Clara, CA, USA) (Hammond *et al.*, 2006).

It has been found that the RNA of one plant species can be used to hybridise to a microarray designed towards a different plant species as a means of studying the transcriptomics of a related species (“cross-species transcriptomics” (Becher *et al.*, 2004; Caceres *et al.*, 2003; Chismar *et al.*, 2002; Enard *et al.*, 2002; Filatov, 2006; Hammond *et al.*, 2005; Higgins *et al.*, 2003; Khaitovich *et al.*, 2004; Uddin *et al.*, 2004; Weber *et al.*, 2004). Of these references several were carried out in animal systems. More relevant to this thesis are those studies that were carried out on plant species. Becher *et al.*, (2004) and Weber *et al.*, (2004) made comparisons between *Arabidopsis halleri*, a plant hyperaccumulator and *Arabidopsis thaliana*, a closely related non-hyperaccumulator. Filatov *et al.*, (2006) compared *A. halleri* with its relative and non- hyperaccumulator, *Arabidopsis petraea*.

Becher *et al.*, (2004) used hydroponically grown plants which, after 4 days, had the hydroponic solution replaced with either “low zinc” (1 μM zinc) or “high zinc” (100 μM or 300 μM zinc). RNA was extracted using a Trizol extraction method which was subsequently used to produce the cDNA for the Microarray, which was carried out on Affymetrix Arabidopsis GeneChips which contained the probes for approximately 8300 genes. The microarray successfully identified significantly higher transcript abundance of several genes in *A. halleri* when compared with *A. thaliana*. In particular the genes highlighted code for proteins that were closely related to the *A. thaliana* proteins AtZIP6 a putative Zn uptake protein which is part of the zinc regulated transporter (ZRT)- iron regulated transporter (IRT)-like protein ZIP family of metal transporters, AtHMA3 a P-type metal ATPase, ZAT/AtCDF1- a

cation diffusion and *AtNAS3* a nicotianamine synthase. Other genes that were found to show significantly higher expression in *A. halleri* was nicotianamine (NA) synthase and a *CDF* gene, a gene that has been found to be related to a *ZAT* gene, which has been linked to tolerance of Zn by *T. caerulescens* (Assunção *et al.*, 2001).

RT-PCR was used to confirm the initial findings of the microarray. Along side this yeast complementation experiments were carried out to determine the function of the genes. Zn hypersensitive yeast mutants were used to test selected genes to determine if the encoded proteins have any function in Zn or Cd detoxification in *A. halleri*. Results suggested that *AtHMA3*, *AhCDF1-3* and *AhNAS3* play some role in Zn detoxification.

Similarly Weber *et al.*, (2004) carried out a microarray comparing *A. thaliana* with *A. halleri*. The same Affymetrix *A. thaliana* GeneChip® was used with probes representing approximately 8000 genes. In this case plants were initially grown hydroponically before being transferred to pots. Before roots were harvested, plants were supplemented for 1 week before harvesting with either “normal” medium (0.8 μM Zn^{2+}), medium without Zn^{2+} or medium containing 30 μM Zn^{2+} . The microarray identified genes that were more highly expressed in *A. halleri* than in *A. thaliana*. Of particular significance was the highly expressed nicotianamine (NA) gene and *ZIP9* gene in *A. halleri* compared with *A. thaliana*. Conclusions from the evidence given in this paper link the presence of NA to Zn homeostasis and Zn hyperaccumulation in *A. halleri* (Weber *et al.*, 2004). This supports other studies that names NA as a

metal chelator and that has been linked to iron uptake in plants (Mori, 1999). *ZIP9*, a member of the ZIP metal transporter family, has previously been associated with Zn^{2+} and Fe^{2+} uptake in yeast and plants (Guerinot, 2000).

Filatov *et al.*, (2006) were interested in the study of evolutionary genetics by comparing how the transcriptome has changed associated with the hyperaccumulation trait. It is important to separate targeted transcriptional changes that are associated with the evolving trait from the unrelated, irrelevant evolutionary changes that have taken place alongside. In order to determine which changes are related to the characteristic studied and which are unrelated, transcriptional changes, interspecies crosses and segregated families of plants are used. In this study, as the test organism was closely related to the sequenced model species, *A. thaliana*, it was possible to use synteny between the two genomes to deduce regions containing quantitative trait loci (QTL). In this study crosses were made between *A. halleri* and *A. petraea* to produce an F_2 population which, segregated extensively for the hyperaccumulation phenotype. Further crosses were made by crossing F_2 plants displaying similar phenotypes to produce an F_3 population that display a range of mean Zn accumulation phenotype. The extreme phenotypes of this F_3 population, displaying phenotypes similar to the parent Zn accumulation phenotype, along with *a. halleri* and *A. petraea* were compared at the transcriptome level using an ATH1GeneChip® (Affymetrix) microarray. This approach is termed the 'bulked segregant analysis' approach (Michelmore *et al.*, 1991, reviewed in Filatov *et al.*, 2006) which enables any gene involved in the hyperaccumulation trait to be identified as any changes in transcript

between the parent species should also be seen between the bulked extremes (non-accumulating and accumulating) of the F₃ generation.

This experiment used hydroponically grown plants, grown on a series of concentrations of increasing Zn concentration (10, 100, 500 and 1000 µM Zn) to determine the Zn accumulation phenotype of the F₂ generation. The grouped F₂ plants were then allowed to cross pollinate naturally within their groups but not between groups.

F₃ generation plants were treated similarly in that they were grown hydroponically along side *A. halleri* and *A. petraea* first at 10 µM Zn for 32 days then 100 µM Zn for 5 weeks to determine their Zn accumulation phenotype.

For the microarray plants were grown on sand until they were large enough to be transferred to a hydroponic system. Two independent microarray experiments were conducted, one to compare *A. halleri* transcriptomes with the transcriptome of *A. petraea* and the second to compare extreme plants from the F₃ population. Results showed 10 % of genes on the GeneChip® were differentially expressed between *A. halleri* and *A. petraea*. When comparing the F₃ generation this figure fell by three or four times. Of this, 141 leaf genes and 140 root genes were shown to be differentially expressed between accumulator and non-accumulator phenotypes. Also consistently greater expression was found in accumulator phenotypes under both high and low zinc conditions for 97 leaf genes and 48 root expressed genes.

The assumption was made that *A. halleri* genes that hybridized to *A. thaliana* genes have similar functions to the corresponding homolog. From this, 8 genes were highlighted as being interesting as they were found in both leaves and roots and therefore suggested a link to the hyperaccumulation trait. Namely, these genes were *AtNRAMP3* (At2g23150) which in *A. thaliana* has been assigned the function of a transport protein for Fe, Mn, Cd and possibly Zn. Previous studies have identified this gene also (Thomine *et al.*, 2000, 2003, reviewed in Filatov *et al.*, 2006 and Weber *et al.*, 2004) and have deduced that its product is located on the tonoplast where it functions as an exporter of iron and cadmium from the vacuole. Similarly to the results found in Becher *et al.*, (2004) the cation transporter *ZIP6* (At2g30080) was found to be constitutively expressed in leaves of all accumulator species and in the roots of the F₃ accumulators in the comparison between *A. halleri* and *A. thaliana*. Other genes that were highlighted are the carbonic anhydrase (CA; At1g23730), a zinc cofactor affected by zinc supply, cytosolic aconitase (At4g35830) and thioredoxin (At1g45145), which has roles in iron and redox homeostasis. The aconitase gene has been identified in mammals where a suggested role has been assigned with iron acquisition despite any previous evidence for such a role within plants it was still considered important to this study. Three further genes, linked to signal transduction, common to both leaves and roots, are an F-box protein (At1g27540), a leucine-rich repeat receptor kinase (At2g20850) and a MAP kinase (AtMPK5, At4g11330). The final two genes have no obvious roles Zn hyperaccumulation; these are a ribosomal 40S protein (At4g34670) and At3g07950.

To confirm the results found in the microarray experiment an RT-PCR was carried out on RNA extracted from *A. halleri* and compared with RNA from *A. petraea*. Of particular significance was the lack of amplification of three genes from genomic DNA of *A. petraea* showing that this species is deficient in the genes, (At2g20850, leucine-rich repeat protein kinase; At1g27540, F-Box protein; and At4g34670, 40S ribosomal protein). The RT-PCR confirmed the expression of *NRAMP3* (At2g23150) and *ZIP6* giving a similar expression pattern to that seen in the microarray.

This paper reported by Filatov *et al.*, (2006) was novel in that the differences in expression observed between *A. halleri* and *A. petraea* were used as heritable markers. By using synteny between the test organisms and *A. thaliana* they identified mapped potential candidate genes to two chromosomal regions on chromosome 3 and 7.

Prior to the commencement of the microarray work described in this chapter to determine differential expression of genes involved in Zn hyperaccumulation a reliable and efficient method was developed using an *Arabidopsis thaliana* Genechip probed with *Brassica oleracea*. This study was novel in that a probe based selection method was developed to study plant genomes for which GeneChips® arrays are not available. (Hammond *et al.*, 2005)

Previous studies have used *Arabidopsis thaliana* GeneChip® arrays (representing 8,300 genes) to study genes involved in Zn transport. The

experiment demonstrated that genes associated with Zn homeostasis and transport were differentially expressed in shoots and roots of the Zn tolerant, Zn hyperaccumulator species *A. halleri* compared with the nonhyperaccumulator *A. thaliana* (Becher *et al.*, 2004; Weber *et al.*, 2004).

Affymetrix high-density oligonucleotide (oligo) GeneChip® arrays are a valuable and extensively used tool for transcriptional profiling (Hennig *et al.*, 2003; Lipshultz *et al.*, 1999). Each array represents every gene from the particular genome and has multiple corresponding oligo probes. Probes representing a gene are collectively called a probe set, each set being made up of between 11 and 20 probe pairs. Each probe pair is made up of a perfect match probe (PM) and a mismatch probe (MM), which has a single mismatch at the 13th base pair from the PM probe. The PM probe consists of a 25-base sequence, complementary to the 3' end of the target sequence. RNA that is fluorescently labelled is hybridised to the array and the signal emitted is imaged and quantified in order to determine the level of transcript in the sample tested. GeneChip® array technology provides reproducible, accurate data at a high throughput rate; data can then be collated and made widely available for all. Within plant biology data from several thousand GeneChip® array studies on the model species *A. thaliana* have been made publicly available. Due to conservation between members of the *Brassicaceae*, the *A. thaliana* chip can be used to determine and compare transcriptomic differences between the nonhyperaccumulator and hyperaccumulator species of *Thlaspi* to highlight putative genes responsible for the trait.

From this experiment a list of over 5000 genes were highlighted as differentially expressed in shoots of *Thlaspi caerulescens* compared with *T. arvense* to further investigate several of these genes qPCR (quantitative real time PCR) was used to verify the difference in expression.

Individual gene mRNA levels are typically quantified by either northern blot analysis or RNase protection assay. The development of the real time quantitative PCR technique has provided an alternative to these traditional methods. This allows the elimination of the use of radiolabelled probes with the added benefit of higher sensitivity. It is possible in fact to quantify RNA from a single cell and in theory it should be possible to detect one mRNA copy (Lockey *et al.*, 1998).

The dye SYBR Green (Applied Biosystems), binds to double stranded DNA which in turn emits a stronger fluorescence. As more double stranded amplicons are produced the fluorescence increases (Fig.3.1).

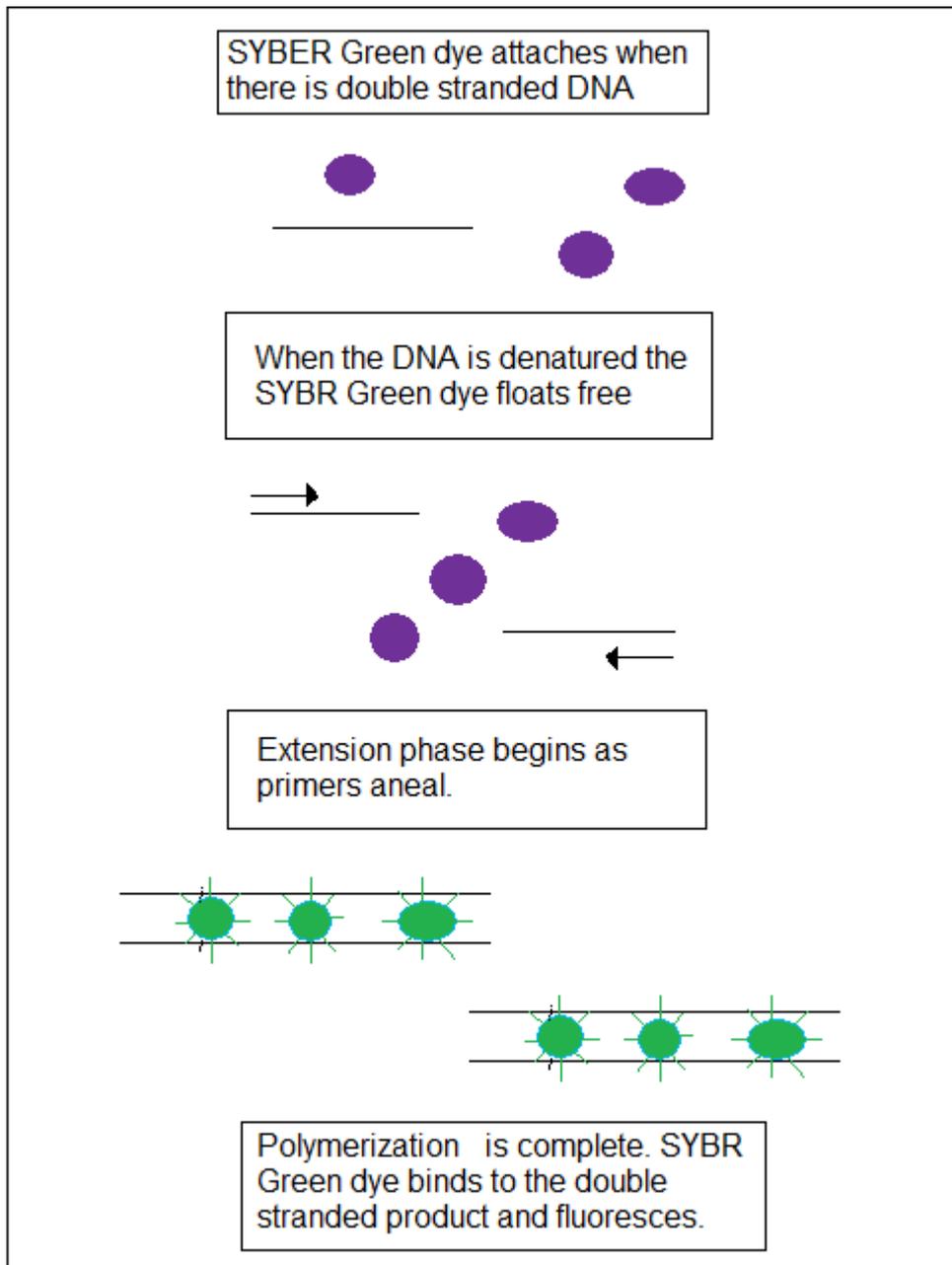


Fig.3.1 SYBR Green assisted qPCR. SYBR Green dye binds to double stranded DNA product and fluoresces which can be visualised and quantified by computers.

The main aim for this study was to identify and confirm genes involved in the hyperaccumulation of Zn using a commercially available GeneChip® array designed for *A. thaliana*. Secondly the aim was to identify and confirm genes

that are differentially expressed between two *Thlaspi* species, *T. caerulescens* and *T. arvense*, a heavy metal hyperaccumulator, and non-hyperaccumulator respectively. Further to the microarray experiment a qPCR would be used to confirm differential expression of potential hyperaccumulating genes identified by the microarray experiment.

3.2 Materials and methods

3.2.1 Plant material and growth conditions

3.2.1.1 For microarray analysis

Seeds of *T. caerulescens* J& C Presl. (Viviez population, France, Reeves *et al.*, 2001) and *T. arvense* L. (collected by AJM Baker, in Toronto, Canada) were surface sterilized with 70 % (v/v) ethanol for 10 min followed by a wash in reverse osmosis purified water. The seeds were then immersed in NaOCl (1 % active chlorine) for 20 min with a final 6 washes in reverse osmosis purified water. Seeds were imbibed for 4 d in sterile deionised water at 4°C to break dormancy. The culture medium used to germinate the seeds was composed of MS basal salts (Murashige and Skoog, 1962) 4.3 g L⁻¹ inorganic salts (Appendix 8.1), 10g L⁻¹ sucrose and 8 g L⁻¹ agar adjusted to a pH of 5.6 with 0.1 M NaOH (MS agar). The MS basal salt agar mix contains 0.03 mM Zn. The medium was autoclaved at 121 °C at 104 kPa for 20 min. Plants were sown in unvented polycarbonate boxes (Sigma-Aldrich, Dorset, UK) on 75 ml of the 0.8 % agar mentioned with approximately 30 seeds per box; the boxes were sealed with Nescofilm® (Bando Chemical Co., Kobe, Japan) to eliminate contamination. The plants were cultured *in vitro* in a growth room at 24 +/- 1

°C in a 16 h photoperiod at an intensity of 50-80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ under 100 W of cool fluorescent tube lighting at plant height (Type 84 Philips, Eindhoven, the Netherlands) and 8 h darkness.

To provide a growth comparison, seeds were also sown in pots containing a mix of 25% sand and 75% (v/v) peat (Shamrock medium grade Sphagnum peat, Scotts, UK, Bromford, Suffolk); the Zn content of the compost mix was 0.2 mg L⁻¹ (+ 0.04 standard error of the mean (SEM); $n=3$). Plants were grown at a constant temperature of 22 °C under a 16 h photoperiod. Plant shoots were harvested 64 days after sowing. Three independent samples from *T. caerulescens* and *T. arvense* plants grown in agar and two samples from plants grown in compost were harvested. Shoot material was then bulked from between three and eight plants per sample, and snap frozen in liquid nitrogen. Samples were stored in sterile screw-cap Eppendorf tubes and stored at -70 °C before RNA extraction was carried out.

To identify constitutive differences in gene expression between shoots of *T. caerulescens* and *T. arvense*, microarray analyses were conducted. Five *T. caerulescens* microarrays were compared with five *T. arvense* microarrays. Three of the five microarray analyses for each species were conducted on agar-grown plants, at a single Zn-level (0.03 mM Zn) and the remaining two using plants grown on compost (at 0.2 mg Zn l⁻¹). Since a cluster analysis (Hammond et al., 2006; Fig.2) revealed that gene expression was affected to a much lesser extent by environment than by species, all downstream

analyses were conducted using the normalised mean of five experimental replicates per species (Hammond et al., 2006).

3.2.1.2 For quantitative, real time PCR (qPCR)

To confirm data collected from the array a qPCR was carried out on *T. caerulescens* and *T. arvense* genes that had their coding sequence available (GenBank®, 13/05/07). Different seed populations were used in this experiment due to supplies. *T. caerulescens* (Ganges population, France) and *T. arvense* (Wellesbourne, collected from Wharf Ground field, Wellesbourne, Warwickshire, UK) were sterilized, with 70 % (v/v) ethanol for 10 min followed by a wash in reverse osmosis purified water. The seeds were then immersed in NaOCl (1 % active chlorine) for 20 min with a final 6 washes in reverse osmosis purified water. Seeds were imbibed for 4 d in sterile deionised water at 4 °C to break dormancy.

The culture medium used to germinate the seeds was composed of MS basal salts (Murashige and Skoog, 1962) 4.3 g L⁻¹ inorganic salts (Appendix 8.1), 10 g L⁻¹ sucrose and 8 g L⁻¹ agar adjusted to a pH of 5.6 with 0.1 M NaOH. The Murashige and Skoog basal salt agar mix (MS agar) contains 0.03 mM Zn. The medium was autoclaved at 121 °C at 104 kPa for 20 min. The seeds were sown on agar supplemented with ZnSO₄ to provide Zn external concentrations in the agar [Zn]_{ext}) of 3, 30, 60, 150, 300, 600, 1200 and 1800 μM. To confirm accurately the quantities of Zn within plant tissues ⁶⁵Zn was supplemented in the agar to a total activity of 2.1 kBq ⁶⁵Zn μmol⁻¹ Zn (section 2.2.3). Plants were sown in unvented polycarbonate boxes (Sigma-Aldrich, Dorset, UK) on

75 ml of the 0.8 % agar mentioned with approximately 30 seeds per box; the boxes were sealed with Nescofilm® (Bando Chemical Co., Kobe, Japan) to eliminate contamination. Plant shoots were harvested 42 days after sowing, the samples were split and a subsample snap frozen in liquid nitrogen and stored at -70 °C before RNA extraction as described. Fresh and dry weights were taken from subsamples and the ⁶⁵Zn γ-emissions were counted for 600 s per samples on an automatic well-type gamma counter (Wallac 1480 Wizard; Perkin-Elmer Life Sciences, Turku, Finland) (section 2.2.3 on determining Zn concentrations from counts per minute).

3.2.2 RNA extraction and hybridisation

RNA was extracted from *T. caerulescens* and *T. arvense* using methods described by Hammond *et al.*, 2005; 2006. Each plant sample had 1 ml of TRIzol® reagent added to it and subsequently manufacturer's instructions were followed to extract total RNA (Invitrogen, Gaithersburg, MD, USA). Several modifications were made to the protocol (i) after homogenisation with TRIzol® reagent, remaining plant material was removed by centrifugation and the remaining supernatant transferred to a new eppendorf tube, (ii) the precipitation of RNA was aided through the addition of 0.25 ml of isopropanol and 0.25 ml of a 1.2 M NaCl solution containing 0.8 M sodium citrate. This protocol maintained the proteoglycans and polysaccharides in a soluble form. Total RNA extracted was cleaned and purified using the 'RNA Cleanup' for RNeasy columns (Quiagen, West Sussex, UK). The quantity and purity of the RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Boblingen, Germany). The first strand of cDNA was synthesized

by reverse transcribing 5 µg of total RNA at 42°C for 1 h. 100 pmol of oligo dT(24) primer was used containing a 5'-T7 RNA polymerase promoter sequence, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (DTT), 10 mM dNTPs and 2000 units SuperScript® II reverse transcriptase (Invitrogen, Gaithersburg, MD, USA). Second strand synthesis was achieved using 10 units of *Escherichia coli* polymerase I, 10 units of *E. coli*. DNA ligase and 2 units of RNase H in the reaction containing 25 mM Tris HCl (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 10 mM (NH₄)₂SO₄, 0.15 mM NAD⁺ and 10 mM dNTPs. The synthesis of the second strand was undertaken at 16 °C for 2 h before 10 units of T4 DNA polymerase was added and allowed to continue reacting for a further 5 min. Termination of the reaction was achieved through the addition of 0.5 M EDTA. The GeneChip® Sample cleanup Module (Affymetrix, Santa Clara, CA, USA) was used to purify the double stranded cDNA products. The synthesized cDNAs were *in vitro* transcribed by T7 RNA polymerase (ENZO BioArray High Yield RNA Transcript Labelling Kit; Enzo Life Sciences Inc, Farmingdale, NY, USA) using biotinylated nucleotides to generate biotinylated complementary RNAs (cRNAs). The GeneChip® Sample cleanup Module (Affymetrix, Santa Clara, CA, USA) was used to purify the cRNAs. This was then followed by random fragmentation of the cRNAs at 94 °C for 35 min by a buffer containing 40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate, producing molecules of approximately 35-200 bp. Affymetrix *A. thaliana* ATH1-121501 (ATH1) GeneChip® arrays were hybridised to 15 µg of fragmented labelled cRNA for 16 h at 45 °C as in the Affymetrix Technical Analysis Manual (Fig.3.1). Streptavidin-Phycoerythrin solution was used to stain the

GeneChip® arrays and these were scanned using an Agilent G2500A GeneArray Scanner (Agilent Technologies, Palo Alto, CA, USA). The program Microarray Analysis Suite (MAS version 5.0; Affymetrix) was used to generate RNA signal intensities.

Chapter 3: *Thlaspi* sp. Transcriptomic Experiments

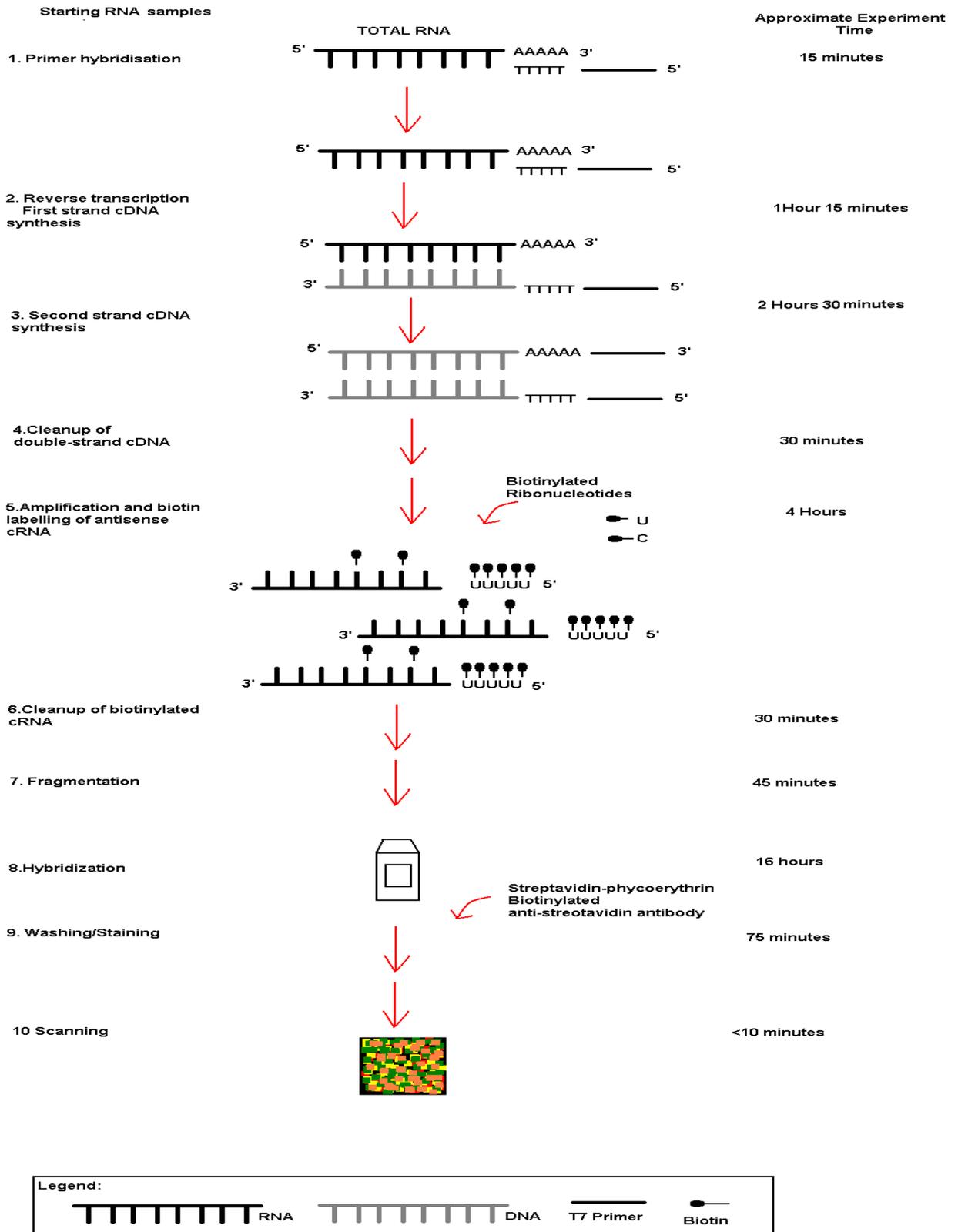


Fig.3.1 Target RNA labelling for GeneChip® probe array (adapted from Affymetrix Inc, www.affymetrix.com).

3.2.3 GeneChip® array manufacture

GeneChip® arrays are produced and made commercially available by Affymetrix, Santa Clara, CA, USA. The Affymetrix *A. thaliana* ATH1-121501 (ATH1), like other similar Affymetrix GenChips®, are constructed via light-directed synthesis. Photolithography and solid-phase DNA synthesis are used to produce high-density DNA probe arrays (Fig.3.2). Quartz wafer is used as the substrate for the oligonucleotides. The wafer is naturally hydroxylated which allows the attachment of linker molecules, which help position the probes on the array. Silane (SiH_4 - a silicon analogue of methane) is used to attach the linker molecules to the quartz; a reaction between the hydroxyl groups and the silane forms a matrix of covalently linked molecules. Photolithographic masks are then used, carrying 18-20 square windows that correspond to the desired sequence of the probes. Ultraviolet light is shone over the mask, unprotecting the exposed linkers, enabling them to become available to nucleotide coupling. A solution containing one single deoxynucleotide group with a removable protection group is washed over the wafer. Deoxynucleotides are able to attach to the activated linkers. The step of deprotection and coupling is repeated until the precise lengths of the probes are reached. In the final step the wafers are deprotected, diced and packaged in flowcell cartridges. (Affymetrix Inc www.affymetrix.com).

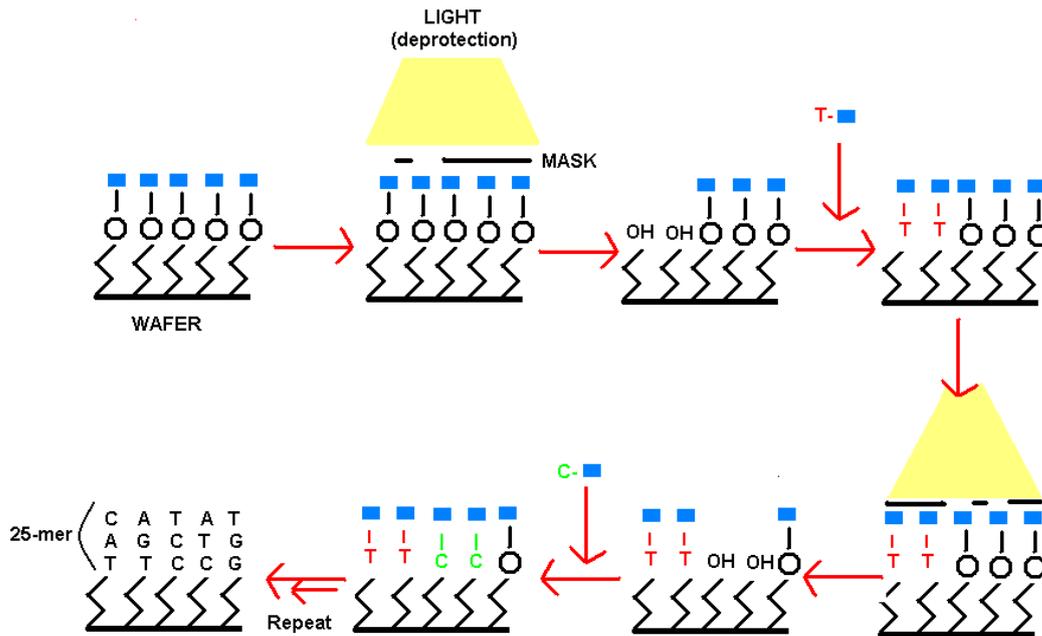


Fig.3. 2 Affymetrix GeneChip array construction. The manufacturing process using the technique, photolithography. A mask covers the wafer to direct oligonucleotide synthesis. (Adapted from Affymetrix Inc, www.affymetrix.com).

3.2.4 Masking strategies employed to allow microarray experiments in heterologous systems

GeneChip® arrays use probe sets to quantify the abundance of a transcript. The probe sets consist of between 11 and 20 probe pairs; each probe-pair is made up of a perfect-match (PM) and a mismatch (MM) probe. The PM probe is a 25-base sequence which is complementary to the target transcript; the MM is identical to the PM with the exception of a single mismatch at the 13th base.

To allow the use of a GeneChip® array designed for one species to be used on a closely related species a novel technique to improve sensitivity of high-

density oligonucleotide arrays was developed (Hammond *et al.*, 2005). This technique involves selecting probe-pairs based on the hybridisation efficiency of the PM oligonucleotide probe with genomic DNA from the target species for which the GeneChip® was not designed. If all probe sets were used sequence polymorphisms between the two species would result in an underestimate of transcript abundance. To determine which probe-set were to be used in subsequent transcript analyses the hybridisation intensities of the PM with the gDNA were assessed and a set threshold level determined.

To provide the gDNA a DNA extraction method described by Thomas *et al.*, (1994) was adapted for both *T. caerulescens* and *T. arvense*. A pestle and mortar was used to macerate cells under liquid nitrogen. Extraction using phenol: chloroform (1:1 v/v) was repeated twice. The BioPrime DNA labelling system (Invitrogen, Gaithersburg, MD, USA) was used according to the manufacturer's instructions to biotinylate the genomic DNA from both *T. caerulescens* and *T. arvense*.

Standard Affymetrix protocols for RNA hybridisation were used to hybridise DNA without the need to fragment the labelled DNA since the labelling reaction produces fragments of the correct length. Hybridisation was carried out at 45 °C for 16 h. Following hybridisation the Affymetrix eukaryotic wash was carried out which included staining of antibodies. Hybridisation was carried out between 0.5 µg of labelled gDNA and the ATH1 GeneChip®. The resulting hybridisation was scanned on a G2500A Gene Array scanner which generated a cell intensity file (.cel file) using Microarray Analysis Suite (MAS

Version 5.0; Affymetrix). Two .cel files were produced which contained the gDNA hybridisation intensities between *T. caerulescens* and *T. arvense* fragments respectively. Probe-pairs from the .cel file were selected for subsequent transcriptome analysis using a .cel file parser script (Xspecies Version 1.1) written in the Perl programming language <http://www.perl.com>. The Perl script was designed to create probe mask (.cdf) files compatible with a range of microarray analysis software packages. Selection of a probe-set was made when it was represented by one or more PM probe pair(s) per probe set, which subsequently means that a minimum of 25 bp of homologous probe sequence of *A. thaliana* was required for future transcriptome analysis of *T. caerulescens* and *T. arvense*. The program was designed to allow a user-defined gDNA hybridisation intensity threshold for probe mask file generation to be set. Therefore the probe mask files were generated by analysing a range of gDNA hybridisation intensity thresholds ranging between 0 and 1000.

3.2.5 Interpretation of the *Thlaspi* transcriptome data

The 10 RNA CEL files created represented replicates of both species grown under both soil and agar conditions. These were analysed using the GeneSpring™ analysis software (GeneSpring™ 7.2; Silicon Genetics, CA, USA) using the Robust Multichip Average (RMA) prenormalisation algorithm, a 3 step normalisation, using the 24 CDF files in turn (Irizarry *et al.*, 2003). This is a 3 step process that involves background correction of data; chip background is taken away from PM values. Secondly data is normalised. Normalisation involves the division of data by a common variable to allow the

effect of this variable to be cancelled out and therefore compare underlying characteristics of the data to be compared. The final step in the RMA analysis is summarisation, which is performed in a log base 2 scale to fit a linear model. Summarisation combines the multiple probe intensities for each probe set to produce a single expression value for each probe set

Prior to analysis using the 24 CDF files, to determine the optimal gDNA hybridisation threshold for interpreting transcriptional data, the non-scaled RNA CEL files were pre-normalised in GeneSpring™ and taken as a whole experimental group using the *A. thaliana* CDF file (i.e. with no probe selection).

Each of the 10 RNA CEL files from the transcriptional analysis was pre-normalised together. From this, 25 sets of data, each containing the 10 grouped RNA CEL files, were produced. These sets of data consisted of one set with no probe selection, 12 with probe selection based on *T. caerulescens* gDNA hybridization and 12 with probe selection based on *T. arvense* gDNA hybridization.

The 25 sets of transcriptional data was reduced to 13 sets by combining the gDNA hybridization thresholds of the *T. caerulescens* RNA CEL files, pre-normalised with a *T. caerulescens* probe mask file (CDF), with the *T. arvense* RNA CEL file, pre-normalised with a *T. arvense* probe mask file (CDF). By combining the data in this way it ensured that only common probe sets were used in all transcriptome comparisons.

Normalisation of the probe- set signal values of each gene, within each of the 13 transcriptome data sets, interpreted at a different gDNA hybridization intensity threshold, in which each data set contained 10 RNA CEL files was applied as follows: Probe-set signal values from *T. caerulescens* and *T. arvense* for each biological replicate and growth condition were standardised to the probe set value of *T. arvense*, as this was considered to be the control. This was achieved by dividing RMA pre-normalised probe-set signal values from *T. caerulescens* by the RMA pre-normalised probe-set signal values from *T. arvense*. Putative genes (i.e. probe-sets) with differential hybridisation intensities were identified between *T. caerulescens* and *T. arvense* using a two-step process. Firstly genes that were shown to be 2-fold up- or downregulated were selected and secondly to determine differential expression between *T. caerulescens* and *T. arvense* a Welch's *t*-test was performed using the Benjamini-Hochberg false discover rate (FDR) multiple testing correction at 0.05 or 0.005 (False discovery rate control is a statistical method used in multiple hypothesis testing to correct for multiple comparisons) (Hammond, *et al.*, .2006; 2005).

3.2.6 *In silico* alignment of *A. thaliana* sequences and PM probes with *Thlaspi* gene sequences

Thlaspi gene coding sequences were downloaded from GenBank® database (www.ncbi.nlm.nih.gov/entrez website 4) which contained 18 *T. caerulescens* and *T. arvense* sequences at the time of study (July 2004). Of these, 9 (seven *T. caerulescens* and two *T. arvense*) *T. caerulescens* [heavy-metal-associated

domain-containing protein 4 (*HMA4*), TCA567384; nitrate reductase 1 (NR1), AY551529; zinc transporter 1 (ZNT1), AF133267; ZNT2/4, AF275752/AF292370; ZNT5, AF292029; carbonic anhydrase (CA), AY551530; nicotianamine synthase 1 (NAS1), TCA300446) and two *T. arvense* genes (chalcone synthase (CHS), AF144535; metal tolerance protein 1 (MTP1), AY483146. Corresponding *A. Thaliana* sequences were identified using the BLAST algorithm against the GenBank® database to find the closest match to the *Thlaspi* spp. For further analysis the sequences were loaded into the Vector NTI program (Version 9.0.0, Invitrogen, Gaithersburg, MD, USA) Sequences were aligned using the ClustralW algorithm.

3.2.7 Quantitative real-time PCR (qPCR)

At the commencement of this thesis the microarray data was available for analysis. To confirm validity of the microarray data qPCR was performed, with assistance from Warwick HRI, on *Thlaspi caerulescens* and *T. arvense* genes for which the coding sequences were available in GenBank™.

3.2.7.1 qPCR primer design

The microarray highlighted over 5000 genes as differentially expressed in *T. caerulescens* compared with *T. arvense*. It was necessary to select a smaller number of genes to confirm their differential expression using qPCR. This selection of genes was limited to those that had sequences available on GenBank®. GenBank® is a genetic sequence database containing a collection of all publically available annotated DNA sequences built by the National Center for Biotechnology Information (NCBI), a division of the National Library

of Medicine (NLM), located on the campus of the US National Institutes of Health (NIH) in Bethesda, MD, USA. The sequences are submitted from individual laboratories and sequencing projects, covering 140,000 organisms (Benson *et al.*, 2004). Through GenBank®, sequences can be searched for using entrez (<http://www.ncbi.nlm.nih.gov/entrez>). Also Sequence similarity searches maybe carried out using BLAST within the GenBank® site.

Seven genes from *T. caerulescens* were chosen to be studied in further detail by qPCR. These genes were selected as they were highlighted as differentially expressed in the microarray in *T. caerulescens* compared with *T. arvense*, the non-hyperaccumulator. Also these genes had sequences available on GenBank® (<http://www.ncbi.nlm.nih.gov/entrez>) at the time of study. The chosen genes were; heavy-metal-associated-domain-containing protein 4 (*HMA4*), TCA567384; nitrate reductase 1 (NRT1), AY551529; zinc transporter 1 (ZNT1), AF133267; ZNT2/4, AF275752/AF292370; ZNT5, AF292029; carbonic anhydrase (CA), AY551530; nicotianamine synthase 1 (NAS1), TCA300446 and two *T. arvense* genes; chalcone synthase (CHS), and Metal Transporter Protein 1 (MTP1). The primers were designed to the cDNA sequences of the genes (Table 3.1) using Primer 3 design tool (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>). An 18s rRNA gene was included as a control; this gene was taken from *Arabidopsis thaliana*. The primer select program (DNASTAR inc., Madison, WI, USA) was used to check for secondary structures. A BLAST search was subsequently carried out using sequences from the GenBank® database on the primer sequences to look for short, near exact sequences (7-20bp) that could cause inaccuracies in the

results and to be sure on *Thlaspi* cDNA was in target. Primers that created loops or dimers or those with homology to other sequences were disregarded and new ones selected.

Gene	Species				
	Arabidopsis	GenBank Identifier	Forward Primer	Reverse Primer	Amplicon length
18S rRNA	thaliana				
CHS	<i>Thlaspi arvense</i>	X16077	CATAAACGATGCCGACCAG	AGCCTTGCGACCATACTCC	108
MTP1	<i>Thlaspi arvense</i>	AF144535	GGACGAGATGAGGAGGAAG	CCGAAACCAAACAAGACAC	84
CA	<i>Thlaspi caerulescens</i>	AY483145	CTCTCGTCTTTTCGGTTATC	CCCTCTCCAGTTTCGTAG	115
<i>HMA4</i>	<i>Thlaspi caerulescens</i>	AY551530	TCTTCTCTTCTCCTCCTC	ATTTCGGTCCCATCTCTTC	136
NAS1	<i>Thlaspi caerulescens</i>	TCA567384	GATAAGAAAAGCCAAGAGAAGG	ACAACAACCAGAGTGAAGATG	81
NR1	<i>Thlaspi caerulescens</i>	TCA300446	ACTCACTCGCTTCTCTCC	TCTCCTCCTTGTTCTATCC	150
ZNT1	<i>Thlaspi caerulescens</i>	AY551529	GGAGGCACAGGGATAACTC	TGCGTAAACCACAAACATCTC	90
ZNT2/4 ^a	<i>Thlaspi caerulescens</i>	AF133267	TGAGAGTAAGCAACAGAGGAAC	CACAACCACGGAAGAAC	88
ZNT5	<i>Thlaspi caerulescens</i>	AF275752/AF292370	GAAGGGAACGATGAGAAAAGTG	TGAGTGTGATTATGTGTATGGTGAG	101

Table 3.1 The primers used in qPCR analysis of *T. caerulescens* and *T. arvensis*

3.2.7.2 RNA extraction and running the qPCR

Total RNA was extracted from the plants described at the start of this chapter as in the microarray paper (Hammond *et al.*, 2006). Residual chromosomal DNA was removed during RNA purification using the Qiagen RNeasy column kit, using the on-column DNase column according to the manufacture's instructions.

Reverse transcription was carried out on 1 μ l of RNA extracted using the ThermoScript RT-PCR system (Invitrogen, Gaithersburg, MD, USA). The synthesis of cDNA was performed using random hexamers (50 ng μ l⁻¹). The primers were designed to the cDNA of nine genes (*ZNT1* (Zinc transporter 1) *ZNT2/4*, *ZNT5*, *HMA4* (heavy-metal associated domain-containing protein 4), *NR1* (Nitrate reductase 1), *CA* (Carbonic anhydrase), *NAS1* (nicotianamine synthase 1), *MTP1* (metal tolerance protein 1), *CHS* (chalcone synthase) and a 18s rRNA control gene using Primer3 (website 4). The program DNASTAR (DNASTAR INC, Madison, WI, USA) was used to check for secondary structures using the PrimerSelect program. The GenBank® database was again used to perform short BLAST sequences to find near match sequences to ensure that the primers were indeed specific to the target *Thlaspi* cDNA sequences.

Quantification of the nine genes was successful in the shoots of *T. caerulescens* and *T. arvense* using the 'standard curve' method of mRNA quantification with normalisation to the control gene 18S rRNA (Wong and Medrano, 2005). The quantification of all nine genes was carried out from plants grown at 3 and 30 μ M

[Zn]_{ext}. The expression of *HMA4*, *ZNT2/4* and *NR1* genes was also quantified at all eight levels of [Zn]_{ext}.

The quantification of genes was achieved by qPCR using the fluorescent dye SYBR® Green (Applied Biosystems, Warrington, UK) and an ABI prism 7900 HT sequence detection system (Applied Biosystems). Triplicate replications were used to ensure accuracy, both biological and technical. The qPCR reactions were carried out in 384-well plates with 15 µl of sample per well, made up of 2 ng of cDNA, 1 µM 5' and 3' primer and 7.5 µl of 2x SYBR® Green PCR master mix (Applied Biosystems). The conditions for the qPCR reaction were 50 °C for 2 min and 95°C for 10 min for 1 cycle, followed by 95 °C 15 s and 60°C for 1 min for 40 cycles. A step consisting of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s was used as a dissociation step for melting curve analysis to allow the detection of primer dimers and non-specific products. Control samples contained either 2 ng of total RNA or Rnase free water to test for gDNA contamination in the samples. A standard curve for each gene allows the quantification of unknown samples. For the standard curves of the genes *HMA4*, *NR1*, *ZNT2/4*, *ZNT1*, *ZNT5*, *CA* and *NAS1* a dilution series of 20, 2, 0.2 ,0.02 ng of reverse transcribed *T. caerulescens* total RNA was used and the same was done for *CHS* and *MTP1* using *T. arvense* total RNA. Transcript levels of each gene were normalised to 18S rRNA and the expression of genes was measured relative to the transcript quantities of *T. arvense* grown at 3 µM Zn.

3.3 Results

The aim of this chapter was to identify genes that were differential expressed in *Thlaspi caerulescens* compared with *Thlaspi arvense*. Secondly the genes that were

highlighted in the microarray as differentially expressed were then studied using qPCR at varying zinc concentrations. Thirdly the microarray that was carried out was novel in that non-model plant species were studied using an Affymetrix *Arabidopsis thaliana* ATH1-121501 Gene Chip®. This was possible due to the sequence homology between the species. A gDNA based probe selection strategy was employed based on the hybridization of *T. caerulescens* and *T. arvense* with *A. thaliana* probes.

3.3.1 Sequence comparison of *A. thaliana* and *T. caerulescens*

Sequences of seven *T. caerulescens* genes and two *T. arvense* genes were chosen to validate the gDNA-based probe selection strategy by identifying homologous *A. thaliana* sequences. Homologous *Thlaspi* and *A. thaliana* sequences were aligned to determine the similarities between the species at the sequence level. The average similarities between these sequences was observed as 81.5 %. The greatest similarity was shown in the carbonic anhydrase gene, with a 89.4 % of the nucleotides in the coding region being identical between *T. caerulescens* and *A. thaliana*. When the PM sequences were aligned with *Thlaspi* gene sequences an average of 87 % of the nucleotides were found to be identical, which is the equivalent of 240 identical nucleotides. The *TcHMA4* gene had the lowest homology to the PM probe sequences.

3.3.2 Masking strategies employed to allow microarray experiments in heterologous systems

Sequence polymorphisms between *Thlaspi* spp. and *A. thaliana* would affect the transcript abundance if all probe sets were used. Therefore a gDNA based probe

selection method was employed by using 13 probe mask files with gDNA hybridisation intensity thresholds ranging from 0 to 1000 to determine an optimum level. It was shown that *A. thaliana* PM probes hybridised extensively to *Thlaspi. sp* genomic DNA (Fig.3.4) as the gDNA hybridisation intensity threshold was increased from 0 to 1000, PM probe retention decreased rapidly. Comparatively retention of whole probe sets, representing transcripts, were less sensitive to increasing gDNA hybridisation intensities, due to the fact that only a minimum of one PM probe was required to retain a whole probe set. With the chosen gDNA hybridisation intensity threshold set at 300 masked >50 % of the PM oligo probes with the loss of 3 % available *A. thaliana* probe-sets. If the threshold level was set higher than 300 too many probe sets are lost.

