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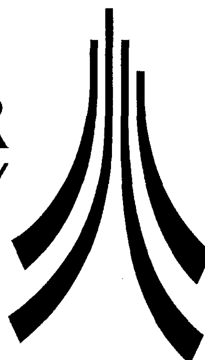

Molecular basis of Calcicole-Calcifuge physiology

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A thesis submitted to Lancaster University for the degree of Doctor of Philosophy

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Declaration

I declare that this thesis and work therein are entirely my own work, unless otherwise stated, and has not been submitted for an award of a higher degree elsewhere. The Ca^{2+} content analysis described in Chapter 3 was performed in collaboration with Mrs. Sue Pritchard.

Sunil C. Cherukuri

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Abstract

Calcium (Ca^{2+}) is an essential plant nutrient that plays a key role in plant growth and development and an essential second messenger that controls a variety of cellular functions. It also plays a role in cellular regulation of plant cell signaling. Plants rely on the unique properties of Ca^{2+} for a range of structural, enzymatic, and signaling functions. Of the many nutrients essential for normal growth and development of plants, Ca^{2+} occupies a unique position both chemically and functionally. Soils vary widely in their Ca^{2+} content and therefore, adaptations shown by plants, mainly to this factor, have led to the emergence of two very distinct ecological divisions termed as calcicoles (grow on calcareous soils) and calcifuges (grow on acidic soils). As excess Ca^{2+} is toxic to plants the sequestering of incoming Ca^{2+} as Ca^{2+} oxalate in epidermal trichomes has been studied in calcicoles from high Ca^{2+} enriched environments (calcareous soils). However, much less is known about the molecular basis of adaptation to this ecological phenomenon as well as the morphological adaptation patterns of calcicoles and calcifuges in response to varied levels of rhizospheric Ca^{2+} .In this study, six ecotypes of *A. thaliana*, comprising two putative wild calcifuges (Glenisla and Penicuik), two putative wild calcicoles (Halifax and Elland) collected from varyingly calcareous regions around UK, and two lab grown ecotypes (Col-4 and Cal-0) were subjected to varied concentrations of Ca^{2+} stress under *invitro* conditions and a difference in growth and morphology of these ecotypes was measured.

Also, DNA microarray analysis was utilized to detect genes that are regulated by Ca^{2+}. A differed pattern in the root growth was observed in all the ecotypes at varied external Ca^{2+} application ranging from 0 mM to 30 mM. The primary root structure and growth differed markedly in Glenisla plants grown at 30 mM Ca^{2+}. TUNEL assay determined the occurrence of apoptosis in the same Glenisla roots. Also, ten repressed Calcium-responsive genes in Cal-0 (Cal-CRGs) at high Ca^{2+} were investigated in all the six ecotypes using Real time RT-PCR, demonstrating their differed expression patterns in putative calcicoles to putative calcifuges. Analysis of SALK T-DNA knockout *Cal-CRG5* and *Cal-CRG17* lines, revealed the potential role of repression of these genes in rhizospheric Ca^{2+} tolerance.

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Abbreviations

^{o}C	degree Celsius
Ca^{2+}	Calcium
$[Ca^{2+}]_{cyt}$	Cytosolic free calcium concentration
$[Ca^{2+}]_{ext}$	Extracellular free calcium concentration
μg	micro gram
μl	Micro litre
ABA	Abscisic acid
ABF	ABRE binding factor
ABRE	Abscisic acid (ABA)-responsive element
ACC	1-aminocyclopropane- 1-carboxylic acid
ACD	Accelerated cell death mutant
AOX	Alternative oxidase
apCAX	*Aphanothece* H^{+}/Ca^{2+} antiporter
APX	Ascorbate Peroxidase
AtCAX	*Arabidopsis thaliana* H^{+}/Ca^{2+} antiporter
AtKH1	*Arabidopsis thaliana* histidine kinase-1
ATP	Adenosine triphosphate
AtSWI3	*Arabidopsis thaliana* encoding SWI3 proteins (SWITCH/SUCROSE NONFERMENTING (SWI/SNF) - like chromatin-remodelling complexes)
bp	Base pair
bZIP	Basic leucine zipper transcription factor
cADPR	Cyclic adenosine 5'-diphosphoribose