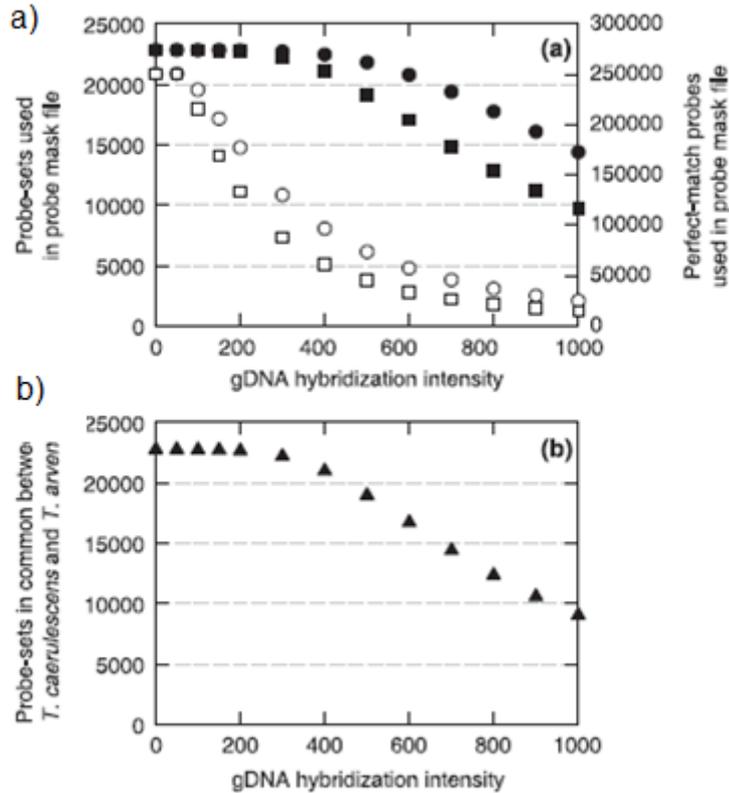


Fig.3.4 (a) Genomic DNA hybridisation intensity values generated from the binding of *Arabidopsis thaliana* perfect-match (PM) probes and probe sets from the Affymetrix *A. thaliana* ATH1-121501 GeneChip® array with the transcriptomes of *Thlaspi caerulescens* and *Thlaspi arvense*, used to generate the probe mask files. Squares represent *T. caerulescens* and circles *T. arvense*. Closed symbols are scaled to the left-hand y-axis (i.e. probe sets used in probe mask files), and open symbols are scaled to the right-hand y-axis (i.e. PM probes retained in probe mask files). (b) gDNA hybridization intensity thresholds to show Probe sets in common between *T. caerulescens* and *T. arvense*, used to generate the probe mask files.

From the growth comparison of genomic DNA between agar and compost grown plants it was found that the transcript profiles were of close association indicating that substrate effect on gene expression was small in comparison to the differential

expression between plant species and that genomic DNA does not change in relation to where it is grown.

Probe selection was used at a gDNA hybridisation intensity threshold level of 300 and probe-set signals were normalised to the median value across all the GeneChip® array data. The use of probe mask files increased the number of genes differentially expressed more or less than 2-fold between *T. caerulescens* and *T. arvense* (Fig.3.5). When no probe selection was used 159 genes were shown to be differentially expressed (>2-fold or >0.5-fold), (72 higher and 87 lower expression) in the shoots of *T. caerulescens* compared with *T. arvense*. With the use of the probe selection at a gDNA hybridisation intensity of 300 the number of genes found to be differentially expressed (>2-fold or >0.5-fold) rose significantly to 5782 genes (3816 higher and 1966 lower expression). This concludes that the use of the probe selection to detect transcript differences between the two species increases the sensitivity of the analysis.

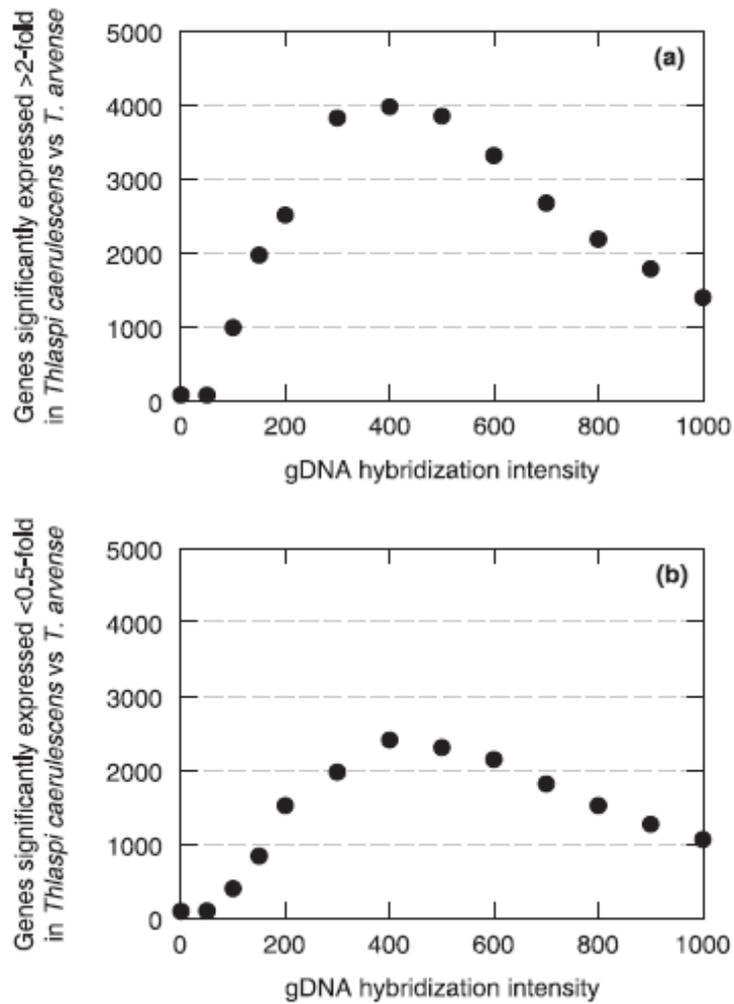


Fig.3.5 Genes differentially expressed in the shoots of *Thlaspi caerulescens* and *Thlaspi arvense* at increasing gDNA hybridization intensity levels used to generate probe mask files for the transcriptome analysis. (a) Genes expressed significantly >2-fold and (b) < 0.5-fold in *T. caerulescens* compared with *T. arvense*.

3.3.3 Microarray analysis to compare differential gene expression between *T. caerulescens* and *T. arvense*

The microarray was successful in several ways. Firstly it showed that whole genome transcriptome profiling can be carried out on a non-model plant species provided there is a GeneChip® array of a model species with enough conservation to give a

feasible hybridisation. This array highlighted in total, 4947 genes that were differentially expressed, both up and downregulated, in *T. caerulescens* compared to *T. arvense*. Of these genes many included those known to be involved in Zn transport and compartmentalisation. 3349 genes were found to be upregulated in the shoots of *T. caerulescens* compared to *T. arvense* and 1598 transcripts were found to be lower. This was found to be true for both agar and compost grown plants. Table 3.1 shows genes which were differentially expressed in *Thlaspi caerulescens* compared with *Thlaspi arvense* (ten of these genes with highest expression and ten with the lowest expression).

ATH1-121501 GeneChip@ array identifier			Normalized expression in shoots of <i>T. caerulescens</i> compared with <i>T. arvense</i>	Standard error	Welch t-test P-value
Highest expression					
257462_at	At1g65740	F-box protein	234.46	15.99	< 0.0001
262632_at	At1g06680	Photosystem II oxygen-evolving complex	192.50	35.30	< 0.0001
255381_at	At4g03510	E3 ubiquitin ligase	124.53	22.19	< 0.0001
262679_at	At1g75830	Plant defensin protein, putative (PDF1.1)	123.16	7.97	< 0.0001
261599_at	At1g49700	Hypothetical protein	91.76	14.05	< 0.0001
259426_at	At1g01470	Expressed protein	84.36	4.65	< 0.0001
262262_at	At1g70780	Expressed protein	81.43	5.56	< 0.0001
252570_at	At3g45300	Isovaleryl-CoA-dehydrogenase	73.56	13.45	< 0.0001
266118_at	At2g02130	Plant defensin protein, putative (PDF2.3)	73.03	2.81	< 0.0001
245650_at	At1g24735	Caffeoyl-CoA 3-O-methyltransferase	72.14	4.93	< 0.0001
Lowest expression					
262684_s_at	At1g76030	Vacuolar ATP synthase subunit	0.024	0.002	< 0.0001
266865_at	At2g29980	Omega-3 fatty acid desaturase (FAD3)	0.024	0.001	< 0.0001
266421_at	At2g38540	Nonspecific lipid transfer protein 1 (LTP 1)	0.023	0.001	< 0.0001
258299_at	At3g23410	Alcohol oxidase-related	0.023	0.004	< 0.0001
256277_at	At3g12120	Omega-6 fatty acid desaturase (FAD2)	0.021	0.003	< 0.0001
251013_at	At5g02540	Short-chain dehydrogenase/reductase	0.021	0.002	< 0.0001
246997_at	At5g67390	Expressed protein	0.020	0.002	< 0.0001
258774_at	At3g10740	Glycosyl hydrolase family 51	0.017	0.002	< 0.0001
261821_at	At1g11530	Thioredoxin family	0.014	0.001	< 0.0001
250862_s_at	At5g04800	40S ribosomal protein	0.012	0.000	< 0.0001

AGI, *Arabidopsis* Genome Initiative.

Table 3.2 Microarray data that shows the ten highest expressed transcripts and the ten lowest transcripts in the shoots of agar and compost grown *Thlaspi caerulescens* compared with *Thlaspi arvense*.

The study highlighted two genes associated with plant defensins, *PDF1.1* (At1g75830) and *PDF2.3* (At2g02130). These were found to be amongst the 10 most highly expressed genes in the shoots of *T. caerulescens* compared to *T. arvense* grown in agar. Also two endoplasmic reticulum–localized fatty acid desaturase genes (*FAD2/FAD3*; At3g12120/At2g29980) and a two-pore calcium channel gene (*TPC1* At4g03560) were found amongst the 10 genes expressed at lower levels in the shoots of *T. caerulescens* compared to *T. arvense*. However it is at present unclear of the biological significance of this (Table 3.2).

Of great importance was the identification of transcript homologous to *A. thaliana* genes known to be members of transport protein families, which have been suggested to be involved in Zn transport. These were found to have a significantly different expression in shoots of *T. caerulescens* compared to *T. arvense*. Specifically three homologs to members of the *A. thaliana* ZIP (ZRT, IRT-like proteins) transporter family were found to have increased expression in *T. caerulescens*, including *AtIRT3* (19 and 6.7 fold difference in agar and compost grown plants respectively; At1g60960), *AtZIP6* (2.3 and 3.4 fold, At2g30080) and in agar grown conditions only, *AtZIP10l* (0.5-fold; At1g31260). The P-type ATPase proteins were found to have higher expression levels in shoots of *T. caerulescens* compared to *T. arvense*: *AtHMA3* (3.5 and 3.9 fold; At4g30120) and *AtHMA4* (2.2 and 2.2 fold; At2g19110), the calcium transporter *AtACA13* (5.4 and 4.7 fold; At3g22920), and the Ca^{2+} *AtACA12* (3.7 fold; At3g63380) in agar grown plants only. The third family of genes identified was the CDF transporters; transcripts to three CDF transporters were shown to have a significantly higher expression in shoots of *T. caerulescens* compared to *T. arvense* (At2g39450, At2g04620 and At3g12100). Three alcohol dehydrogenases including cinnamyl-alcohol dehydrogenases (up to 26 fold ; At1g09500), a histidinol dehydrogenase (up to 5 fold; At5g63890), a beta-lactamase (2.1 fold; At5g63420), two carbonic anhydrases (including At4g20990, up to 18 fold), a metalloprotease (6.2 fold; At2g32480), a metallothionein (2.5 fold; At3g15353) and several genes involved in glutathione metabolism (up to 11 fold for *AtGST16*; At2g02930) were all found to be higher in shoots of *T. caerulescens* compared to *T. arvense* in at least one growth condition. Along side the genes identified over 1770 of the genes with a significantly different level of expression between the two plant species have no known function.

3.3.4 Quantitative PCR to confirm differential gene expression between *T. caerulescens* and *T. arvense*

To confirm this differential expression, qPCR was adopted to analyse this further. Nine genes, seven from *T. caerulescens* and two from *T. arvense* were chosen according to sequence data that was publicly available. Fig.3.5 shows the shoot transcript abundances of the nine chosen genes in *T. caerulescens* and *T. arvense* at 3 and 30 μM $[\text{Zn}]_{\text{ext}}$, normalized to *Thlaspi arvense* at 3 μM $[\text{Zn}]_{\text{ext}}$. Of particular interest are the genes *ZNT2/4*, *HMA4* and *NR1*. The expression of these genes are significantly greater in *T. caerulescens* than *T. arvense*. There is also a marked difference in expression of these genes between concentrations of $[\text{Zn}]_{\text{ext}}$. This could be studied further by looking at the spatial expression and distribution of these genes. Using the same qPCR data, Fig.3.6 shows the average transcript abundance of the biological replicates for *Thlaspi caerulescens* compared with *Thlaspi arvense* for each of the 9 genes separately. Represented this way, *HMA4* and *NR1* expression is interesting; there was a overall significant difference between the expression of of *T. caerulescens* and *T. arvense* with *T. caerulescens* showing considerably higher expression than *T. arvense*. Looking at the expression of *HMA4* and *NR1* in *T. caerulescens* at the two Zn concentrations it can be seen that the expression doubles at 30 μM $[\text{Zn}]_{\text{ext}}$ compared with 3 μM $[\text{Zn}]_{\text{ext}}$. Also of interest is the expression of *ZNT2/4* genes. Again comparing the expression in *T. caerulescens* with *T. arvense* there is a significantly higher expression shown in *T. caerulescens* over *T. arvense*. Looking at the expression of the gene in *T. caerulescens* at 3 and 30 μM $[\text{Zn}]_{\text{ext}}$ it can be seen that the gene appears to be down regulated at higher zinc concentrations. For *T. arvense* the opposite can be seen with expression of the *ZNT2/4* genes being upregulated at higher Zn levels. The expression of *ZNT1* shows

a similar pattern with higher expression overall in *T. caerulescens* than in *T. arvense*. Expression in *T. caerulescens* is again downregulated at elevated Zn levels though the difference in transcript abundance between 3 and 30 μM $[\text{Zn}]_{\text{ext}}$ is closer for *ZNT1* than *ZNT2/4*. The expression in *ZNT5* is unlike any other genes studied here, the gene is downregulated in *T. caerulescens* at high Zn levels, with the opposite occurring in *T. arvense* where the gene is upregulated at higher Zn levels. The expression of the gene in *T. arvense* at 30 μM $[\text{Zn}]_{\text{ext}}$ is greater than that found in *T. caerulescens* at 30 μM $[\text{Zn}]_{\text{ext}}$. The transcript abundance of the *CA* gene was interesting in that for *T. caerulescens* expression remained low at both Zn levels, with only a small elevation at 30 μM $[\text{Zn}]_{\text{ext}}$ compared with 3 μM $[\text{Zn}]_{\text{ext}}$. However for *T. arvense* there was a significant increase at elevated Zn levels, over double that of *T. caerulescens*. For *NAS1* expression was significantly higher in *T. caerulescens* compared with *T. arvense* with the expression becoming reduced in both plant species at increase Zn concentrations. The expression of the *CHS* gene was higher in *T. arvense*. When comparing the expression of *CHS* at the different Zn concentrations it can be seen that for both *T. arvense* and *T. caerulescens* the expression was downregulated at 30 μM $[\text{Zn}]_{\text{ext}}$ when compared with 3 μM $[\text{Zn}]_{\text{ext}}$. Lastly the expression of the *MTP1* gene was similar to that of *CHS* in that expression was highest in *T. arvense* and that expression was downregulated at higher Zn levels.

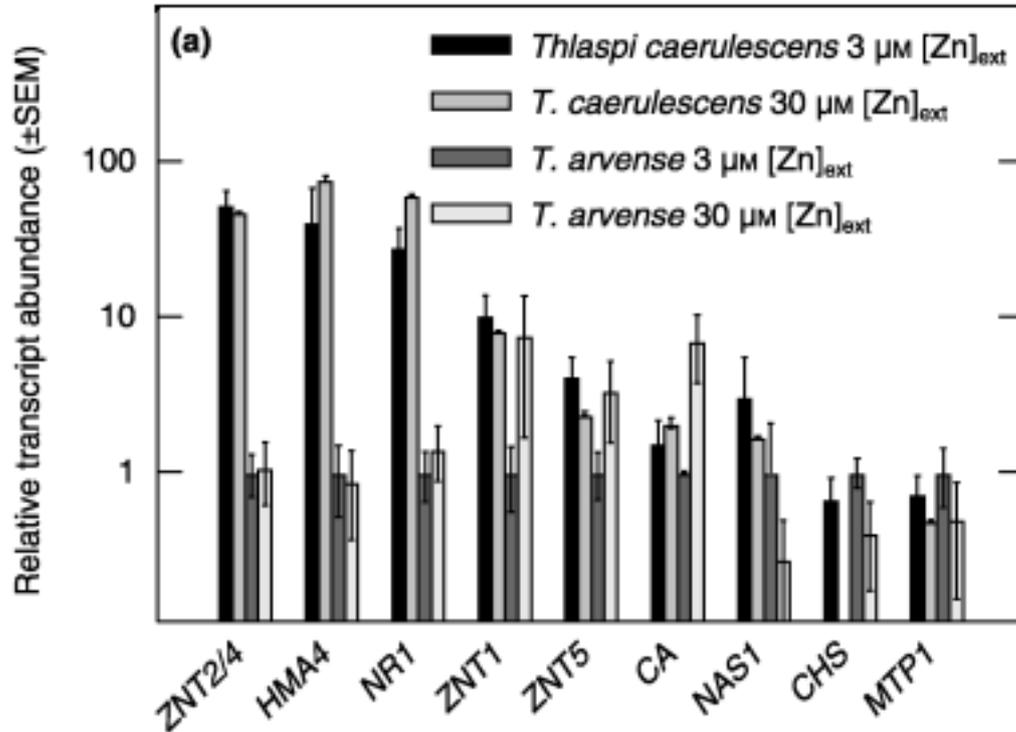
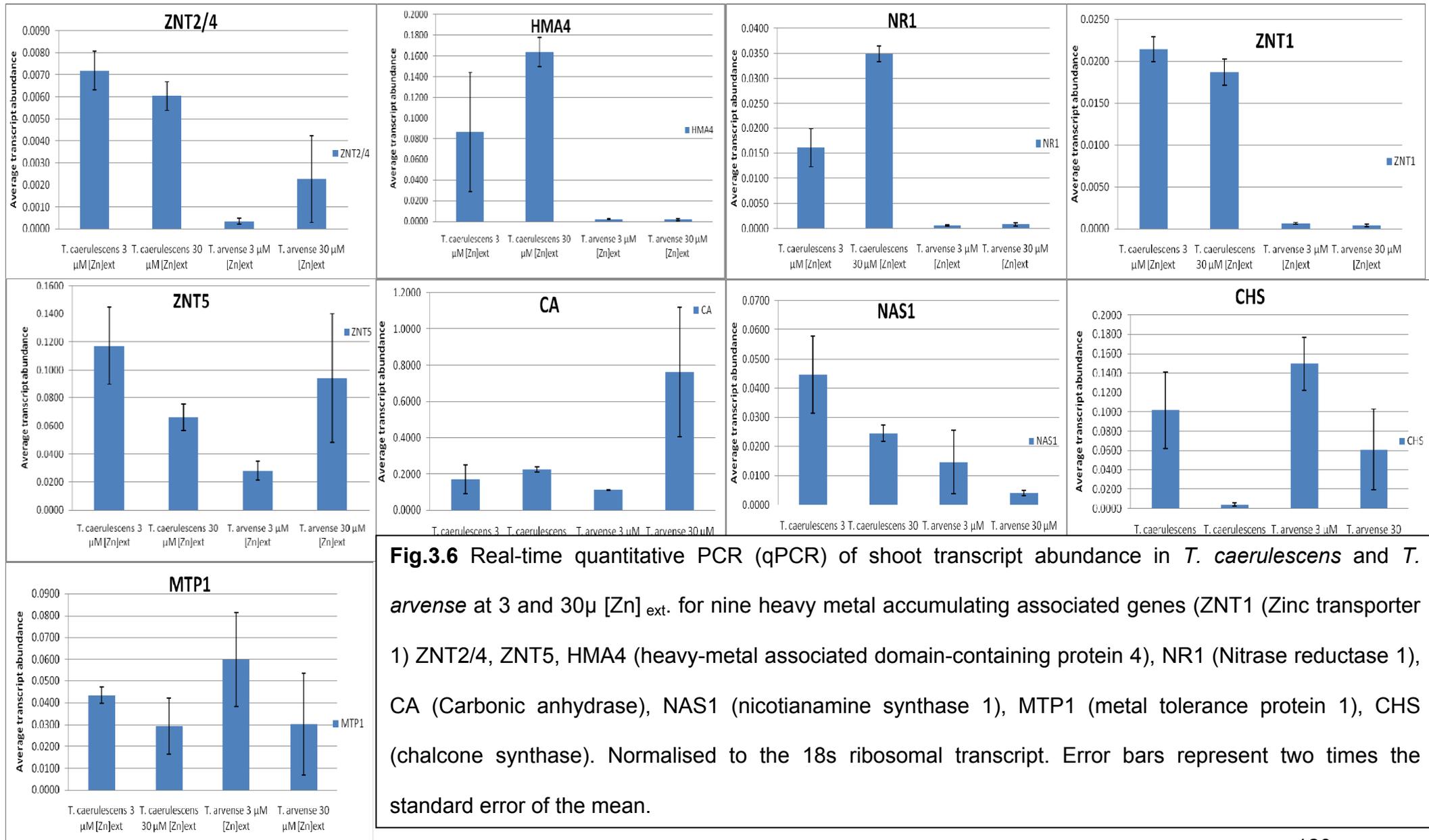


Fig.3.5 Real-time quantitative PCR (qPCR) of shoot transcript abundance in *T. caerulescens* and *T. arvense* at 3 and 30 μM $[\text{Zn}]_{\text{ext}}$. for nine heavy metal accumulating associated genes (*ZNT1* (Zinc transporter 1) *ZNT2/4*, *ZNT5*, *HMA4* (heavy-metal associated domain-containing protein 4), *NR1* (Nitrate reductase 1), *CA* (Carbonic anhydrase), *NAS1* (nicotianamine synthase 1), *MTP1* (metal tolerance protein 1), *CHS* (chalcone synthase). The data represents the average abundance normalized to *T. arvense* at 3 μM $[\text{Zn}]_{\text{ext}}$. Error bars represent \pm the standard error of the mean.



3.3.4.1 Expression of *HMA4*, *ZNT 2/4* and *NR1* in *T. caerulescens* under increasing external zinc concentrations

Following the above results a further experiment looked in more detail at the expression of *HMA4*, *ZNT 2/4* and *NR1* this time at increasing zinc concentrations from 3 to 3000 μM . These three genes were chosen as they were highlighted in the first qPCR experiment to have a considerably higher expression in *T. caerulescens* than *T. arvense*. The results for this experiment (plus physiological results for comparison) are shown in Fig.3.7. The results for each of the genes were varied. First looking at the expression of *HMA4* there was little difference between 3 and 30 μM $[\text{Zn}]_{\text{ext}}$ however a significant increase in expression occurred at 150 and 300 μM $[\text{Zn}]_{\text{ext}}$ before returning to basal levels at 600 μM $[\text{Zn}]_{\text{ext}}$. When looking at the expression data collected for *ZNT2/4* a very different picture is given. Between 3 and 30 μM $[\text{Zn}]_{\text{ext}}$ the level of expression rises slightly then at levels above 30 μM $[\text{Zn}]_{\text{ext}}$ drops significantly. Considering *NR1* last, the expression data suggests that this gene was less affected by increased zinc levels. However at levels above 1200 μM $[\text{Zn}]_{\text{ext}}$ there is a significant rise in its expression.

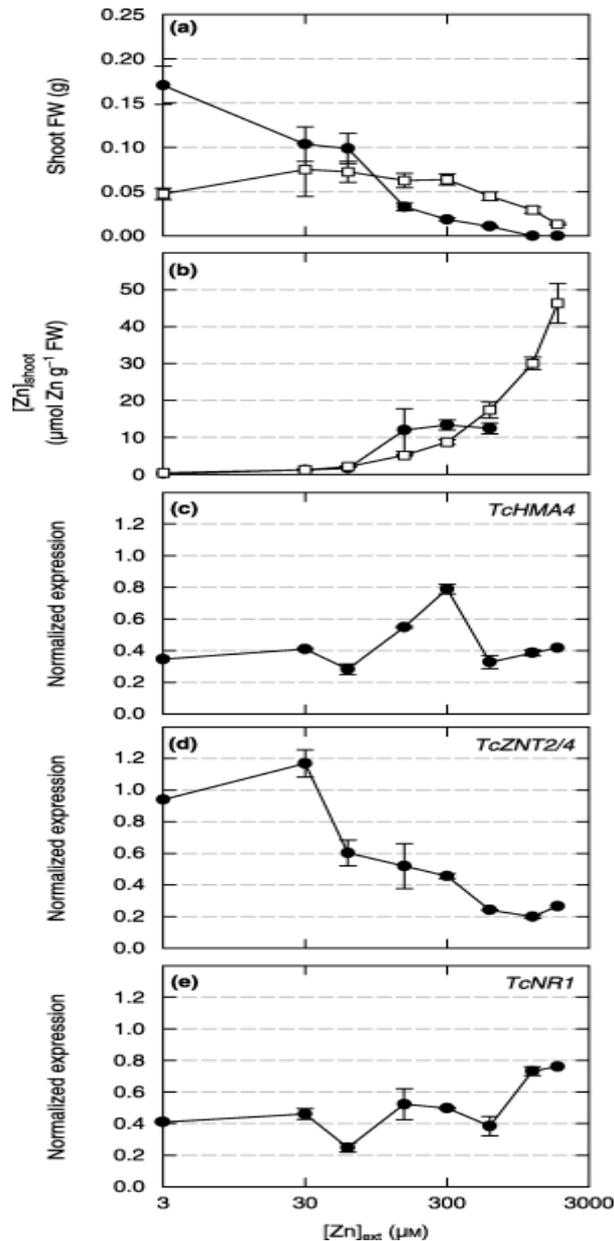


Fig.3.7 a) Shows the shoot fresh weight (FW) values and b) shoot zinc (Zn) content of *Thlaspi caerulescens* (white squares) and *Thlaspi arvense* (black circles) to increasing external zinc concentrations $[Zn]_{ext}$. (mean \pm standard error of the mean (SEM) n=3).c-e) shows shoot expression of 3 genes, *T. caerulescens* heavy metal associated domain-containing protein 4 (*HMA4*), zinc transporter 2/4 (*ZNT2/4*) and Nitrate reductase 1 (*NRT1*), measured via quantitative real time PCR (qPCR). Primers were designed to published data sequences and data normalised to an 18s rRNA control (mean \pm SEM n=3).

3.4 Summary

The aim of this chapter was to identify genes that were differentially expressed in *Thlaspi caerulescens* compared with *T. arvense* through a cross species microarray approach. Secondly the aim was to confirm the differential expression of some of these genes via qPCR.

The microarray was successful in that it showed that whole genome transcriptome profiling can be carried out on non-model plant species, provided there is a GeneChip® array of a model species with enough conservation to give feasible hybridization. The microarray was successful at identifying several thousand genes that were differentially expressed in *Thlaspi caerulescens* compared with *T. arvense*. In total 3349 genes were found to be upregulated in *T. caerulescens* and 1598 were down regulated. Of particular interest were nine genes that had their sequences available in GenBank®. These genes were heavy-metal-associated-domain-containing protein 4 (*HMA4*), TCA567384; nitrate reductase 1 (*NRT1*), AY551529; zinc transporter 1 (*ZNT1*), AF133267; *ZNT2/4*, AF275752/AF292370; *ZNT5*, AF292029; carbonic anhydrase (*CA*), AY551530; nicotianamine synthase 1 (*NAS1*), TCA300446 and two *T. arvense* genes chalcone synthase (*CHS*) and Metal Transporter Protein 1 (*MTP*).

qPCR experiments confirmed the findings of the microarray, that these genes were differentially expressed in *T. caerulescens* compared with *T. arvense*. The data from both studies were found not to be significantly different from each other. Of greatest importance were the *ZNT2/4*, *HMA4* and *NR1* genes that showed significant upregulation in *T. caerulescens*.

From these experiments it was decided to study *HMA4* further *in planta*. The reason for this was it showed significantly different upregulation in *T. caerulescens*, sequenced data was available and previous studies had placed this gene in a potential role in heavy metal transport in other organisms, such as *Arabidopsis thaliana* and *Saccharomyces cerevisiae* (Hussain *et al.*, 2004; Mills *et al.*, 2003). This will now be discussed in the following two chapters.

4. Design of a *HMA4* RNAi construct for transformation of *T. caerulescens*

4.1 Introduction

Previous chapters have detailed how a candidate gene involved in the hyperaccumulation of Zn in *Thlaspi caerulescens* has been identified by a microarray experiment. Confirmation of the differential expression of this gene was achieved through qPCR. This chapter discusses the design of an RNAi construct to the *HMA4* candidate gene. The aim of designing and creating this construct was to study the effects of silencing the gene *in planta*. The following chapter will outline steps made in the attempt to transform *Thlaspi caerulescens* using this construct. A paper reporting on successful transformation of *Thlaspi caerulescens* using only reporter genes indicated that transformation should be feasible. The transformation experiments sought to test different constructs for floral dip and tissue culture methods for *Thlaspi caerulescens* and *T. arvense*.

4.1.1 Heavy metal transporter genes

Transition metals or heavy metals such as Zn, Fe, Cu and Mn are essential to plant growth however at high concentrations they become toxic to plants. Therefore for the plant to remain healthy it must develop a way to successfully uptake and transport them around the plant whilst carefully monitoring their concentrations within plant cells and organelles (Hall and Williams, 2003). It is thought that a complex process involving many different genes is involved

mainly in membrane transport. This area of research is key to this thesis and has been studied in great detail by others in the past decade. Much progress has been made through the use of yeast, *Saccharomyces cerevisiae*, using complementation experiments with candidate genes and also through the study of candidate genes in model plants species, for example *Arabidopsis thaliana* (Hussain *et al.*, 2004; Mills *et al.*, 2003). The number of genes that have been highlighted as playing a putative role is large and includes, eight heavy metal ATPases, six Nramps and 15 ZIPs (Hall and Williams, 2003). This chapter will now focus on one gene, *HMA4* a member of the P_{1B}-ATPase family, a sub family of the superfamily P-type ATPases.

4.1.1.1 P-type ATPase gene family

The P-type ATPases are enzymes that are found in all living organisms from bacteria to humans and are known to pump a range of cations across membranes using ATP against their electrochemical gradients. The name P-type refers to the mechanism by which they work which is conserved amongst all within the family. This mechanism involves a phosphorylated reaction cycle intermediate. All of the members of this family pump cations across membranes using the energy generated from exergonic ATP hydrolysis. Sequencing of the rice (*Oryza sativa*) and *Arabidopsis thaliana* genomes has identified 8 genes among the P-type ATPase *HMA* gene family. In comparison other eukaryotes only normally possess one or two of these genes. Work carried out on *A. thaliana* has found that *HMA1-4* are involved in Zn, Cd Pb and Co transport and *HMA5-8* are involved in the transport of Cu and Ag (Verret *et al.*, 2004).

P_{1B}-ATPases have eight transmembrane helices, a CPx/SPC motif in transmembrane domain six, with possible transmembrane binding domains at the N or C-termini (Williams and Mills, 2005) (Fig.4.1).

Knockout mutants have been successfully used to determine their function in the plant while lethal effects experienced through deleting more than one gene shows the importance of their role in transition metal transport (Williams and Mills, 2005). A number of metals transported by these P_{1B}-ATPases have been revealed through their expression in *E. coli* and yeast, while methods of this metal transportation have been shown in yeast, bacteria and *Arabidopsis* e.g. *AtHMA6/PAA1* and *AtHMA8/PAA2* are both closely related and involved in transporting Cu to some chloroplast proteins. Mutant studies involving gene knocks out have proved invaluable in determining their function and importance e.g. *paa1 paa2* double mutants are seedling lethal (Abdel-Ghany *et al.*, 2005). They also demonstrated that *AtHMA6/PAA1* was present in both shoots and roots, indicating a function in both green and non-green plastids. *AtHMA8/PAA2* however was thought to have a function in supplying metals to the thylakoid membrane of chloroplasts (Abdel-Ghany *et al.*, 2005).

4.1.1.2 P_{1b} ATP HMA4

Studies of the *HMA4* gene have taken place in *A. thaliana*, *Saccharomyces cerevisiae* and the *Escherichia coli zntA* mutant. Initially *HMA4* was isolated and cloned from *A. thaliana* (Mills *et al.*, 2003) and found to cluster phylogenetically with Zn/Co/Cd/Pb pumps. Sequencing analysis confirmed that

it possessed the characteristic groups of a P_{1b}-ATPases including the eight transmembrane domains, metal binding sites and a CPx/SPC motif located in the sixth transmembrane domain. In this same study the gene was expressed in *E. coli* to determine which metals were transported or bind to this putative metal pump. The *AtHMA4* gene was expressed in *E. coli* mutants and a wild type strain. The *E. coli* mutants contain a disruption in *ZntA* or *CopA*. *ZntA* had previously been designated a P-type ATPase that catalyses the efflux of Zn, Cd and Pb and *CopA* a Cu-translocating efflux pump. The mutants were exposed to a variety of metals and differing concentrations and found to be extremely sensitive to Zn at 200 µM compared with the wild type. This sensitivity was almost completely complemented in the mutant when transformed with the *AtHMA4* gene. No significant differences between the wild type and transformed mutants were observed when studying the response to other metals such as Cu, Co, Cd, Mn and Pb. Similarly the *AtHMA4* was unable to reverse the sensitivity to Cu in the *CopA* mutants grown on a range of Cu concentrations. Mills *et al.*, (2003) also tested metal specificity of the *AtHMA4* gene in yeast of all the metals tested (Zn, Cu, Cd, Co, Ni, Mn) the most significant difference in metal sensitivity between the wild type and yeast transformed with the *AtHMA4* was in the presence of Cd. It was found that the expression of the *AtHMA4* gene decreased the sensitivity to Cd. Finally this study looked at the localization of this gene within *Arabidopsis* plants. Tissue specific expression of the *HMA4* gene was achieved using RT-PCR and concluded that the gene was expressed in all tissues studied however highest expression was observed in roots. The expression of the gene was analysed in the presence of several metals and

was found to be upregulated at elevated Zn and Mn but downregulated by Cd. These studies concluded that the genes may have a function in Zn efflux in the plasma membrane (Mills *et al.*, 2003)

Mutant plants were created to study this gene. A double mutant for *hma2 hma4* was produced. This showed that these genes had putative roles with zinc homeostasis in plant cells. In order to rescue the mutant exogenous applications of Zn were required and later promoter-*GUS* constructs demonstrated that *AtHMA4* was expressed in cells surrounding the root vascular tissue (Hussain *et al.*, 2004). This suggested that *AtHMA2* and *AtHMA4* were involved in xylem loading of Zn to be transported to the shoot (Hussain *et al.*, 2004).

Yeast complementation studies have revealed other putative functions for *AtHMA4* such as metal detoxification of Cd and Zn (Mills *et al.*, 2003). The *AtHMA* genes are thought to transfer high Zn and Cd from the roots to the leaves where they have less potential to cause damage. The transcription of the pump involved in sending high Zn levels to the shoots is regulated by metals, however it is not known if it is post-transcriptionally controlled (Williams and Mills, 2005).

There have been no transgenic studies on the *HMA4* gene in *T. caerulescens* however other studies have been carried out; the *TcHMA4* gene was first identified using complementation tests in yeast under Cd exposure (Bernard, 2004). This gene has already been sequenced and is available for public use,

which allowed primers for this sequence to be designed (Papoyan and Kochian, 2004). A structure for the *HMA4* gene has also been identified and as discussed previously in this chapter is made up of eight transmembrane domains, and two potential metal binding domains at the N and C termini (Fig.4.1).

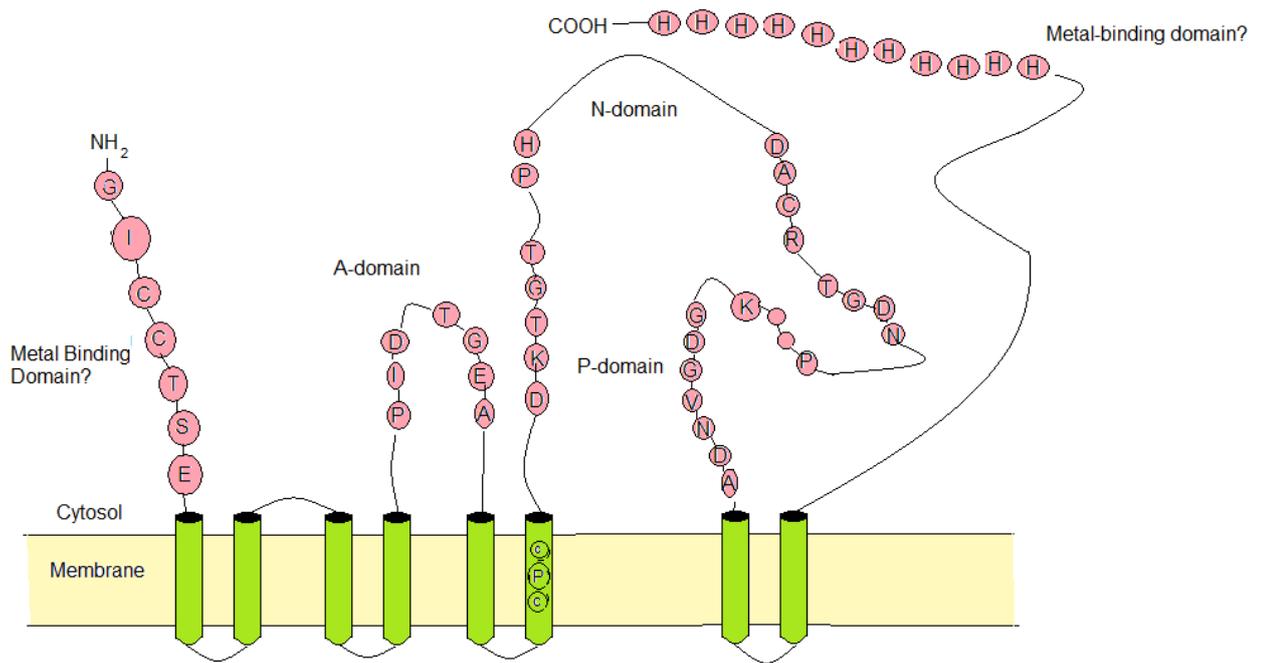


Fig.4.1 Structure of the P_{1B}-ATPase *HMA4* gene showing eight transmembrane domains (green cylinders), possible metal-binding domains at the N and C termini and a CPx/SPC motif in transmembrane domain six.

Most recently the *HMA4* gene has been studied in *A. halleri*, a hyperaccumulating plant related to *A. thaliana*. Hanikenne *et al.*, (2008) used a RNAi gene silencing method to study the gene *in planta* and also transfer the gene to the non- hyperaccumulator *A. thaliana*. The *AhHMA4* gene was isolated from *A. halleri* cDNA using proofreading polymerase to carry out the

PCR. The *HMA4* sequence (2541-2997 bp) was inserted into a Gateway binary vector, pJAWOHL8 by site directed recombination. This vector generates a hairpin construct that consists of an antisense *AhHMA4* fragment-intron-sense *AhHMA4* fragment which is downstream from a CaMV 35S promoter.

RT-PCR was used to assess the transcript levels of *HMA4* in *A. halleri* transformed with the RNAi construct. It was found that transcript levels of *HMA4* were reduced by between 45% and 10% compared with wild types. Morphologically these plants were deemed normal by comparing the root elongation with that of wild types when grown hydroponically on solution supplemented with increasing Zn concentrations. The levels of Zn contained within the transformed plants were closer to those found in the non-hyperaccumulator *A. thaliana*, between 12 and 35% lower. Transformed lines were found to contain higher levels of Zn in the roots (49-137 fold higher) than the shoots, a characteristic found in *A. thaliana*. In wild type *A. halleri* the opposite is normally observed, which suggests a root to shoot mechanism of metal translocation. This evidence suggests that *AhHMA4* is required for efficient root to shoot flux.

In this same study, fluorescent imaging was used to determine the effect *HMA4* has on Zn localisation within the roots of *A. halleri*, using the fluorescent indicator Zinpyr-1. This study showed that wild type plants had the majority of its Zn localised in the xylem vessels, inwards of the vascular pericycle. Comparatively, the RNAi transgenic lines had the majority of Zn localised in

the pericycle cell layer. A similar feature was observed in the *A. thaliana* *HMA4* mutant compared with wild type plants. This suggests that the silencing of the *AhHMA4* gene inhibits the movement of Zn from the root symplast to the apoplastic xylem vessels which is in fact the primary route of solutes from the roots to the shoots which explains the lack of Zn in the shoots of the RNAi transgenic plants.

Also in this study is the reported transfer of *HMA4* mini-gene into *A. thaliana*, a non-hyperaccumulator, to determine whether increased *HMA4* activity is necessary for altered heavy metal accumulation. This mini-gene consisted of an *AhHMA4* cDNA linked to the *AhHMA4-1* promoter. Transformants were found to show moderately elevated levels of *HMA4* transcript levels. (2.44 ± 0.08 and 2.85 ± 0.89 fold in roots and shoots, respectively), compared with the wild type levels. Localisation studies using the fluorescent indicator Zinpyr-1 showed high levels of Zn in xylem vessels which is what can be found in wild type *A. halleri* plants. This concludes that transformation of *A. thaliana* with *AhHMA4* is adequate to provide Zn distribution typical of *A. halleri* in the roots of *A. thaliana*.

This leads to Zn partitioning in the xylem vessels and transcriptional upregulation of Zn deficiency response genes. *A. thaliana* also contained increased transcript levels of the Zn deficiency response genes *ZIP4* and *IRT3*, again similar to that found in wild type *A. halleri*. It is therefore suggested that Zn flux into the xylem is *HMA4* dependent and that this flux

depletes symplastic pools of Zn which in turn triggers the upregulation of Zn deficiency response genes in the roots.

A. thaliana transformed with the *AhHMA4* were grown in media supplemented with toxic levels of 150 μM Zn or 40 μM Cd. They were shown to have leaf chlorosis and smaller rosettes, signs of Zn and Cd hypersensitivity. In comparison when grown on 5 μM Zn, transgenic lines were deemed healthy and were shown to accumulate higher levels of Zn than non-transgenic lines. These results suggest that *AhHMA4* is required for more efficient transfer of metals from roots to leaves however due to the sensitivity of shoots it is thought that additional genes are required for metal detoxification in order for the plants to survive elevated *HMA4* dependent flux into the shoots of *A. halleri*.

As well as to the silencing the *AhHMA4* gene Hanikenne *et al.*, (2008) also studied the effect of over expression of the *AhHMA4* gene in *A. halleri*. Effect of this overexpression was monitored by RT-PCR to determine how the expression of Zn deficiency genes changed in relation to the overexpression of *HMA4*. The roots of the RNAi lines were shown to have a positive correlation of the expression of *IRT3* and *ZIP4* genes in relation to the transcript abundance of *HMA4*. The Zn deficiency genes mentioned belong to the same family of proteins associated with the uptake of Zn. It has therefore been proposed that the expression of these genes in the roots of wild type *A. halleri* is an outcome of the increased *HMA4* activity and as a result further increases metal accumulation.

4.1.2 RNA interference-a method of gene silencing using a GATEWAY™ cloning system

The proposed method of creating the RNAi construct is through the use of the GATEWAY™ cloning system (Invitrogen, Gaithersburg, MD, USA). This relatively new method of cloning is able to provide a fast and reliable alternative to more traditional cloning methods. The GATEWAY™ cloning system eliminates the need for traditional ligase mediated cloning due to using the bacteriophage lambda, site-specific recombination system. The process that occurs between *E. coli* and bacteriophage lambda in which phage lambda becomes integrated into *E. coli* DNA or is excised from *E. coli* DNA involves steps that have been adapted for the GATEWAY™ system. Integration occurs between the *attP* (242 bp) of bacteriophage and the *attB* site (25 bp) of the *E. coli*. As a result bacteriophage lambda is flanked by the *attL* (100 bp) and *attR* (168bp) sites (the BP reaction). In the reverse reaction, to excise the phage DNA, recombination occurs between *attL* and *attR* sites (LR reaction). The BP reaction requires two enzymes to work; the phage integrase (Int) and the *E. coli* integration host factor (IHF) (Nakagawa *et al.*, 2009).

The site specific recombination system of the GATEWAY™ cloning method allows sequences to be moved between plasmids containing compatible recombination sites. The first step in creating the construct involves using topoisomerase-mediated cloning which eliminates any conventional DNA ligase mediated cloning. This involves using amplification of the target gene using a forward primer that includes the sequence CACC at the 5' end. This sequence enables integration into Invitrogen's pENTR/D-TOPO entry vector

(Earley *et al.*, 2006; Shuman, 1994). The recombinant plasmid produced has the target gene DNA flanked by the *attL* recombination sequences. The *attL* recombination sites allow recombination with *attR* sites using the LR clonase reaction mix (Invitrogen, Gaithersburg, MD, USA). The reaction with the LR clonase mix (which contains the enzymes phage lambda integrase, the *E. coli* integration host factor and the phage protein excisionase (Xis)) transfers the target DNA into the destination vector. The destination vectors contain a gene, *ccdB*, which is lethal to most *E. coli* strains; this feature is used as a method of selection during the transformation. 'Empty' destination vectors are selected against upon transformation with *E. coli* cells in the recombination reaction. This negative selection method is used alongside a positive selection for antibiotic resistance to ensure that colonies grown contain only plasmids that have undergone recombination (Fig.4.2). Although the topoisomerase-mediated cloning is the most popular method for producing the entry vector there are two alternative methods. The first is to use traditional ligase-mediated insertion into the entry vector at the multiple cloning sites that are flanked by the *attL* sites. The second option is to use BP clonase reaction mix (Invitrogen, Gaithersburg, MD, USA) to recombine a PCR product of the target gene, flanked by *attB* sites, into the donor vector containing the *attP* recombination site. The recombination reaction involving the BP clonase mix (containing the phage lambda integrase enzyme and the *E. coli* integration host factor enzyme) results in a target sequence that is flanked by *attL* sequences. These sequences allow further recombinations to occur with the destination vector chosen. The destination vector contains *attR* sites which recombine with the entry clone. The Products from the LR reaction between

an entry vector and a destination vector are an expression clone with *attB* recognition sites and a by-product with *attB* sites. The BP reaction is in effect the reverse reaction when the *attB* sites recombine with the *attP* sites to create vectors with *attL* and *attR* sites. Plant destination vectors are available for a variety of different purposes including, promoter fusion analysis, protein localisation, gene overexpression, analysis of protein/protein interaction and gene knockdown by RNA interference (as discussed in this chapter) (Earley *et al.*, 2006; <http://www.invitrogen.com>).

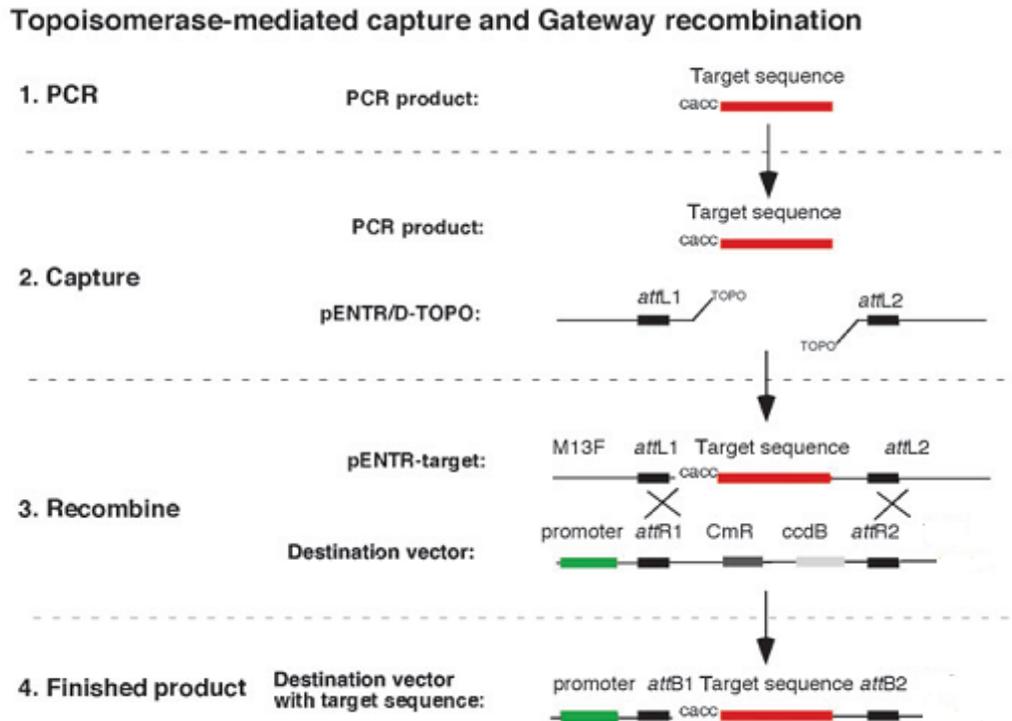


Fig.4.2 Overview of GATEWAY cloning involving topoisomerase-mediated capture and recombinational cloning. 1) The gene of interest is amplified by PCR using a forward primer containing the sequence CACC to facilitate the cloning into the pENTR/D-TOPO vector. 2) PCR products are mixed with the pENTR/D-TOPO vector. This vector has topoisomerase molecules that catalyse the ligation of the target gene and vector sequences. The *attL1* and *attL2* sites that flank the cloning site initiate following recombination reactions. 3) The target gene is recombined into the chosen destination vector using the LR clonase reaction enzyme mix (Invitrogen, Gaithersburg, MD, USA); this reaction mix contains the necessary enzymes required for the recombination between *attL* and *attR* sites. The gene *CmR*, responsible for chloramphenicol resistance, is located between the *attR* sites of the destination vector. This gene is lethal to most strains of *E. coli* and therefore only *E. coli* strains containing plasmids that have undergone successful recombination are able to survive. 4) The finished product (Earling *et al.*, 2006).

Now many genomes of different organisms have been sequenced it is possible to determine the function of these genes. RNA interference (RNAi) is a post transcriptional gene-silencing technology that has been successfully used to assign gene functions in many different organisms. RNAi is a process in which the translation of a cell's messenger RNA (mRNA) sequences is prevented due to the presence of double stranded RNA sequences. Under normal circumstances within a cell, RNA exists as a single stranded molecule. Within a cell's nucleus mRNA is produced and transported to the ribosomes within the cytoplasm as a single strand. Double stranded RNA (dsRNA) is normally only found within the cell under circumstances that pose a threat, for example during an infection by a dsRNA virus. In these circumstances the cell detects the dsRNA and uses endoribonuclease enzymes (termed dicer) to cleave the RNA into small fragments (20-25 bp long), known as small interfering RNAs (siRNAs) which results in inactivation of the RNA and in the case of viruses, inhibits viral replication, which ultimately protects the cell from harm. Alongside the degradation of dsRNA these fragments of mRNA prevent any further expression of mRNA containing the same sequence and therefore the protein it encodes will not be translated, in effect the gene is turned off. It is thought that the dsRNA produced from the initial cleavage act is involved in further cleavage of mRNA. One strand of the dsRNA, known as the guide strand, is incorporated into the RNA-induced silencing complex (RISC) where it targets complementary mRNA strands.

The RISC complex uses the siRNA as a template for recognizing complementary mRNA. The guide strands are bound to the argonaute

proteins with the RISC; this protein has endonuclease activity. When the RISC complex with the bound siRNA finds a complementary strand of mRNA, it activates RNase (ribonuclease) activity and cleaves the mRNA into more siRNA strands. At present it is unclear how the RISC complex locates the complementary mRNA (Ambros, 2001; Gura, 2000; Rueyling, and Avery, 1999). (Fig.4.3)

This mechanism is self-sustaining as new fragments can then target any new mRNA produced. These argonaute proteins as well as being partially responsible for the guide strand selection are also responsible for the degradation of the passenger strand of the siRNA (the other half of the siRNA sometimes called the anti-guide strand). It is because of this ability to downregulate genes that this mechanism has been exploited for research of plant genes (Ambros, 2001; Gura, 2000; Rueyling, and Avery, 1999). The mechanisms for plant gene research are much the same in that double stranded RNA is introduced into the plant through the insertion of a sequence of DNA that consists of the gene of interest in the forward and reverse direction separated by an intron. Upon transcription into mRNA the strand folds round on itself producing a hairpin structure which in effect is a dsRNA strand which leaves the nucleus and is detected by the cell and cleaved as described above in the siRNA fragments. The siRNA fragments as described knock-down the genes expression which silences the gene allowing its function in the plant to be studied.

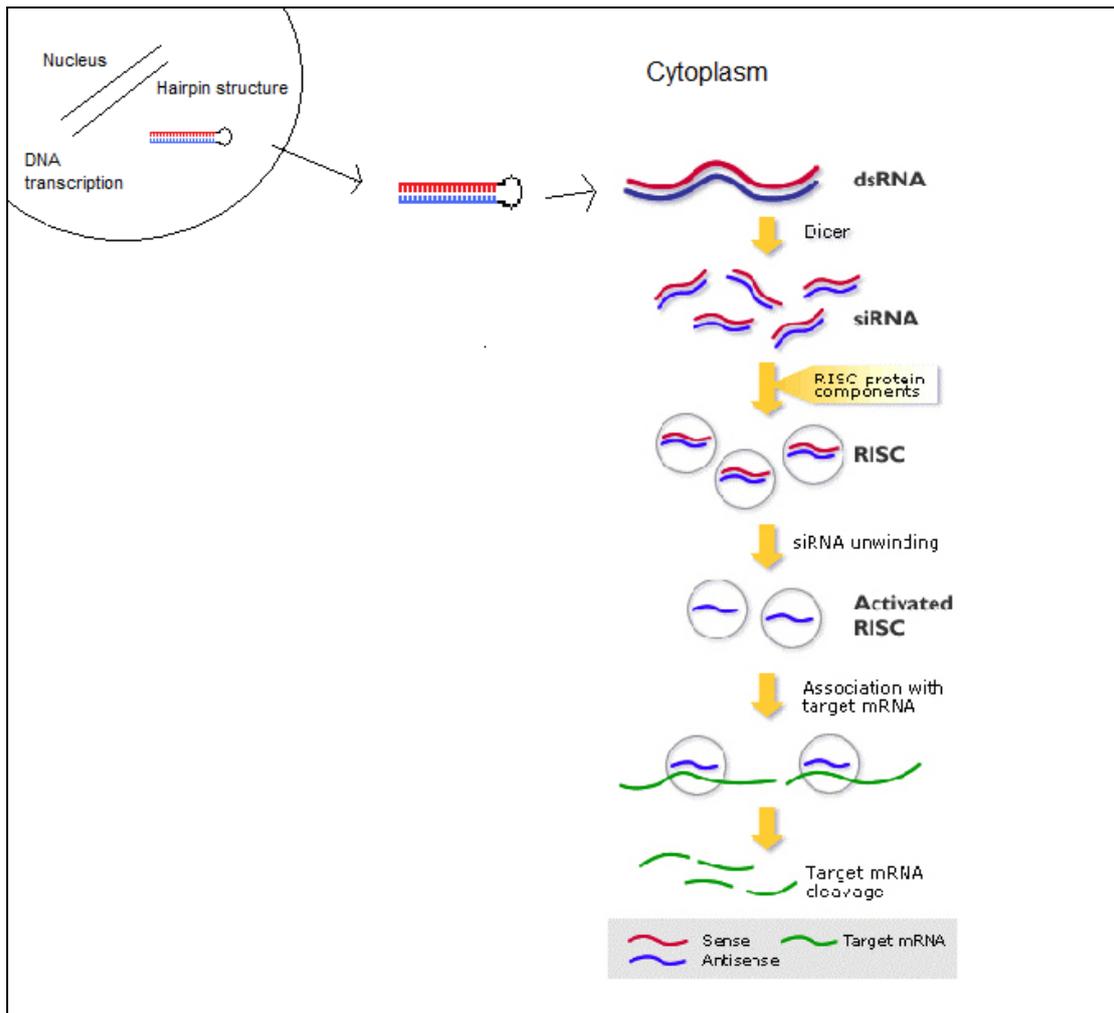


Fig. 4.3 Overview of the RNAi mechanism. DNA is transcribed into mRNA within the nucleus. The transformed plants contain a sequence of DNA that consists of the forward and reverse sequence of the target gene separated by an intron. Upon transcription the mRNA forms a hairpin structure which in effect is a dsRNA strand. This moves into the cytoplasm where it is cleaved into siRNA fragments (20-25 bp) by an endonuclease (sometimes called dicer). One strand of the siRNA fragments, the guide strand, becomes associated with the argonaute protein with the RISC (RNA induced silencing complex) which targets complementary mRNA strands. The RISC complex degrades complementary mRNA strands along with the other siRNA strand, the passenger strand. The degradation of any complementary mRNA strand

inhibits any protein synthesis and therefore silences or down-regulates the gene (Adapted from Guru, 2000).

4.2. Materials and methods

4.2.1 Primer Design

The *HMA4* Gene sequence published by Papoyan and Kochian (2004) was some 1185 bp long and showed a high degree of conservation to the *Arabidopsis thaliana HMA4* gene sequence (Fig.4.4). For the construct, primers were designed to anneal to a region that lacked introns that produced a PCR product size of 420 bp (using Primer3 (www.primer3.com)). The small size was necessary for the cloning procedure. Added to the beginning of the chosen left or forward primer was the short sequence CACC, which is the recognition site required in the cloning system. The primers were ordered online from MWG (<http://www.eurofinsdna.com/home.html>).

Left or Forward Primer:

5' CACCGCTAGGGAATGCTTTGGATG 3'

Right or Reverse Primer:

5' CTTCTCTCGCAGAAGCAACA 3'

Chapter 4: HMA4 RNAi Construct Design

<i>AtHMA4</i>	109	CAA-AACAAAGAAGAAGAGAAAAAGA-AAGTGAAGAAGTTGCAAAAAGAGTTACTTTCGATG	166
<i>TcHMA4</i>	21	CAAGAACAAAGAAGAAGATAAAAAGACAA-AGAAGAAGTGGCAGAAGAGTTACTTTCGACG	79
<i>AtHMA4</i>	167	TTCTCGGAATCTGTTGTACATCGGAAG-TTCTATAATCGAGAATATTCTCAAGTCACTT	225
<i>TcHMA4</i>	80	TTTTGGGAATCTGTTGTACATCGG-AGATTCTCTGATCGAGAATATTCTCAAGTCTCTC	138
<i>AtHMA4</i>	226	GACGGCGTTAAAGAATATTCGTCATCGTTCCCTCGAGAACCGTGATTGTTGTTCCAGC	285
<i>TcHMA4</i>	139	GACGGCGTTAAGGAATATACCGTCATCGTTCCGTCGAGAACCGTGATCGTTGTCCACGAC	198
<i>AtHMA4</i>	286	AGTCTCCTCATCTCTCCC-TTCCAAATGCTAAGGCACTAAACGAAGCTAGGTTAGAAGC	344
<i>TcHMA4</i>	199	AGTCTCCTCATC-CCCCGTTCCAAATGCTAAGGCACTGAACCAAGCGAGGTTAGAAGC	257
<i>AtHMA4</i>	345	AAACGTGAGGGTAAACGAGAAACTAGCTTCAAGAACAATGGCCGAGCCCTTTCGCGGT	404
<i>TcHMA4</i>	258	AAACGTGAAAGTAAACGAGAAACCAGCTTCAAGAATAAATGGCCGAGCCCTTTCGCGGT	317
<i>AtHMA4</i>	405	AGTTTCCGGCTTACTT-CTCCTCCTATCCTTCCTAAAGTTTGTCTACTCGCCT-TTACGT	462
<i>TcHMA4</i>	318	GGTTTCCGGCATA-TTCCTCCTCCCTCCTTCTTAAAATTTGTATAACCACCTCTT-CGA	375
<i>AtHMA4</i>	463	TGGCT--C-GCCGTGGCAGCAGTTGCCGCCGTATCTATCCGATTCTTGCCAAAGCCTTT	519
<i>TcHMA4</i>	376	TGGCTAGCTGTCTGTGG--GC-GTCGCTGCTGGTATTTATCCGATTCTTGCCAAAGCCGTC	432
<i>AtHMA4</i>	520	GCTTCCATTAA-AAGCCTAGGATCGACATCAACATATTGGTCATAATAACCGTGATTGC	578
<i>TcHMA4</i>	433	GCTTCTA-TAAGAAGGCTTAGGGTCGACATCAACATCCTAATCATTATCACAGTGGCTGC	491
<i>AtHMA4</i>	579	AACACTTGCAATGCAAGATTTCATGGAGGCAGCAGAGTTGTTCCTATTACCATATC	638
<i>TcHMA4</i>	492	AACACTTGCAATGCAAGATTACATGGAGGCTGCAGCAGTTGTCTTCTATTACCACAGC	551
<i>AtHMA4</i>	639	CGACTGGCTCGAAACAAGAGCTAGCTACAAGGCGACTCGGTAATGCAGTCTCTGATGAG	698
<i>TcHMA4</i>	552	TGACTGGCTGGAACAAGAGCTAGCTACAAGGCCAACTCGGTGATGCAGTCTCTGATGAG	611
<i>AtHMA4</i>	699	CTTAGCTCCACAAAAGGCTA-TAATAGCAGAGACTGGTGAAGAAGTTGAAGTAGATGAGG	757
<i>TcHMA4</i>	612	CTTAGCTCCACAAAAGGC-AGTCATAGCAGAGACTGGAGAAGAAGTTGAAGTAGATGAGG	670
<i>AtHMA4</i>	758	TTAAGGTTGATACAGTTGTAGCAGTTAAAGCTGGTGAACCATACCAATTGATGGAATTG	817
<i>TcHMA4</i>	671	TTCAGCTCAACACAATCATAGCAGTTAAAGCCGGTGAACCATACCTATTGATGGAATTG	730
<i>AtHMA4</i>	818	TGGTGGATGGAACCTGTGAGGTAGACGAGAAAACCTTAACGGGCGAAGCATTTCCTGTGC	877
<i>TcHMA4</i>	731	TGGTCGATGGAACCTGTGAAGTAGACGAGAAAACCTTAACGGTGAAGCATTTCCTGTGC	790
<i>AtHMA4</i>	878	CTAAACAGAGAGATTCTACGGTTTGGGCTGGCACCATCAATCTAAATGGTTACATATGTG	937
<i>TcHMA4</i>	791	CTAAACAGAGAGATTCTACGGTTTGGGCTGGAACATGAATCTAAATGGTTATATAAGTG	850
<i>AtHMA4</i>	938	TGAAAACAACCTCTTTAGCGGGTGATTGCGTGGTTGCGAAA-ATGGCTAAGCTAGTAGAA	996
<i>TcHMA4</i>	851	TGAACACAACCTGCTTTAGCTAGTGATTGCGTGGTTGC-AAAGATGGCTAAGCTCGTAGAA	909
<i>AtHMA4</i>	997	GAAGCTCAGAGCAGTAAAACCAATCTCAGAGACTAATAGACAAATGTTCTCAGTACTAT	1056
<i>TcHMA4</i>	910	GAAGCTCAGGGCAGTAAAACCAATCTCAGAGACTAATAGACAAATGTTCTCAGTACTAT	969
<i>AtHMA4</i>	1057	ACTCCAGCAATCATCTTAGTATCAGCTTGCCTTGCCATTGTCCCGTTATAATGAAGGTC	1116
<i>TcHMA4</i>	970	ACTCCAGCAATCATCATAATATCGGCTGGCTTTGCGATTGTCCCGCTATAATGAAAGTT	1029

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<i>AtHMA4</i>	1117	CACAACCTTAAACATTGGTTCCACCTAGCATTAGTTGTGTTAGTCAGTGGTTGTCCCTGT 	1176
<i>TcHMA4</i>	1030	CACAACCTCAACCATGGTTTCATTAGCACTGGTTGTGTTAGTCAGTGGTTGTCCCTGT 	1089
<i>AtHMA4</i>	1177	GGTCTTATCCTCTCTACACCAGTTGCTACTTTCTGTGCACTTACTAAAGCGGCAACTTCA 	1236
<i>TcHMA4</i>	1090	GGTCTTATCCTCTCTACACCAGTAGCTACATTTCTGTGCACTTACTAAAGCGGCAACTTCA 	1149
<i>AtHMA4</i>	1237	GGGCTTCTGATCAAAAAGTGCTGATTATCTTGACACTCTCAAAGATCAAGATTGTTGCT 	1296
<i>TcHMA4</i>	1150	GGGCTTCTGATCAAAAAGTGCTGGTCATCTTGACACTCTTCAAAGATCAAAATCGCTGCT 	1209
<i>AtHMA4</i>	1297	TTCGATAAAACTGGGACTATTACAAGAGGAGAGTTTCATTGTCATAGATTTCAGTCACTC 	1356
<i>TcHMA4</i>	1210	TTTGACAAAACCGGAACCTACTAGAGGAGAGTTTCATTGTCATAGAATTCAAGTCACTC 	1269
<i>AtHMA4</i>	1357	TCTAGAGATATAAACCTACGCAGCTTGCTTTACTGGGTATCAAGTGTGAAAGCAAATCA 	1416
<i>TcHMA4</i>	1270	TCTAGAGACATAAGCCTACGCAGCTTGCTTTACTGGGTGTCAGTGTGAAAGCAAATCA 	1329
<i>AtHMA4</i>	1417	AGTCATCCAATGGCAGCAACAATCGTGGATTATGCAAATCTGTTTCTGTTGAGCCTAGG 	1476
<i>TcHMA4</i>	1330	AGTCACCCAATGGCAGCAACGATTGTGGACTATGCTAAATCTGTTTCTGTTGAGCCTAGG 	1389
<i>AtHMA4</i>	1477	CCTGAAGAGTTGAGGATTACCAGAACTTCCAGGTGAAGGAATCTACGGGAAGATTGAT 	1536
<i>TcHMA4</i>	1390	AGTGAAGAGTTGAGGATTATCAGAACTTCTCGGTGAAGGAATCTATGGGAAGATTGAT 	1449
<i>AtHMA4</i>	1537	GGTAACGATATCTT-CATTGGGAACAAAAGATAGCTTCTCGAGCTGGTTGTTCAACAGT 	1595
<i>TcHMA4</i>	1450	GGGAACAATGT-TTACATTGGGAACAAAAGATTGCTTACGAGCTGGTTGTTCAACAGT 	1508
<i>AtHMA4</i>	1596	TCCAGAGATTGAAGTTGATACCAAAGCGGGAAGACTGTTGGATACGCTCTATGTAGGTGA 	1655
<i>TcHMA4</i>	1509	TCCAGAGATTGAGGTTGATACCAAAGGAAAGACTGTCGGATACGCTCTATGTAGGTGA 	1568
<i>AtHMA4</i>	1656	AAGACTAGCTGGATTTTCAATCTTTCTGATGCTTGTAGATCTGGTGTCTCAAGCAAT 	1715
<i>TcHMA4</i>	1569	AAGATTAGCTGGAGTTTCAATCTTTCCGATGCTTGTAGATCCGGAGTAGCTCAAGCAAT 	1628
<i>AtHMA4</i>	1716	GGCAGA-ACTGAAATCTCTAGGAATCAAAACCGCAATGCTAACGGGAGATAATCAAGCCG 	1774
<i>TcHMA4</i>	1629	GA-AGGGACTCAAAGATCTTGAATCAAAACCGCAATGCTAACAGGAGATAATCAAGATT 	1687
<i>AtHMA4</i>	1775	CGGCAATGCATGCTCAAGAACAGCTAGGGAATGTTTTAGATGTTGTACATGGAGATCTTC 	1834
<i>TcHMA4</i>	1688	CAGCAATGCAAGCTCAAGAACA GCTAGGGAATGCTTTGGATGTTGTTTCATGGAGAGCTTC 	1747
<i>AtHMA4</i>	1835	TTCCAGAAGATAAGTCCAGAATCATACAAGAGTTTAAAGAAAGAGGGACCAACC-GCAATG 	1893
<i>TcHMA4</i>	1748	TTCCAGAAGACAAATCCAAATCATACAAGAGTTTAAAGAAAGAGGGACCAACTTGTA-TG 	1806
<i>AtHMA4</i>	1894	GTAGGGGACGGTGTGAATGATGCACCAGCTTTAGCTACA-GCTGATATTGGTATCTCCAT 	1952
<i>TcHMA4</i>	1807	GTAGGAGATGGTGTGAATGATGCACCAGCTTTAGCTA-ATGCTGATATTGGTATCTCCAT 	1865
<i>AtHMA4</i>	1953	GGGAATTTCTGGCTCTGCTCTTGCAACACAAACTGGTAATATTATTCTGATGTCTAATGA 	2012
<i>TcHMA4</i>	1866	GGGGATTTCTGGCTCTGCGCTCGCGACGCAGACTGGTCATATCATTTCTCATGTCAAATGA 	1925
<i>AtHMA4</i>	2013	TATAAGAAGGATACCACAAGCGGTGAAGCTAGCGAGAAGAGCAC-GACGCAAAGTTGTTG 	2071
<i>TcHMA4</i>	1926	TATCAGAAGGATACCACAAGCGATAAAGCTAGCAAGAAGAGCTCAG-CGGAAAGTTCTTC 	1984
<i>AtHMA4</i>	2072	AAAACGTGTGTC-TATCGATCATTTTAAAAGCAGGAATACTCGCTTTGGCATTGCTGGT 	2130
<i>TcHMA4</i>	1985	AAAACGTGA-TCATCTCCATCACTTTGAAAGTAGGGATACTGGTTTTAGCATTTGCTGGT 	2043
<i>AtHMA4</i>	2131	CATCCTTTGATTTGGGCTGCGGTTCTTGTGTGATGTAGGGACTTGTCTGCTTGTGATTTTC 	2190
<i>TcHMA4</i>	2044	CATCCTTTGATTTGGGCTGCGGTTCTTACTGATGTAGGGACTTGCCTGATTTGTGATTTCTC 	2103
<i>AtHMA4</i>	2191	AATAGTATGTTGCTGCTGCGAGAGAAGAAAAGAT-TGGGAACAAAAGTGTACAGGGC 	2249

<i>TcHMA4</i>	2104	AACAGTATGTTGCTTCTGCGAGAGAAGGATAA-ATCTAAGATCAAGAAGTGTACAGG--	2160
<i>AtHMA4</i>	2250	TTCTACATCTAAGTTGAATGGTAGGAACTTGAAGGCGAT-GATGATTATGTTGTGGACT	2308
<i>TcHMA4</i>	2161	----A-A-----G---AA---A-----CTTGAAGGCG-TCGATGACCAAGGCCCTTGACT	2197
<i>AtHMA4</i>	2309	TAGAAGCAGGCTTGTTAACAAAGAGCGGGAATGGTCAATGCAAATCAAGCTGTTGTGGAG	2368
<i>TcHMA4</i>	2198	TAGAAGCAGGTTGTTATCAAAGAG-----T---CAATGCAACTCAGGATGTTGTGGTG	2248
<i>AtHMA4</i>	2369	ATAAGAAAAATCAAGAGAACGTTG-TGATGATGAAACCAAG-TAGTAAAACAGTTCTGA	2426
<i>TcHMA4</i>	2249	ATCAGAAAAGCCAAGAGAAGGT-GATGTTGATGAGACCA-GCTAGTAAAACAGTTCTGA	2306
<i>AtHMA4</i>	2427	TCATTCT-CACCCTGGTTGTTGTGGCGATAAGAAGGAAGAAAAAGTGAAGCCGCTTGTGA	2485
<i>TcHMA4</i>	2307	CCAT-CTTCACTCTGTTGTTGTGGTGAAAAGAAGCAAGAGAGTGTAAAGC---TTGTGA	2362
<i>AtHMA4</i>	2486	AAGATGGCTGTTGCAGTGAGAAAACTAAGAAATCAGAGGGAGATATGGTTTCATTGAGCT	2545
<i>TcHMA4</i>	2363	AAGATAGCTGTTGCGGTGAGAAAAGTAGGAAACCAGAGGGAGATATGGCTTCACTGAGCT	2422
<i>AtHMA4</i>	2546	CATGTAAGAAGTCTAGTCATGTCAAACATGACCTGAAAATGAAAGTGGTTCAGGTTGTT	2605
<i>TcHMA4</i>	2423	CATGCAAGAAGTCTA---A---CAA---TGACCTGAAAATGAAAGTGGTTCAGGTTGTT	2473
<i>AtHMA4</i>	2606	GTGCTAGCAAAAATGAGAAAGG-GAAGGAAGTAGT-G--GCAAAGAGCTGTTGTGA	2657
<i>TcHMA4</i>	2474	GTGCTAGTAAAATGAGAA-GCTGAAGGAAGCAGTAGTAGCAAAGAGCTGCTGTGA	2528

Fig. 4.4 Sequence alignment of the *TcHMA4* gene to the *AtHMA4* gene. The genes show 85% alignment. The primer positions are shown in red, the 420bp region is highlighted in green.

4.2.2 Plant genomic DNA isolation

Genomic DNA was extracted from *T. caerulea* sp. (Ganges) and *T. arvense*. (Wellesbourne) using the GeneElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich GmbH, Steinheim, Germany). Plants taken for DNA extraction were grown on a mixture of Levington’s seed compost (Scotts U.K. Professional, Ipswich, U.K.), vermiculite and perlite (William Sinclair Horticulture Ltd., Lincoln, U.K.) at a 3:1:1 (v/v) ratio, and supplemented with 50 ml L⁻¹ compost of systemic insecticide “Intercept” [70% (w/w) Imidacloprid] prepared at 0.2 g L⁻¹ to prevent scarid fly infestation (Monro South, Wisbech, U.K.). Trays were then placed in a miniature incubator in a growth room at 20

$\pm 2^{\circ}\text{C}$ for a 16 h photoperiod, at a light intensity of $50 - 80 \mu\text{mol m}^{-2} \text{s}^{-1}$ produced by 58 W white halophosphate fluorescent tubes (Cooper Lighting and Security, Doncaster, UK). Following 8-12 days seedlings were transferred to the glasshouse and cultured at $24 \pm 2^{\circ}\text{C}$ with a 16 h photoperiod maintained by light supplementation from 600 W luminaries using 600 W pressure sodium lamp (Philips® Sun-t Pia Green Power, Philips Electricals UK Ltd., Guildford, U.K.) for a six to eight weeks.

Leaf discs of plant tissues were frozen in Eppendorf tubes in liquid nitrogen and then ground to a fine powder using a micropestle. The genomic DNA was released by adding 350 μl of Lysis Solution A and 50 μl of Lysis Solution B followed by vortexing for 3 s inversion and an incubation period of 10 minutes at 65°C . The debris was removed by adding 130 μl of Preparation Solution and mixed by inversion followed by 5 minutes incubation on ice. The debris was pelleted out by 5 minutes of centrifugation. The supernatant was then transferred to the Blue Filtration Column and spun for 1 minute.

The Binding Column was prepared by adding 700 μl of Binding Solution to the filtrate and mixed by inversion. 700 μl of the mix was added to the binding column and spun for 1 minute at 1300 rpm and the flow-through discarded. This was repeated for the remainder of the mix. The column was then transferred to a new tube.

Subsequently, 500 μl of Wash Solution was added and spun for 1 minute. Again the column was transferred to a new tube. Another 500 μl of Wash

Solution was added and spun for a further 3 minutes. Finally the column was transferred to a new tube and the DNA was eluted by adding 100 µl of Elution Solution (warmed to 65°C) to the column. This was spun for 1 minute and then repeated. The DNA concentration was checked with a NanoDrop® (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Wilmington, DE 19810, USA) and stored at -20°C.

4.2.3 PCR DNA amplification

In order to increase the DNA concentration of DNA samples were amplified by PCR. The PCR reaction was carried out at a range of concentrations to determine the optimum requirements to amplify the DNA (Table 4.1). The PCR was run for 2 min at 94 °C followed by thirty cycles of 94 °C for 20 s, 56 °C for 20 s and 72°C for 40 s. Completed with 10 min at 72 °C.

Reagents	<i>T. caeruleus</i> µl				<i>T. arvense</i> µl	
H ₂ O	12.2	9.2	6.2	3.2	8.2	1.2
10 x biobuffer	2	2	2	2	2	2
50 mM MgCl ₂	0.6	0.6	0.6	0.6	0.6	0.6
10 mM dNTPs	0.4	0.4	0.4	0.4	0.4	0.4
Left primer	0.8	0.8	0.8	0.8	0.8	0.8
Right primer	0.8	0.8	0.8	0.8	0.8	0.8
DNA (3.3 µg/µl)	3	6	9	12	7	14
BioTaq (Bioline)	0.2	0.2	0.2	0.2	0.2	0.2

Table 4.1. PCR mixture made up to determine the optimum concentrations to give an increase in DNA concentration.

4.2.4 Agarose gel electrophoresis of DNA

All PCR products were visualised by running samples on a 0.7% (w/v) Agarose gel for 30 min at 94 V in 1X TBE buffer. The 1X TBE buffer was made up using 100 ml of 10XTBE buffer (108 g TrisBase, 55 g Boric Acid, 20 mL 0.5 M EDTA, make up to 1 L with water) and 900ml water, containing 5 µl of 10 mg ml⁻¹ ethidium bromide.

4.2.5 DNA purification from agarose gels.

Gel electrophoresis bands were visualised under a short wavelength UV transilluminator. Bands of the correct size i.e. those containing the DNA

fragment of interest were excised from the gel using a sterile scalpel, placed into Eppendorf tubes and flash frozen in liquid nitrogen.

The DNA extracted using a MinElute Qiagen Gel Extraction kit (Qiagen Ltd, Crawley, West Sussex) using a microcentrifuge. The fragment in the agarose was weighed and to it a volume of Buffer QG; three times the weight of the fragment was added. This was incubated in a water bath at 50 °C for 10 minutes or until the gel had dissolved, during incubation the tube was inverted every 2-3 minutes to aid dissolving. Once the dissolving was complete, the colour of the tube contents were checked to confirm they remained yellow, an indication that the contents is at optimum pH. One times the gel volume of isopropanol was added to the tube and mixed by inversion several times. A MinElute column supplied in the kit was placed in a 2 ml collection tube (also supplied). The DNA extracted from the gel was transferred to the MinElute column and centrifuged at 10,000 x g for 1 minute to bind the DNA to the column. The flow through liquid was discarded and the MinElute column containing the bound DNA was retained in the collection tube. 500 µl of Buffer QG was added to the spin column and centrifuged at 10,000 x g for 1 minute. Again the flow through was discarded and the column and tube retained. A wash was carried out by adding 750 µl of Buffer PE to the column and spinning at 10,000 x g for 1 minute. The flow through was discarded and the tube spun for an additional 1 minute at 10,000 x g. The MinElute column was placed in a clean 1.5 ml collection tube and 10 µl of reverse osmosis purified water was added to the column to elute the DNA. This was left to stand for one min and then centrifuged at 10,000 x g for 1 minute.

4.2.6 Overview of the cloning strategy used to produce an RNA interference construct

To produce a RNAi *HMA4* construct for transformation of *T. caerulescens* and *T. arvense*, the GATEWAY® recombination cloning technology was to be used. The protocol involves inserting the fragment of interest into the entry vector, pENTR/D- TOPO® vector, this is made possible by the addition of a CACC sequence at the 5' end of the target DNA and the *attL* sites that flank the cloning sites to allow recombination. Following successful recombination, confirmation by PCR and sequencing. the target gene is recombined into the chosen destination vector (PK 7GW1WG2 GATEWAY® vector for RNA interference) using the LR clonase reaction enzyme mix (Invitrogen, Gaithersburg, MD, USA).

In this study the initial insertion of the *HMA4* fragment into the entry vector, pENTR/D- TOPO, was unsuccessful. Therefore a more traditional approach using pGEM® T-EASY vector was used prior to the GATEWAY recombination cloning technology. The following is the procedure outlined in more detail.

4.2.7 Ligation with pGEM®-T Easy Vector

Before the transformation of the extracted 420 bp fragments into the pGEM T-Easy vector (Fig.4.5) A overhangs were added through the addition of a single Adenine molecule using a thermocycler to carry out the elongation reaction; the reaction mix is displayed in Table 4.2.

Reagents	<i>T. caerulescens</i> μ l	<i>T. arvense</i> μ l
H ₂ O	0	5
10 x biobuffer	1	1
50 mM MgCl ₂	0.4	0.4
dATPs	1	1
DNA fragment	6.6	5
BioTaq (Bioline)	1	1

Table 4.2 The reaction mix used to add the series of Adenine molecules required to produce blunt ends. The reaction mix was placed in a thermocycler and run at 70°C for 30 minutes.

The 420 bp DNA fragment with blunt ends was ligated into the pGEM® T-Easy Vector System 1 at two concentrations to determine an optimum to allow the ligation to take place. DNA ligase was added alongside the reaction buffer and water (Table 4.3). The samples were left in the cold room at 4°C for 2 days to allow ligation.

Reagents	<i>T. caeruleus</i> 1:1 μ l	<i>T. caeruleus</i> 3:1 μ l	<i>T. arvense</i> 1: 1 μ l	<i>T. arvense</i> 3: 1 μ l
dATP reaction	0.7	2.1	0.7	2.1
pGEM-Teasy	1	1	1	1
H ₂ O	2.3	0.4	2.3	0.4
2x rapid buffer	5	5	5	5
T ₄ DNA ligase	1	1	1	1

Table 4.3 showing the reaction mixture used to carry out the ligation of the fragment with the pGEM T-Easy vector.

Prior to the ligation procedure two LB/ampicillin/IPTG/X-Gal plates were produced for each ligation reaction, plus two plates for determining transformation efficiency. The plates were equilibrated to room temperature prior to plating. The tubes containing the ligation reactions were centrifuged to collect contents at the bottom of the tube. 2 μ l of each ligation reaction was added to a sterile 1.5 ml microcentrifuge tube and set on ice. Another tube was placed on ice with 0.1ng of uncut plasmid for determination of the transformation efficiency of the competent cells.

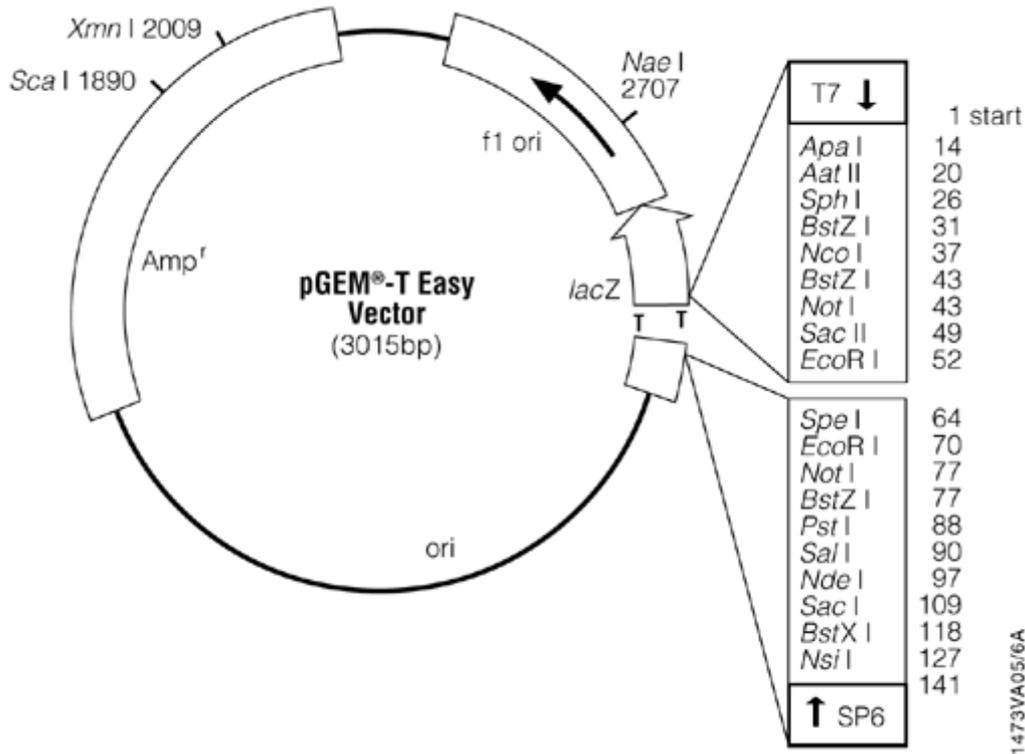


Fig.4.5 Map of the pGEM® T-Easy Vector displaying restriction sites and selectable marker genes.

4.2.8 Transformation into competent *E. coli* cells.

Ligation mixes of plasmids and the *HMA4* fragments were ligated into DH 52 *E. coli* competent cells. The ligation products were added to competent *E. coli* cells, stored on ice for 10 minutes before being placed in a water bath at 42°C for 90 s. This provided a heat shock that creates pores in the bacterial membrane to allow fragments of DNA to pass through. The tubes were then again placed on ice for 1 minute before adding 600 µl of liquid LB medium (10 g L⁻¹ Bacto-tryptone, 10 g L⁻¹ Yeast extract and 10 g L⁻¹ NaCl with pH adjusted to 7.2 using 0.1 M HCl or 0.1 M NaOH), then the culture was shaken at 37°C for 1 hour.

The cultures were plated at 200 μl and 150 μl on to solid LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin at 37°C overnight, to obtain single colonies.

4.2.9 Selection of positive *E. coli* clones

Following transformation into competent *E. coli* cells positive colonies were identified in one of two ways depending on the vector the *HMA4* fragment was inserted in.

4.2.9.1 Selection of positive *E. coli* clones containing the pGEM® T-Easy Vector

The plates produced blue and white bacterial colonies of which only the white are useful as these show they contain the *HMA4* DNA insert which has disrupted the gene that would normally produce the blue pigment (Lac Z gene which under normal conditions produces β -galactosidase, which is able to turn the substrate X-gal into a blue product). Approximately 6 white colonies were picked from each plate and grown separately overnight in 20 μl of liquid LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin at 37°C.

The positive clones were analysed by PCR reaction for the presence of the *HMA4* fragment of the correct size. Prior to the PCR amplification the extracted plasmid was incubated with the enzyme Not1 to release the fragment from the plasmid (Table 4.4). The incubation period was 1 h at 37°C.

Reagents	<i>T. caerulescens</i> μ l	<i>T. arvense</i> μ l
H ₂ O	16.0	13.0
10x reaction buffer	3	3
DNA	10	13
<i>Not1</i>	1.0	1.0

Table 4.4 The PCR used to determine the presence of the *HMA4* gene in the positive clones including the addition of the *Not1* enzyme to release the fragments from *E. coli*.

4.2.9.2 Selection of positive *E. coli* clones containing the pENTR/D-TOPO Vector

Positive clones were chosen from the overnight culture (i.e. those that grew) named TOPO-G and TOPO-W. The plasmid contains a *ccdB* gene that is lethal to most *E. coli* cells. Upon recombination this gene is removed allowing only *E. coli* cells containing plasmids that have successfully undergone recombination to grow. Master plates of these colonies were made on solid LB supplemented with the same antibiotics as previously. To test for the positive presence of the *HMA4* gene PCRs were set up containing very minute amounts of bacteria picked up with a pipette tip run with the *HMA4* primers.

4.2.10 Extraction of plasmid DNA

To allow the fragment to be moved into subsequent plasmids the construct was first removed from the current host using the Qiagen Spin Miniprep kit

(Qiagen Ltd, Crawley, West Sussex). The liquid overnight cultures were spun down into individual pellets, firstly the pellet was re-suspended in 250 µl Buffer P1 and transferred to a microcentrifuge tube. To that, 250 µl Buffer P2 was added and inverted 4-6 times to mix. 350 µl Buffer N3 was added and immediately but gently inverted 4-6 times to produce a cloudy mix. This was centrifuged for 10 minutes at 17,900 x g to form a compact white pellet. The supernatants from the previous step were applied to a QIAprep spin column by decanting or pipetting and centrifuged for 30-60 s, the flow through was discarded. The QIAprep spin column was washed by adding 0.5 ml of Buffer PB and spun for 30-60 s; again the flow through was discarded. The step removes the trace nuclease activity. Another wash step was carried out by adding 0.75 ml Buffer PE and centrifuging for 30–60 s at 10,000 x g. After discarding the flow through an additional spin for 1min removed the residual wash buffer. The QIAprep column was transferred to a clean 1.5 ml microcentrifuge tube to carry out DNA elution. 50 µl reverse osmosis purified water was added to the center of each QIAprep column. This was left to stand for one min and then centrifuged at 10,000 x g for 1 minute.

4.2.11 Ligation with pENTR/D-TOPO vector

Following the positive identification of *HMA4* fragments within the pGEM® T-Easy Vector by PCR and sequencing the *HMA4* TV-G (*T. caerulea*) and TV-W (*T. avense*) sequences were cut from the pGEM® T-Easy vector with *Not1* before ligation. TOPO A6 vector was also cut with *Not1* and dephosphylated with CIAP (Calf Intestinal Alkaline Phosphatase) enzyme (Table 4.5) The incubation period was carried out at 37°C for 1 h.

	<i>T.caerulescens</i> μ l	<i>T.arvense</i> μ l
H ₂ O	15.5	12.5
10x buffer 3	3	3
DNA	10	13
<i>Not1</i>	1.5	1.5

Table 4.5 The reaction mix used to transform the fragment into the PENTR/D-TOPO Vector

The fragments were inserted into the Invitrogen Gateway entry vector pENTR/D-TOPO through ligation over a two day period in a cold room according to the manufacturer's protocol using the reaction mix (Table 4.6) (Invitrogen, Gaithersburg, MD, USA). Following this the ligation product was transformed into competent *E. coli* cells as described in section 4.2.8. Clones which survived were grown overnight on solid LB medium, supplemented with 50 μ g ml⁻¹ kanamycin at 37°C, followed by PCR with *HMA4* primers and sequencing to confirm the presence of *HMA4*. The plasmid constructs were identified according to the origin of the DNA i.e. TOPO-G and TOPO-W from *T. caerulescens* (Ganges) and *T. arvense* (Wellesbourne) DNA respectively.

Reagents	<i>T. caerulea</i> Ganges μ l	<i>T. arvensis</i> Welesborne μ l	Control μ l
H ₂ O	1	0	2
2x buffer	5	5	5
TOPO	2	2	2
DNA Fragment	1	2	0
Ligase	1	1	1

Table 4.6 The reaction mix used for the ligation of the fragment into the PENTR/D-TOPO Vector.

4.2.12 Ligation into the PK 7 GATEWAY vector for RNA interference

Overnight cultures of the pENTR/D-TOPO G1 (*T. caerulea*) and pENTR/D-TOPO-W1 (*T. arvensis*) were produced as described previously and the plasmid DNA extracted using the Qiagen Spin Miniprep kit (Qiagen Ltd) as described in section 4.2.10. The samples were again cut with *Not1* enzyme as before and the ligation between the fragment and the PK7GW1WG2 GATEWAY RNAi destination vector (Fig. 4.6) was carried out as with the ligation between the fragment and the pENTR/D-TOPO vector but this time using the LR Clonase Enzyme Mix (Invitrogen, Gaithersburg, MD, USA).

The ligation was left in the cold room for 2 days, in the dark to ligate. On completion of the ligation process Proteinase K (Invitrogen, Gaithersburg, MD, USA) was added (1 μ l) and incubated for 10 minutes at 37°C. This step improves transformation efficiency.

The ligation reaction mix was transformed into competent *E. coli* cells as described in section 4.2.8. Positive colonies were identified by PCR and sequencing and a master plate made up of successful colonies as previously discussed.

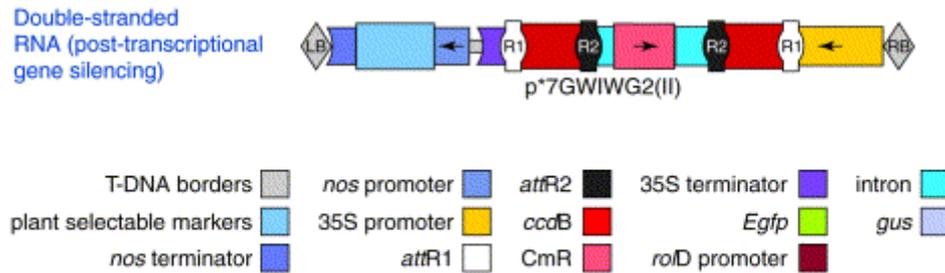


Fig.4.6 Map of GATEWAY™ destination vector Pk7GW1WG2 showing the *attR* recombination sites, the *ccdB* gene that inhibits growth in *E. coli* that is removed and replaced by the chosen DNA fragment (Karimi *et al.*, (2002).

4.2.13 Transformation into *Agrobacterium tumefaciens*

Two different strains of *A. tumefaciens* were chosen, LBA4404 for the tissue culture plant transformation and C58 for the floral dip method. Overnight cultures of the *T. caerulescens* (sample G4) and *T. arvense* (sample W8), which were successfully sequenced, were grown up from the master plates in LB medium supplemented with 50 $\mu\text{g ml}^{-1}$ of spectinomycin. The plasmids were then extracted using the Qiagen extraction kit (4.2.9). Then 50 ng of the *HMA4* in the pk7 GW1WG2 Gateway vector was mixed gently with 50 μl of competent *Agrobacterium tumefaciens* cells. The mixture was frozen in liquid nitrogen for 3 minutes and thawed by incubation in a water bath at 37 °C for 5 minutes to apply a heat shock to the cells. This heat shock induces pores in

the membranes to allow uptake of the vector into the cells. Before incubation, 600 μl of APM medium (5 g L^{-1} yeast extract, 0.5 g L^{-1} casein hydrolysate acid, 8 g L^{-1} Mannitol, 2 g L^{-1} Ammonium sulphate, 5 g L^{-1} NaCl, 0.427 g L^{-1} MgCl_2 with pH adjusted to 6.6 using a pH meter) was added and incubated in the dark at 29°C for 2-4 h whilst being shaken.

The incubated suspension was spread on solid LB medium plates supplemented with 50 $\mu\text{g ml}^{-1}$ spectinomycin, 50 $\mu\text{g ml}^{-1}$ streptomycin and 35 $\mu\text{g ml}^{-1}$ rifampicin. The plates were incubated in the dark for 2-3 days at 29°C. Positive clones could then be tested by PCR. Positive clones were confirmed by PCR and sequencing and glycerol stocks produced for the *A. tumefaciens* C58 (*T. caerulescens* and *T. arvense*) constructs and *A. tumefaciens* LBA4404 (*T. caerulescens* and *T. arvense*) constructs. 1.5 ml Eppendorf tubes containing 0.75 ml aliquots of bacterial culture were mixed with 0.75 ml of 40% (v/v) glycerol in Luria broth (containing 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 litre distilled water Adjust to pH 7.5), stored at -70°C.

4.2.14 Sequencing of plasmids

To determine that the gene had entered the plasmids and that there were no errors in the sequences the plasmids were sequenced using a Beckman CEQ ceq8000 DNA sequencer (Beckman Coulter, Inc, Fullerton, California). For the final construct, sequencing was carried out using primers for the 35S promoter in the RNAi construct with the *HMA4* left primer for the *T. caerulescens* construct and the primers for the 35S promoter in the RNAi construct with the *HMA4* right primer for the *T. arvense* construct.

4.3 Results

4.3.1 Plant genomic DNA isolation

Using the primers designed for the *HMA4* fragment (420 bp) PCR amplified the fragments from *T. caerulescens* (G1, G2 and G3 represent biological replicates of different *T. caerulescens*) and *T. arvense* (W1 and W2 represent biological replicates of different *T. arvense* plants) genomic DNA, despite the low levels of DNA extracted (at concentrations of $3.3 \text{ ng } \mu\text{L}^{-1}$ and $1.5 \text{ ng } \mu\text{L}^{-1}$ respectively) (Fig.4.7).

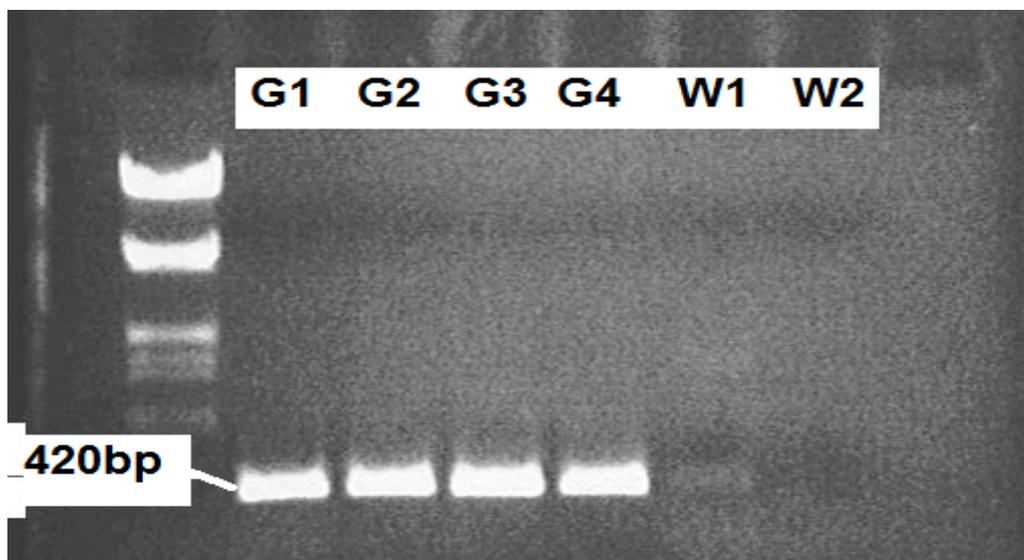


Fig. 4.7 PCR amplification of four *T. caerulescens* (Ganges) (G1-4) and two *T. arvense* (Wellesborne) samples (W1-2) DNA using the *HMA4* primers. Positive bands for Ganges samples 1 to 4 and very faint bands at W1 and W2. This shows that the primers designed amplify the expected 420 bp from Ganges. The very faint band in the Welesborne samples may be due to the lower copy number of the *HMA4*, which may explain the lack of the hyperaccumulating trait. The replicates (G1, G2, W1 and W2) refer to DNA templates extracted from individual plants.

4.3.2 Ligation with the pGEM® T-EASY vector

Initial attempts to insert the *HMA4* fragments into the GATEWAY™ pENTR/D TOPO vector were not successful. Reasons for this are unknown however they may be due to one or more of several factors including efficiency of competent cells, the enzyme used, the enzyme may have denatured somehow possibly due to storage or maybe the problem lay with the DNA fragment itself.

The steps taken to overcome this problem involved using a more traditional cloning approach using the pGEM® T-EASY vector. Following positive selection of *E. coli* colonies by blue and white selection, PCR amplification of the plasmids was carried out using the *HMA4* forward and reverse primers, following an incubation period with the enzyme *Not1*. This PCR of positive *E. coli* clones containing the *T. caerulescens* (G1-G6) and *T. arvense* (W1-W6) fragments respectively and showed that all colonies possessed the 420 bp *HMA4* fragment (Fig.4.8) However some fragments appeared more prominent than others therefore one *T. caerulescens* containing colony and one *T. arvense* containing colony was chosen to be sent for sequencing (Appendix 8.1.1, 8.1.2).

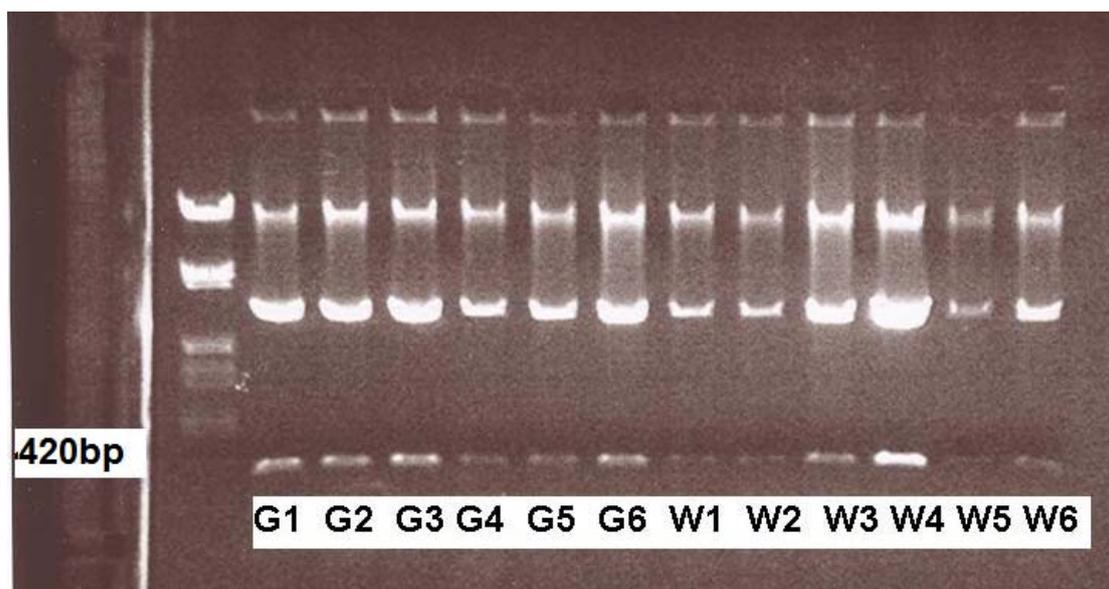


Fig.4.8 Gel electrophoresis of amplified PCR products of the positive *E. coli* colonies. The PCR results showed all colonies possess the *HMA4* gene. However some are more prominent bands than others. As only one *T. caerulescens* and one *T. arvense* clone is required two strong bands were chosen and these were sent for sequencing: these were named TV-G and TV-W.

4.3.3 Ligation into the pENTR/D- TOPO vector

Follow the ligation into the GATEWAY™ pENTR/D-TOPO vector positive colonies were analysed by PCR and sequencing to confirm correct insertion of the 420 bp *HMA4* fragment. Fig.4.9 confirms that 2 positive bands at 420 bp can be seen for *T. caerulescens* (G) and *T. arvense* (W) which concludes that the *HMA4* correctly inserted into the pENTR/D-TOPO vector. Colonies containing the *HMA4 T. caerulescens* fragment, G1 and *HMA4 T. arvense* fragment, W1 were chosen for sequencing and used in subsequent reactions (Appendix 8.2.5 and 8.2.6).

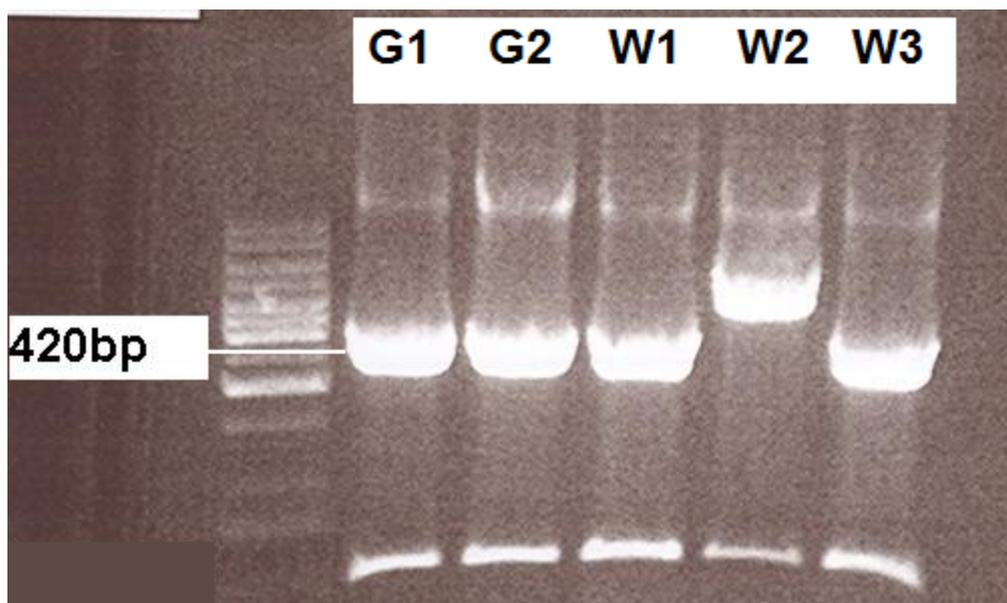
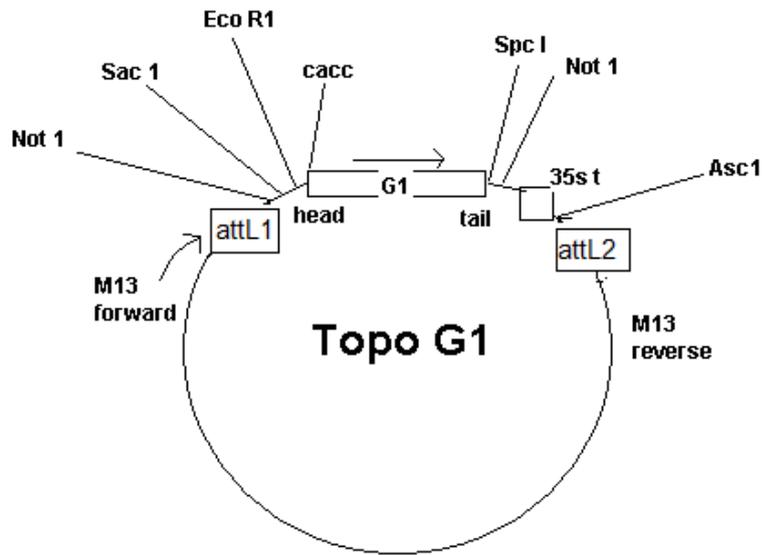
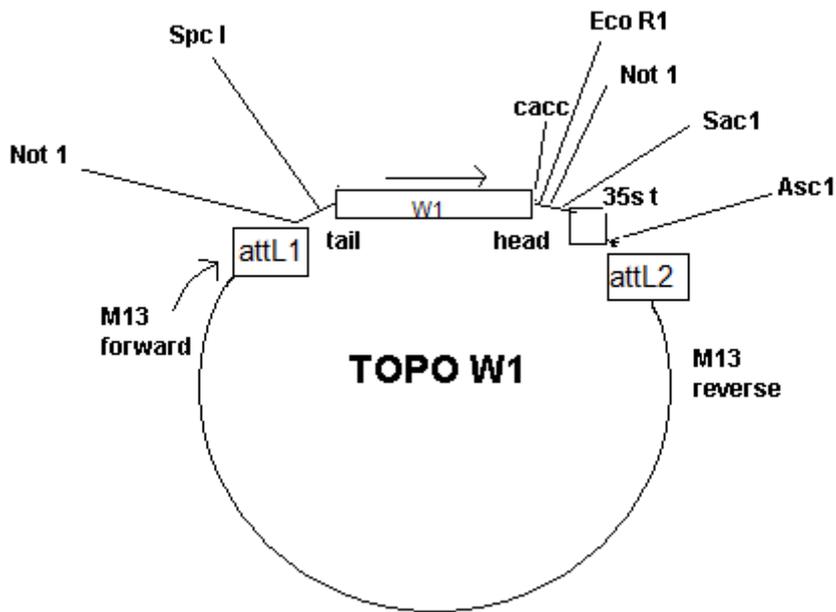


Fig.4.9 The electrophoresis of the PCR to amplify *E. coli* containing the PENTR/D TOPO vector carrying the *HMA4* gene. G1 (*T. caerulescens*) and W1 (*T. arvense*) were chosen for sequencing. The results were positive for *T. caerulescens* (G1, G2) and *T. arvense* (W1, W3) (*T. arvense* W2 has an unusually large band).

From the sequencing results it was noted that the *T. arvense HMA4* sequence was inserted in the reverse orientation to the *T. caerulescens HMA4* sequence. This was interesting to know however it should not affect the final dsRNA product that this construct ought to produce. Fig.4.10 shows an overview of how the *HMA4* (either G1 or W1) appeared in the pENTR/D-TOPO vector showing additional features such as the *att* recognition sites.



a)



b)

Fig.4.10a Map of TOPO-G1 construct showing orientation of the *HMA4* insert in comparison to the TOPO-W1 (**Fig.4.10b**) construct where the insert is in the reverse orientation

4.3.4 Ligation into the PKGW1WG2 GATEWAY vector for RNAi

Successful recombination between the entry clone and the destination vector, in this case PKGW1WG2 RNAi vector, was confirmed again by PCR and sequencing of positive clones. The PCR was carried out with different combinations of primers in this case. Using the forward *HMA4* primer and either the 35S promoter or terminator primer from the PKGW1WG2 RNAi vector gave the results shown in Fig.4.11. Three strong positive clones containing the *HMA4* fragment were identified. Replicate G2 was chosen to be sequenced (Appendix 8.2.7, 8.2.8) The PCR had to be repeated as no positive results were identified for *T. arvensis* (Fig.4.12). This time the *HMA4* reverse primer was used with the 35S promoter or terminator primer from the PKGW1WG2 RNAi vector. Five positive clones were identified with replicate W8 and sent for sequencing (Appendix 8.1.11) along with a second *T. caerulescens* replicate G4 (Appendix 8.2.9, 8.2.10).

From the sequencing and the PCR results it was determined that indeed the *T. arvensis HMA4* sequence had entered the destination vector in the opposite orientation to the *T. caerulescens HMA4* sequence as outlined by Fig.4.13.

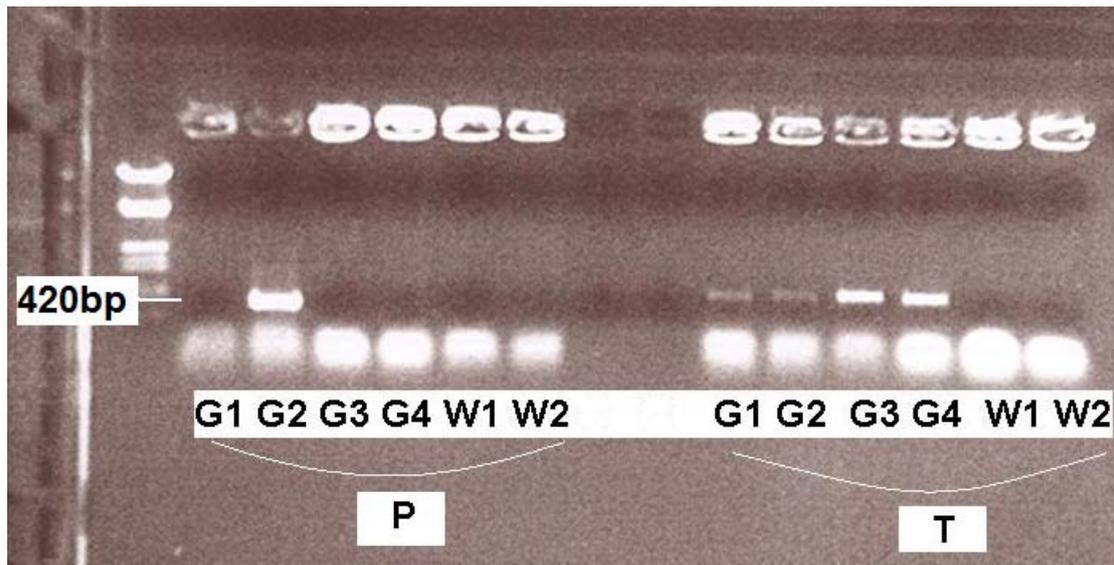


Fig.4.11 The electrophoresis results from a PCR run using two primer sets, *HMA4* forward primer plus either the 35S promoter (P) or terminator (T) primer only G2 (*T. caerulea*) promoter and G3 and G4 terminator worked well. *T. arvense* (W) appeared not to work at all. G2 was sent for sequencing.

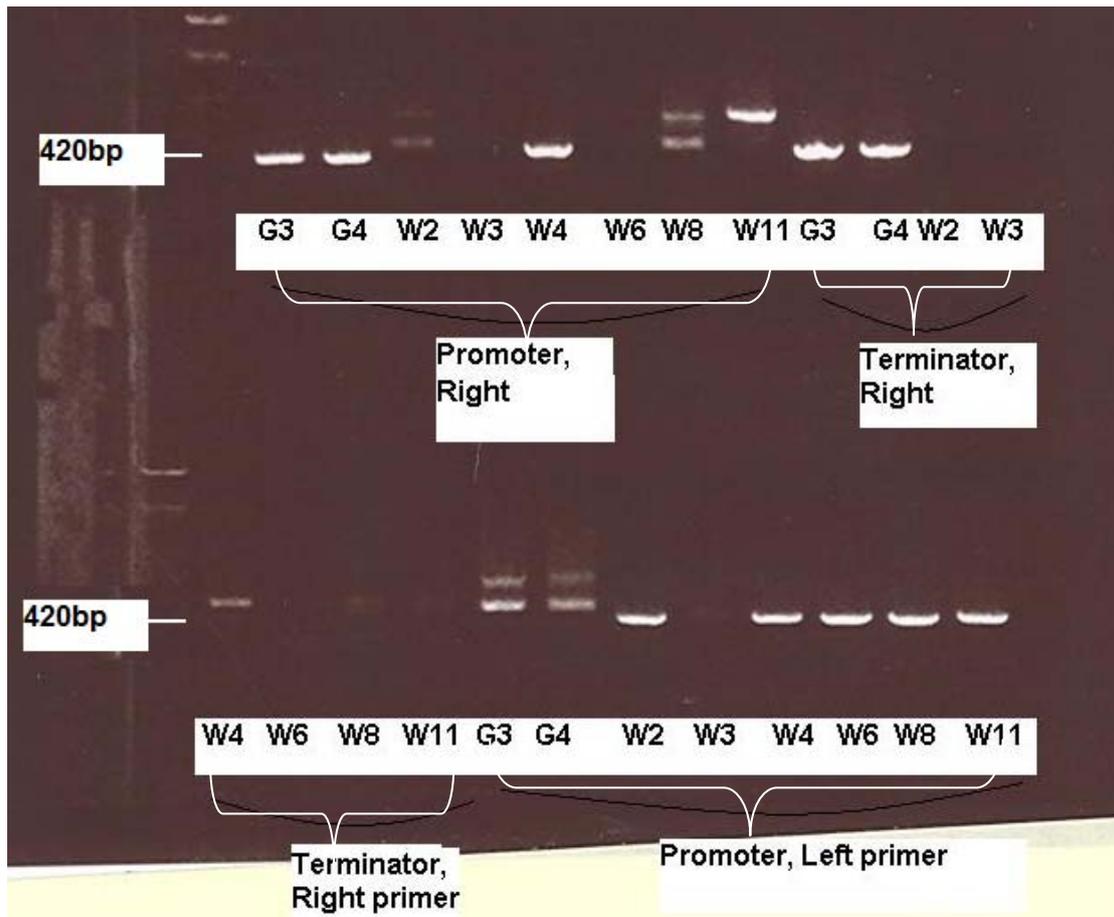


Fig.4.12 Electrophoresis results from the PCR run to determine that the *HMA4* gene has entered the Gateway vector. *T. arvense* samples; W4, W6, W8, W11 amplified with the promoter primer and left primer are positive as are W6 promoter with right primer. *T. caerulea* (G4) *T. arvense* W8 were sent for sequencing.

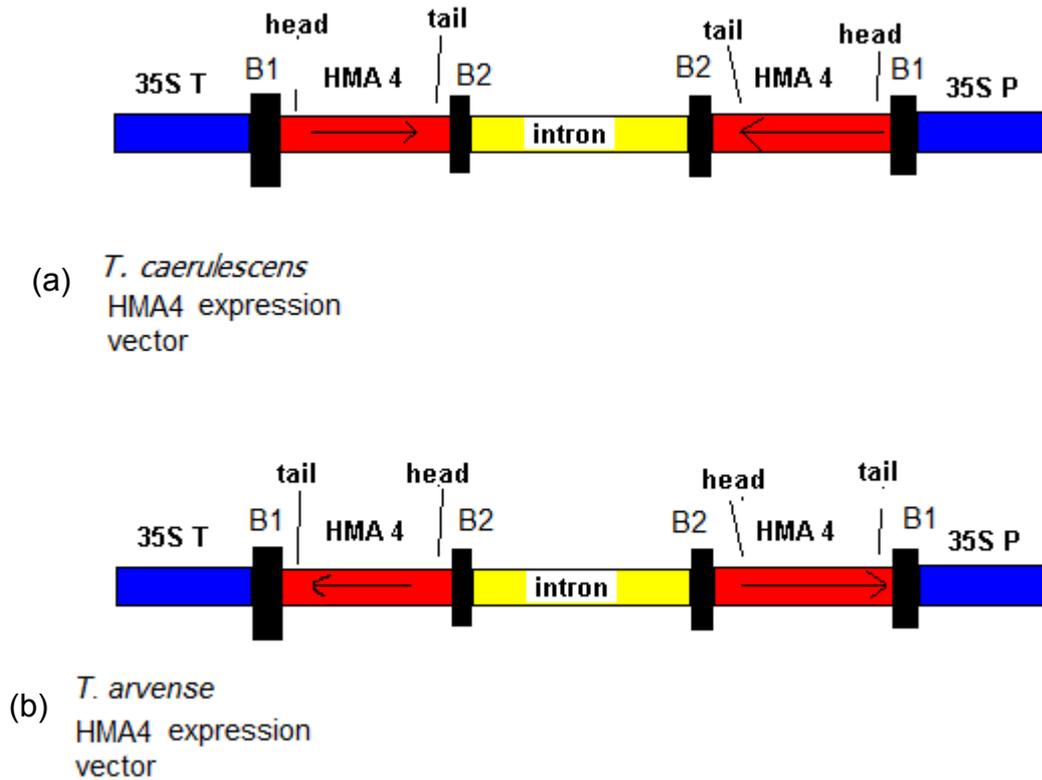


Fig.4.13 Showing the orientation of the *HMA4* gene around the intron region in the *T. caerulescens* *HMA4* construct (a) *T. caerulescens* *HMA4* construct is the opposite of *T. arvensis* *HMA4* construct (b). When compared to the entry vector it can be seen that the *HMA4* fragment has replaced the *ccdB* fragment that inhibits the growth of the majority of *E. coli* cells. In the LR reaction the entry clone (pENTR/D-TOPO vector) with attL sites recombine with the destination vector (PK7GW1WG2 RNAi vector) with attR sites to form the expression clone with attB sites and a by product with attP sites.

4.3.5 Transformation into *A. tumefaciens*

With the *HMA4* fragments from both *T. caerulescens* and *T. arvense* successfully confirmed to be contained within the RNAi destination vector, the last step was to transform the plasmids into the two strains of *A. tumefaciens*, C58 and LBA4404. The following figures (Fig.4.14 and Fig.4.15) confirm that *T. caerulescens* Ganges (G) was successfully transformed into both strains of *Agrobacterium tumefaciens* (C58 and LBA4404) with all colonies tested being positive. *T. arvense* Wellesborne (W) was successfully transformed into *Agrobacterium tumefaciens* strain C58, however the bands for the LBA4404 strain were a lot more faint, apart from sample number 6. However this was not an issue as only one replicate of *T. caerulescens* and *T. arvense* was required for the production of glycerol stocks and to be sequenced to confirm the correct sequence.

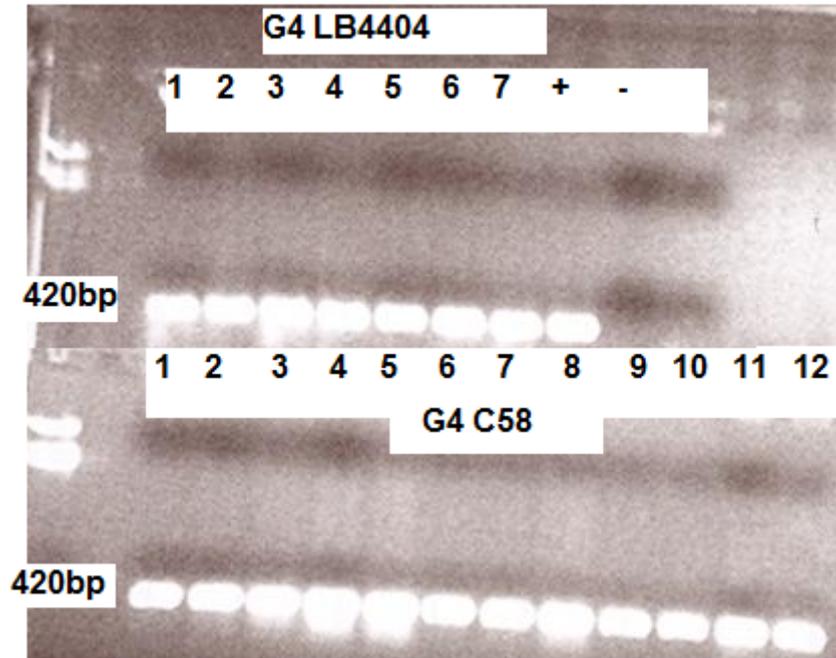


Fig.4.14 Electrophoresis results of the final PCR of the *T. caerulescens* samples to confirm the presence of the *HMA4* gene in the *Agrobacterium tumefaciens* strains LBA4404 and C58. 7 positive clones were identified for the LBA4404 strain and 12 for the C58 strain.

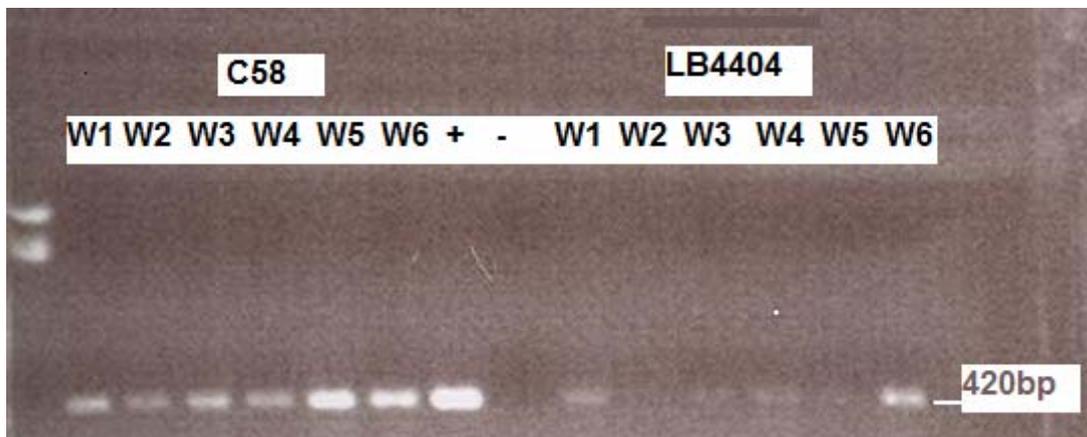


Fig.4.15 Electrophoresis results of the final PCR of the *T. arvense* samples to confirm the presence of the *HMA4* gene in the *Agrobacterium tumefaciens* strains LBA4404 and C58. 6 positive clones were identified for the LBA4404 strain and 6 for the C58 strain.

4.3.6 Sequencing of the final constructs

The end result of this work was the production of two constructs (*HMA4* of *T. caerulescens* and *T. arvense*) in two different strains of *A. tumefaciens*, C58 and LBA4404. The final confirmation of the production of these constructs was their sequencing (Fig.4.16, 4.17). It is possible to see that the *T. caerulescens HMA4* sequence lies between bases 269-689 (CACCGCT....TGCGA). In contrast the *T. arvense HMA4* sequence lies between bases 129-553 (CTTCTCT....TAGCGGTG) as it was shown the gene has inserted in the reverse orientation. (Other electropherograms produced for the making of the construct can be found in the appendices).

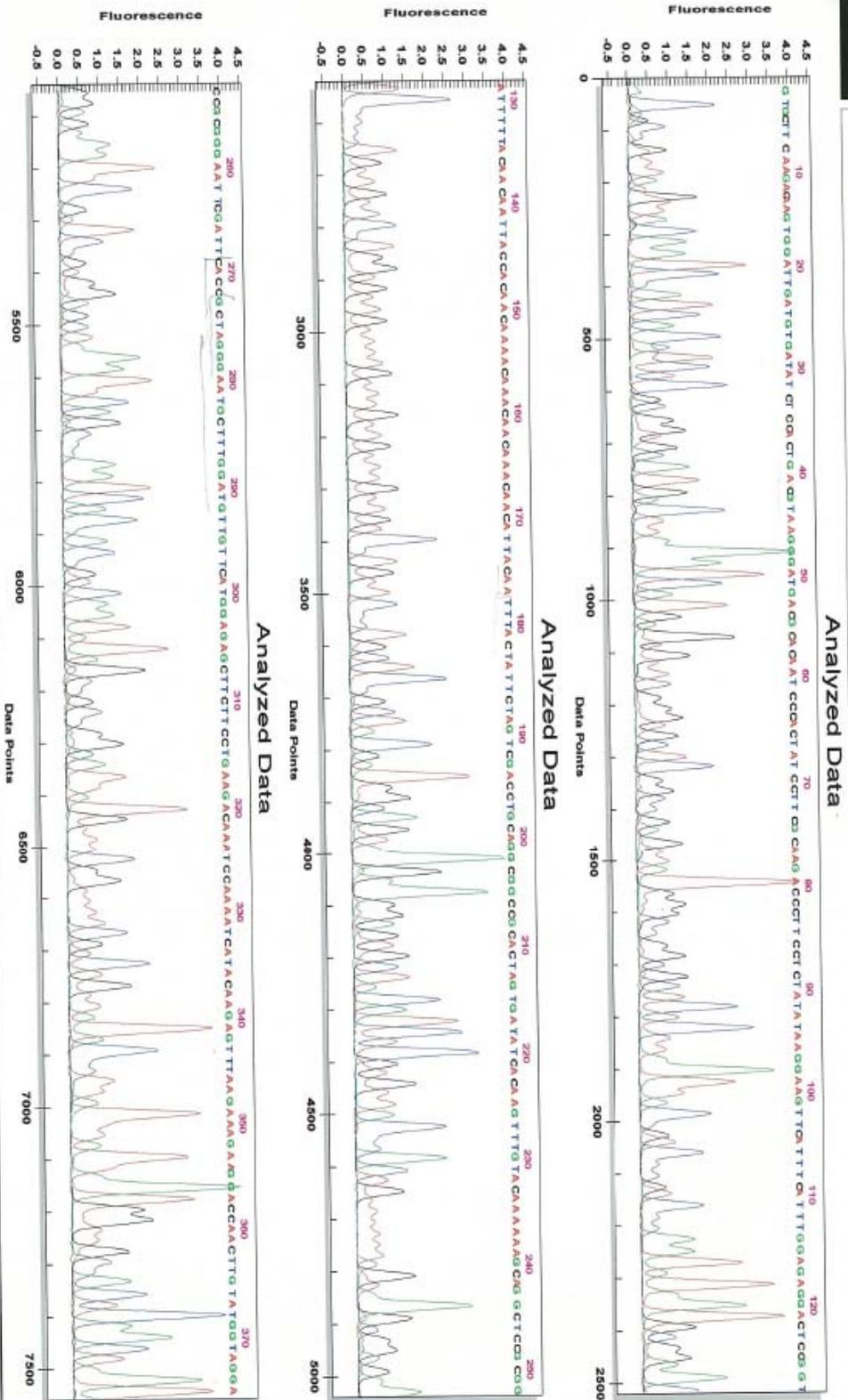


Project : Default
 Sample : PK7G4,PK735SP.H01_05081111YD
 Result : PK7G4,PK735SP.H01_05081111YD

System : CEQ System 3166768

Instrument : CEQ System 3066768 (Ver. 8.0.52)

Operator :



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Page: 1

Fig. 4.16 Sequence data for HMA4 gene of *T. caerulescens* in *Agrobacterium* cells. The sequence can be seen between bases 269-689 (CACC CGCT...TGCGA).

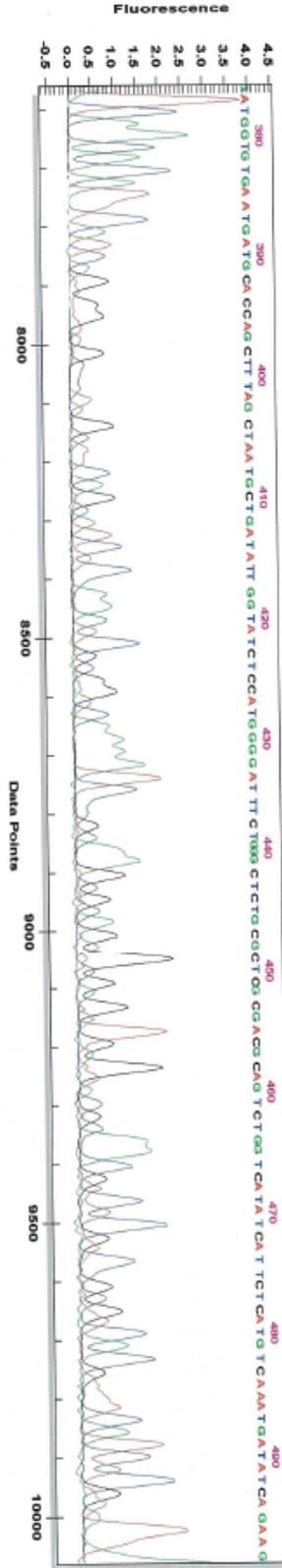


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 Result : PK7G4,PK735SP.H01_05081111YD

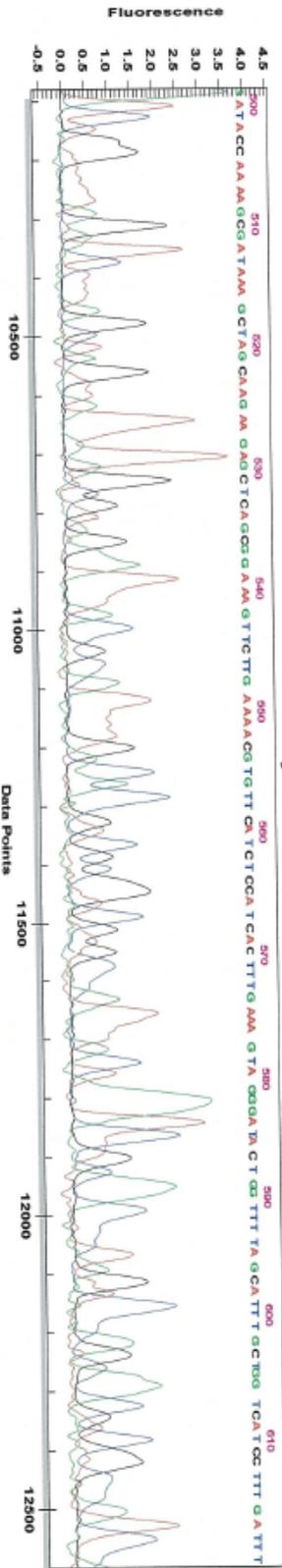
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Operator :
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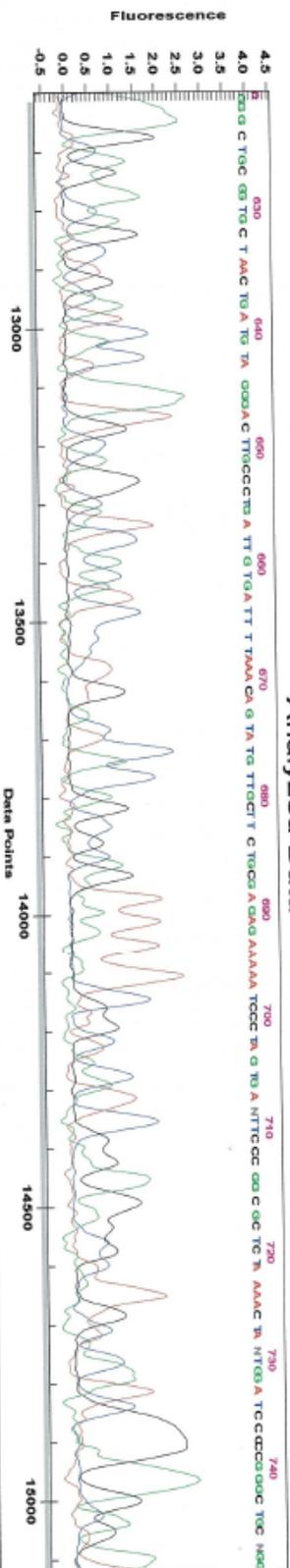
Analyzed Data



Analyzed Data



Analyzed Data



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Fig. 4.16 (cont.)

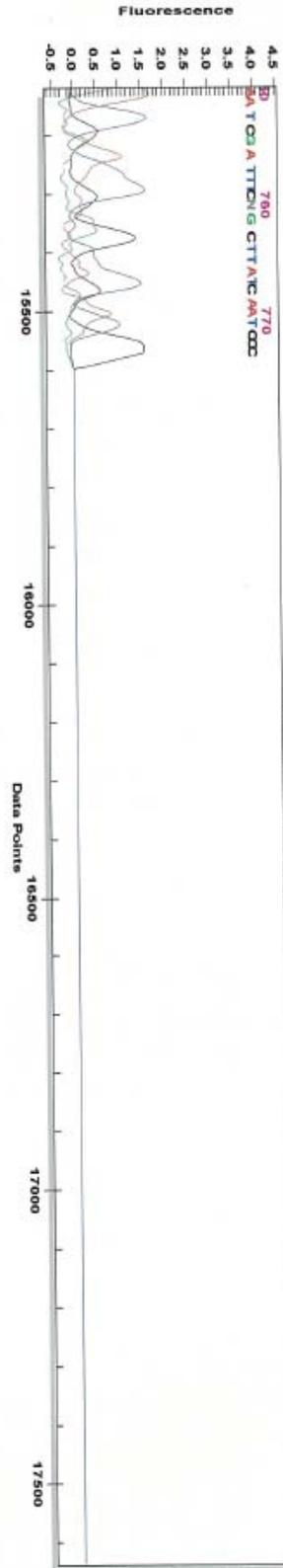


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Operator :
Instrument : CEQ System 3066768 (Ver. 8.0.52)

Analyzed Data



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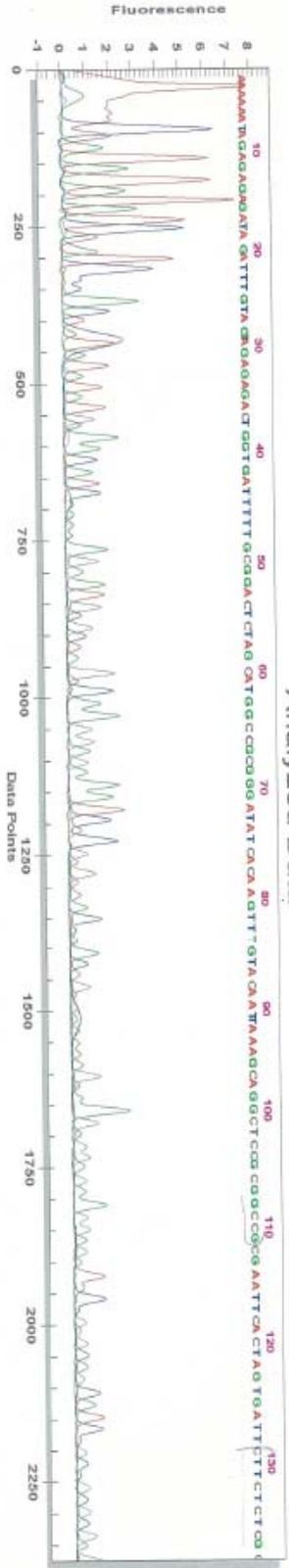
Fig. 4.16 (cont.)



Project : Default
 Sample : PK7W8,PK7FTSZTF.B01_05092011B
 Result : PK7W8,PK7FTSZTF.B01_05092011B

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



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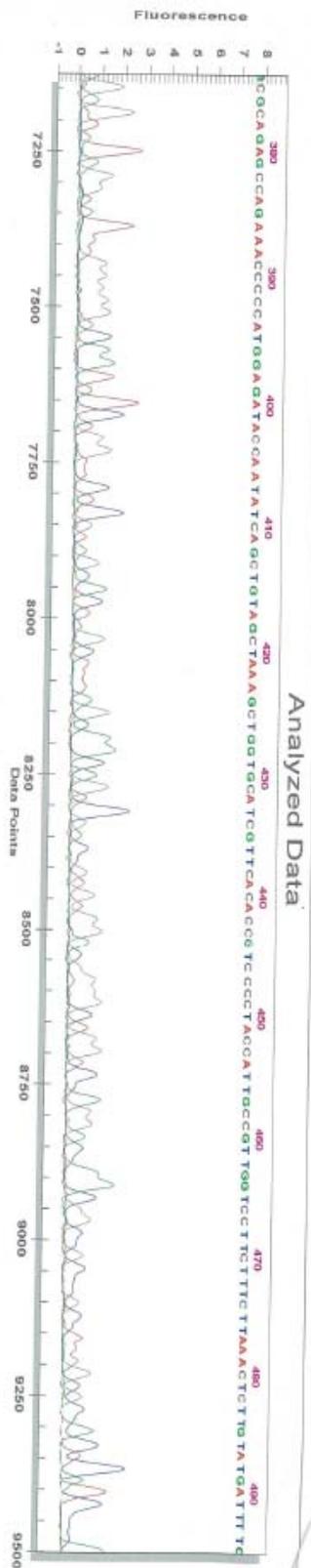
Fig. 4.17 Sequence data for HMA4 gene of *T. arvensis* in *Agrobacterium* cells. The sequence can be seen, starting at base number 129- 553 (CTTCTCT...TAGGGGTG) As it was shown the gene has inserted in the reverse orientation.



Project : Default
 Sample : PK7W8,PK7FTSZTF.B01_05092011IB
 Result : PK7W8,PK7FTSZTF.B01_05092011IB

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.5Z)



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Fig. 4.17 (cont.)

4.4 Results summary

The aim of this chapter was to produce an RNAi construct to the *T. caerulescens* *HMA4* gene. This construct could then be used to transform *T. caerulescens* either by floral dip methods or by tissue culture.

Two constructs were produced in two different *A. tumefaciens* strains, *Agrobacterium tumefaciens* C58 construct, one containing the *HMA4* sequence from *T. caerulescens* and one containing the sequence from *T. arvense*. These constructs will allow the transformation of *Thlaspi* sp. through floral dip methods. The final constructs were *Agrobacterium tumefaciens* LBA4404 containing the sequence from *T. caerulescens* and one containing the sequence from *T. arvense*. These can be used to transform *Thlaspi* sp. *in vitro*.

The following chapter discusses the approaches taken to transform *T. caerulescens* both by floral dip and tissue culture methods. The chapter also describes the successful transformation of *Arabidopsis thaliana* using the construct described in this chapter. This is consistent with the construct being successfully created despite the difficulties encountered when producing it.

5. Transformation of *Thlaspi* species through floral dip and tissue culture methods.

The aim of this chapter was to produce an efficient protocol for the transformation of *Thlaspi caerulescens* and *T. arvense*. In previous chapters it has been described that potential candidate genes had been identified by microarray experiments by showing differential expression in *Thlaspi caerulescens* in comparison with *T. arvense*. These genes were then confirmed as being differentially expressed by qPCR. Taking the information gained from these experiments and from published literature a candidate gene was selected for further study, *HMA4*. The previous chapter described how the RNAi construct was designed with the aim to silence the *HMA4* gene *in planta*. This chapter follows on and describes the work carried out to develop an efficient protocol for transformation by floral dip or tissue culture methods.

5.1. Introduction

To study the function of these genes in greater detail would be beneficial to be able to transform *Thlaspi* sp. in order to knockout and up-regulate genes to determine the effect this would have on plant phenotype. At the start of this project there was a reported efficient transformation of *Thlaspi caerulescens* with a *GFP* reporter gene by floral dip methods. It therefore seemed a sensible approach to test the candidate gene *in planta* as it was thought the transformation procedure would be fairly straightforward (Peer *et al.*, 2003). Also with *Thlaspi* spp. being a close relative of the model plant species

Arabidopsis thaliana which is easily transformed it was thought that using methods similar to protocols used on *Arabidopsis thaliana* that transformation would be possible (Bechtold *et al.*, 1993; Clough and Bent, 1998; Labra *et al.* 2004).

Prior to this project there had been no reported literature on studying heavy metal genes in *Thlaspi* spp. via transformation methods. All previous work on gene manipulation of putative heavy metal hyperaccumulation genes has been done in *Arabidopsis* spp. and yeast complementation with *Saccharomyces cerevisiae* (Bernard *et al.*, 2004; Papoyan and Kochian, 2004; Pence *et al.*, 2000).

5.1.1 Plant Transformation

The genetic manipulation of plants has become an established tool to today's scientists. Some of the first useful applications of genetic manipulation of microorganisms included the transformation of bacteria to produce hormones such as insulin to treat diabetics. More recently, transformed microorganisms have been used to produce plants that are able to cope with adverse conditions (e.g. saline tolerance) and to produce plants that are able to synthesize pharmaceuticals and novel products (Flowers, 2004; Hellwig *et al.*, 2004). Genetic manipulation has provided a tool to explore the function of genes within both eukaryotes and prokaryotes. Currently plant transformation is achieved by one of four main techniques: *Agrobacterium tumefaciens* mediated transformation, biolistic DNA delivery, electroporation and polyethylene glycol mediated uptake of DNA by protoplasts (Barton *et al.*,

1983; Fromm *et al.*, 1986; Herrera-Estrella *et al.*, 1983; Klien *et al.*, 1987; reviewed in Christou 1995).

The transformation of plants via *A. tumefaciens* originally involved lengthy tissue culture stages. This technique relies on the plant tissue's totipotency and its ability to regenerate. More recently, the development of non-tissue culture based *A. tumefaciens* transformation has been favoured, in particular via floral dip resulting in approximately 1% transformation efficiency (Clough and Bent, 1998).

5.1.1.1 Direct DNA Uptake

The first transformants were produced using protoplasts and direct DNA uptake using either PEG (Polyethelene glycol) or electroporation. Problems with this method however include somaclonal variation (genotypic or phenotypic variation that arises in plants produced through tissue culture), albino plants (plants that lack chlorophyll), multicopy integrations along side the fact that this technique is extremely laborious, difficult to achieve fertile regenerants and genotype-dependent. This explains why this method became rarely used upon the advent of new gene delivery methods (Tyagi and Mohanty 2000).

5.1.1.2 Biolistics

Biolistics, also known as microprojectile bombardment or particle gun delivery, was used on rice embryos almost immediately after this method became available (Christou *et al.*, 1991). This technique involves coating gold particles

in DNA and firing them at the target tissues. The DNA coated gold particles enter the plant where the DNA can be incorporated into the plant genome (Komari *et al.*, 1998). Several tissue types have been successfully transformed including cell suspensions, immature embryos and leaf tissue (reviewed in Hiei *et al.*, 1997) For some time biolistics was the most widely used technique for the production of transgenic monocotyledonous plants such as rice and other cereals as *Agrobacterium* favoured dicotyledonous plants such as the Solanaceae. However the problems with biolistics include multi copy number of inserts and gene rearrangements (Komari *et al.*, 1998).

5.1.1.3 *Agrobacterium*-Mediated Transformation

Agrobacterium-mediated transformation is the most widely used technique for transforming dicotyledons due to the higher transformation efficiency, high occurrence of single gene insertions and limited gene rearrangements (Hiei *et al.*, 1997; Tyagi and Mohanty, 2000). However due to the fact that monocotyledons were outside the natural host range of the *Agrobacterium tumefaciens*, transformation by this method was initially over looked and research was focused on the other systems already mentioned. More recently *Agrobacterium*-mediated methods of plant transformation have been favoured as the most efficient method, in particular via floral dip transformation. The floral dip transformation of *Arabidopsis thaliana* has been described as having approximately 1% transformation efficiency (Clough and Bent, 1998).

Agrobacterium tumefaciens is a gram negative, soil borne pathogen, responsible for Crown Gall disease. Virulent strains of the bacterium contain

one or more large plasmids that carry the genes for tumour induction; this is known as the Ti plasmid (tumour inducing). The Ti plasmid contains genes that are responsible for the symptoms and control the host range. A small number of genes are transferred to the host plant; this is referred to as the T-DNA (Transfer DNA) (Dumas *et al.*, 2001). The T-DNA is flanked by a region of 25bp of repeat sequence (left border (LB) and right border (RB)); any DNA contained within these will be transferred to the plant. In wild type strains the DNA in this region encodes enzymes for the synthesis of cytokinins and auxins, which, once produced in the plant, are responsible for the production of the gall tumors. Secondary the T-DNA contains the genes responsible for production of opines that *Agrobacterium* is able to utilise as a carbon and nitrogen source. The ability of the T-DNA to be transferred to the host DNA is due to the presence of *vir* (virulence) genes which are also located on the Ti plasmid (reviewed in Zupan and Zambryski, 1995; Zhu *et al.*, 2000). The infection of *Agrobacterium* begins when a plant gets wounded and releases phenolic defense compounds that stimulate the expression of *vir* genes. These *vir* genes trigger the replication of the T-DNA to produce a T- strand, which along with Vir proteins get transferred to the host plant cell through a transport channel. Once inside the plant cell the Vir proteins carry out a second role, combining with the T-strand to form a T-complex where the complex targets the host nucleus. The transferred DNA is then integrated into the plant genome where, along with plant genes, the bacterial genes are expressed (Fig.5.1) (Gelvin, 2005).

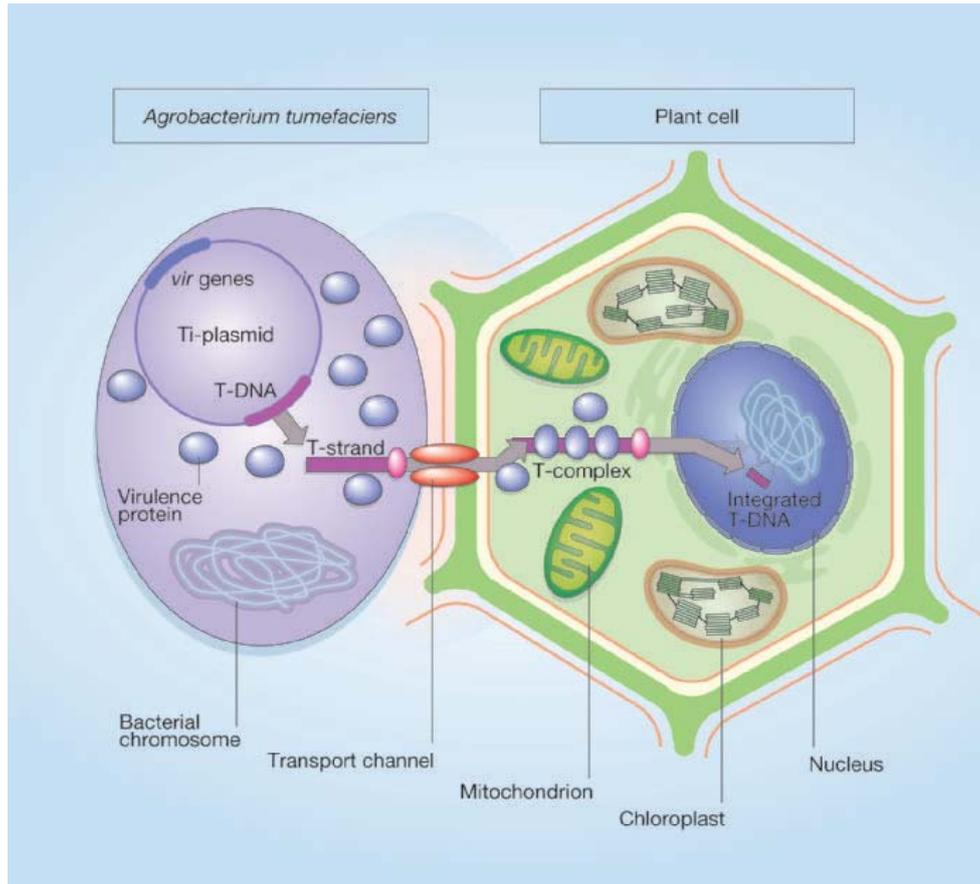


Fig.5.1 *Agrobacterium*, nature's genetic engineer. *Agrobacterium* contains a Ti-plasmid which contains *vir* genes which allow the T-DNA to be transferred to the host plant where it becomes integrated into the plant genome (Gelvin, 2005).

To utilize *Agrobacterium* as a tool for genetic engineering, the natural processes of T-DNA transfer is harnessed. Using the bacterium in its wild type state however had several issues. The large size of the Ti-plasmid and low copy number made it difficult and cumbersome to manipulate and insert the gene of interest, particularly as it would not replicate in *E. coli*. Also the presence of the tumour inducing oncogenes and opine producing genes were not required. To overcome this, DNA contained within the LB and RB is removed without compromising on the bacterium's ability to transfer the DNA

to the plant but does remove its facility to produce tumours; they are therefore termed “disarmed” *Agrobacterium* strains. In the place of the deleted genes a gene of interest can be placed in the T-DNA region. A major development was made in 1983 by Hoekema *et al.*, and de Framond *et al.* (reviewed in Lee and Gelvin, 2008) who identified that it was possible to locate the *vir* genes and the T-DNA on different replicons as long as they were located within the same *Agrobacterium* cell; this system later became known as a binary system. This new system allowed genes of interest to be inserted into vectors through manipulation within *E. coli* cells. The vector containing the T-DNA is known as the binary vector, containing also the origins of replication which are able to play a role in both *Agrobacterium* and *E. coli* and the antibiotic resistance genes that allow selection for the binary systems. The *vir* genes are contained on a replicon known as the *vir* helper (Fig.5.2) (Lee and Gelvin, 2008). Since this initial development the binary system has evolved to give a range of binary system to suit the particular project, containing different selectable markers that allow projects such as the one described in this chapter to be carried out.

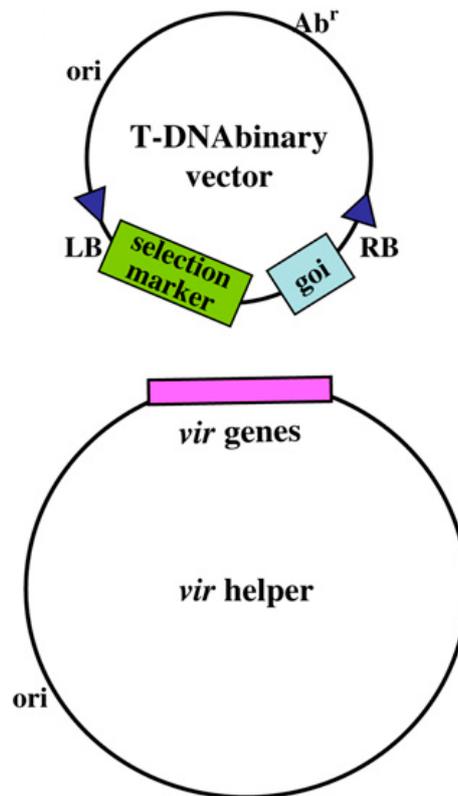


Fig.5.2 The binary system showing the two replicons, the binary vector containing the gene of interest (goi), antibiotic resistance genes and origin of replication. The *vir* helper replicon allows the T-DNA to be transferred to the plant cell whilst keeping the size of the binary vector small which allows easier manipulation (Lee and Gelvin 2008).

5.1.2 Reported transformation of *Thlaspi caerulescens*

Peer *et al.*, (2003) reported efficient transformation of *Thlaspi caerulescens* accession St. Félix de Pallières, France via floral dip using *Agrobacterium tumefaciens*. It was documented that *T. caerulescens* has been transformed using a construct containing both green fluorescent protein (GFP), a reporter gene and *bar* as a selectable marker. Finer details however of how the method was optimised were not included in the literature. This work however

gave the basis that the work aimed to be carried out to study the *HMA4* gene was feasible despite the accession of *T. caerulescens* used having a higher seed production than *T. caerulescens* (Ganges).

5.2. Materials and Methods

5.2.1 Plant Materials, Culture media and Culture Conditions

Seeds of *T. caerulescens* c.v. (Ganges) were surface sterilized with 70 % (v/v) ethanol for 10 min followed by a wash in reverse osmosis purified water. The seeds were then immersed in 50% (v/v) Domestos™ bleach solution (Diversey Levre, Northampton, UK) for 20 min with a final 6 washes in reverse-purified osmosis water.

The culture medium used to germinate the seeds was composed of MS basal salts (Murashige and Skoog, 1962) 4.3 g L⁻¹ inorganic salts (Appendix 8.1), 10 g L⁻¹ sucrose and 8 g L⁻¹ agar adjusted to a pH of 5.6 with 0.1 M NaOH. The medium was autoclaved at 121°C at 104 kPa for 20 minutes. Plants were sown in polycarbonate boxes with approximately 30 seeds per box; the boxes were sealed with Nescofilm® (Bando Chemical Co., Kombe, Japan) to eliminate contamination. The plants were cultured *in vitro* at 24 ± 1°C on a 16 h photoperiod at an intensity of 50-80 μmol m⁻² s⁻¹ under 58 W of white halophosphate fluorescent tube lighting (Crompton Lighting, Germany) and 8 h darkness.

5.2.2 Plant material for floral dip transformation

Seeds of *T. caerulescens* c.v. Ganges (France) and *T. arvense* c.v. Wellesbourne (Wellesbourne, Warwick, UK) were sown in a 3:1 (v/v) ratio of Levington's seed compost (Scotts UK Professional, Ipswich, UK) and vermiculite (Sinclair, Lincoln, UK). An insecticide named, "Intercept (70 % (w/w) Imidacloprid)" was used to control scarid fly at a concentration of 0.75 g kg⁻¹. Twelve well seed trays were used to germinate the seeds, with 3-4 seeds planted per well to ensure at least one see germinated. Seeds were germinated in growth room conditions in the glasshouse, at 20± 1°C with a 16 h photoperiod at 50-80 μmol m⁻² s⁻¹ intensity under 58 W of white halophosphate fluorescent tube lighting (Crompton Lighting, Germany) and 8 h darkness. After four weeks the young plants were transferred to a growth room at a lower temperature of 4± 1°C with a 16 h photoperiod at 50-80 μmol m⁻² s⁻¹ intensity under 58 W of white halophosphate fluorescent tube lighting (Crompton Lighting, Germany) and 8 h darkness. with a 16 h photoperiod at 50-80 μmol m⁻² s⁻¹ intensity under 58 W of white halophosphate fluorescent tube lighting (Crompton Lighting, Germany) and 8h darkness, a vernalisation period to induce flowering. Following the four-week vernalisation period, plants were transferred back to the glasshouse with a 16 h photoperiod using 600 W pressure sodium lamp (Phillips® Sun-t Pia green power, Phillips Electrics UK Ltd., Croydon, UK).

5.2.3 *Agrobacterium* Cultivation

Two separate strains of *Agrobacterium tumefaciens* were maintained: one, LBA4404 for the tissue culture based transformation and C58 for the floral dip method. C58 was used containing the pAch5 Ti plasmid to provide *in trans* the *vir* functions for the transfer of the integrated T-DNA from a disarmed pBIN19 based binary cloning vector with *Thlaspi HMA4* gene under the control of CaMV 35S promoter and terminator and the *nptII* gene encoding neomycin phosphotransferase under control of the *nos* promoter and terminator (Bevan, 1984; Hellens *et al.*, 2000). LBA4404 was used containing the pAch5 Ti plasmid to provide *in trans* the *vir* functions for the transfer of the integrated T-DNA from a disarmed pBIN19 based binary cloning vector with an *Arabidopsis* FtsZ gene (for chloroplast division) under the control of a CaMV 35S promoter and terminator.

Strain LBA4404 was maintained on APM medium (Table 5.2) supplemented with 500 $\mu\text{g ml}^{-1}$ Streptomycin (Sigma Chem. Co., Steinheim, Germany) and 50 $\mu\text{g ml}^{-1}$ Spectinomycin (Sigma Chem. Co., Steinheim, Germany). Strain C58 was maintained on LB medium (Table 5.1) supplemented with 50 $\mu\text{g ml}^{-1}$ Spectinomycin (Sigma Chem. Co., Steinheim, Germany), 35 $\mu\text{g ml}^{-1}$ Rifampicin (Sigma Chem. Co., Steinheim, Germany). Overnight liquid cultures were made prior to transformation in 1 ml amounts followed the next day by transformation into fresh media to a volume of 20 ml. Overnight cultures were shaken at a temperature of $28 \pm 1^\circ\text{C}$ in the dark on an orbital shaker (180 rpm).

Two different strains of *Agrobacterium tumefaciens* were chosen due to the differences in their nature and through past suitability of the strain to its method of transformation.

A. tumefaciens C58 is a wild type nopaline strain that has been adapted in many ways for its use in transformation of plants (Goodner *et al.*, 2000 Woods, *et al.*, 2000). It is considered a more robust strain than LBA4404 and therefore more suitable to *in planta* transformation procedures. It has successfully been used to transform *Arabidopsis* in this way (Clough and Bent, 1998) also *Brassica napus* (Wang *et al.*, 2003) In comparison, *A. tumefaciens* LBA4404 is an octopine strain that has been successfully used to transform plants via tissue culture methods including sugar beet (Bekheet and Solliman, 2007), rice (Hiei *et al.*, 1994) and maize (Ishida *et al.*, 1996).

Medium Type	Components
APM	5 g L ⁻¹ yeast extract, 0.5 g L ⁻¹ Casein hydrolysate acid, 8 g L ⁻¹ Mannitol , 2 g L ⁻¹ Ammonium sulphate, 5 g L ⁻¹ NaCl, 0.427 g L ⁻¹ Mg Cl ₂ with pH adjusted to 6.6 using a pH meter and 0.1 M HCl or 0.1 M NaOH.
LB agar medium	(Luria-Bertani medium) 10 g L ⁻¹ Bacto-tryptone, 10 g L ⁻¹ Yeast extract, 10 g L ⁻¹ NaCl and 18 g L ⁻¹ agar with pH adjusted to 7.2 using a pH meter and 0.1 M HCl or 0.1 M NaOH.
LB liquid medium	10 g L ⁻¹ Bacto-tryptone, 10 g L ⁻¹ Yeast extract and 10 g L ⁻¹ NaCl with pH adjusted to 7.2 using 0.1 M HCl or 0.1 M NaOH.
MS0 solid medium	MS0 liquid medium contained 8 g L ⁻¹ Agar, 4.3 g L ⁻¹ MS inorganic, 30 g L ⁻¹ sucrose, pH adjusted to 5.7 using 0.1 M HCl or 0.1 M NaOH. and no added plant growth regulators.

Table 5.1 Shows various media referenced in this chapter and the rest of the thesis.

5.2.4 Determination of optimum level of selection of plants on kanamycin

In order to pick out positively transformed plants from either floral dip or tissue culture based transformed plants a kanamycin resistance gene is included in the construct. This allows transformed plants to grow on agar supplemented on kanamycin when wild type plants would normally perish. To determine the

level of Kanamycin needed to kill off wild type plants a kill curve experiment must first be executed. A range of MS agar media were prepared supplemented with kamamycin at 30, 40, 50, 55, 60, 65 $\mu\text{g ml}^{-1}$ using a 10 $\mu\text{g ml}^{-1}$ stock solution of kanamycin. Seeds were sterilised as for previous experiments and plated on Petri dishes containing the kanamycin supplemented agar at a concentration of 10 seeds per plate. The plants were cultured *in vitro* at 24 ± 1 °C on a 16 h photoperiod at an intensity of 50-80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under 58 W of white halophosphate fluorescent tube lighting (Crompton Lighting, Germany) and 8 h darkness for 14 days. The results from this initial kill curve suggested that the highest level of kanamycin was not sufficient to kill wild type plants. A second kill curve experiment was carried out at elevated kanamycin levels of 65, 70, 80, 90, 100, 150, 200 $\mu\text{g ml}^{-1}$ using a 10 $\mu\text{g ml}^{-1}$ stock solution of kanamycin. The seeds were cultured in the same way as the first kill curve experiment. Results were recorded as a percentage germination at each concentration and an average taken of the three replications carried out at each concentration.

5.2.5 Floral dip transformation

Agrobacterium tumefaciens cultures adjusted to an optical density (O.D.) of 1 in MS medium supplemented with Silwet L-77 to a concentration of 0.05% (500 $\mu\text{l L}^{-1}$) were used to carry out the floral dip of *Thlaspi* species. The *Agrobacterium* suspension was applied to young buds using a pipette (Gilson Inc., Wisconsin, USA) to apply small droplets. Plants were covered with a plastic sleeve to limit transfer of bacteria to neighbouring plants. Plants

were kept in darkness for 12-24 h at $23 \pm 1^\circ\text{C}$ to allow bacterial growth and increase the chances of gene insertion. Plants were then placed under glasshouse conditions as described in section 5.2.1 and allowed to set seed in approximately 1 month. Plants were dried before seeds were harvested.

5.2.6 Plant Regeneration

To develop a tissue culture based transformation procedure for *Thlaspi caerulescens* it was necessary to first develop a plant regeneration protocol from *T. caerulescens* explants; this is described here. Plants were grown as described in section 2.2.1 on agar without any addition of external zinc. Following a three-week growth period, *T. caerulescens* plantlets were removed from the polycarbonate boxes, in sterile conditions and cut into explants of cotyledons, stems and roots. These were cultured in 25-well plates (Bibby Sterilin Ltd., Staffs, UK), one explant per well. Each well-contained 2 ml of MS medium as described in section 2.1 supplemented with a combination of auxin and cytokinin. The auxins used were IAA (3- Indoleacetic acid) and NAA (α -naphthaleneacetic acid) and the cytokinins, BAP (6-benzylaminopurine), TDZ (Thiadiazuron, N-phenyl, N-1, 2, 3-thiadiazol-5-yl urea) and Zeatin (Table 5.2). Grids were made with a range of auxin $0\text{-}4 \mu\text{l ml}^{-1}$ in a well in one direction and cytokinins at a range of $0\text{-}4 \mu\text{l ml}^{-1}$ in a well in the other direction (Fig.5.3). Three replicates of each hormone combination (total combinations 6) and the three tissue types gave a total of 54 hormone grids.

Hormone	Components and Preparation
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Stock	
IAA	100 mg IAA (3- Indoleacetic acid) dissolved in 70 ml ethanol and 30 ml reverse osmosis water gives a final concentration of 1 mg ml ⁻¹ .
NAA	100 mg NAA (α-naphthaleneacetic acid) (dissolved in 70 ml ethanol and 30 ml reverse osmosis water to give a concentration of 1 mg ml ⁻¹ .
BAP	100 mg BAP (6-benzyl amino purine), dissolved in a few drops of 1.0 M HCl, left for 10 min, add 100 ml reverse osmosis water to give a concentration of 1 mg ml ⁻¹
TDZ	100 mg TDZ (Thiadiazuron, N-phenyl, N-1, 2, 3-thiadiazol-5-ylurea) dissolved in 1 ml 1.0 M KOH, add 99 ml water to give a volume of 100 ml and a concentration of 1 mg ml ⁻¹ .
Zeatin	100 mg Zeatin (Z), dissolved in 100 ml reverse osmosis water to gave a concentration of 1 mg ml ⁻¹ .

Table 5.2 Plant hormone stock solution (Sigma Chem. Co., Steinheim, Germany) methods. All stock solutions were filter sterilised and stored at 4°C.

Cytokinin µg ml⁻¹

	0	1	2	3	4
0					
1					
2					
3					
4					

Fig.5.3 Hormone grid plates showing the ratios tested for giving optimum plant regeneration.

Explants were cultured in the grids sealed with Nescofilm® under the same growth conditions as the germinated seeds. Explants were left for a minimum of 14 days to allow regeneration. Regenerated explants were individually transferred to jars containing MS 0.8 medium with no extra hormones to allow root development. Plants were maintained in the jars for a further 14-30 days to develop into plantlets. They were then transferred to the glasshouse and planted in Levington's seed compost (Scotts UK Professional, Ipswich, UK), covered by a propagator to allow acclimatization and hardening to the less humid conditions of the glasshouse ($25 \pm 1^\circ\text{C}$ and 16 h photoperiod with 600 W luminaries using 600 W high pressure sodium lamp (Philips® Sun-t Pia Green power, Philips Electronics UK Ltd., Croydon, UK).

5.2.7 Regeneration frequency testing of *Thlaspi caerulescens*

The hormone combinations that gave regeneration were tested for frequency to determine which would ultimately be the best combination to use in subsequent experiments. This was determined by preparing Petri dishes (Bibby Sterilin Ltd., Staffs, UK) containing the selected hormone ratios and adding 10 explants to each Petri dish. Three replicates at each ratio were carried out. By determining how many out of the 10 explants germinated an average, percentage frequency of regeneration could be determined.

5.2.8 Molecular analysis of putative transgenic plants

Thlaspi species and *Arabidopsis thaliana* plants were transformed via floral dip using the *HMA4* RNAi C58 *Agrobacterium* cultures. Described here is the molecular analysis of putative transformants via polymerase chain reaction (PCR).

5.2.8.1 DNA isolation and Polymerase Chain Reaction (PCR) analysis of *Thlaspi* species

Those plants that survived the kanamycin screen were replanted on soil and allowed to grow under greenhouse conditions (as described in section 5.2.2). Plants (of which there were few that survived to this stage) were analysed by PCR for the presence of the *nptII* gene (kanamycin resistance). DNA from plants transformed by floral dipping, was extracted using the Sigma-Aldrich Plant DNA extraction kit (Sigma-Aldrich, Steinheim, Germany) (as Chapter

4.2.3) DNA samples were quantified using NanoDrop® software (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Wilmington, DE 19810, USA).

The website Primer 3 was used to produce the primers, below, to amplify a 690 bp sequence of the *nptII* gene, which are shown below.

The sequences of the primers were:

Left/Forward Primer: 5'- AATATCACGGGTAGCCAACG -3'

Right/Reverse Primer: 5'- TCGAGGCATGATTGAACAAG -3'

The plasmids (pK7- G or pK7-W) were used as positive controls for putatively transformed *Thlaspi species* depending on the species. Following PCR amplification using the conditions described below, a gel electrophoresis was run to highlight the PCR products containing the *nptII* fragment (690 bp).

Master Mix for 1 PCR sample:

H₂O: 6.2 µl

Red Taq: 0.2 µl

Forward primer: 0.8 µl

Reverse primer: 0.8 µl

DNA 9.0 µl

PCR conditions:

Initialization: 94°C 3 mins.

Denaturation: 94°C 48 s.

Annealing: 56°C 48 s.

Extension: 72°C 1 mins.

Extension 72°C 10 mins.

Hold 4°C hold

} 35 cycles

5.2.8.2 DNA isolation and Polymerase Chain Reaction (PCR) analysis of *A. thaliana*

Molecular analysis was performed to demonstrate the existence of *nptII* genes in putative transgenic *A. thaliana* which were transformed using both *HMA4* (pK7-G and pK7-W) designed for floral dip transformation of *T. caerulescens* and *T. arvense* respectively. DNA from plants transformed by floral dipping, was extracted using the Sigma-Aldrich Plant DNA extraction kit (Sigma-Aldrich, Steinheim, Germany) (as Chapter 4.2.3). DNA samples were quantified using NanoDrop® software (NanoDrop® ND-1000 UV-Vis Spectrophotometer, Wilmington, DE 19810, USA). Primer 3 was used to produce the primers, below, to amplify a 260 bp sequence of the *nptII* gene. The sequences of the primers used to produce the *nptII* fragment were:

Forward primer: 5'- AGA CAA TCG GCT GCT CTG AT -3'

Reverse primer: 5'- ATA CTT TCT CGG CAG GAG CA -3'

The plasmids (pK7- G or pK7-W) were used as positive controls for putatively transformed *A. thaliana* 'Colombia' depending on what construct had been used to carry out the transformation. Following PCR amplification using the conditions described below, a gel electrophoresis was run to highlight the PCR products containing the *nptII* fragment (260 bp).

Master Mix for 1 PCR sample:	PCR conditions:	
H ₂ O: 3.5 µl	Initialization: 94°C 5mins.	
Red Taq: 7.5 µl	Denaturation:94°C 1 min.	} 35 cycles
Forward primer: 1.0 µl	Annealing: 48°C 1 min.	
Reverse primer: 1.0 µl	Extension: 72°C 1 min.	
DNA: 2.0 µl	Extension 72°C 10 min.	
	Hold 4°C hold	

5.3 Results

5.3.1 Plant regeneration of *T. caerulescens*

T. caerulescens explants were grown on a range of hormone combinations to determine the optimum conditions for plant regeneration, to be used for the subsequent transformation of *T. caerulescens*. All plates were scored for calli induction after a 4 week growth period. The tissue type that produced the most calli independent of hormones added was stem followed by roots. Leaf tissue was relatively unsuccessful and most often died after one week incubation period, therefore would not be a considered tissue type for transformation (Fig.5.2). Many of the hormone combinations were successful in producing calli in both roots and shoots, with thickening of explants seen as soon as one week after incubation (Fig.5.3) The most successful combinations, i.e. those that produced three out of three calli were then repeated on petri dishes with ten explants per plate (Table 5.3).

Hormone concentrations ($\mu\text{g ml}^{-1}$)											
NAA	BAP	NAA	TDZ	NAA	Zeatin	IAA	BAP	IAA	TDZ	NAA	Zeatin
0	4	1	3	1	4	2	1	3	2	1	0
1	0	2	0	2	3	2	2	4	1	1	1
1	2	2	1	4	2	2	3			1	3
1	3	2	4			3	1			2	1
2	0	3	1			3	2			3	0
2	4	3	4			4	1			3	1
3	0	4	0			4	2			4	0
3	2	4	2							4	1
3	3	4	3								
4	0	4	4								

Table 5.3 Table showing the hormone combinations that were successful in producing calli of *T. caerulescens* for all three replicates carried out. All combinations were in 2 ml of MSO full strength media. These successful combinations were then repeated on plates containing 10 explants per plate. From the successful combinations highlighted from the initial hormone grid experiment, the same combinations were set up in Petri dishes containing 20 ml of MSO full strength medium and the relevant hormone combinations. From this experiment it was found that $2 \mu\text{l ml}^{-1}$ NAA without cytokinins gave 98% production of callus (Fig.5.4). When the calli was transferred to larger jars containing MSO medium roots were produced but although many media types have been tried no shoots have been successfully regenerated (Fig.5.5). It was due to the lack of shoot regeneration that the tissue culture

based transformation was given way to the floral dip transformation as without the regeneration of whole plants the method of transformation is futile.

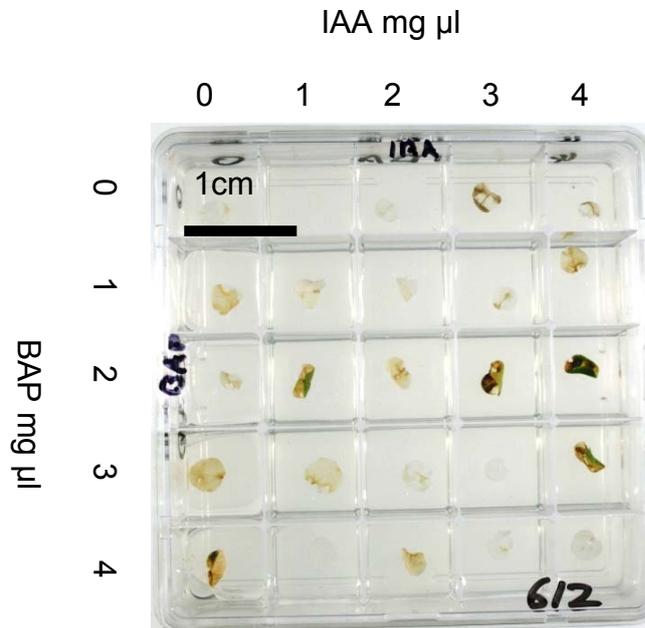


Fig.5.4 An example of *T. caerulescens* leaf explants in a hormone grid, after 4 weeks incubation, showing that this explant type was unsuccessful in the regeneration of callus. All 25x well culture plates contained 25 combinations of auxin and cytokinin concentrations. Each well contained 2 ml MS0 basal medium (8 g L⁻¹ agar with full strength MS macro and micro salts and vitamins, 30g L⁻¹ sucrose, without growth regulators, pH 5.8). Scale bar represents 1 cm.

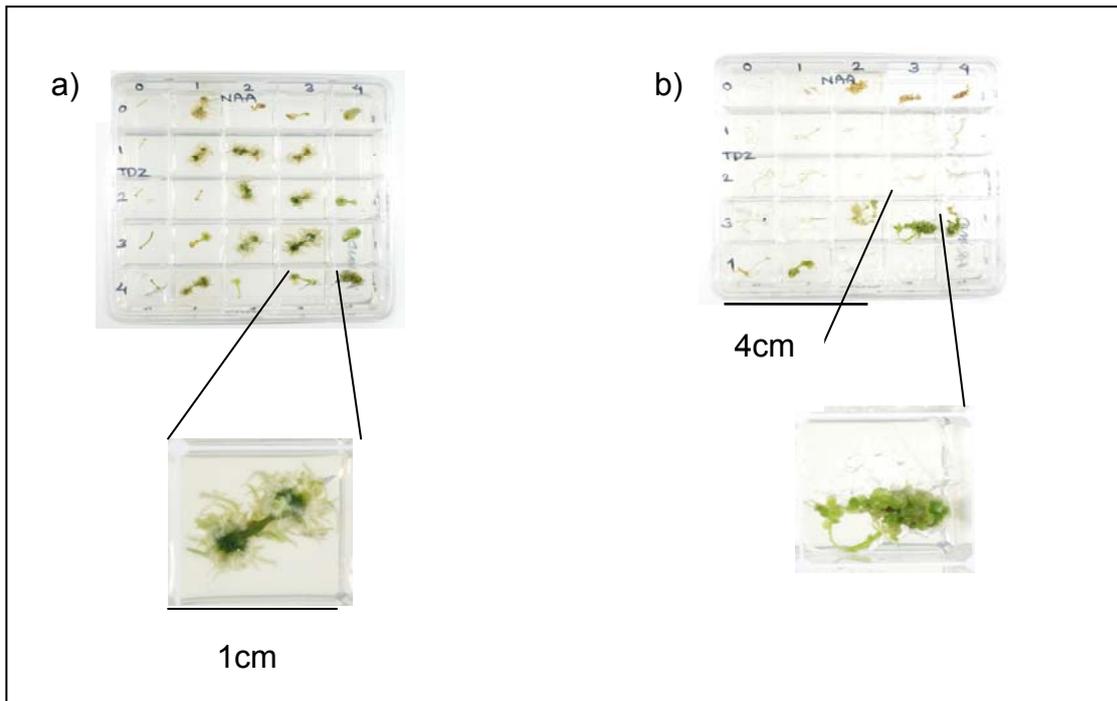


Fig.5.5 Example of the *T. caerulescens* tissue culture hormone grids with callus production. In this case it is for NAA/TDZ. Showing a) explants type-roots and b) explants type-shoots.

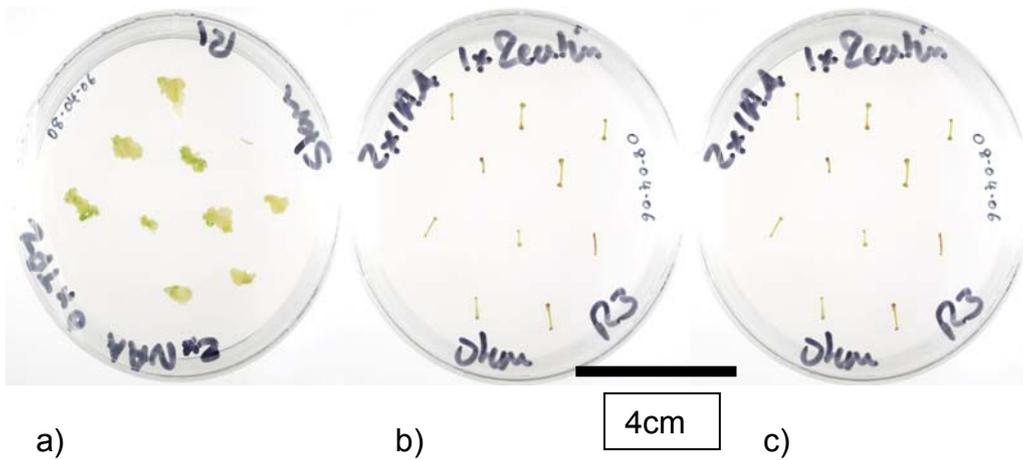


Fig.5.6 Samples taken from the final callus frequency test using *T. caerulescens* ‘Ganges’ stem explants maintained on MS0 basal medium with, a)= $2 \mu\text{g ml}^{-1}$ NAA, b)= $2 \mu\text{g ml}^{-1}$ NAA + $4 \mu\text{g ml}^{-1}$ TDZ and c)= $2 \mu\text{g ml}^{-1}$ IAA + $2 \mu\text{g ml}^{-1}$ Zeatin. Scale bar represents 4 cm. Combination a) gave an overall frequency of 97% which was the most successful combination.

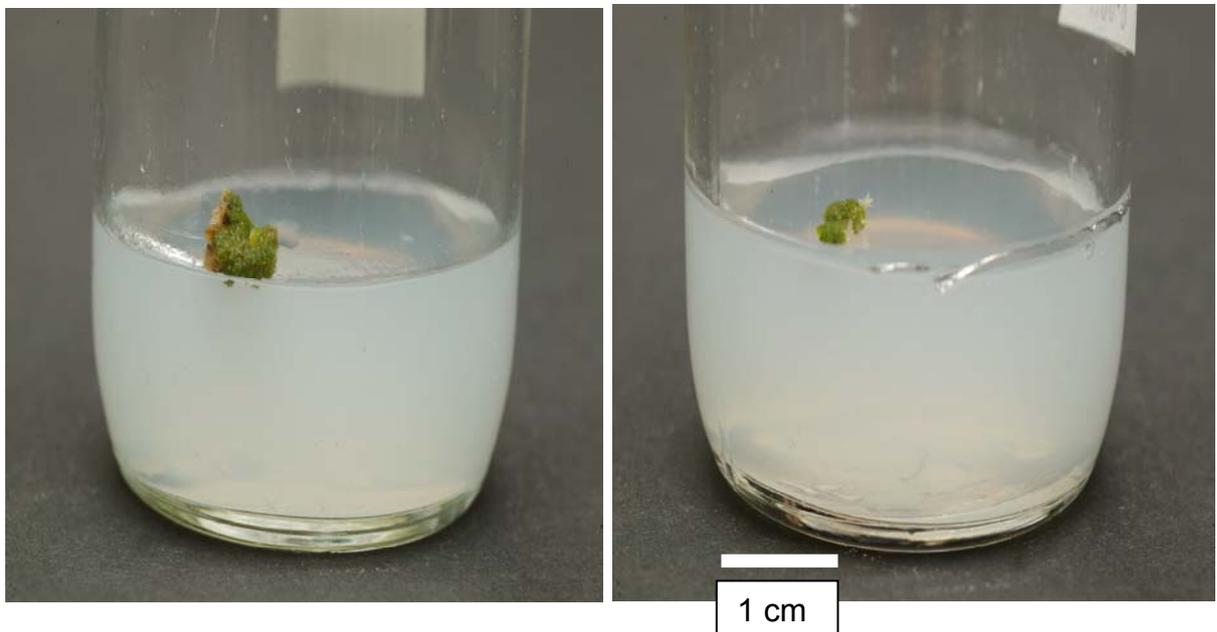


Fig.5.7 *T. caerulescens* calli regeneration on MS0 medium. From *T. caerulescens* shoot explants on $2 \mu\text{l ml}^{-1}$ NAA. The photo on the right shows roots just beginning to form.

5.3.2 Growth response of plants to kanamycin

In order to screen potential transformants from floral dip it is necessary to have a reliable kanamycin screen. These results show the development of a screen to select transformants. Wild type seeds were germinated on agar supplemented with increasing concentrations of kanamycin and scored for percentage seedling viability at 14 days post sowing. The results shown in Fig.5.6 shows that overall viability of seedlings dropped as kanamycin concentration increased. *T. caerulescens* showed a higher viability overall than *T. arvense* though this could be accounted for by a high seed viability under any conditions, or may be accounted for by the fact that this species is able to accumulate, translocate and detoxify toxic compounds with more efficiency. Between the range of 5 and 80 $\mu\text{g ml}^{-1}$ *T. caerulescens* showed higher resistance to kanamycin than *T. arvense*.

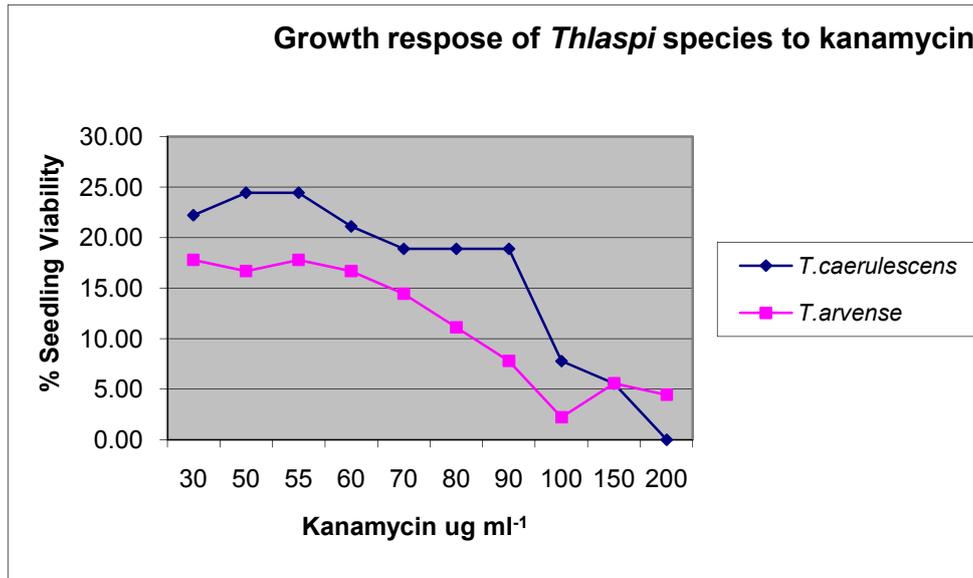


Fig.5.8 Graph showing the percentage seedling viability of *T. caerulescens* and *T. arvense* on increasing kanamycin concentration. Overall for both *T. caerulescens* and *T. arvense* the viability of seedling decreased as kanamycin concentration increased.

Fig.5.9 shows the decrease in seedling viability and also the decrease in seed germination as kanamycin concentration increases. From the graph and visual evaluation $70 \mu\text{g ml}^{-1}$ was chosen as a workable concentration as from this picture the majority of seedlings after this point looked unhealthy and unlikely to survive, therefore kanamycin resistant plants would stand out in a screen at this concentration.

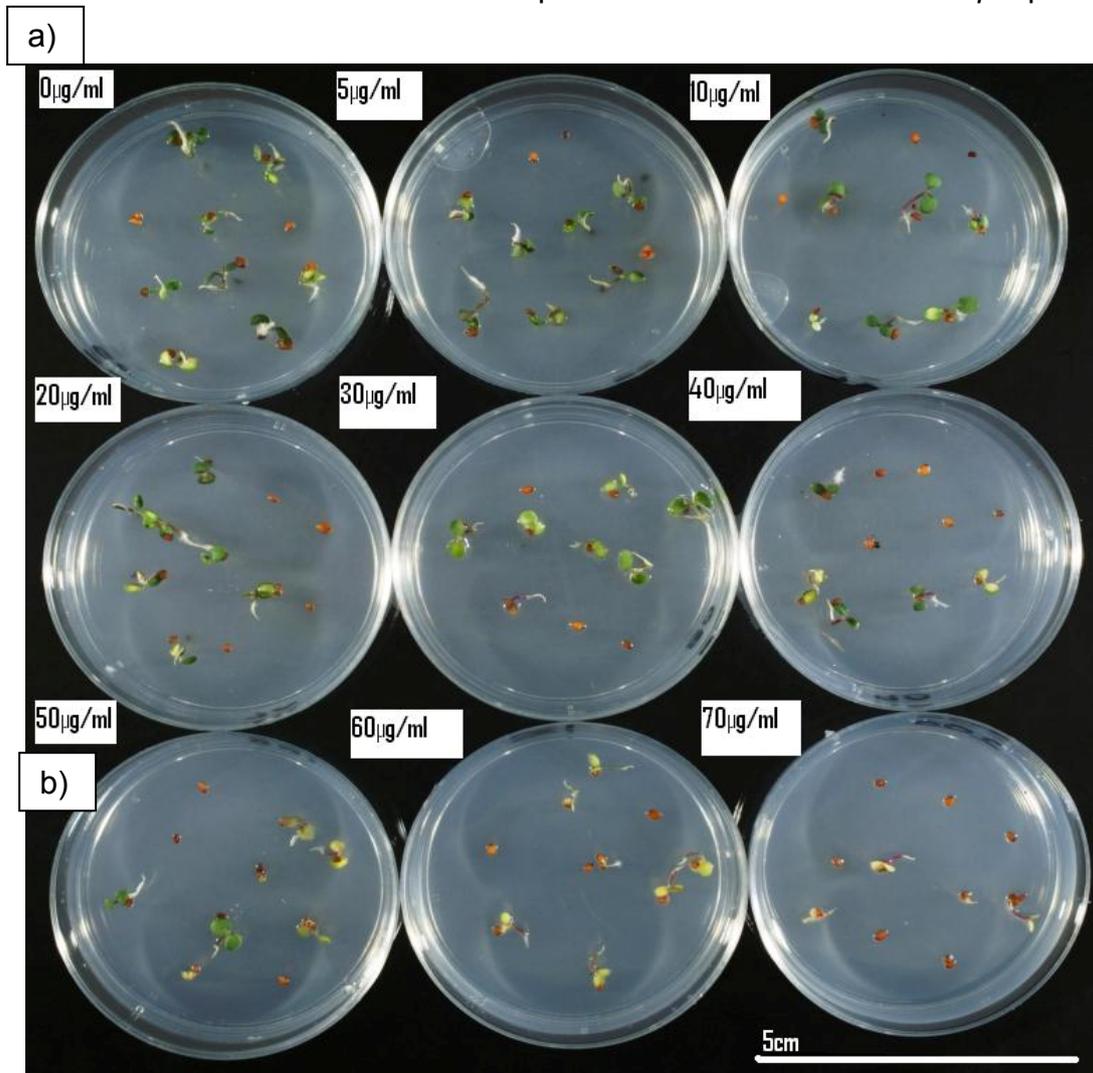


Fig.5.9 a) Growth response of *T. caerulescens* (Ganges) on increasing kanamycin concentration within full strength MS0 basal medium (4.3 g L^{-1} MS basal salts with no plant growth regulators [PGRs]). The scale bar represents 5 cm.

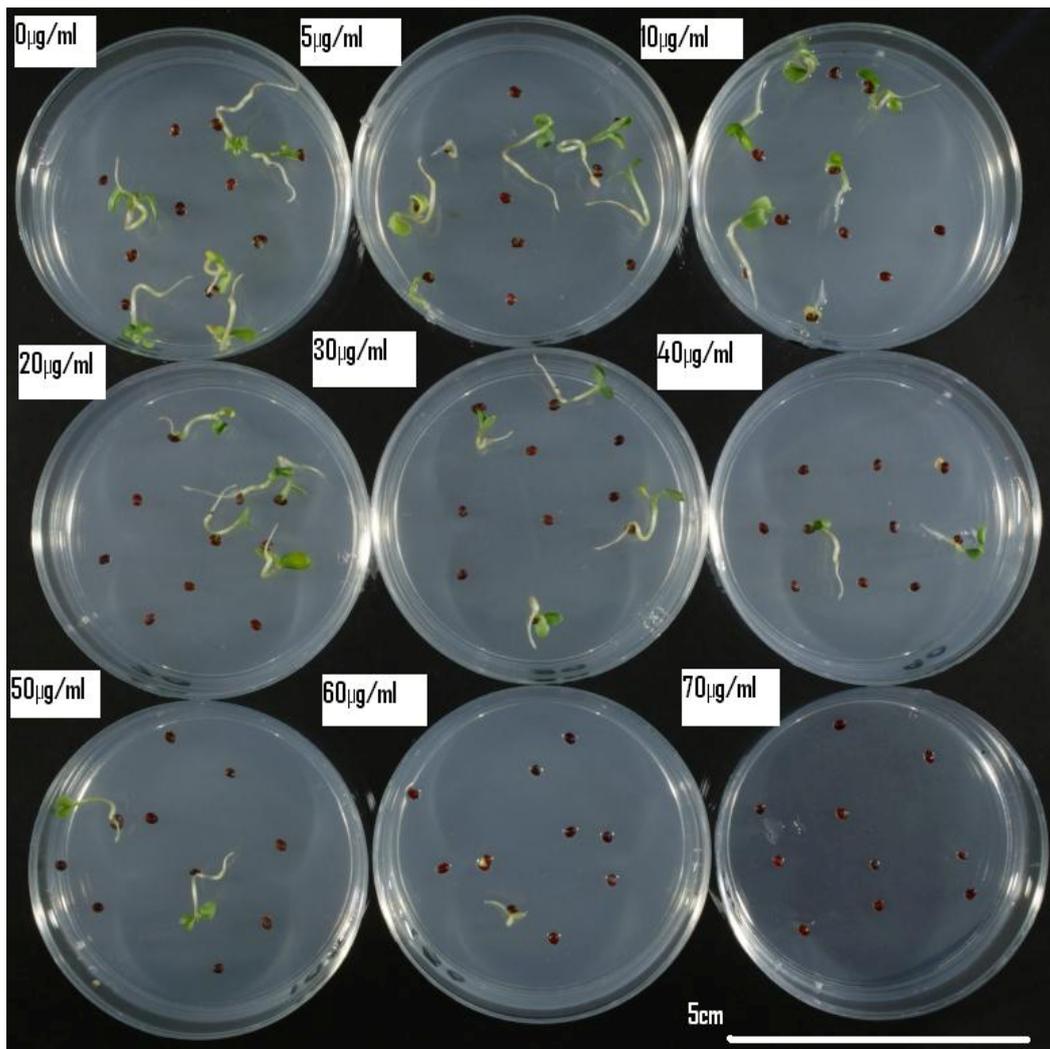


Fig.5.10 b) Kanamycin kill curve of *T. arvense* (Wellesbourne) on increasing kanamycin concentration within full strength MS0 basal medium (4.3g L^{-1} MS basal salts with no plant growth regulators [PGRs]). The scale bar represents 5 cm.

5.3.3 Potential transformants

With the tissue culture method of transformation unsuccessful, all focus and attention was put into floral dip method of transformation of *T. caerulescens* and *T. arvense*. Despite many rounds of floral dip no successful *T. caerulescens* and *T. arvense* transformants were produced. Limitations of this method were the slow seed to seed time of these plants. Initially there were potential transformants identified through a kanamycin screen and PCR however further analysis on these plants did not validate these results (Fig.5.8).



Fig.5.11 Initial PCR results suggested that plants in lanes 2, 3, 5 and 9 were potential transformants using the *HMA4* RNAi construct. However further PCR analysis did not back this up and it was therefore decided that these bands must be artefacts.

5.3.4 Transformation of *Arabidopsis thaliana* using the *HMA4* RNAi construct and molecular analysis of putative transformants

Transformation of *Thlaspi caerulescens* and *T. arvense* was not successful in this instance. However successful transformation of *Arabidopsis thaliana* by floral dip using the *HMA4* RNAi construct described in Chapter 4 was achieved (pers. com. Claire Eustace). *Arabidopsis thaliana* “Columbia” was transformed using the *HMA4* RNAi construct (pK7-G and pK7-W) to test the construct itself. Molecular analysis of *A. thaliana* ‘Colombia’ was performed through PCR to demonstrate putative transgenic T1 plantlets obtained from floral dip mediated transformation. Pooled samples of plantlets which were inoculated with *A. tumefaciens* containing the *HMA4* RNAi (pK7-G), *HMA4* RNAi (pK7-W) constructs and which survived kanamycin selection (Fig.5.9) were analysed using PCR. All pooled samples showed the presence of *nptII* confirming the validity of the kanamycin selection results and their putative transgenic status and also confirming that the construct itself was functioning as expected (Fig.5.10).

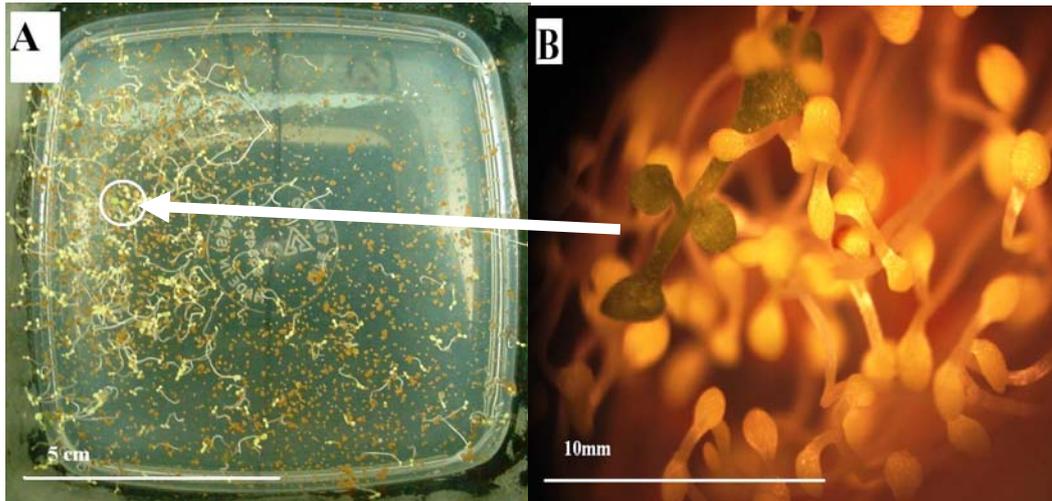


Fig.5.12 Close up image of one putative transgenic *Arabidopsis* plantlet (**B**) with its position in the box (**A**) indicated by the white arrow. Plantlets grown on MS-0 supplemented with $50 \mu\text{g ml}^{-1}$ kanamycin sulphate. Scale bars for A and B are 5 cm and 10 mm respectively.

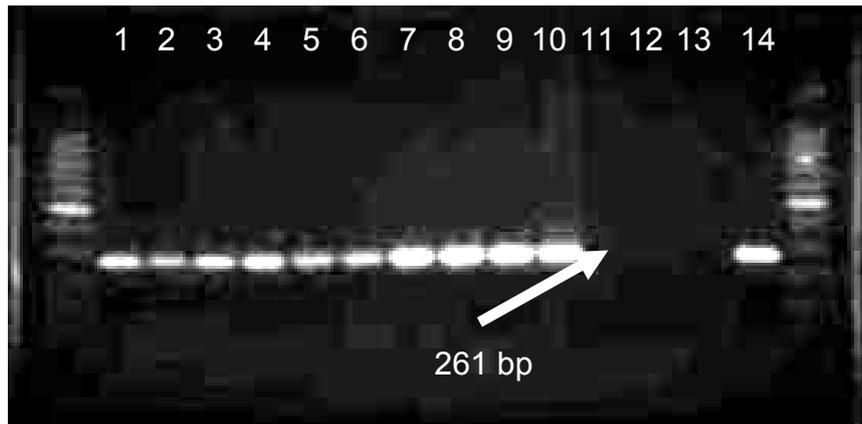


Fig.5.10 Agarose gel electrophoresis of PCR amplified products from pooled samples of putative transgenic *A. thaliana* 'Colombia'. Numbers indicate lanes. Lanes 1-4 and 5-6 contained pooled *A. thaliana* 'Colombia' DNA transformed with *HMA4* RNAi (pK7-G) and (pK7-W) respectively. Lanes 7-10 contained *A. thaliana* 'Colombia' DNA transformed with the *GUS:nptII* construct (not relevant to this thesis). Lanes 12, 13 and 14 contained wild type (wt) *Arabidopsis* DNA, negative control (water) and positive control (construct) for *nptII* (white arrow) (construct plasmid DNA containing *nptII*) respectively. Ladder – 100 bp *NPTII* fragment = 260 base pairs (bp). Agarose 1.5%

Gel electrophoresis of PCR products from individual members of pooled samples transformed with *HMA4* RNAi (pK7-G), showed that three plants contained the *nptII* gene insert (Fig.5.11). A similar analysis was performed on individual samples transformed with *HMA4* RNAi (pK7-W), revealing that two plants contained the *nptII* gene insert (Fig.5.12).

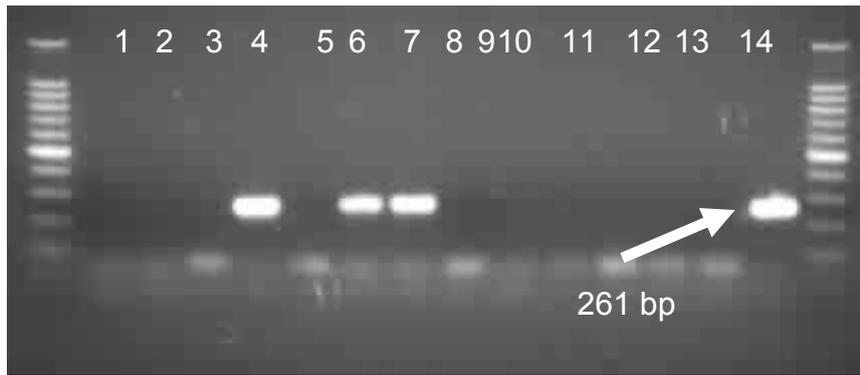


Fig.5.11 Agarose gel electrophoresis of PCR amplified products from individual samples of putative *HMA4* RNAi (Pk7-G) transgenic *A. thaliana* 'Colombia'. Numbers indicate lanes. Lanes 1-11 contained individual DNA PCR products from *A. thaliana* 'Colombia' that survived *in vitro* kanamycin screening. Lanes 12 and 13 contained wild type (wt) *Arabidopsis* DNA, negative control (water) and a positive control (construct) for *nptII* (white arrow) respectively. Ladder = 100 bp, *nptII* fragment = 261 bp. Agarose – 1.5%.



Fig.5.12 Agarose gel electrophoresis of PCR amplified products from individual samples of putative *HMA4* RNAi (pK7-W) transgenic *A. thaliana* 'Columbia.' Numbers indicate lanes. Lanes 1-11 contained individual DNA PCR products from *A. thaliana* 'Columbia' that survived *in vitro* kanamycin screening. Lanes 12 and 13 contained wild type (wt) *Arabidopsis* DNA, negative control (water) and a positive control (construct) for *nptII* (white arrow) respectively. Ladder = 100 bp, *nptII* fragment = 260 bp. Agarose 1.5%.



Fig.5.13 PCR results of putative *Arabidopsis thaliana* plants transformed floral dip using the *HMA4* RNAi construct. Lanes 1-11 contained pooled *A. thaliana* 'Colombia' DNA transformed with *HMA4* RNAi (pK7-G) and (pK7-W) respectively. Lanes 12, 13 and 14 contained wild type (wt) *Arabidopsis* DNA, negative control (water) and positive control for *nptII* (white arrow) (construct plasmid DNA containing *nptII*) respectively. *NPTII* fragment = 261 bp.

5.4 Summary

To summarise the results of Chapter 5 attempts were made to develop an efficient protocol for the transformation of *Thlaspi caerulescens* by tissue culture or floral dip methods. Progress was initially made with the tissue culture, managing to successfully regenerate *T. caerulescens* callus on a number of different hormone combinations. Particularly promising was the generation of 98% efficiency on medium supplemented with $2 \text{ mg } \mu\text{l}^{-1}$ NAA. However when the callus was sub cultured it was possible to regenerate roots but no shoot production was observed despite numerous attempts and differing hormone combinations. Floral dip was also not successful in generating transformants despite numerous attempts; this was hindered by the slow time between seed to seed production.

It was thought that the floral dip transformation of *Thlaspi caerulescens* could be feasible due to its similarity to *A. thaliana*, which is successfully transformed in this way to a 1% efficiency rate. Bent (2000) reviews the development of transformation without the use of tissue culture, beginning with the work of Feldmann and Marks (1987); Feldmann (1992) where they applied *Agrobacterium* to seeds, grew them without any selection methods, collected the progeny and grew them on media containing antibiotics. These procedures were repeated by others with varying results; however with persistent rounds over several years successfully produced thousands of transgenic lines (insertional mutagenesis lines) that contributed to the analysis of the *Arabidopsis* genome. Following this, Bent, (2000) reviews a method developed by Chang *et al.* (1994) and Katavic *et al.* (1994) called “clip ‘n squirt” where inflorescences are cut off and *Agrobacterium* applied to the centre of the rosette, new inflorescences grew and the process was repeated again. The plants were then allowed to go to seed and the transformants selected. This procedure was more productive than the initial seed method but still not as productive as tissue culture methods. The third major development reviewed here that resulted in the 1% transformation efficiency was termed “vacuum infiltration” (Bechtold *et al.*, 1993 reviewed in Bent, 2000). This method involved placing uprooted plants at the early stages of flowering in a bell jar with *Agrobacterium* and applying a vacuum to the jar. On release of the vacuum air any trapped air leaves allowing the *Agrobacterium* to take its place within the plant. Plants were then replanted in soil and allowed to mature and set seed, which was subsequently, screened using a method suitable to the selectable marker gene present. This method of *in*

planta (called *in planta* or *in vivo* transformation because genes are generally delivered into intact plants (Hansen and Wright, 1999), transformation is favourable over tissue culture methods despite the low efficiency because of the large number of seeds returned to screen compared with explants from tissue culture. Though this method is far less technically demanding than tissue culture methods it still involves the uprooting and replanting of plants following vacuum infiltration. A simplified method of transformation was reported by Clough and Bent (1998) in which they described the dipping of developing floral tissues into a solution of *Agrobacterium*, sucrose and a surfactant called Silwet L-77, to lower surface tension to allow the bacterium to enter plant tissues. They also commented on the importance of covering plants after dipping to increase humidity which in turn doubled transformation rates. This report investigated several parameters in the transformation procedure for example plant growth stage. It has been reported that the most effective growth stage was primary buds clipped with secondary buds between 2-10 cm, with some open flowers. The least susceptible growth stage was mature bolts with many siliques already produced. This data may explain why the transformation of *Thlaspi* spp. was not successful. *Thlaspi* spp. plants were transformed at the stage of primary bolts with a few open flowers. It was found that the presence of the surfactant Silwet L-77 (0.02%) was crucial to transformation efficiency. In comparison when transforming *Thlaspi* spp. Silwet L-77 was also added (0.05%), the levels of this and sucrose were not in anyway adjusted to attempt to determine an optimum level. In further experiments, Clough and Bent (1998) used Silwet L-77 at a level of 0.05% but noted that high levels can contribute to necrosis of plant

tissue, something that may have affected transformation rates in *Thlaspi* spp. Finally they reported on using the *Agrobacterium* strain LBA4404 (used for tissue culture transformation in this thesis) and GV3101 (pMP90), which is based on C58 and pTiC58, successfully to transform *Arabidopsis* via floral dip. The strain could be something that could be changed in the transformation of *Thlaspi*. In previous work, the ecotype of *Arabidopsis* was changed to analyse any differences in transformation efficiency between ecotypes. Successful transformation was reported in several ecotypes including Col-0, Ws-0, Nd-0, No-0 however Ler0 was more difficult and with varying results. The reasons for the difficulty may be relevant to *Thlaspi*, a potential incompatibility or low compatibility with *Agrobacterium* or a differential growth of the inflorescences.

In conclusion the main variables that affect transformation efficiency are growth stage of the plant, presence of a surfactant and sucrose concentration. The application of some kind of covering to the plant for 12-24 hours assists transformation rates by maintaining humidity to provide conditions to allow the mobility of *Agrobacterium* to enable them to reach target cells. Although the full process of floral dip transformation is not understood indications suggest that the ovules or cells that later become ovules are targets of T-DNA in transformed *Arabidopsis* and therefore probably similar for other plant species (reviewed in Wang *et al.*, 2003). Floral dip transformation has since been successfully reported in other species besides *Arabidopsis* for example Curtis and Nam, (2001) report of the successful transformation of radish (*Raphanus sativus* L. *longipinnatus* Bailey) by floral dip. Here, as before, the importance

of the surfactant, Silwet L-77 (0.05% v/v), was reported giving a transformation efficiency of 1.4%. In addition to this Pluronic F-68 and Tween 20 were analysed as potential alternatives with little success compared with Silwet L-77. Again the development stage of the plant was important to efficiency rates, with the best being primary bolting stage over secondary and lastly tertiary.

Prior to this and with less success was the reported transformation of *Brassica napus* and *Beta vulgaris* (Siemens and Scheiler, 1996 reviewed in Wang *et al.*, 2003). The application of vacuum infiltration to *Brassica rapa* L. (Chinese cabbage 50-60 cm in size) returned only two transgenic plants from the screening of 20,000 seeds harvested from 30-50 plants (Liu *et al.*, 1998, reviewed in Wang *et al.*, 2003). Wang *et al.* (2003) reported on the *in planta* transformation of *Brassica napus* using the C58 CIRifR containing the Ti plasmid pGV3101 strain of *Agrobacterium tumefaciens*. Several variations of treatment were applied including application by floral dip or vacuum infiltration. Another parameter that was changed was the time the plants were dipped for in the inoculation medium. It was found that an inoculation time of 5 min compared with 2 or 3 min gave higher transformation efficiency, a parameter that was not studied in *Thlaspi* spp.

In planta transformation technologies have been applied to Kenaf Plants (*Hibiscus cannabinus* var. Aokawa) by applying *A. tumefaciens* to the meristems of young plants. Three strains of *A. tumefaciens* were used, an avirulent M-21 mutant strain, two LB14404 strains, one with a binary vector

modified to allow for the rescue of the T-DNA and flanking host chromosomal DNA, the second with no binary vector. Transformation was successfully achieved and supported by the recovery of the plasmid containing T-DNA from the host plant (Kojima *et al.*, 2004).

Therefore, in light of other successful and less successful *in planta* transformations of other plant species, potential problems with the transformation of *Thlaspi* spp. could be due to the flowering time at which the dipping took place, the amount of surfactant or sucrose, the time length of inoculation or the fact that the inoculation was not repeated at a later date. All of these parameters could be investigated at a later date.

Several papers described that covering the plants post inoculation increased efficiency by maintaining humidity for plant cuticle repair and bacterial introgression. However in this case plastic 'sleeves' were used during floral dipping; these sleeves reduce airflow around the plant, resulting in a number of diseases such as downy mildew (*Peronospora farinosa*). In order to prevent this and reduce plant mortalities, sleeves should be removed 4-5 days following floral dipping. However it was possible to successfully transform *Arabidopsis thaliana* plants using the *HMA4* construct which proves that the *HMA4* RNAi construct was working and that this was not the limiting factor in the transformation of *Thlaspi caerulescens*.

6. General Discussion

This thesis aimed to cover several aspects related to Zn accumulation in *Thlaspi caerulescens*. Firstly the aim was to confirm that the model plant, *Thlaspi caerulescens* could accumulate high levels of Zn and that elevated external Zn concentrations could be tolerated without impeding growth. The initial studies aimed to quantify the levels of Zn that could be accumulated and compare this to the published data and with a non-hyperaccumulating species. Secondly this thesis aimed to confirm the differential expression of genes using qPCR. These genes had previously been identified as differentially expressed by a microarray. Using the results from these experiments a potential candidate gene involved in the hyperaccumulating trait was to be selected for further investigation. The aim was then to study the candidate gene *in planta* using transformation techniques. Previously published evidence was given on the transformation of *Thlaspi caerulescens* using reporter genes which indicated that it should be feasible to attempt to transform this plant. There had been however no reports of any other manipulation of genes within *Thlaspi* spp. The published evidence of its transformation however made it seem a feasible and novel approach to take. The first step to this approach was to develop an efficient and successful transformation procedure for *Thlaspi caerulescens*. Secondly it was necessary to create a construct for the silencing of the candidate gene, *HMA4*. This chapter will now discuss the results reported in this thesis.

6.1 Comparing the accumulation and tolerance of zinc in *T. caerulescens* and *T. arvense*

6.1.1 Zn accumulation levels

The aim of this chapter was to compare published data of the levels of accumulation and tolerance in *Thlaspi caerulescens* with actual data collected from the plants grown *in vitro*. Secondly data was collected of the accumulation and tolerance of *T. caerulescens* with its relative, non-accumulating *T. arvense*. Maximum recorded levels of Zn hyperaccumulation in *Thlaspi caerulescens* have reached up to 3% d. wt. in the shoots (Reeves and Baker, 2002) or 25000 $\mu\text{l Zn g}^{-1}$ shoot d. wt. when grown hydroponically (Brown *et al.*, 1995). These levels greatly exceed the 300 $\mu\text{l g}^{-1}$ which is considered to be toxic to plants. The results from these experiments gave a maximum 21,671 $\mu\text{g Zn g}^{-1}$ shoot d. wt. in *Thlaspi caerulescens* which is similar to the levels observed by Brown *et al.*, (1995).

The increase in concentration of Zn in the shoots of *T. caerulescens* was accompanied over much of the range by an increase in the substrate Zn level. When concentration declined, so did content. From Fig 2.5a and 2.6b it appears that the maximum shoot Zn concentration was not yet obtained under the treatments investigated as concentration appears to still be rising. The pattern of accumulation shown in the graph is linear and under those concentrations tested does not fit the generalised model of hyperaccumulation proposed by Baker and Walker (1990). If hyperaccumulation were to follow the pattern suggested by Baker and Walker (1990), the curve on the graph should eventually flatten over a higher, albeit unknown, range of treatment

concentrations. (Fig.6.1). The conclusion was there exists three basic patterns of metal uptake: the “Excluder”; which keeps the level of metal within the plant at a low level. When levels in the soil reach critical levels toxicity occurs due to uncontrolled metal transport into the plant. The “Indicator” plants which uptake and transport metals into the shoots either passively or by regulated means. The internal concentrations directly reflect external concentrations. Finally the “Hyperaccumulator” that actively maintains a high level of internal Zn across the full range of external Zn concentrations. The pattern expressed by these plants suggests a complex mechanism inside the plant exists to execute this process. In comparison Figures 2.5 a and 2.6 a show that the Zn content of *T. arvense* shoots peaked at the 300 μM external Zn concentration and then declined markedly, whilst the external Zn concentration continued to rise. Therefore it can be suggested that the rise in Zn concentration of *T. arvense* did not result from hyperaccumulation but rather it was an artefact of the rate of growth declining faster than the rate of Zn uptake and transport to the shoots.

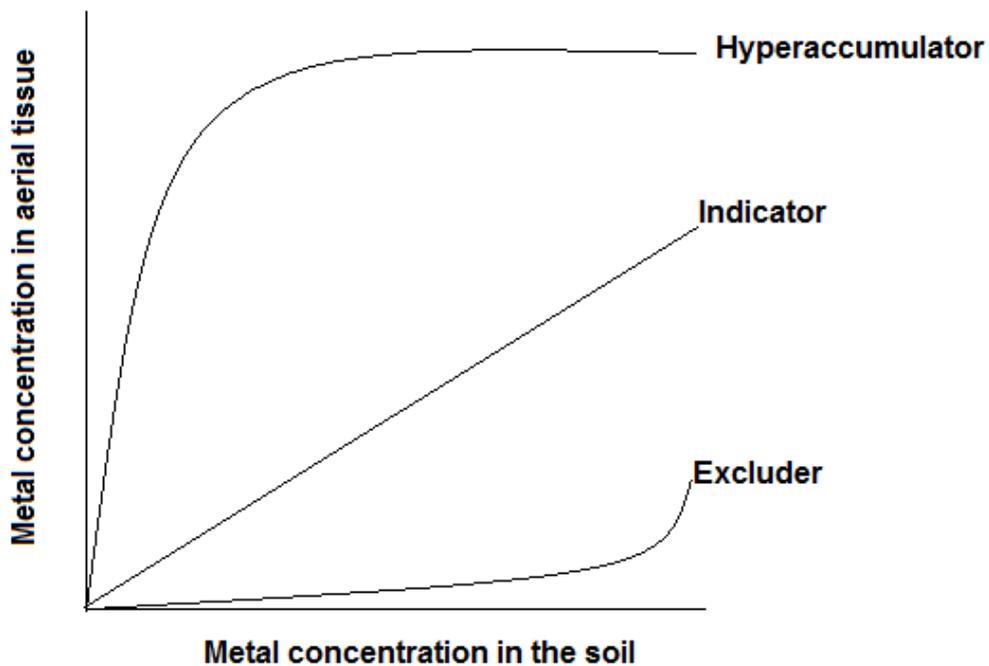


Fig.6.1 Idealised diagram of the physiology of hyperaccumulator plants compared to other uptake responses to heavy metals showing the three characteristics observed by plants on a range of Zn soils. (Reproduced from Baker 1981 reviewed in Baker and Walker, 1990).

Zinc accumulation in the roots did not follow the linear pattern of the shoots instead the root data created a curve with results increasing to a certain degree then declining in internal concentrations at the higher external Zn concentrations. This may be the result of root sequestration organelles becoming saturated with Zn and hence the Zn concentration within the root tissue plateauing. The sequestration capacity of the shoot is far greater than that of the roots however and whilst the roots continue to transport excess Zn into the shoots the shoot Zn concentration continues to rise as its saturation point is yet to be reached. This data supported that found by Shen *et al.*

(1997) who also found Zn concentration was higher in the shoots than the root.

This accumulation of Zn in the roots of *T. caerulescens* is a better fit to the Baker model which was characterised by an initially steep curve, flattening in accordance with the model (Figs. 2.5b and 2.6b). This indicates that the uppermost limits of hyperaccumulation had been reached. However, the large standard error of the final point compared to its neighbours suggests that it may be anomalous. The high error bar suggests there was a great deal of variability in the individual metal contents despite the plants being closely controlled and each treatment block having been planted on homogeneous agar.

Lasat *et al.*, (2001) showed that it was sequestration of the Zn in the root vacuole of *T. arvense* that retarded its translocation to the shoot. This resulted in approximately 2.5-fold more Zn accumulating in leaf sections obtained from *T. caerulescens* than from *T. arvense*. In this study *T. caerulescens* only accumulated 1.6-fold more Zn in its shoots than *T. arvense*. This difference in shoot Zn accumulation by *T. caerulescens* is likely to be attributable to the increased root accumulation seen at the higher Zn concentrations within this study. From the results of Lasat *et al.*, (2001) it is possible to suggest that if this sequestration within *T. arvense* roots was to be in some way inhibited, or the capacity reduced, that the Zn would then be free for translocation to the shoot tissue; if for example the root vacuoles become saturated with Zn at 60 μM it is possible that any further increase in Zn concentration within the root

would therefore be transported out to the shoots as was seen with *T. arvense* at 150 μM .

6.1.2 Zn tolerance of *Thlaspi* species

Alongside looking at the accumulation of the two *Thlaspi* sp. tolerance of the plants to increasing external Zn concentration was evaluated by measuring growth. The results confirmed that there was differential growth between *Thlaspi caerulescens* and *T. arvense* at increasing zinc concentrations. *Thlaspi caerulescens* grew well at all concentrations of zinc except the highest concentrations (1800 μM), which is 34 times higher than the basal salts provide for normal plant growth *in vitro*. In comparison to *T. arvense* managed growth at 3 and 30 μM Zn; however all levels above this growth decreased sharply. At levels above 600 μM no seeds were able to germinate. Previous literature has recorded *T. caerulescens* as possessing tolerance to elevated Zn concentrations (Brown *et al.*, 1995; Shen *et al.*, 1997; Whiting *et al.*, 2000). In comparison several studies have included *T. arvense* as a non-hyperaccumulating species. These reported projects have been comparable to the data collected here (Lasat *et al.*, 1998).

The findings from these experiments showed that *T. arvense* produced some root biomass at external Zn concentrations below 150 μM . Above this level root biomass was difficult to measure due to the restricted growth. Above 600 μM the seeds failed to germinate. *T. caerulescens* in comparison showed less detrimental effect of increasing Zn concentration. Whiting *et al.*, (2000) compared *T. caerulescens* with *T. arvense* and found that *T. caerulescens* had highly branched roots, high root: shoot biomass ratio, highly specific root

length and extremely long root hairs (>2.1 mm). These variables were not measured in this study however it is possible to conclude that *T. caerulescens* was more tolerant of elevated Zn levels than *T. arvense*.

6.2 Transcriptomic analysis of *Thlaspi* spp.

Prior to the commencement of this project a microarray experiment had been conducted, comparing the expression of *T. caerulescens* and *T. arvense* genes on different external Zn concentrations. Several thousand genes were identified as differentially expressed. A select few of these genes were chosen to confirm their differential expression through qPCR. These genes were chosen due to published data linking them to metal transport (Pence, 2000; Assunção *et al.*, 2001; Zhao *et al.*, 2003). The qPCR was successful in confirming differential expression of nine genes (*ZNT1* (Zinc transporter 1), *ZNT2/4*, *ZNT5*, *HMA4* (heavy-metal associated domain-containing protein 4), *NR1* (Nitrate reductase 1), *CA* (Carbonic anhydrase), *NAS1* (nicotianamine synthase 1), *MTP1* (metal tolerance protein 1), *CHS* (chalcone synthase).

6.2.1 Microarray analysis of *T. caerulescens*

The use of an *Arabidopsis thaliana* gene chip to test the transcription of *T. caerulescens* and *T. arvense* (non-model plant species) was developed as described in this chapter. The aim was to design probes to the target species without including polymorphisms that can occur between the target and model plant species. The experimental results were confirmed using qPCR. It was found that on average there was an 81.5% similarity between *A. thaliana* and

T. caerulescens or *T. arvense* coding regions. Previous studies have found a similar level of homology (87-88%) (Peer *et al.*, 2003).

Comparitively Becher *et al.* (2004), Weber, *et al.* (2004) and Filatov *et al.* (2007) used microarray analysis to compare expression of plant hyperaccumulators with closely related non-hyperaccumulating species. The study carried out by Filatov *et al.* (2007) was different in that they used a combination of genomics and classical genetics to identify candidate genes. This study concluded that there appeared to be two QTLs for zinc accumulation located on chromosomes 3 and 7. Genes that were identified here included *AtNRAMP3* which encodes a protein that is responsible for Fe, Mn, Cd and possibly Zn transport; similarly this gene was identified in the study carried out by Weber *et al.* (2004). The gene *ZIP6* was identified in both the Becher *et al.* (2004) and the the Filatov *et al.* (2004) study. A gene that was found to be differentially expressed in this microarray, by Filatov *et al.* (2006) and Becher *et al.* (2004) was the carbonic anhydrase gene (CA), which has a cofactor and which changes in activity relative to zinc supply.

6.2.2 qPCR analysis of *T. caerulescens*

Of the nine genes studied three genes were of particular interest, the *ZNT 2/4*, *HMA4* and *NR1* genes. These genes were expressed significantly greater in *T. caerulescens* than *T. arvense*. This data supports that reported by Assunção *et al.* (2001) who cloned two Zn transporter genes, *ZNT2* and *ZTP1*, from *T. caerulescens*. These genes showed increased expression in *T.*

caerulescens compared to *T. arvense*. In *T. caerulescens*, the expression of *ZNT2* was barely responsive to the Zn supply, whereas their expression in *T. arvense* occurred exclusively under the conditions of Zn deficiency. These results suggest that Zn transporter genes may play an important role in enhanced Zn uptake and Zn hyperaccumulation in *T. caerulescens* (Zhao *et al.*, 2003).

It has previously been noted that *TcZNT1*, *TcZNT2* and *TcZTP1* have roles in Zn hyperaccumulation and it has been shown that the expression is constitutively higher in both roots and shoots of *T. caerulescens* compared to *T. arvense* (Pence *et al.*, 2000; Assunção *et al.*, 2001).

Sequence and structural homology has been noted between *TcZNT1* and *TcZNT2* with members of the ZIP family that have previously been associated with accumulation of essential metals and detoxification of harmful ones in many species including homologues of five genes found in *A. thaliana*. Alongside these genes other proteins that are able to control Zn ionic fluxes in hyperaccumulators include cation diffuser facilitator families (CDF) (Gaither and Eide, 2001; Hammond *et al.*, 2006; Kim *et al.*, 2004) and certain P_{1b}-type ATPases (HMA- heavy metal associated domain containing proteins) (Hammond *et al.*, 2006; Hussain *et al.*, 2004; Papoyan and Kochian, 2004; Williams *et al.*, 2000; Williams and Mills, 2005).

Assunção *et al.* (2001) have previously shown that the expression of *TcMTP1* is higher in shoots and roots of three ecotypes of *T. caerulescens* compared

to *T. arvense*; however this study showed the opposite. Kim *et al.* (2004) have shown that *TgMTP1* has a role in Zn efflux into cells in *Thlaspi goesingense*, a Zn and Ni hyperaccumulator. Papoyan and Kochian (2004) have functionally analyzed *TcHMA4* and shown it plays a role in xylem loading of Zn. This remains consistent with the findings in the *A. thaliana* double mutant *hma2hma4* that accumulates less Zn than the wild type whereas over expression of the *AtHMA4* in *A. thaliana* showed increased Zn and Cd accumulation in the shoot (Hammond *et al.*, 2006; Hussain *et al.*, 2004; Verret *et al.*, 2004).

6.3 Design of the *HMA4* RNAi construct for transformation of *T. caerulescens*

The aim of this chapter was to design a suitable construct to allow the transformation of *Thlaspi caerulescens* to study the function of the *HMA4* gene through RNAi silencing. The *HMA4* gene had been chosen as the candidate gene to study following a microarray experiment that identified its differential expression in *T. caerulescens* on elevated Zn concentrations. Its differential expression was then confirmed by qPCR. Previous studies of the *HMA4* gene in yeast (Papayon and Kochian, 2004) and *Arabidopsis* (Hussain *et al.*, 2004; Verret *et al.*, 2004) have suggested it plays a role in the hyperaccumulation of Zn. However no studies had been carried out on the gene itself in *Thlaspi caerulescens*. This made it an ideal candidate for the study through RNAi gene silencing. Mills *et al.*, (2005) further confirmed the role played by *HMA4* using a knockout strategy. In this example T-DNA insertion mutants were used to study the effect of the downregulation of the

gene. Results from this study showed that increased sensitivity to Zn and Cd occurred as the gene was silenced. This suggested that *HMA4* played a role in metal detoxification.

A recent report, with a similar aim to the one attempted in this thesis has been published. It involved the silencing of *HMA4* in *Arabidopsis halleri* (a hyperaccumulator) using an RNAi approach (Hanikenne *et al.*, 2008). The aim was to determine whether *AhHMA4* functions in the heavy metal accumulation or heavy metal tolerance of *A. halleri*. *A. halleri* L. (accession Langilsheim) and *A. thaliana* plants were grown hydroponically or on solid agar medium containing the same hydroponic solution.

Proofreading polymerase was used to amplify by PCR the *AhHMA4* sequence (2541-2997 bp) from cDNA. This was first cloned into the pENTR/D TOPO vector before being inserted into the Gateway binary vector, pJAWOHL8 by site directed recombination. The pJAWOHL8 binary vector generates a hairpin construct consisting of antisense-*AhHMA4* fragment intron-sense *AhHMA4* fragment which is downstream from a CaMV 35S promoter.

Results of the transformation of *A. halleri* with the RNAi construct showed a decrease in the transcript levels of *HMA4* by between 45% and 10% compared with wild types, determined by RT-PCR. These plants were deemed morphologically normal by comparing root elongation of lines grown hydroponically on solution supplemented with increasing Zn concentrations. Similarly, plants were shown to contain between 12-35% of the Zn normally

found in wild type *A. halleri*. These levels were similar to those observed in the non-hyperaccumulator *A. thaliana*. The levels of Zn found in *A. halleri* roots are normally lower than shoots which indicates root-shoot metal translocation. However in the RNAi transformed lines 49-137 fold higher levels of Zn are found in the roots, again levels similar to those found in *A. Thaliana*. This evidence suggests *AhHMA4* is required for efficient root to shoot flux.

In the same study fluorescent imaging using the Zn fluorescent indicator, Zinpyr-1 was used to determine the effect *HMA4* has on the localisation of Zn within the roots of *A. halleri*. In the wild type Zn was localised mainly in the xylem vessels, inwards of the vascular pericycle. In the RNAi transgenic lines Zn localisation was most intense in the pericycle cell layer. Similarly this pattern of Zn localisation has been reported in the *A. thaliana HMA4* mutant when compared to wild type plant. This suggests that the silencing of the *AhHMA4* gene inhibits the movement of Zn from the root symplast to the apoplastic xylem vessels which is in fact the primary route of solutes from the roots to the shoots which explains the lack of Zn in the shoots of the RNAi transgenic plants.

This study has similarities to this thesis in that it was choosing to down regulate the *HMA4* gene in a known hyperaccumulator using a construct produced using the GATEWAY cloning system. The differences that led to this being successful and the study in this thesis not achieving transformed plants lies not with the construct necessarily, even though different binary

vectors were used. The construct in this thesis appeared to work in that it was delivered successfully into *A. thaliana*, confirmed via PCR however any action of the RNAi silencing was not investigated. The limiting factor may be the hyperaccumulator plant chosen to study the gene expression, *T. caerulescens*. The transformation of *A. halleri* by floral dip may have less technical difficulties associated with it due to it being more closely related to *A. thaliana*, which has an efficient transformation protocol. To further confirm the correct assembly of the RNAi *HMA4* construct produced during this PhD it could be used to transform *A. halleri* following the method outlined by Hanikenne *et al.* (2008). Plants were cultured from seed on 0.5x MS medium supplemented with 1% (w/v) sucrose, 0.05% (w/v) MES (2-N-morpholino ethanesulphonic acid), pH 5.7 and 0.75% (w/v) agar (Sigma Agar M) for 6-7 weeks. For the tissue culture based transformation root explants were grown on MS medium containing 2% (w/v) sucrose, 0.05% (w/v) MES, pH 5.7 and 0.75% (w/v) agar containing hormones to induce callus production including 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg L^{-1} kinetin for 7 days prior to inoculation with *A. tumefaciens* for 3 days. Regeneration was achieved by culturing on the previously mentioned medium with the necessary hormones to stimulate regeneration (1 mg L^{-1} 6-benzylaminopurine, 0.5 mg L^{-1} α -naphthaleneacetic acid, 125 mg L^{-1} ticarcillin disodium/potassium clavulanate). Similarly, the regeneration of *T. caerulescens* accomplished by Guan *et al.*, (2008) (refer to section 6.4) was achieved using the hormones NAA and BAP, a combination attempted in this PhD but succeeded by NAA alone using shoot tissue. Selection of potential transformants was achieved

on the above mentioned medium with the addition of 50 mg L⁻¹ kanamycin, 25 mg L⁻¹ phosphinotricin, or 10 mg L⁻¹ hygromycin. Root induction was then achieved by excised shootlets by cultivation on medium containing 1 mg L⁻¹ indole-3-acetic acid, 125 mg L⁻¹ ticarcillin disodium/potassium clavulanate.

Hanikenne *et al.* (2008) state that the *HMA4* gene is required for full tolerance of *A. halleri* to Zn and Cd. Also reported is the transfer of *HMA4* gene into *A. thaliana*, a non-hyperaccumulator. This leads to Zn partitioning in the xylem vessels and transcriptional upregulation of Zn deficiency response genes. This study confirms the feasibility of choosing this gene for study.

Secondly to the silencing of the *AhHMA4* gene Hanikenne *et al.* (2008) also studied the effect of overexpression of the *AhHMA4* gene in *A. halleri*. The effect of this overexpression was monitored by RT-PCR to determine how the expression of Zn deficiency genes changed in relation to the overexpression of *HMA4*. The roots of the RNAi lines were shown to have a positive correlation of the expression of *IRT3* and *ZIP4* genes in relation to the transcript abundance of *HMA4*. The Zn deficiency genes mentioned belong to the same family of proteins associated with the uptake of Zn. It has therefore been proposed that the expression of these genes in the roots of wild type *A. halleri* is an outcome of the of the increased *HMA4* activity and as a result further increases metal accumulation.

To summarise, *AhHMA4* is thought to be responsible for naturally selected Zn hyperaccumulation and Cd and Zn hypertolerance in *A. halleri*. Elevated levels of *HMA4* within the plant is attributable to a high copy number of the gene and due to this the gene it is found that there is increased Zn flux from root symplasm to the xylem vessel.

The results from the work carried out by Hanikenne *et al.* (2008) provide key leads to evaluate the work carried out in this thesis and give ideas for new research in future related to the *HMA4* RNAi construct produced. The construct produced was successfully used to transform *A. thaliana*, to test that the construct was working. However due the difficulty encountered in the transformation of *Thlaspi caerulescens* (section 6.4) effect of the RNAi silencing was not observed. *A. halleri* could possibly be a potential candidate for transformation using the assembled construct to evaluate the effectiveness of the construct itself.

6.4 Transformation of *T. caerulescens*

The transformation of *Thlaspi caerulescens* was attempted as a means of studying the *HMA4* gene *in planta*, a novel approach. Reports of *Thlaspi caerulescens* previously being transformed by floral dip methods with reporter genes meant that it was thought that this would be relatively straight forward. (Pence *et al.*, 2000). However this was not to be the case. Unfortunately there were no successful transformants produced from this project despite numerous attempts using both my floral dip and tissue culture methods. The construct used was successfully transformed into *Arabidopsis thaliana*

suggesting that the construct was not the limiting factor. Factors that may have contributed to the inability to transform *T. caerulescens* include contamination of the plants by mildew, an effect of covering the plants with plastic bags following floral dipping. A low efficiency (less than 1% as observed in *A. thaliana*) would mean obtaining transformants would be very low frequency. The cycle of seed to seed in *T. caerulescens* is longer than in *A. thaliana*. This combined with a low frequency could make the chances of obtaining a transformant very low (Ebbs *et al.*, 1997).

At the time of completing the thesis a paper was published that confirmed transformation of *Thlaspi caerulescens* by *Agrobacterium tumefaciens* (Guan *et al.*, 2008). They reported on the successful insertion of a GUS reporter gene, *nptII* selectable marker and a foreign catalase gene through a tissue culture based transformation system. Seeds of *T. caerulescens* were grown on sterile agar for seven to fourteen days. Cotyledons, hypocotyls and shoots (10-20 mm) with nodes were excised from these plantlets and transferred on to MS media containing 300 mg L⁻¹ lactalbumin hydrolysate (LH), 200 mg L⁻¹ inositol, 30 g L⁻¹ sucrose, and 6 g L⁻¹ agar, supplemented with factorial combinations of 1 mg L⁻¹ benzylaminopurine (BA) and 0.2 mg L⁻¹ α-naphthaleneacetic acid (it was subsequently determined that the cotyledons and hypocotyls were unsuccessful at regenerating). In comparison the regeneration conditions that favoured *T. caerulescens* in this PhD was 2 μl ml⁻¹ NAA without cytokinins which gave 98% production of callus from shoot explants. As discussed the successful regeneration of plants from callus was never achieved in this study however Guan *et al.* (2008) discussed how it was

achieved in their study. After allowing tissue to differentiate for 1 week the nodes from several shoots were transferred to MS medium containing 200 mg L⁻¹ inositol, 30 g L⁻¹ sucrose, and 7 g L⁻¹ agar, supplemented with (1 or 2 mg L⁻¹) kinetin (KT), (1 or 2 mg L⁻¹) benzylaminopurine BA, and (0.5 or 1 mg L⁻¹) NAA for regeneration of shoot cluster, at three week intervals this medium was replaced with recordings of regenerated roots recorded.

Guan *et al.*, (2008) used a construct containing a CaMV 35S promoter/catalase/GUS cds/30NOS expression cassette within a pBI121 binary vector inserted into *Agrobacterium tumefaciens* strain EHA105. The transformation conditions were optimised by carrying out several experiments changing various variables including inoculation time and optical density of the *A. tumefaciens* inoculum. The final transformation procedure involved pre-culturing shoot clusters for 4 days whilst a single bacterial colony was cultured in 5 ml f LB medium supplemented with 50 mg L⁻¹ kanamycin and 50 mg L⁻¹ rifampicin and grown over night at 28°C. The bacterial growth was monitored until it reached an optical density (OD₆₀₀) of 0.5. The culture was subsequently centrifuged for 5 min at 4000 rpm and diluted with liquid MS to a 1:3 ratio containing 100 µM acetosyringone. Plant explants were submerged in the culture for 10 mins with continuous shaking. Following inoculation explants were blotted onto sterile filter paper and culture on agar for 4 days at 28°C in the dark.

Following culture with the *A. tumefaciens* inoculums, the explants were washed with sterile water three times and once with MS liquid medium

containing 500 mg L⁻¹ carbenicillin to inhibit bacterial growth. The explants were then transferred to selection medium that contained 250 mg L⁻¹ carbenicillin (and the growth hormones; 2 mg L⁻¹ NAA, 2 mg L⁻¹ BA, 2 mg kinetin and 200 mg inositol) for 5-7 days. A second selection medium was used following this containing 60 mg L⁻¹ kanamycin in addition to the carbenicillin and growth hormones previously used). Regeneration was initiated by repeated culturing on medium as laid out above with the addition of a further 0.5 mg L⁻¹ GAs for 6 weeks. Explants were excised from callus and transferred to MS medium containing 250 mg L⁻¹ carbenicillin and 30 mg L⁻¹ kanamycin. This medium was changed at 3 week intervals and surviving explants were recorded at 6 weeks. Elongated shoots were then transplanted to fresh medium to allow root regeneration. Boxes containing rooted plantlets had the lids removed for three day prior to transplantation into the green house.

Transformation was confirmed in potential transformants through GUS analysis and PCR amplification of the *nptII* gene and Southern analysis which confirmed that the T-DNA had integrated singularly. RT-PCR confirmed the presence of the catalase gene (1479 bp). At the protein level, analysis of the catalase gene expression was observed by measuring catalase activity. The result showed that significantly higher catalase activity was found in the transgenic plant compared with the wild type.

Guan *et al.*, (2008) discuss the parameters that made this transformation successful which allows assumptions to be made on why the transformation

system used in this thesis was not successful. The development of plants used for explants is important due to the hormone levels that vary within the plant at different phases of development. This also links into the explant type used as different tissues exhibit different hormones at the same stage in development. The study of Guan *et al.* (2008) varied from the thesis study mostly in the hormones and media used. This is a vital factor in the successful regeneration of plants and the main inhibitor in the method used in this thesis. Similarly the bacterial density is important to successful transformation; too high a density inhibits transformation. As important is the optimisation of inoculation time; too long and the bacterial growth takes over the plant and that the carbinicillin is unable to completely eliminate, a problem which was observed during the floral dip method used in this thesis. If any higher levels of antibiotics are used damage can be caused to the plant tissue by the antibiotic itself. Guan *et al.* (2008) discussed OD for the transformation of other species for example basmati indica rice or *Rhizalidopsis gaertneri* which is generally carried out between 0.3-0.6, which is consistent with the transformation of *Thlaspi caerulescens* here. Future work on the transformation of *Thlaspi* spp. would involve reproducing these methods reported by Guan *et al.*, (2008) to confirm the method described, with particular focus on regeneration medium used and the inoculation OD and incubation time.

6.5 Future work

This thesis was successful in confirming that *Thlaspi caerulescens* possesses the ability to accumulate and tolerate high levels of Zn and the results obtained were significantly different to those recorded for the non hyperaccumulator *T. arvense*. Secondly this study successfully confirmed the differential expression of several genes in *Thlaspi caerulescens* that had been highlighted in a microarray experiment. From this a candidate gene, *HMA4*, a member of the P-type ATPase family was chosen for further study. A construct designed to silence *HMA4*, *in planta*, was produced that although was not successfully transformed into *Thlaspi caerulescens*, was used to transform *A. thaliana*. This was done to confirm that the construct was not the inhibiting factor in the difficulty of transformation of *Thlaspi caerulescens*. It was not possible to transform *Thlaspi* spp. in this report and to date the lab has not successfully achieved this despite rigorous attempts being made. Limitations of the transformation procedure include the slow seed to seed cycle of the plant. Work since that has been considered after this thesis include rapid cycling of *Thlaspi caerulescens*. This includes experimenting with the vernalisation period to induce flowering earlier in the plants. Recorded efficiency of transformation of *Arabidopsis thaliana* is 1% (Clough and Bent, 1998) if efficiency is lower than this in *T. caerulescens*, combined with the slow cycle of *T. caerulescens* the occurrence of transformants will be less.

Several recent papers have provided areas of future work namely the work carried out by Hanikenne *et al.* (2008) in which they transformed *A. halleri*

with a RNAi *HMA4* construct to assess its activity within the plant. Using the methods described in this paper *A. halleri* could be transformed using a RNAi *HMA4* construct produced in this thesis, or similar construct. This would assess the successful production of the construct within a hyperaccumulator and also to compare the results with those reported by Hanikenne *et al.*, (2008).

Recently methods described by Guan *et al.* (2008) to transform *T. caerulescens* by tissue culture methods could be attempted have been described. It would be interesting to attempt to repeat these methods with a reporter gene such as GUS, to assess the efficiency of transformation and also with the RNAi *HMA4* construct produced to assess the *HMA4* gene within *T. caerulescens* itself.

7. References

Abdel-Ghanya SE, Müller-Mouléb P, Niyogib KK, Pilona M, Shikanaic T (2005) Two P-type ATPases are required for copper delivery in *Arabidopsis thaliana* chloroplasts. *Plant Cell* **17** 1233 – 1251.

Aharoni A, Vorst O (2001) DNA microarrays for functional plant genomics. *Plant Molecular Biology* **48** 99-118.

Alloway BJ (2003) *Zinc in Soils and Crop Nutrition* Brussels, Belgium: International Zinc Association.

Alloway BJ (2001) Zinc – the vital micronutrient for healthy, high-value crops. Brussels, Belgium: International Zinc Association.

Alloway BJ (1990) *Heavy metals in soils*. Blackie Academic and Professional.

Alloway BJ, Ayres DC (1997) Chemical principles of environmental pollution. Chapman & Hall.

Ambros V (2001) Development: Dicing up RNAs. *Science* **293** 811-813.

Assunção AGL, Pieper B, Vromas J, Lindhout P, Aarts MG, Schat H (2006) Construction of a genetic linkage map of *Thlaspi caerulescens* and quantitative trait loci analysis of zinc accumulation. *New Phytologist* **170** 21-32.

Assunção AGL, Da Costa Martins P, De Folter S, Vooijs R, Schat H, Aarts MGM (2004) Elevated expression of metal transporter genes in three

accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant Cell and Environment* **24** 217-226.

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

Assunção A GL, Bookman WMT, Nelissen HJM, Voojjs R, Schat H, Ernst WHO (2003b) A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159** 383-390.

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

Auld DS (2001) Zinc coordination sphere in biochemical zinc sites. *Biometals* **14** 271-313.

Baker AJM, Reeves RD, Hajar ASM (1994) Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. Presl (Brassicaceae). *New Phytologist* **127** 61-68.

Baker AJM, Proctor J, van Balgooy MMJ, Reeves RD (1992) The vegetation of ultramafic (Serpentine) soils: Proceedings of the First International Conference on Serpentine Ecology; Hyperaccumulation of nickel by the flora of the ultramafics of Palawan, Republic of the Philippines. In Baker AJM, Reeves RD, eds ed, Vol pp Intercept, Baker AJM, Proctor J, Reeves RD eds pp Intercept 291-304.

Baker AJM, Walker PL (1990) Ecophysiology of metal uptake by tolerant plants Chapter 11 in *Heavy Metal Tolerance in Plants: Evolutionary Aspects* Ed Shaw AJ CRC Press, London UK pp 155-179.

Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements-a review of their distribution, ecology and phytochemistry. *Biorecovery* **1** 81-126.

Barak P, Helmke PA (1993) The chemistry of Zinc. In Robson AD *Zinc in soil and plants*. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp1-13.

Barton KA (1983) regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny *Cell* **32** 1033-104.

Becher M, Talke IN, Krall L, Krämer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37** 251-268.

Bechtold N, Ellis J and Pelletier G. (1993) *In plant Agrobacterium-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. C.R. Acad. Sci. Paris Life Sciences* **316** 1194-1199.

Bekheet S, Solliman M (2007) An efficient transformation system for sugarbeet mediated by *Agrobacterium tumefaciens*. *Journal of Food, Agriculture & Environment* **5** 193 - 197.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. (2004) GenBank: update. *Nucleic Acid Research*. **1** D23-D26.

Bent AF (2000) Arabidopsis in Planta Transformation. Uses, Mechanisms, and Prospects for Transformation of Other Species. *Plant Physiology* **124** 1540-1547.

Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N (2004) A novel CPx ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569** 140–148.

Bert V, Bonnin I, SAumitou-Laprade P, de LAguerie P, Petit D (2002) Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? *New Phytologist* **146** 225-233.

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research* **12** 8711 – 8721.

Borevitz JO, Chory J (2004) Genomics tools for QTL analysis and gene discovery *Current Opinion in Plant Biology* **7** 132-136.

Bouché N, Bouchez D (2001) *Arabidopsis* gene knockout: phenotypes wanted. *Current Opinion in Plant Biology* **4** 111-117.

Boyd RS, Martens SN (1992) The raison d'être for metal hyperaccumulation by plants. *The vegetation of ultramafic (serpentine) soils* edited by AJ Baker, J Proctor and RD Reeves. Andover, UK, Intercept 279-289.

Brait JF, Lebrun M (1999) Plant responses to metal toxicity. *Comptes Rendus de l'Académie des Sciences - Series III - Sciences de la Vie* **322** 43-54.

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

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

Brooks RR (1998) *Plants that hyperaccumulate heavy metals*. Wallingford, UK CAB International. pp380.

Brooks R (1994) *Plants the hyperaccumulate heavy metals*. In *Plants and Chemical Elements*. Ed Farago ME ed VCH, Weinheim, Germany 87-105.

Brooks RR, Lee J, Reeves RD, Jaffré T (1977) Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration* 7 49-57.

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

Burd GI, Dixon DG, Glick BR (1998) Plant growth promoting bacteria that decrease nickel toxicity in seedlings. *Applied Environmental Microbiology* 64: 3663–3668.

Burd GI, Dixon DG, Glick BR (2000) Plant growth promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal Microbiology* 46: 237–245.

Cacres M, Lacher J, Zapala MA, Redmond JC, Kudo L, Geschwind DH, Lockhart DJ, Preuss TM, Barlow C (2003) Analysis of results variability from high-density oligonucleotide arrays comparing same-species and cross species hybridisations. *Biotechniques* 33 516-524.

Cakmak I (2004) Plant nutrition research: Priorities to meet human needs for food in a sustainable ways. *Plant and Soil* 247 3-24.

Cakmak I (2002) Identification and correction of widespread zinc deficiency in Turkey-a success story. *Proceedings of the International Fertilizer Society* **552** 1-26.

Chaney RI (1993) Zinc phytotoxicity. In Robson AD *Zinc in soil and plants*. Dordrecht, The Netherlands: Kluwer Academic Publishers, pp135-150.

Chismar JD, Mondala T, Fox HS, Roberts E, Langford D, Masliah E, Salomon DR, Head AR (2002) Analysis of results variability from high-density oligonucleotide arrays comparing same-species and cross-species hybridisations. *Biotechniques* **33** 516-524.

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Current Biology* **6** 325-330.

Christou P (1995) Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. *Euphytica* **85** 13-27.

Christou P, Ford TL and Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of endogenous DNA into immature zygotic embryos. *Biotechnology* **9** 957-962.

Clemens S (2000) Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212** 475-486.

Clemens S (2001) Evolution and function of phytochelatin synthases. *Journal of Plant Physiology* **163** 319-332.

Clough SJ and Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16** 735-43.

Cobbett C (2003) Heavy metals and plants-model systems and hyperaccumulators. *New Phytologist* **159** 289-293.

Colangelo EP, Guerinot MN (2006) Put the metal to the petal: metal uptake and transport throughout plants. *Current Opinion in Plant Biology* **9** 322-330.

Collins CD. (1999) Strategies for minimizing environmental contaminants. *Trends in Plant Science* **4** 45.

Cosio C, Martinoia E and Keller (2004) Hyperaccumulation of Cadmium and Zinc in *Thlaspi caerulescens* and *Arabidopsis halleri* at the leaf cellular level. *Plant Physiology* **134** 716-725.

Curtis IS, Nam HG (2001) Transgenic radish (*Raphanus sativus* L. longipinnatus Bailey) by floral-dip method – plant development and surfactant are important in optimizing transformation efficiency. *Transgenic Research* **10** 363-371.

Deniau AX, Pieper B, Bookum WMT, Lindhout P, Aarts MGM, Schat H (2006) QTL analysis of cadmium and zinc accumulation in the heavy metal hyperaccumulator *Thlaspi caerulescens*. *Theoretical and Applied Genetics* **113** 907-920.

Donson, J, Fang Y, Espiritu-Santo G, Xing W, Salazar A, Miyamoto S, Armendarez v, Volkmuth W (2002) Comprehensive gene expression analysis by transcript profiling. *Plant Molecular Biology* **48** 75-97.

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

Dumas F, Duckely M, Pelczar P, Van Gelder P, Hohn B. (2001) An *Agrobacterium* VirE2 channel for transferred-DNA transport into plant cells. *Proceedings of the National Accademy of Science of the United States of America* **98 (2)** 485-490.

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics *The Plant Journal* **45** 616-629.

Ebbs S, Lau I, Ahner B, Kochian L (1997) Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl) *Planta* **214** 635-640.

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

Enard W, Khaitovich P, Klose J, Zöllner S, Heissig F, Giavalisco P, Nieselt-Struwe K, Muchmore E, Varki A, Ravid R, Doxiadis GM, Bontrops RE, Pääbo S (2002) Intra-and interspecific variation in primate gene expression patterns. *Science* **296** 340-343.

Ernst WHO, Verkleij JAC Achat H (1992) Metal tolerance in plants. *Acta Botanica Neerlandica* **41** 229-248.

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury HJ, Macnair MR (2007). A quantitative trait loci analysis of zinc hyperaccumulation in *Arabidopsis halleri* *New Phytologist* **174** 580-590.

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury HJ, Macnair MR (2006) Comparison of gene expression in segregating families identifies

genes and genomic regions involved in a novel adaptation, zinc hyperaccumulation. *Molecular Ecology* **15** 3045-3049.

Flowers TJ (2004) Improving crop salt tolerance *Genetics of Plant Mineral Nutrition* **55** 307-319.

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

Fromm M, Taylor LP and Walbot V (1986) Stable transformation of maize after gene transfer by electroporation. *Nature* **319** 791-793.

Gelvin SB (2005) Gene exchange by design. *Nature* **433** 583-584.

Graham RD, Ascher JS, Hynes SC (1992) Selecting zinc-efficient cereal genotypes for soils of low zinc status. *Plant and Soil* **146** 241-250.

Grotz N, Fox TC, Connolly E, Park W, Gurinot ML, Eide D (1998) Identification of a family of zinc transporter genes from *Arabidopsis* that respond to zinc deficiency. *Proceedings of the National Academy of Sciences, USA* **95** 7220-7224.

Gonzaga MIS, Gonzaga Santos JA, Ma LQ (2006) Arsenic phytoextraction and hyperaccumulation by fern species. *Science Agriculture (Piracicaba, Brazil)*. **63** 90-101.

Goodner G, Hinkle G, Gattung S, Miller N, Blanchard M, Qurollo B, Goldman BS, Cao Y, Askenazi M, Halling C, Mullin L, Houmiel K, Gordon J, Vaudin M, Iartchouk O, Fang Liu A, Wollam C, Allinger M, Doughty D, Scott C, Lappas C, Markelz B, Flanagan C, Crowell C, Gurson J, Lomo C, Sear C, Strub G, Cielo C, Slater S (2001) Genome Sequence of the Plant

Pathogen and Biotechnology Agent *Agrobacterium tumefaciens* C58. *Science* **294** 2323-2328.

Guan ZQ, Chai TY, Zhang XY, Zu J, Wei W, Han L, Cong L (2008) Gene Manipulation of a Heavy Metal Hyperaccumulator Species *Thlaspi caerulescens* L. via *Agrobacterium*-mediated Transformation. *Molecular Biotechnology* **40** 77-86.

Guerinot ML, Salt DE (2001) Fortified foods and phytoremediation. Two sides of the same coin. *Plant Physiology* **125** 164-167.

Guerinot ML (2000) The ZIP family of metal transporters. *Biochimica et Biophysica Acta* **1465** 190–198.

Gura T. (2000) A Silence that Speaks Volumes *Nature* **404** 804-808.

Hacisalihoglu G and Kochian LV (2003) How do some plants tolerate low levels of soil zinc? Mechanisms of zinc efficiency in crop plants. *New Phytologist* **159** 341-350.

Hall JL, Williams LE (2003) Transition metal transporters in plants. *Journal of Experimental Botany* **54** 2601-2613.

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

Haines BJ (2002) Zincophilic root foraging in *Thlaspi caerulescens*. *New Phytologist* **155** 363-372.

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

Hammond JP, Broadley MR, Craigon DJ, Higgins J, Emmerson Z, Townsend H, White PJ, May ST (2005) Using genomic DNA-based probe-

selection to improve the sensitivity of high-density oligonucleotide arrays when applied to heterologous species. *Plant Methods* **1** 1-10.

Hammond SM, Caudy AA, Hannon GJ (2001) Post transcriptional gene silencing by double stranded RNA. *Nature Reviews Genetics* **2** 110-119.

Hannikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymanns J, Weigel D, Krämer U (2008) Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of *HMA4*. *Nature* **453** 391-396.

Hansen G, Wright MS (1999) Recent advances in the transformation of plants. *Trends in Plant Science* **4** 226-231.

Hellens R, Mullineaux P, Klee H (2000) 'Technical focus: A guide to *Agrobacterium* binary Ti vectors'. *Trends in Plant Science* **5** 446 – 451.

Hellwig S, Drossard J, Tyman RM, Fischer R (2004) Plant cell cultures for the production of recombinant proteins *Nature Biotechnology* **22** 1415-1422.

Hennig L, Meneges M, Murray JA, Gruissen W (2003) *Arabidopsis* transcript profiling on Affymetrix GeneChip® arrays. *Plant Molecular Biology* **53** 457-465.

Herrera-Estrella L, Depicker, van Montagu, M, Schell J (1983) Expression of chimeric genes transferred into plants using a Ti-plasmid-derived vector. *Nature* **303** 209-213.

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *The Plant Journal* **6** 271-282.

Higgins MA, Berridge BR, Mills BJ, Schultze AE, Gad H, Searfoss GH, Baker TK, Ryan TP (2003) Gene expression analysis of the acute phase response using a canine microarray. *Toxicology Sciences* **74** 470-484.

Hirschi KD, Zhent RG, Cunningham KW, Reat PA, Fink GR (1996) CAX1, an H⁺/Ca²⁺ antiporter from *Arabidopsis* *Proceedings of the National Academy of Sciences*.

Hodge A (2004) The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytologist* **162** 9-24.

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

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS P-type ATPases heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis* *Plant Cell* **16** 1327 –1339.

Hüttermann A, Arduini I, Godbold DL (1999) Metal pollution and forest decline. In Prasad NMV, Hagemeyer J. eds. *Heavy metal stress in plants: from molecules to ecosystems*. Berlin: Springer-Verlag pp 253-272.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. (2003) Exploration, normalization, and summaries of highdensity oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. *Nature Biotechnology* **14** 745-750.

Jentschke G, Godbold DL (2000) Metal toxicity and ectomycorrhizas. *Physiologia Plantarum* **109** 107-116.

Jones L (1991) *Biotechnological Improvements in Crop Production*. (1st edition) Butterworth-Heinemann Ltd, Oxford pp290.

Kertulis-Tartar GM, Ma LQ, Tu C, Chirenje T (2006) Phytoremediation of an arsenic-contaminated site using *Pteris vittata* L. A two year study. *International Journal of Phytoremediation*. **8** 311-322.

Khaitoich P, Weiss G, Lachmann M, Hellmann I, Enard W, Muetzel B, Wirkner U, Ansorge W, Pääbo S (2004) A neutral model of transcriptome evolution. *Public Library of Science Biology* **2** 682-689.

Khoshgiftar AH, Shariatmadari H, Karimaian N, Kalbasi M, van der Zee Seadm, Parker DR (2004) Salinity and zinc application effects on phytoavailability on cadmium and zinc. *Soil Science Society of America Journal*. **68** 1885-1889.

Klein T (1987) High velocity microprojectiles for delivering nucleic acids into living cells. *Nature* **327** 70-73.

Krämer U (2005) Phytoremediation: novel approaches to cleaning up polluted soils. *Current Opinion in Biotechnology* **16** 133-141.

Krämer U, Pickering IJ, Prince RC, Raskin I, Salt DE (2000) Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. *Plant Physiology* **122** 1343–1353.

Krämer U, Smith RD, Wenzel WW, Raskin I, Salt DE (1997) The Role of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense* Hálácsy. *Plant Physiology* **115** 1641-1 650.

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

Kobae Y, Uemuraa T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M (2004) Zinc transporter of *Arabidopsis thaliana* ATMTTP1 is localized to the vacuolar membranes and implicated in zinc homeostasis. *Plant Cell Physiology* **45** 1749-1758.

Kojima M, Shioiri H, Nogawa M, Mozue M, Matsumoto D, Wada A, Saiki Y, Kiguchi K (2004) In Planta Transformation of Kenaf Plants (*Hibiscus cannabinus* var. aokawa No. 3) by *Agrobacterium tumefaciens*. *Journal of Bioscience and Bioengineering* **98** 136-139.

Komari T, Hiei Y, Ishida Y, and Kubo T (1998) Advances in cereal gene transfer. *Current Opinion in Plant Biology*. **1** 161-165.

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

Küpper H, Lombi H, Zhao FJ, Wieshammer G, McGrath SP (2001) Cellular compartmentation of nickel in the hyperaccumulators *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*. *Journal of Experimental Botany*. **52** 2291-2300.

Küpper H, Zhao FJ, McGrath SP (1999) Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **119** 305-311.

Labra M, Vannini C, Grassi F, Bracale M, Balsemin M, Basso B, and Sala F (2004) Genomic stability in *Arabidopsis thaliana* transgenic plants obtained by floral dip. *Theoretical and Applied Genetics* **109** 1512-1518.

Lasat MM, Baker AJM, Kochain LV (1996) Physiological characterisation of root Zn²⁺ absorption and translocation to shoots in Zn hyperaccumulator and non-accumulator species of *Thlaspi*. *Plant Physiology* **112** 1715-1722.

Lasat MM, Baker AJM, Kochain LV (1998) Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in hyperaccumulation in *Thlaspi caerulescens*. *Plant Physiology* **118** 875-883.

Lasat, MM & Kochian LV (2000) Physiology of Zn hyperaccumulation in *Thlaspi caerulescens*. In: *Phytoremediation of Contaminated Soil and Water*, Terry, N & Bañuelos, G (Eds.). Lewis Publishers, Florida, USA. pp. 159-169.

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. *Journal of Experimental Botany* **51** 71-79.

Lasat MM, Pence NS, Letham DLD, Kochian LV (2001) Zinc phytoextraction in *Thlaspi caerulescens*. *International Journal of Phytoremediation* **3** 129-144.

Lee LY, Gelvin SB (2008) T-DNA binary vectors and systems. *Plant Physiology* **146** 325-332.

Li T, Yang X, Meng F, Lu L (2007) Zinc adsorption and desorption characteristics in root cell wall involving zinc hyperaccumulation in *Sedum alfredii* Hance. *Science Letters* **8** 111–115.

Lin R, Leon A (1999) RNA Interference. Policing Rogue Genes. *Nature* **402** 128-129.

Lipshutz RJ, Fodor SPA, Gingeras TR, Lockhart DJ (1999) High-density synthetic oligonucleotide arrays. *Nature Genetics* **21** 20-24.

Lockey C, Otto E, Long Z (1998) Real time fluorescence detection of a single RNA molecule. *Biotechniques* **24** 744-746.

Lucca P, Hurrell R, Potrykus I (2001) Genetic engineering approaches to improve the bioavailability and the level of iron in rice grains. *Theoretical and Applied Genetics*. **102** 392-397.

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED (2001) A fern that hyperaccumulates arsenic. *Nature* **409** 579.

Macnair MR (2003) The hyperaccumulation of metals by plants. *Advances in Botanical Research* **40** 63-105.

Macnair MR (1993) the genetics of metal tolerance in vascular plants. *New Phytologist* **124** 541-559.

Maret W (2005) Zinc coordination environments in proteins determine zinc functions. *Journal of Trace Elements in Medicine and Biology* **19** 7-12.

Martens SN, Boyd RS (2002) The defensive role of Ni hyperaccumulation by plants: a field experiment. *American journal of Botany* **89** 998-1003.

Marschner H (1995) Mineral Nutrition of Higher Plants (2nd Edition) Academic Press Ltd, London.

Mazé P (1915) Détermination des éléments minéraux rares nécessaires au développement du maïs. *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences* **160** 211-214.

Meerts P, Van Isacker N (1997) Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133** 221-231.

McGrath SP, Zhao FJ, Lombi E (2001) Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils. *Plant and Soil* **232** 207-214.

McGrath SP, Lombi E, Gray C, W Caille, N Dunham, SJ, Zhao FJ (2006) Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri*. *Environmental Pollution* **141** 115 - 125.

McNair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D (1999) Zinc tolerance and zinc hyperaccumulation are genetically independent characters. *Proceedings of the Royal Society of London* **266** 2175-2179.

Mills RF, Krijger GC, Baccarini PJ, Hall JL, Williams LE (2003) Functional expression of *AtHMA4*, a P_{1B}-type ATPase in the Zn/Co/Cd/Pb subclass. *Plant Journal* **35** 164 – 176.

Milner MJ, Kochian LV (2008) Investigating heavy metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany* **102** 3-13.

Mori S (1999) Iron acquisition by plants. *Current Opinion in Plant Biology* **2** 250-253.

Mulligan CN, Yong RN, Gibbs BF (2001) Remediation technologies for metal-contaminated soils and ground water: an evaluation. *Engineering Geology* **60** 193-207.

Nakagawa T, Ishiguro S, Kimura T (2009) GATEWAY vectors for plant transformation. *Plant Biotechnology* **26** 275-284.

Ni TH, Wei YZ (2003) Subcellular distribution of cadmium in mining ecotype *sedum alfredii*. *Acta Botanica Sinica* **45** 925-928.

Palmgren MG, Clemens S, Williams LE, Krämer U, Borg S, Schjørrings JK, Sanders D (2008) Zinc biofortification of cereals: Problems and solutions. *Trends in Plant Science* **13** 464-473.

Papoyan A, Kochain LV (2004) Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **136** 3814-3823.

Peer WA, Mamaudian M, Lahner B, reeves RD, Murphy AS, Salt DE (2003) Identifying model metal hyperaccumulating plants: germplasm analysis of 20 Brassicaceae accessions from a wide geographical area. *New Phytologist* **159** 421-430.

Pence NS, Larsen, PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D, and Kochain LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proceedings in the National Academy of Science* **95** 4956-4960.

Persans MW, Nieman K, Salt DE (2001). Identification of a novel family of metal transporters uniquely expressed in metal hyperaccumulating *Thlaspi goesingense* (Hálácsy). *Proceedings in the National Academy of Science* **98** 9995–10000.

Pollard AJ, Powell KD, Harper FA, Smith JA (2002) The genetic basis of metal hyperaccumulation in plants. *Critical Review in Plant Sciences* **21**(6) 539-566.

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

Pollard AJ, Baker AJM (1996) Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytologist* **132** 113-118.

Reeves RD, Schwartz C, Morel JL, Edmondson J (2001) Distribution and metal-accumulating behaviour of *Thlaspi caerulescens* and associated metallophytes in France. *International Journal of Phytoremediation* **3** 145-172.

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In Raskin I, Ensley BD, eds. *Phytoremediation of toxic metals: Using plants to clean up the environment*. New York, USA John Wiley & Sons Inc pp193-229.

Reeves RD (1992) The hyperaccumulation of nickel by serpentine plants. In: *The Vegetation of Ultramafic (Serpentine) Soils*, Intercept, Andover pp. 253–277.

Ross SM (1994) *Toxic metals in soil-plant systems*. Wiley Publishers, Chichester, UK.

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. *Annual review of Plant Physiology and Plant Molecular Biology* **49** 643-668.

Samach A, Coupland G (2000) Time measurement and the control of flowering in plants. *BioEssays* **22** 38-47.

Schat H, Llugany M, Bernhard R (2000) Metal-specific patterns of tolerance, uptake and transport of heavy metals in hyperaccumulating and non-hyperaccumulating metallophytes. In: Terry N, Banuelos G ed. *Phytoremediation of Contaminated Soils and Water*. Boca Raton, FL, USA: CRC Press 171-188.

Schena, M (1996) Genome analysis with gene expression microarrays. *BioEssays* **18** 427-431.

Schwartz C, Moril JL, Saumier S, Whiting SN, Baker AJM (1999) Root development of the zinc-hyperaccumulator plant *Thlaspi caerulescens* as affected by metal origin, content and localization in the soil. *Plant and Soil* **208** 103-115.

Sharma HC, Crouch JH, Sharma KK, Seetharama N and Hash CT (2002) Applications of biotechnology for crop improvement: prospects and constraints. *Plant Science* **163** 381-395.

Shen ZG, Zhao FJ, McGrath SP (1997) Uptake and transport of zinc in the hyperaccumulator *Thlaspi caerulescens* and the non-hyperaccumulator *Thlaspi ochroleucum*. *Plant Cell and Environment* **20** 898-906.

Shuman S (1994) Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. *The Journal of Biological Chemistry* **269** 32678-32684.

Skoog F (1940) Relationships between zinc and auxin in the growth of higher plants. *American Journal of Botany* **27** 239-951.

Sommer AL, Lipman CB (1926) Evidence on the indispensable nature of zinc and boron for higher green plants. *Plant Physiology* **1** 231-249.

Thomas CM, Jones DA, English JJ, Carroll BJ, Bennetzen JL, Harrison K, Burbridge A, Bishop GJ, Jones JDG (1994) Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain reaction. *Molecular and General Genetics* **242** 573-585.

Tyagi AK. and Mohanty A (2000) Rice transformation for crop improvement and functional genetics. *Plant Science* **158** 1-18.

Uddin M, Wildman DE, Liu GZ, Xu WB, Johnson RM, Hof PR, Kapatos G, Grossman LI, Goodman M (2004) Sister grouping chimpanzees and humans as revealed by genome-wide phylogenetic analysis of brain gene expression profiles. *Proceedings of the National Academy of Sciences, USA* **101** 2957-2962.

Vacchina V, Mari S, Czernic P, Marqués L, Pianelli K, Schaumlöffel D, Lebrun M, Łobiński R (2003) Speciation of nickel in a hyperaccumulating plant by high-performance liquid chromatography-inductively coupled plasma mass spectroscopy and electrospray MS/MS assisted by cloning using yeast complementation. *Analytical Chemistry* **75** 2740–2745.

Van Assche F, Clijsters H (1990) Effect of metals on enzyme activity in plants. *Plant Cell and Environment* **13** 195-206.

van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JAC, Hooykaas PJJ (1999) Overexpression of a Novel Arabidopsis Gene Related to Putative Zinc-Transporter Genes from Animals Can Lead to Enhanced Zinc Resistance and Accumulation. *Plant Physiology* **119** 1047–1056.

Vázquez MD, Poschenrider CH, Barceló J, Baker AJM, Hatton P, Kopp GL (1994) Compartmentation of zinc in roots and leaves of the zinc hyperaccumulator *Thlaspi caerulescens* J&C Presl. *Botanica Acta* **107** 243-250.

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

- Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume N, Vavasseur A, Richaud P** (2004) Overexpression of *AtHMA4* enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576** 306-312.
- Wang WC, Menon G, Hansen G** (2003) Development of a novel *Agrobacterium*-mediated transformation method to recover transgenic *Brassica napus* plants. *Plant Cell Reproduction* **22** 273-281.
- Weber M, Harada E, Vess C, van Roepenack-Lahaye E, Clemens S** (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthetase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. *Plant Journal* **37** 269-281.
- Welch RM, Graham RD** (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany* **55** 353-364.
- White JG, Zasoski RJ** (1999) Mapping soil micronutrients. *Field Crop Research* **60** 11-26.
- White PJ, Broadley MR** (2005) Biofortifying crops with essential mineral elements. *Trends in Plant Science* **10** 586-593.
- White PJ, Broadley MR** (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. *New Phytologist* **182** 49-84.
- Whiting SN, Leake JR, McGrath SP, Baker AJ** (2000) Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **145** 199-210.

Whiting SN, Broadley MR, White PJ (2003) Applying a solute transfer model to phytoextraction: Zinc acquisition by *Thlaspi caerulescens*. *Plant and Soil* **249** 45-56.

Williams, LE, Pittman JK, Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. *Biochimica et Biophysica Acta (BBA). Biomembranes* **1465** 104-126.

Wong ML, Medrano JF (2005) Real time PCR for mRNA quantification. *Biotechniques* **39** 75-85.

Wood D, Setubal C, Kaul R, Monks D, Kitajima J, Okura V, Zhou Y, Chen L, Wood G, Almeida Jr. N, Woo L, Chen Y, Paulsen I, Eisen J, Karp P, Bovee Sr. D, Chapman P, Clendenning J, Deatherage G, Gillet W, Grant C, Kutuyavin T, Levy R, Li M, McClelland E, Palmieri A, Raymond C, Rouse G, Saenphimmachak C, Wu Z, Romero P, Gordon D, Zhang S, Yoo H, Tao Y, Biddle P, Jung M, Krespan W, Perry M, Gordon-Kamm B, Liao L, Kim S, Hendrick C, Zhao Z, Dolan M, Chumley F, Tingey F, Tomb J, Gordon M, Olson M, Nester E (2001) The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294** 2317-2322.

Xing JP, Jiang RF, Ueno D, Ma JF, Schat H, McGrath SP, Zhao FJ (2008) Variation in root-to-shoot translocation of cadmium and zinc among different accessions of the hyperaccumulators *Thlaspi caerulescens* and *Thlaspi praecox* *New Phytologist* **178** 315-325.

Yang XE, Long XX, Ni WZ, Ni SF (2001) Zinc tolerance and hyperaccumulation in a new ecotype of *Sedum alfredii* Hance. *Advances in Phytoecology in China* **25** 670-677.

Ye X, Al-Babili S, Klóti A, Zhang J, Lucca P, Beyer P, Potrykus I (2000) Engineering the provitamin A (β -Carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287** 3030-305.

Zhang H, Forde BG (1998) An *Arabidopsis* MADS box gene that controls nutrient-induced changes in root architecture. *Science* **279** 407-409.

Zhao H, Eide D (1996) The yeast *ZRT1* gene encodes the zinc transporter protein of a high affinity uptake system induced by zinc limitation. *Proceedings of the National Academy of Sciences, USA* **93** 2454-2458.

Zhao H, Butler E, Rodgers J, Spizzo T, Dusesterhoeft S, Eide D (1998) Regulation of zinc homeostasis in yeast by binding of the ZAP1 transcriptional activator to zinc-responsive promoter elements. *Journal of Biological Chemistry* **273** 28713-28720.

Zhu J, Oger P, Schrammeijer B, Hooykaas PJJ, Farrand SK, Winans SC (2000) The basis of crown gall tumourigenesis. *Journal of Bacteriology* **182** 3885-3895.

Zupan JR, Zambryski P. (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. *Plant Physiology* **107** 1041-1047.

Websites

IUBMB <http://www.chem.qmul.ac.uk/iubmb/enzyme/>13/05/07

perl www.perl.com13/05/07

GenBank www.ncbi.nlm.nih.gov/entrez/13/05/07

Primer 3 www.primer3.com 13/05/07

US Census <http://www.census.gov/> 20/08/08

Population Clock <http://www.worldometers.info/population> 20/08/08

MWG Primers <http://www.eurofindna.com> 12/09/08

United Nations www.un.org 14/08/08

8. Appendices

Appendix 8.1 Murashige and Skoog, 1962

MS inorganic salts:

Components (mg L ⁻¹)	Concentration
Calcium chloride dihydrate	440.0
Ammonium nitrate	1650
Potassium nitrate	1900
Potassium iodide	0.830
Cobalt (II) chloride hexahydrate	0.025
Potassium dihydrogen orthophosphate	170.0
Boric acid	6.200
Sodium molybdate dihydrate	0.250
Magnesium sulphate tetrahydrate	370.0
Copper (II) sulphate pentahydrate	22.30
Zinc sulphate heptahydrate	0.025
Iron (II) sulphate heptahydrate	27.85
Ethylenediamine tetra acetic acid disodium salt	37.25
Glycine	2.000
Inositol	100.0
Nicotinic acid	0.500
Pyridoxine hydrochloride	0.500
Thiamine hydrochloride	0.100
Sucrose	30000
Agar	8000.0
pH	5.7-5.8

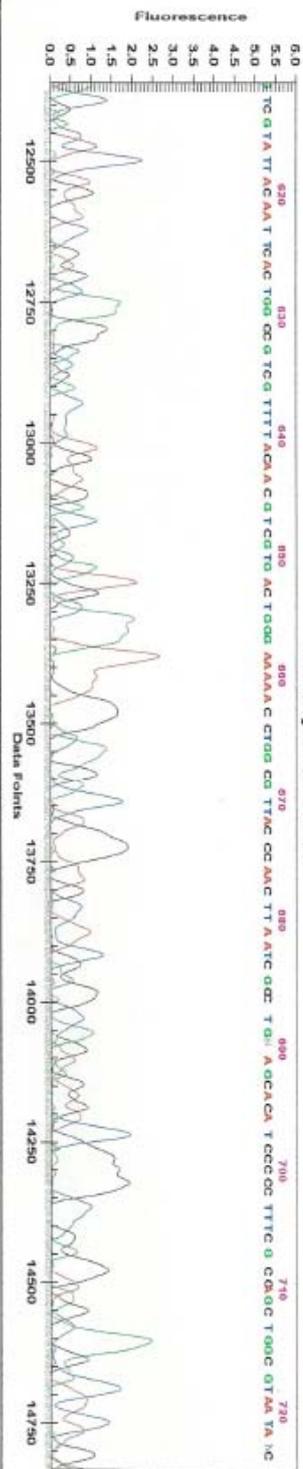
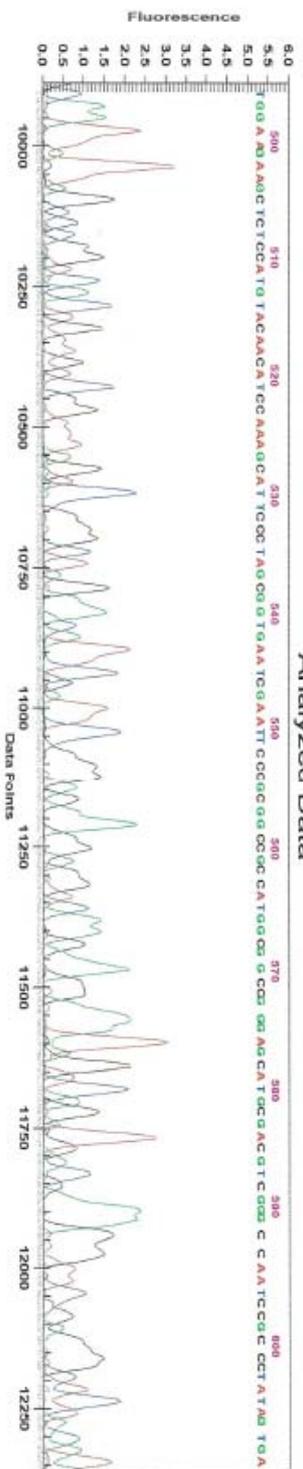
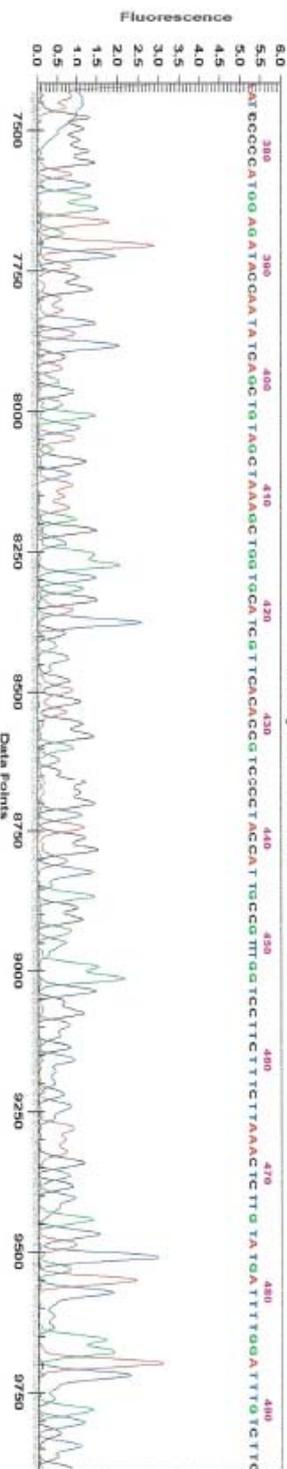
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Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



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8.2.1 Sequence data of the *T. arvensis* HMA4 gene within the pGEM Teasy vector sequence using the HMA4 left primer and the plasmid terminator primer

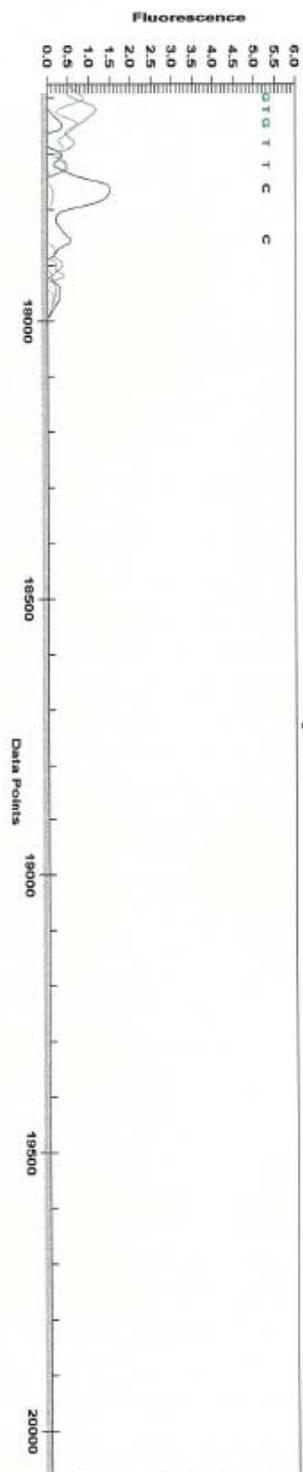
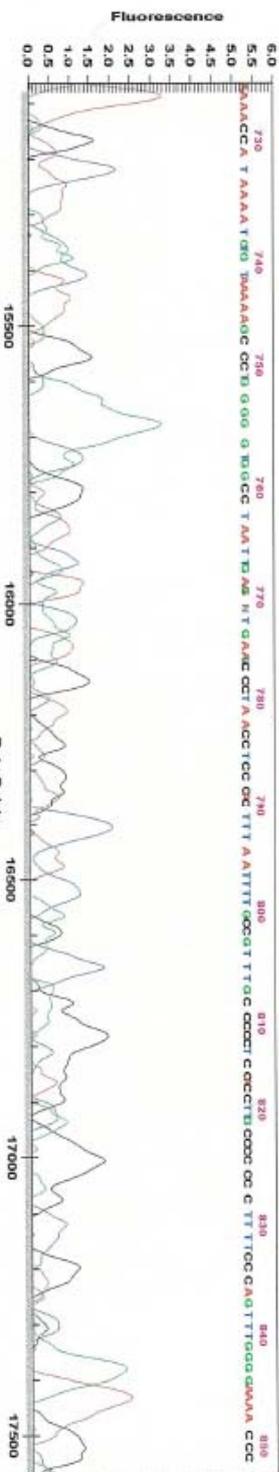


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Analyzed Data



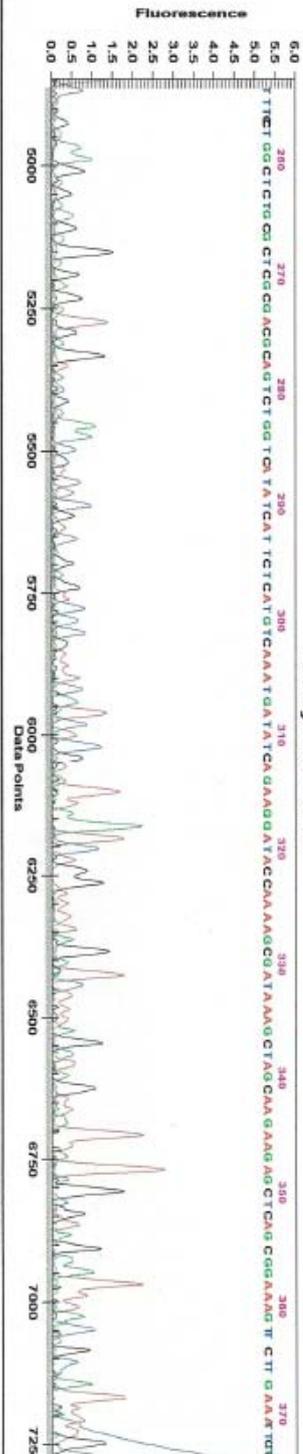
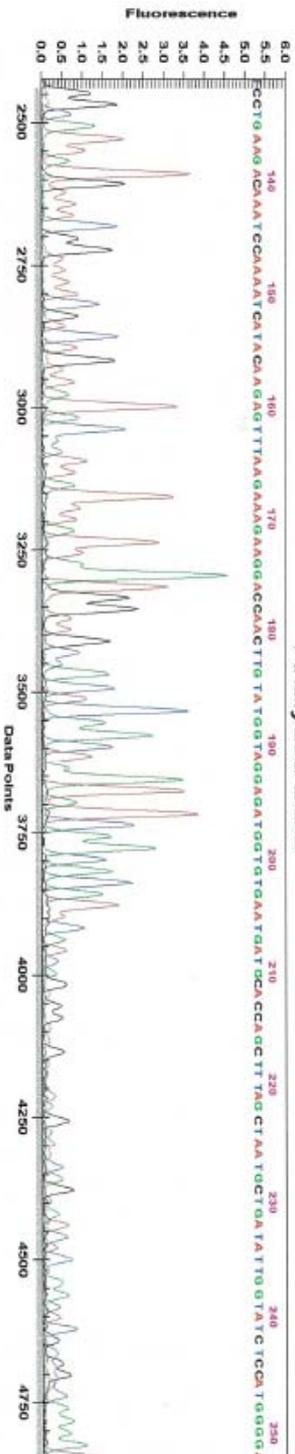
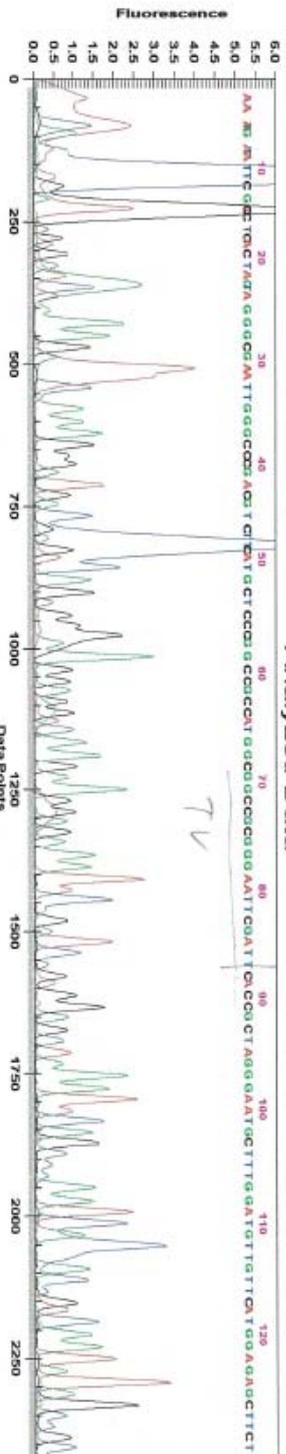
8.2.2 Sequence data of the *T. arvensis* HMA4 gene within the pGEM Teasy vector sequence using the HMA4 left primer and the plasmid terminator primer



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Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



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8.2.3 Sequence data of the *T. caerulescens*HMA4 gene within the pGEM Teasy vector sequence using the HMA4 left primer and the plasmid terminator primer



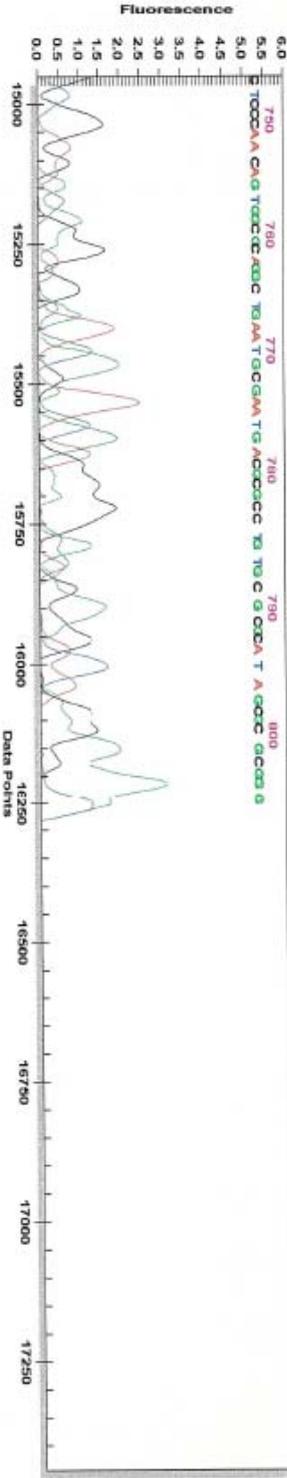
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Operator :

Analyzed Data



File 05/27/05 10:38:13

8.2.4 Sequence data of the *T. caerulescens* HMA4 gene within the pGEM Teasy vector sequence using the HMA4 right primer and the plasmid terminator primer



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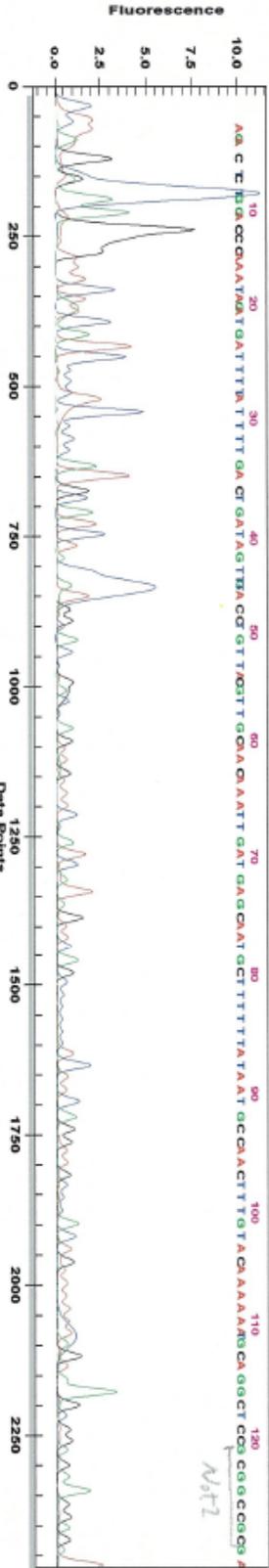
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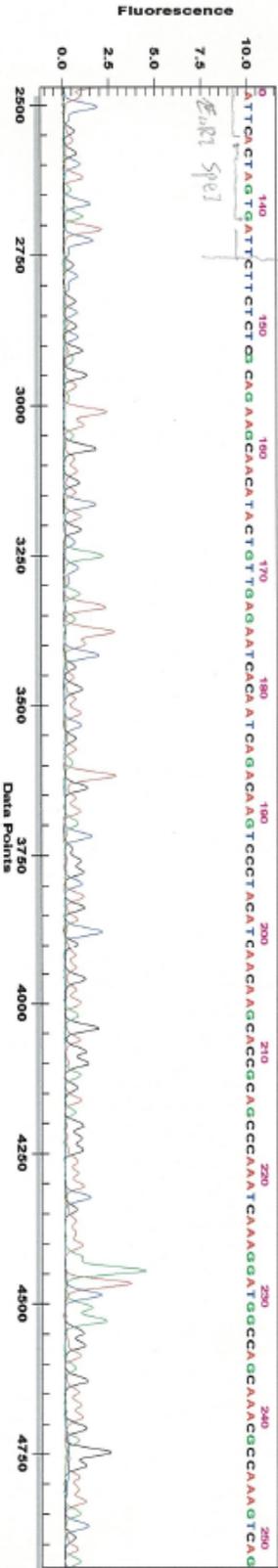
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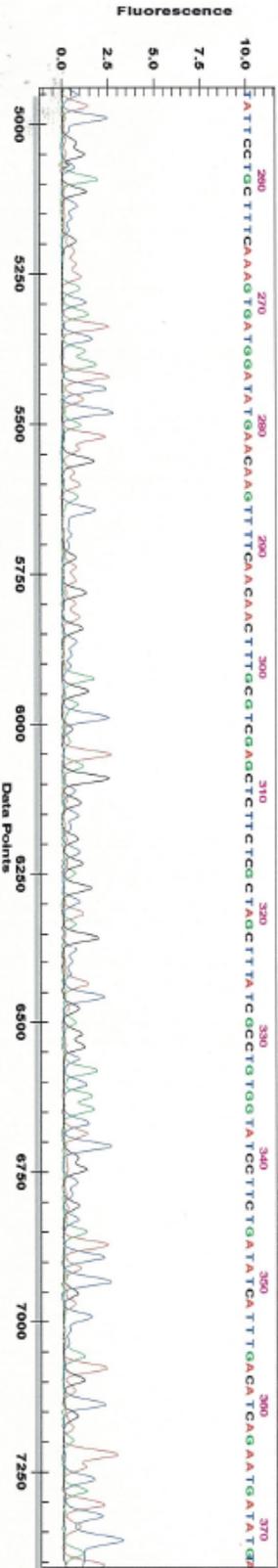
Analyzed Data



Analyzed Data



Analyzed Data



Thu 06/23/05 16:57:19

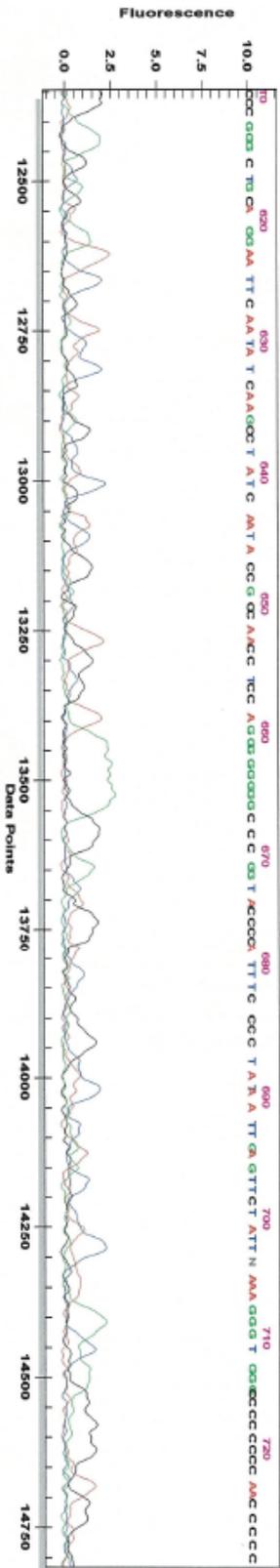
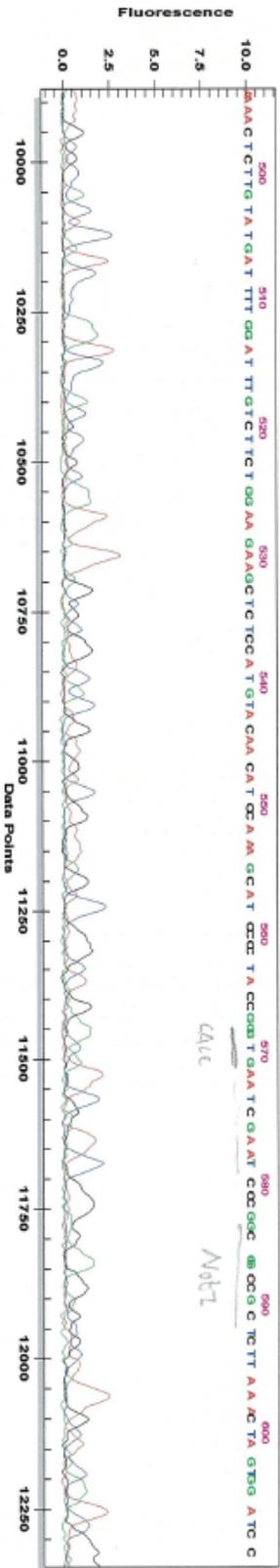
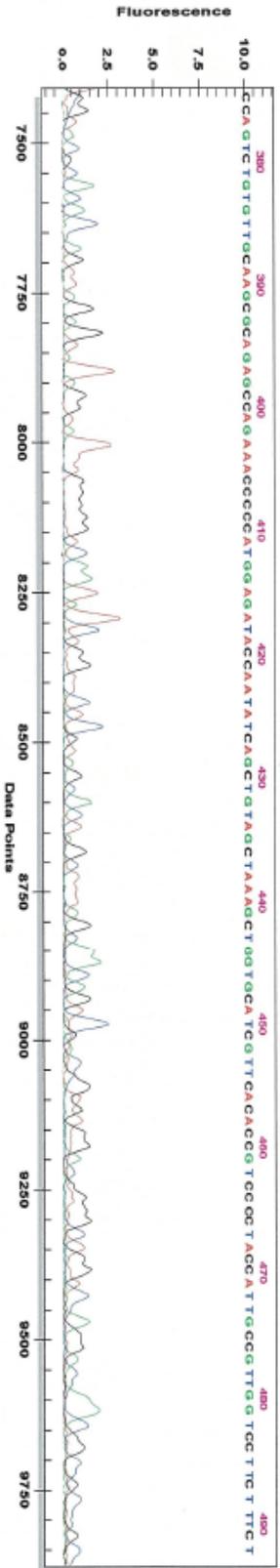
8.2.5 Sequence data of the *T. arvensis* HMA4 gene within the TOPO vector sequence using the HMA4 left primer and the plasmid terminator primer



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Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



Thu 06/23/05 16:57:19

8.2.5 Sequence data of the *T. arvensis* HMA4 gene within the TOPO vector sequence using the HMA4 left primer and the plasmid terminator primer

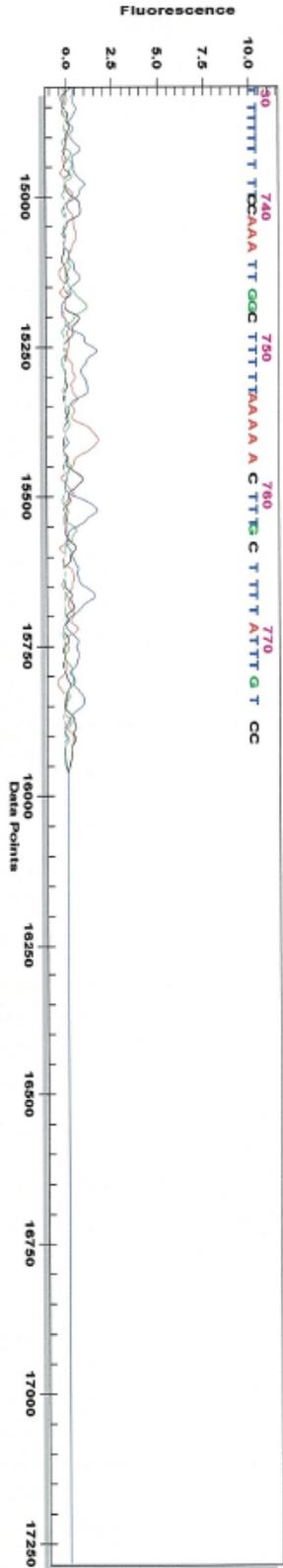


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Operator :
Instrument : CEQ System 3066768 (Ver. 8.0.52)

Analyzed Data



Thu 06/23/05 16:57:19

Page 3

8.2.5 Sequence data of the *T. arvensis* HMA4 gene within the TOPO vector sequence using the HMA4 left primer and the plasmid terminator primer

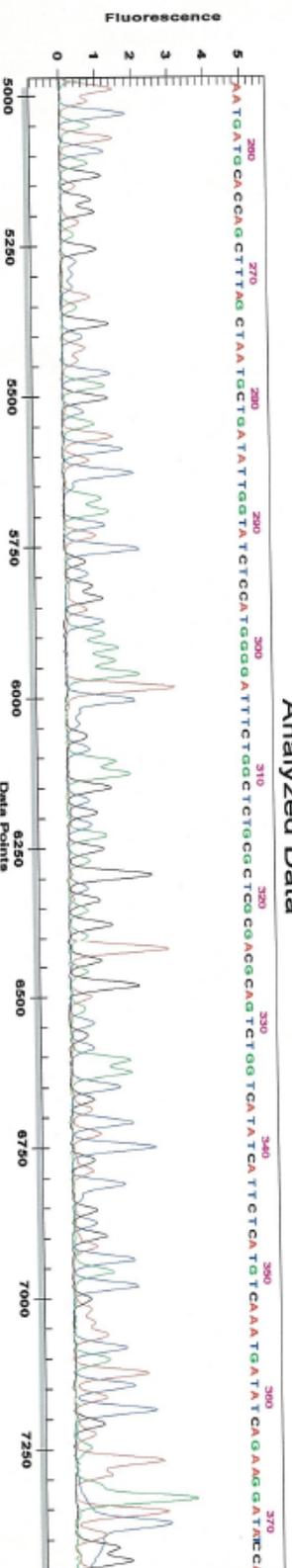
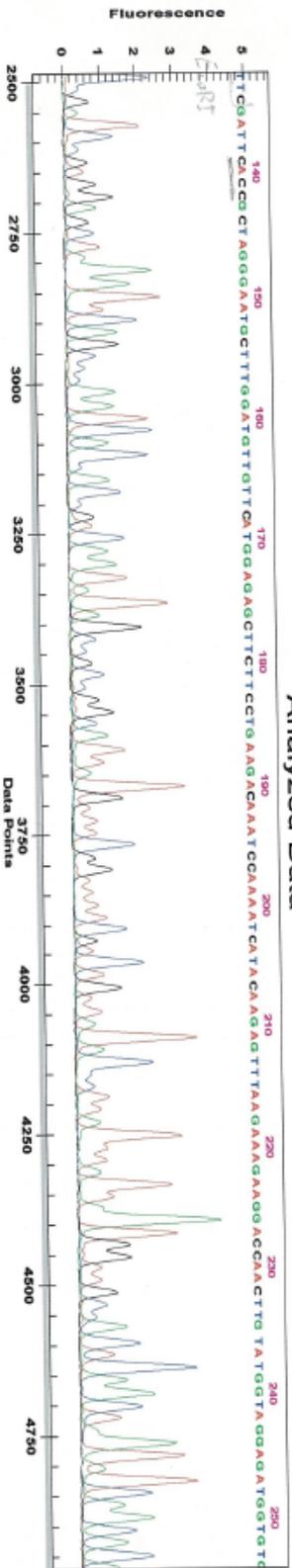
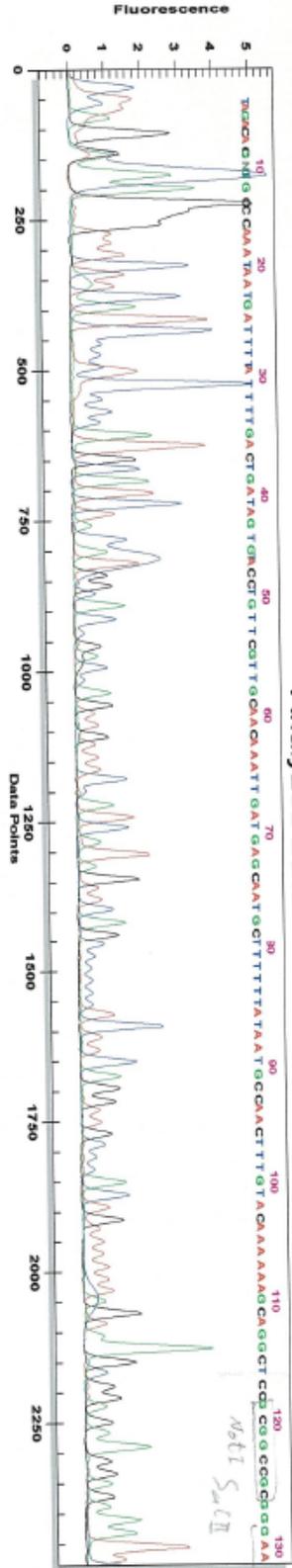


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8.2.6 Sequence data of the *T. caerulescens* HMA4 gene within the TOPO vector sequence using the HMA4 left primer and the plasmid terminator primer

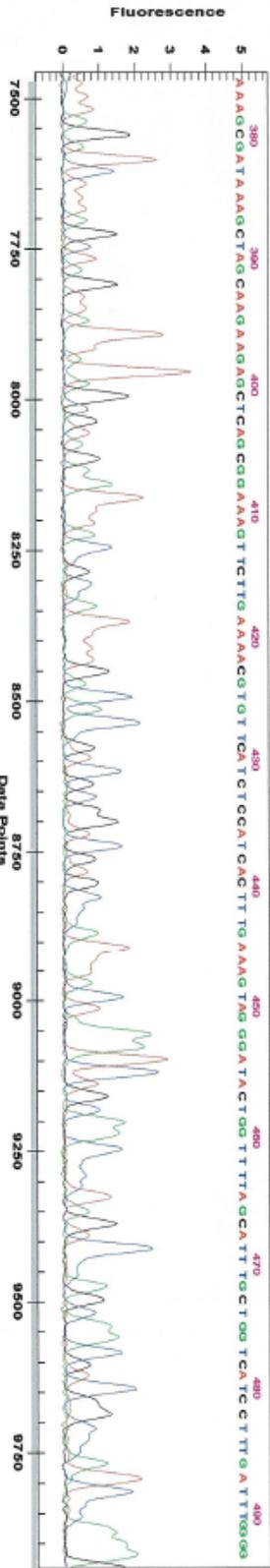


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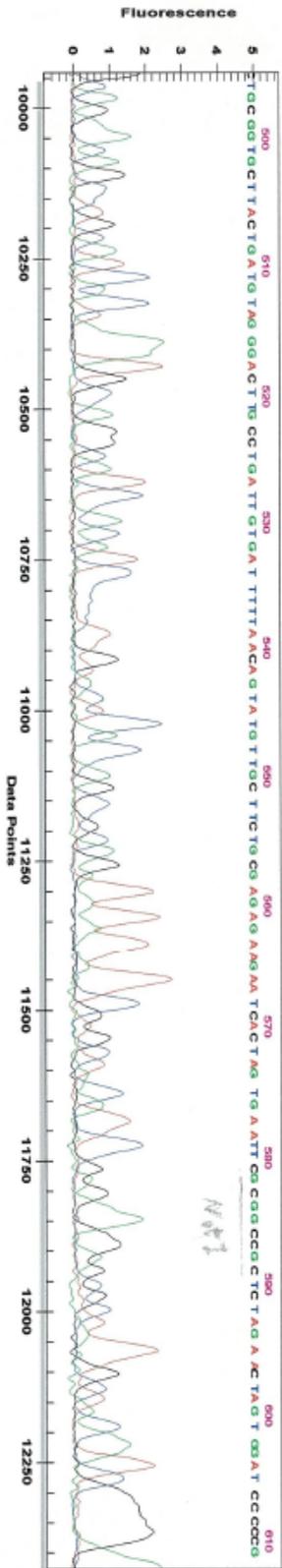
System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)

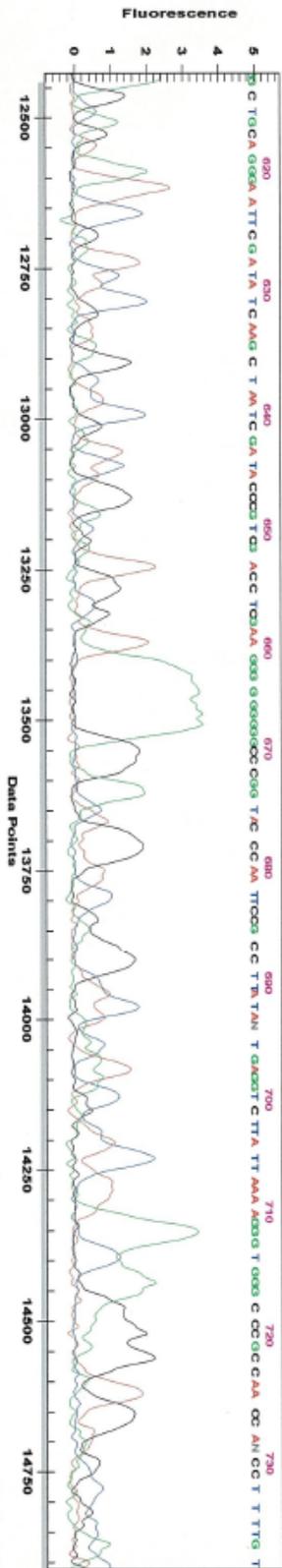
Analyzed Data



Analyzed Data



Analyzed Data



Thu 06/23/05 16:57:10

8.2.6 Sequence data of the *T. caerulescens* HMA4 gene within the TOPO vector sequence using the HMA4 left primer and the plasmid terminator primer

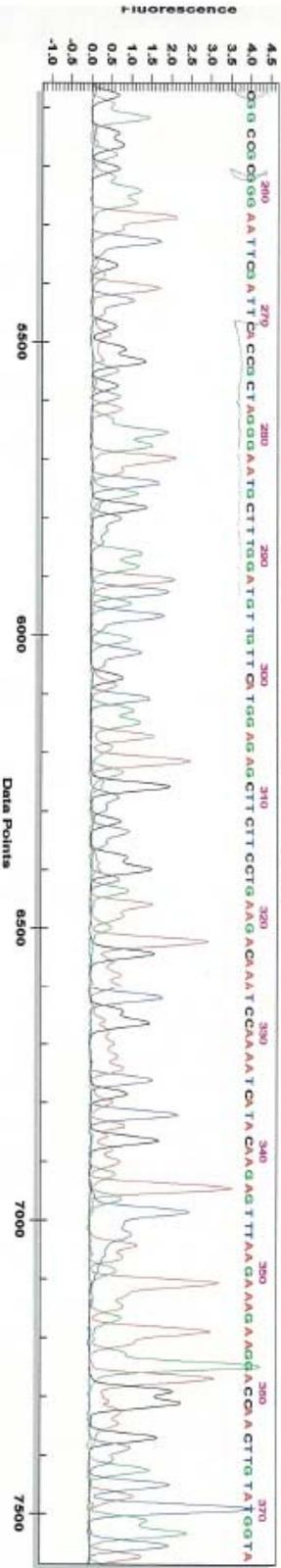
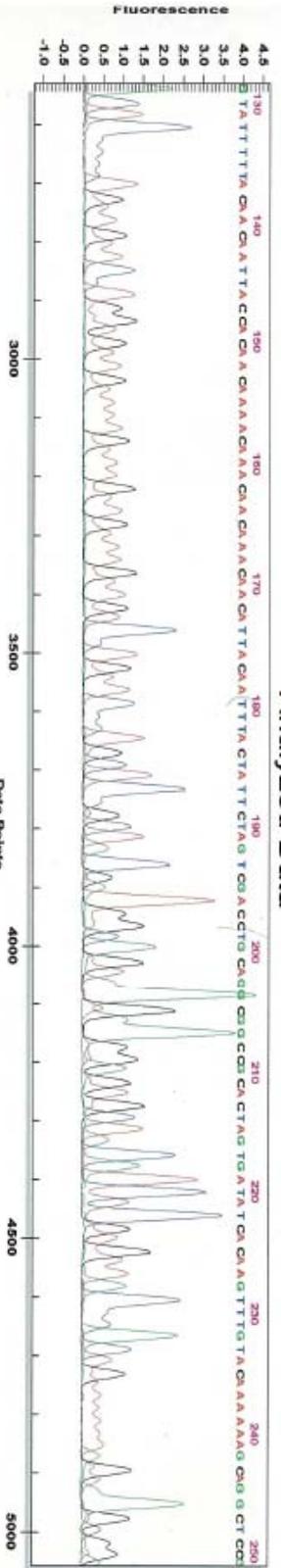
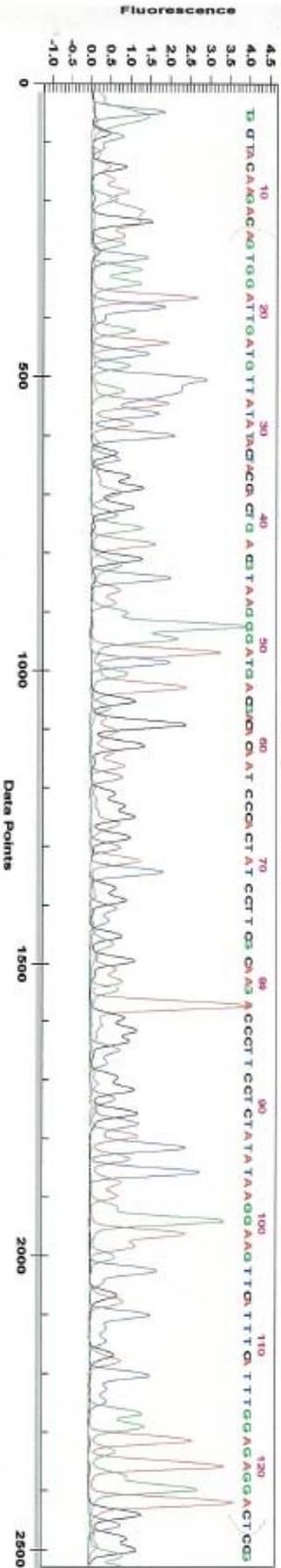


Project : Default
 Sample : PK7G2,PK735ST.E01_05072613FS
 Result : PK7G2,PK735ST.E01_05072613FS

35SP
 355

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



je 07/26/05 15:13:11

8.2.7 Sequence data of the *T. caeruleus* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 left primer and the plasmid 35S promoter primer

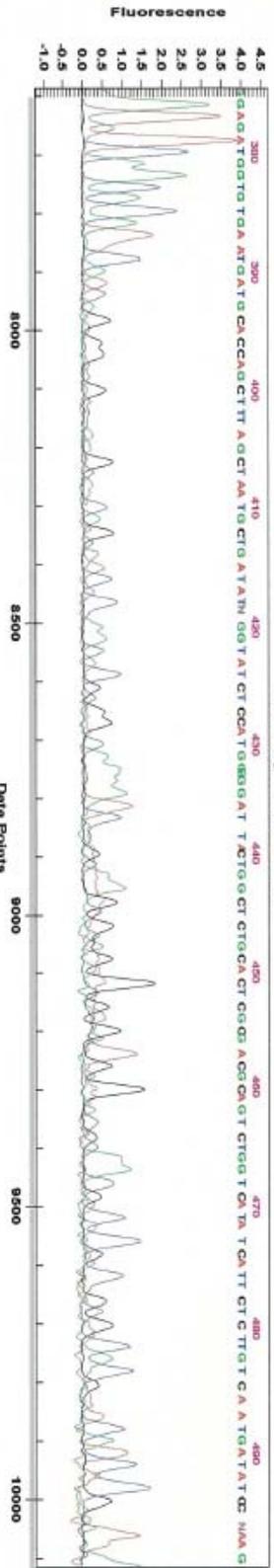


Project : Default
 Sample : PK7G2,PK7355T.E01_05072613FS
 Result : PK7G2,PK7355T.E01_05072613FS

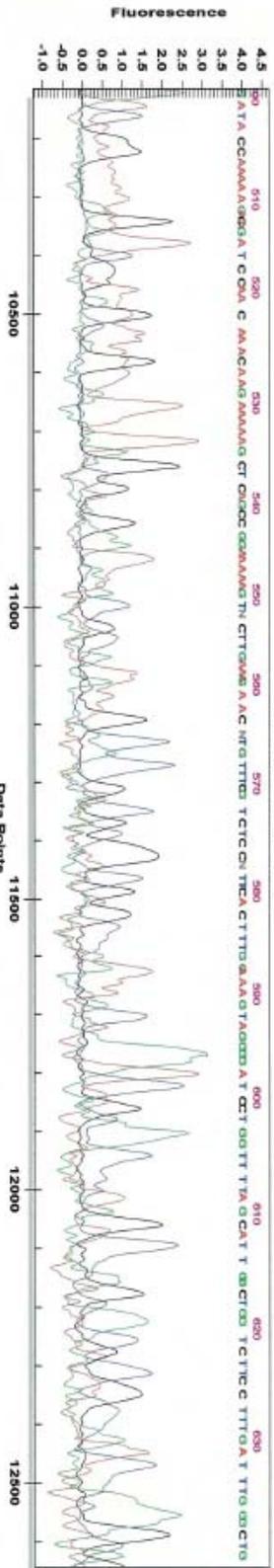
System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)

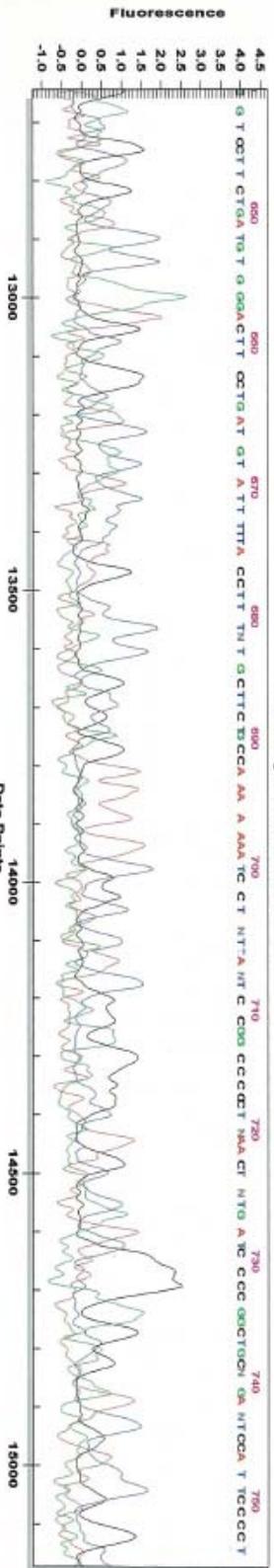
Analyzed Data



Analyzed Data



Analyzed Data



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8.2.7 Sequence data of the *T. caerulescens* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 left primer and the plasmid 35S promoter primer

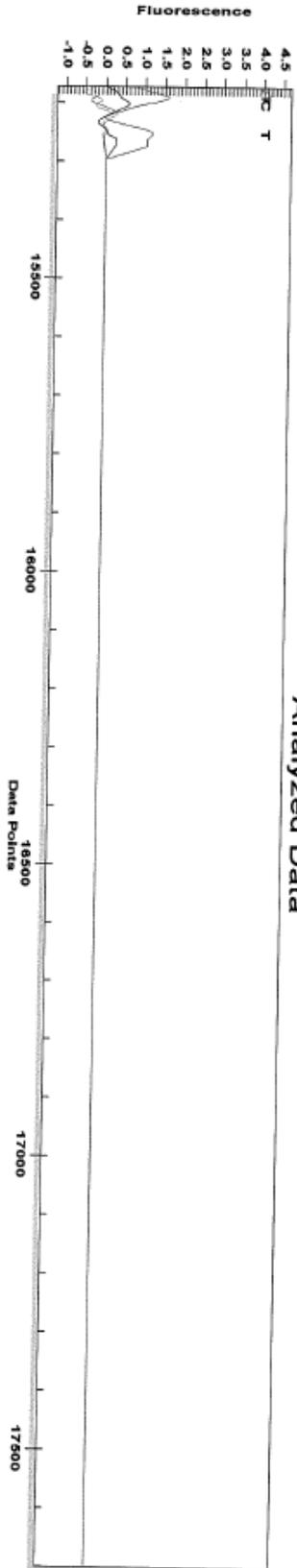


Project : Default
Sample : PK7G2,PK735ST.E01_05072613FS
Result : PK7G2,PK735ST.E01_05072613FS

System : CEQ System 3066768

Operator :
Instrument : CEQ System 3066768 (Ver. 8.0.52)

Analyzed Data



Tue 07/26/05 15:13:11

8.2.7 Sequence data of the *T. caeruleus* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 left primer and the plasmid 35S promoter primer

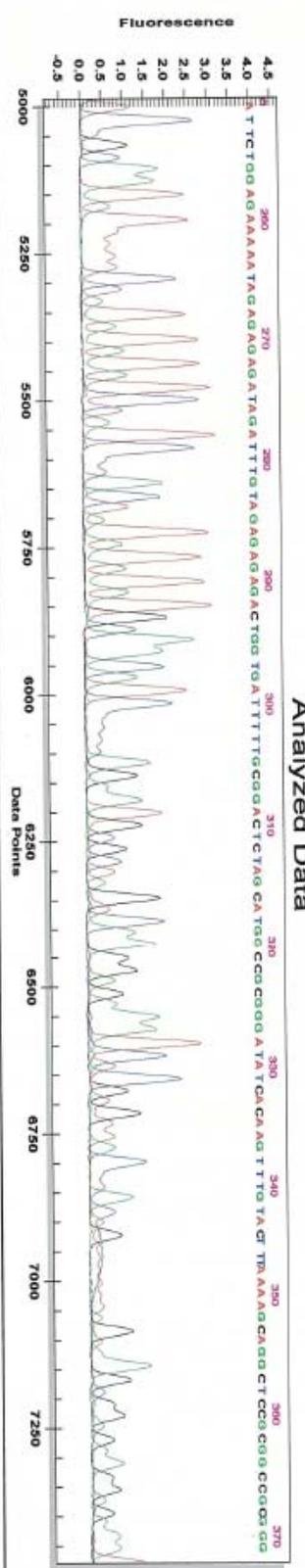
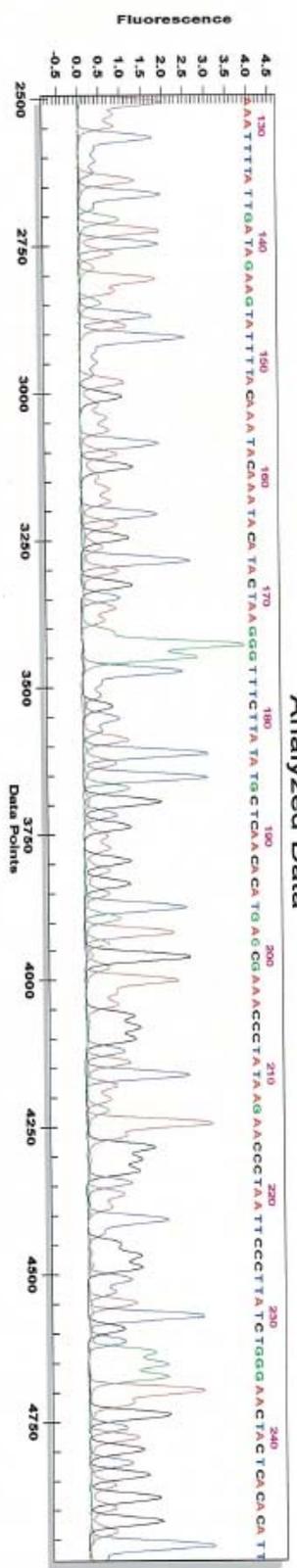
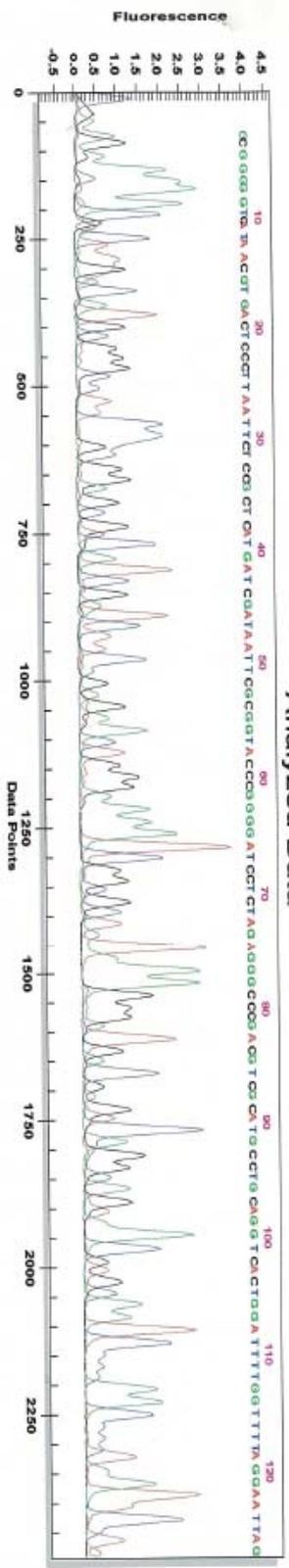


Project : Default
 Sample : PK7G2,PK735ST.F01_05072613FS
 Result : PK7G2,PK735ST.F01_05072613FS

355T

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



Tue 07/26/05 15:13:19

8.2.8 Sequence data of the *T. caerulescens* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 right primer and the plasmid 35S terminator primer

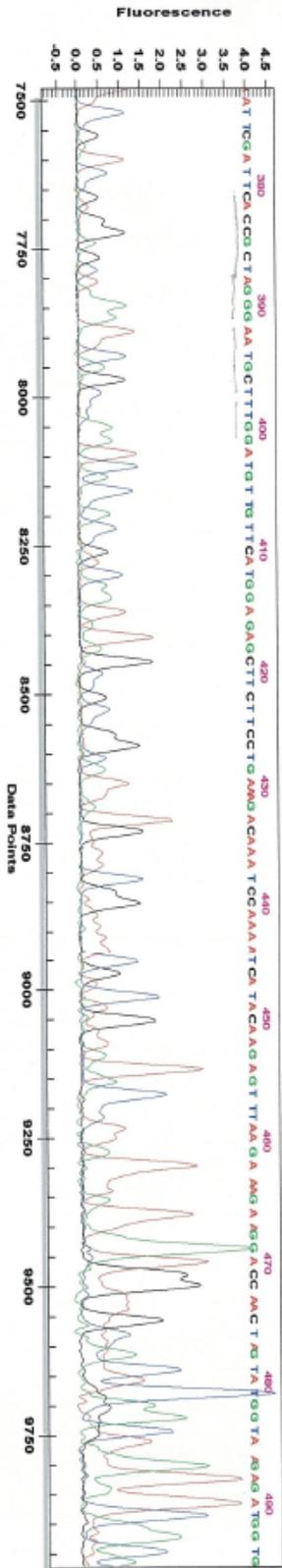


Project : Default
 Sample : PK7G2,PK735ST.F01_05072613FS
 Result : PK7G2,PK735ST.F01_05072613FS

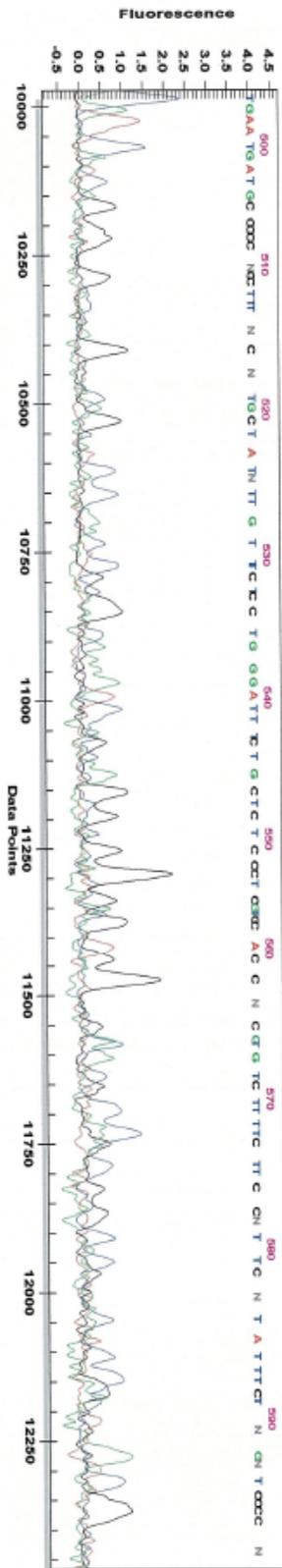
System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)

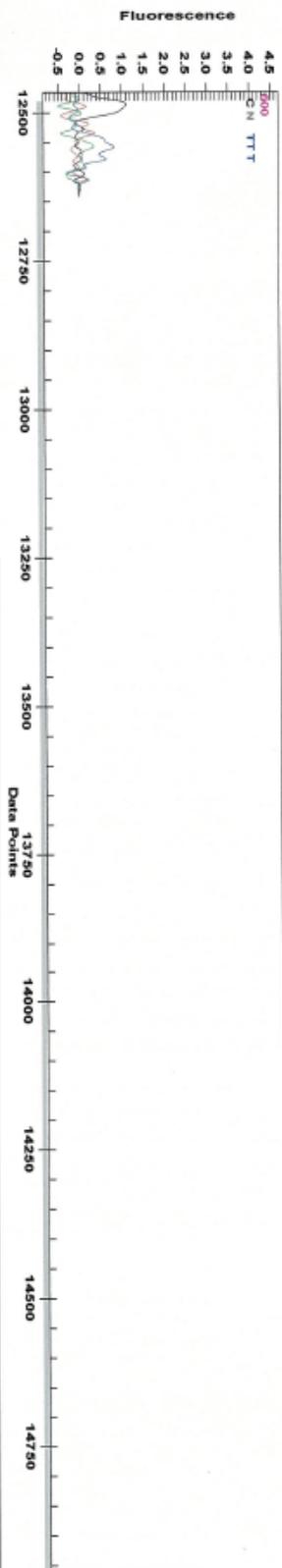
Analyzed Data



Analyzed Data



Analyzed Data



Tue 07/26/05 15:13:19

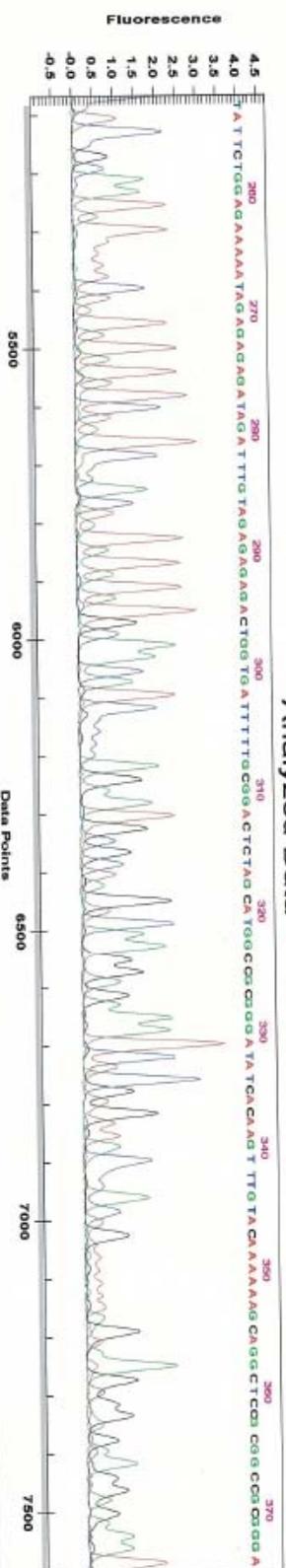
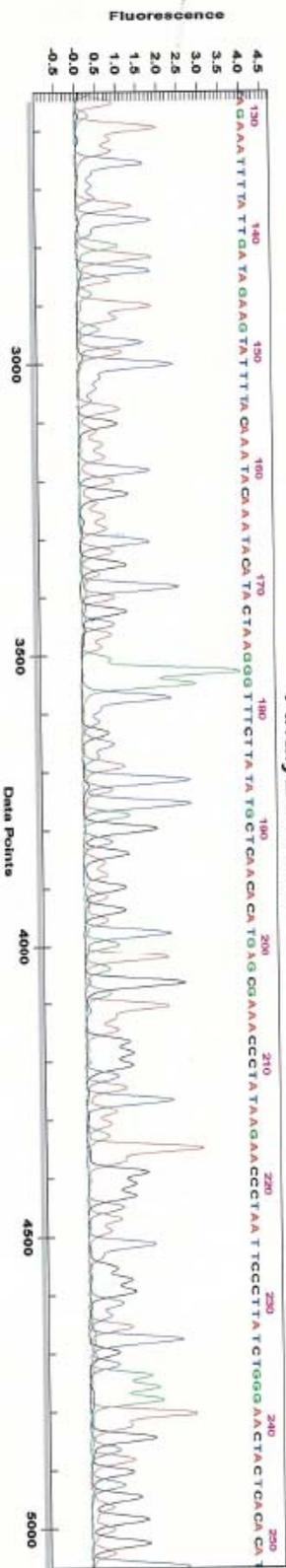
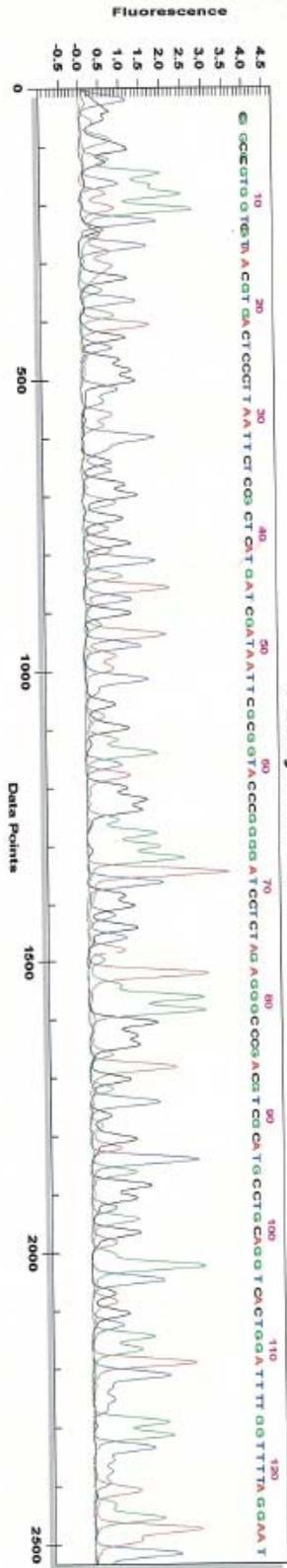
8.2.8 Sequence data of the *T. caerulescens* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 right primer and the plasmid 35S terminator primer



Project : Default
 Sample : PK7G4,PK735ST_A02_05081113Q2
 Result : PK7G4,PK735ST_A02_05081113Q2

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



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8.2.9 Sequence data of the *T. caerulescens* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 right primer and the plasmid 35S terminator primer (Repeat of previous this chosen as final)

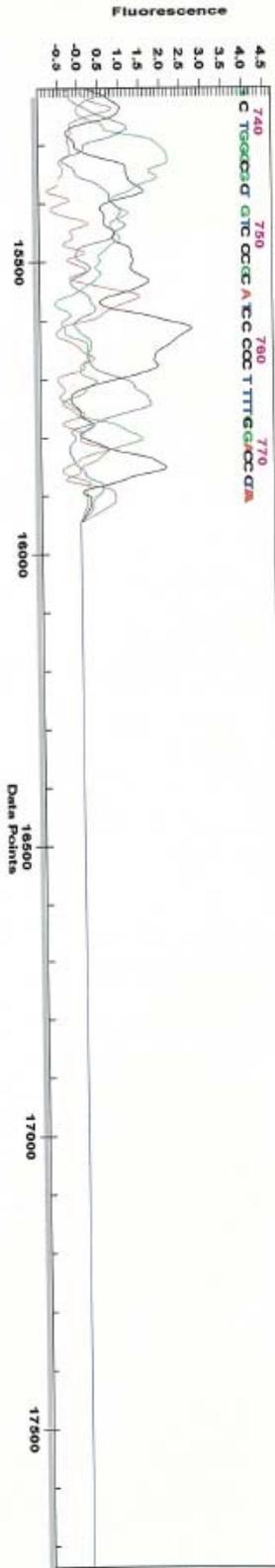


Project : Default
Sample : PK7G4,PK735ST.A02_05081113Q2
Result : PK7G4,PK735ST.A02_05081113Q2

System : CEQ System 3066768

Operator :
Instrument : CEQ System 3066768 (Ver. 8.0.52)

Analyzed Data



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8.2.9 Sequence data of the *T. caerulescens* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 right primer and the plasmid 35S terminator primer (Repeat of previous this chosen as final)

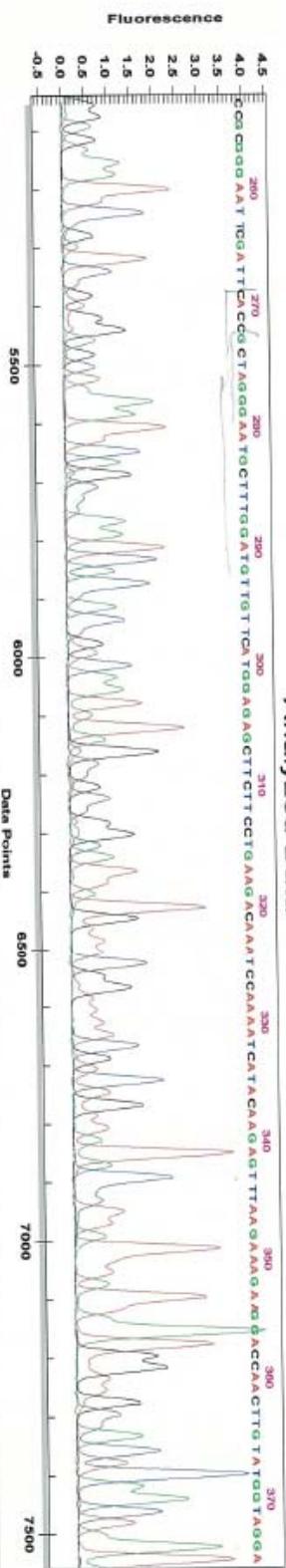
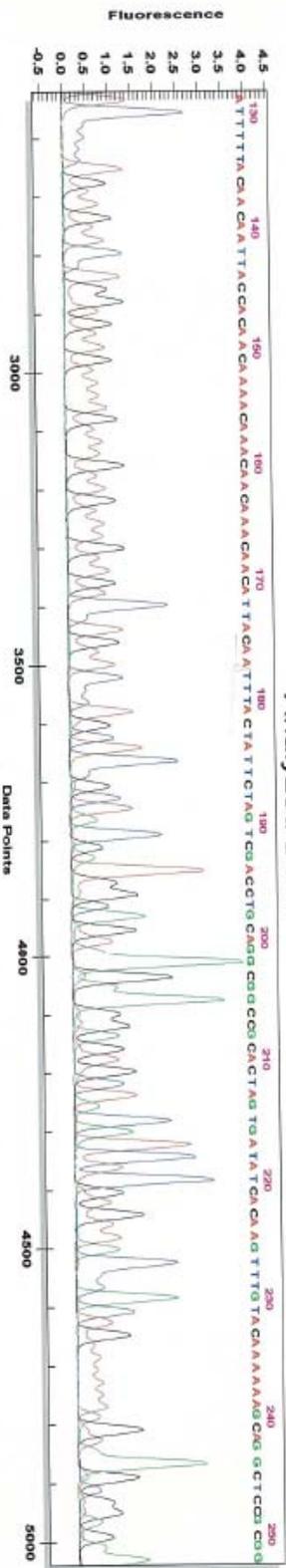
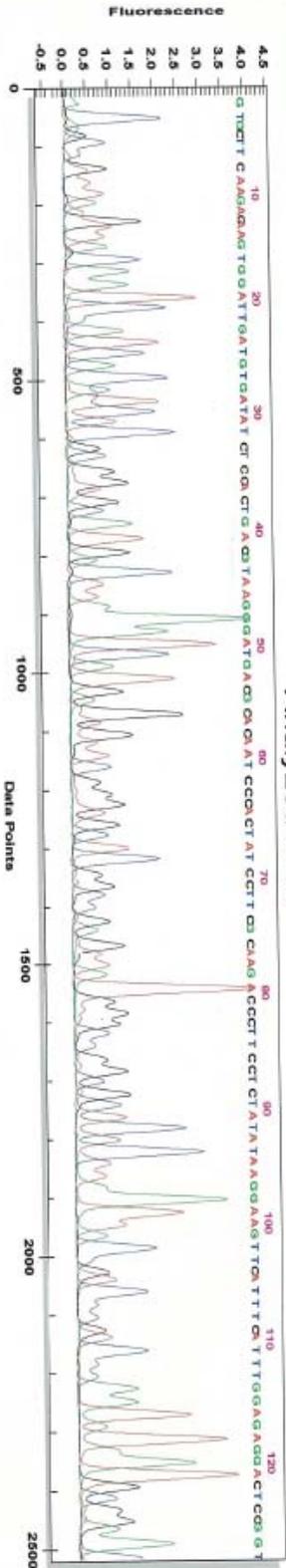


Project : Default
 Sample : PK7G4,PK735SP.H01_05081111YD
 Result : PK7G4,PK735SP.H01_05081111YD

System : CEQ System 3066768

Instrument : CEQ System 3066768 (Ver. 8.0.52)

Operator :



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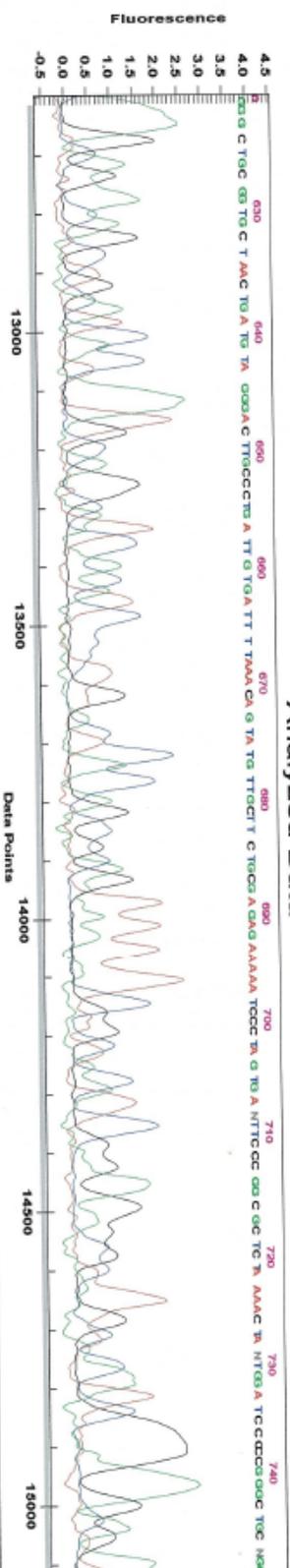
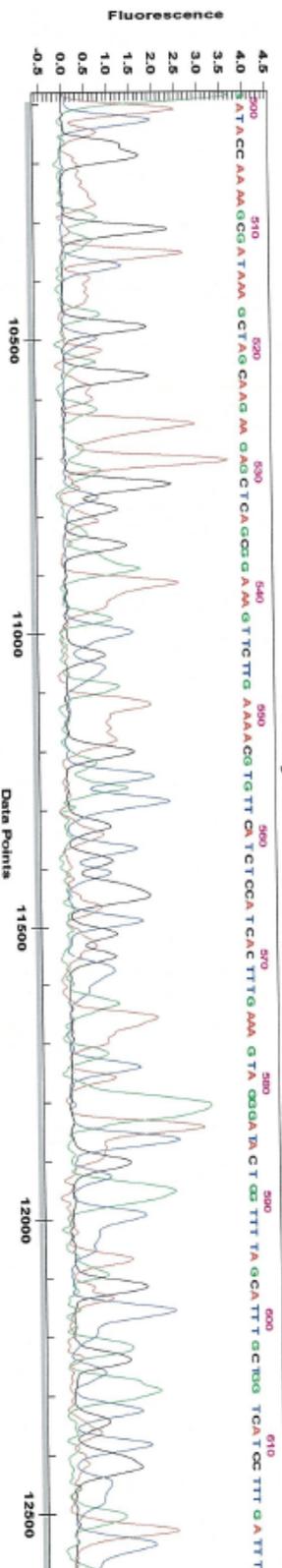
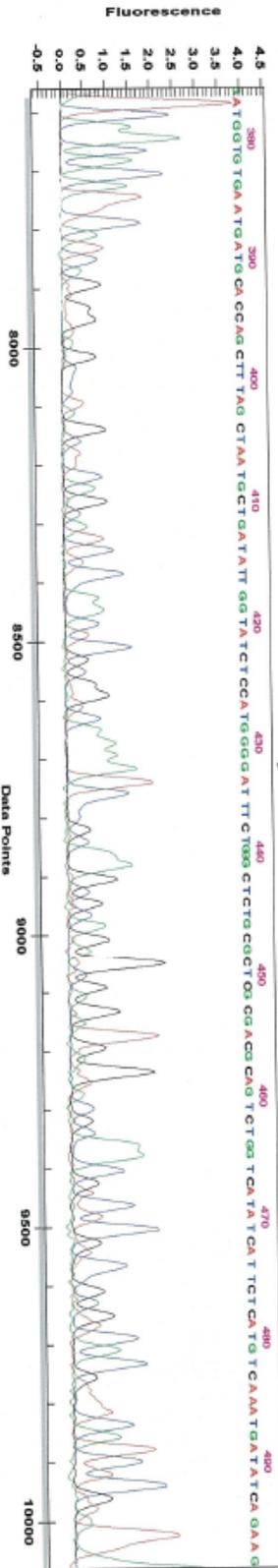
8.2.10 Sequence data of the *T. caeruleus* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 left primer and the plasmid 35S promoter primer (Repeat of previous this chosen as final)



Project : Default
 Sample : PK7G4,PK735SP.H01_05081111YD
 Result : PK7G4,PK735SP.H01_05081111YD

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)



Thu 08/1/05 13:44:35

8.2.10 Sequence data of the *T. caeruleus* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 left primer and the plasmid 35S promoter primer (Repeat of previous this chosen as final)

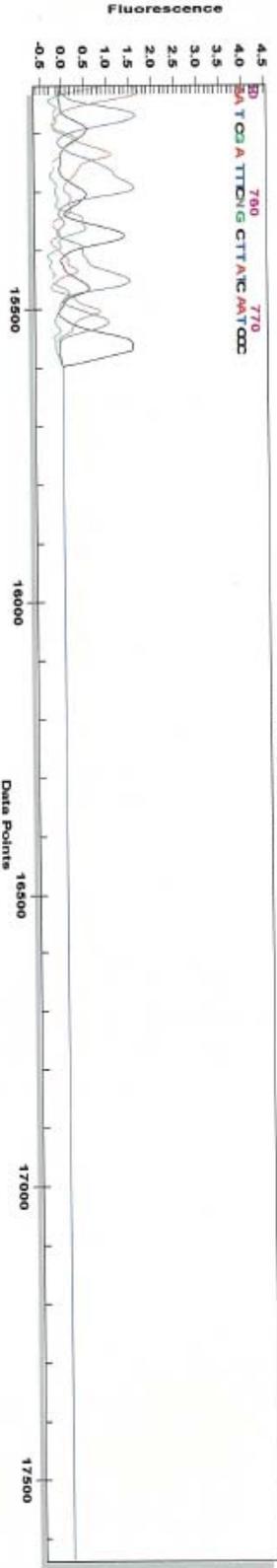


Project : Default
Sample : PK7G4,PK735SP,H01_05081111YD
Result : PK7G4,PK735SP,H01_05081111YD

System : CEQ System 3066768

Operator :
Instrument : CEQ System 3066768 (Ver. 8.0.52)

Analyzed Data



Thu 08/11/05 13:44:35

8.2.10 Sequence data of the *T. caeruleus* HMA4 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HMA4 left primer and the plasmid 35S promoter primer (Repeat of previous this chosen as final)

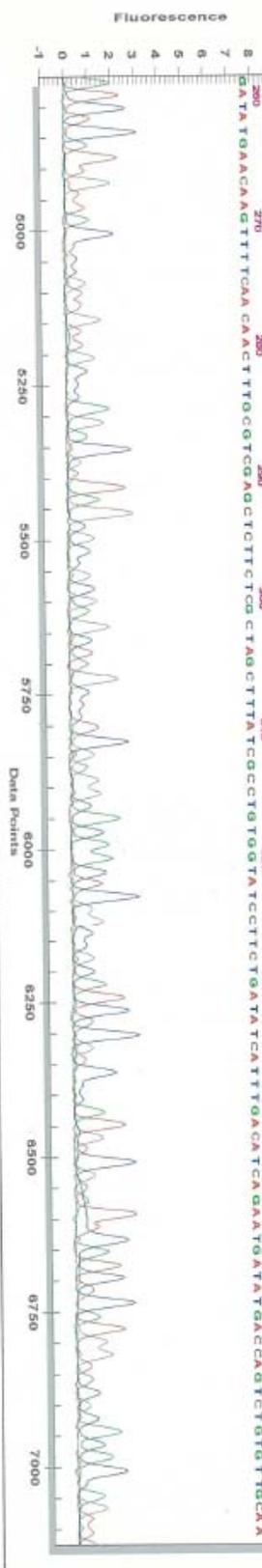
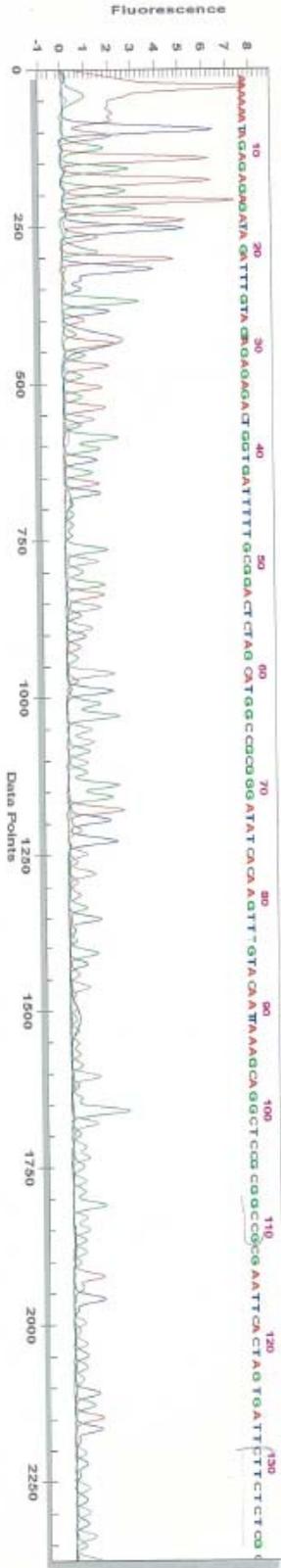


Project : Default
 Sample : PK7W8,PKFTSZTF.B01_05092011B
 Result : PK7W8,PKFTSZTF.B01_05092011B

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)

Analyzed Data



Tue 09/20/05 13:20:01

8.2.11 Sequence data of the *T. caerulescens* HM44 gene within the final destination vector, within the *Agrobacterium* cells sequenced using the HM44 right primer and the plasmid 35S promoter primer



Project : Default
 Sample : PK7W8,PK7FTSZTF.B01_05092011IB
 Result : PK7W8,PK7FTSZTF.B01_05092011IB

System : CEQ System 3066768

Operator :
 Instrument : CEQ System 3066768 (Ver. 8.0.52)