CAX	Calcium exchanger
CBL	Calcineurin-B-like-protein
CBF	CRT (C-repeat)/DRE (drought-responsive element) binding factor
cDNA	Complementary DNA
CDPK	Calcium-dependant protein kinase
CICR	Calcium-induced calcium release
CNGC	Cyclic nucleotide-gated channel
COR	Cold-responsive protein
CRG	Calcium-responsive gene
Ct	Cycle number when threshold reached
conc.	Concentration
DACC	Depolarisation-activated Ca^{2+} channel
dATP	deoxyadenosine triphosphate
dChip	DNA-Chip analyser
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DREB	Dehydration-responsive element binding
dTTP	deoxythymidine triphosphate
ECA	ER-type calcium ATPase
EDTA	ethylenediamine tetra acetic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag

GARNet	Genome Arabidopsis resource network
GDP	Guanosine diphosphate
GLRs-	Glutamate receptors
GO	Gene Ontology
HACC	Hyperpolarisation-activated Ca^{2+} channel
HCl	Hydrochloric acid
Hsp	Heat shock protein
IAA	Indole acetic acid
ICE	Inducer of CBF expression
IP_3	Inositol 1,4,5-triphosphate
IP_6	Inositol hexakiphosphate
JA	Jasmonic acid
kb	kilo base (pair)
LEA	Late embryogenesis abundant
M	molar (conc.)
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
Mb	Mega bases
mg	Milligram
mM	Mill molar
MIPS	Martinsreid institute for protein sequences
mRNA	Messenger RNA
NAADP	Nicotinic acid adenine dinucleotide phosphate
NaCl	sodium chloride

NADPH	Nicotinamide adenine dinucleotide phosphate
NASC	Nottingham Arabidopsis stock centre
ng	nano gram
PCD	Programmed cell death
PCR	polymerase chain reaction
pH	puissance *de* hydrogen (ion conc.)
PI	Propidium iodide
PLD	Phospholipase D
ppm	parts per million
PtdOH	Phosphatidic acid
PX	Peroxidase
qRT-PCR	Quantified reverse transcription polymerase chain reaction
RAPD	random amplified polymorphic DNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RFLP	restriction fragment length polymorphism
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard Error of Mean
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SOS	Salt overly sensitive
SP1	Stable protein 1
SSC	Standard saline citrate
SV	Slow-acting vacuolar channel

synCAX	*Synechocystis* H^+/Ca^{2+} antiporter
TAE	Tris acetate EDTA buffer
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris borate EDTA buffer
T-DNA	Transfer DNA
TE	Tris EDTA buffer
TEMED	N, N, N', N'-tetra methyl ethylene diamine
Tm	Melting temperature
TPC	Two-pore calcium channel
Tris	tris (hydroxy methyl) amino methane
uv	ultra violet
V	volts
VICC	Voltage-independent calcium channel
Wt	Wild type

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Chapter 1

General Introduction

1.1 Research into Plant Stress Biology

The perseverance of a plant community at a given location is dependent upon the interaction of plant and environment throughout the whole of the life-cycle, at stages of germination, establishment, vegetative growth, flowering and the setting of viable seeds (Clapham, 1956). Plant growth and development is controlled by hormonal and environmental signals (Finkelstein and Gibson, 2002). Under most favourable conditions, an optimal assessment for metabolism is homeostasis, and it is seldom achieved by plants because of the influence exerted by external stress factors: e.g. climatic, biotic, and nutrient imbalances (Bohnert *et al.*, 2006). Plants have an outstanding ability to cope with highly variable environmental stresses (*i.e.* abiotic stress), including cold (Chinnusamy *et al.*, 2006), drought (Chaves *et al.*, 2002), and soils with changing salt and nutrient concentrations (Grubb, 1998). Plants, unlike animals, are immobile and therefore have developed mechanisms to sense and respond to these biotic and abiotic stresses so that they can be adapted better to their environment (Reddy, 2001).

Environmental stress is a dominant force influencing the evolution of plant populations in nature as well as a key factor restricting economic yields, with abiotic stresses being the primary cause of crop loss worldwide (Boyer, 1982), reducing average yields for most major crop plants by more than 50% (Bray et al., 2000). In contrast, the estimated yield loss caused by pathogens in major crops is typically around 10% to 20% (Baker, 1997). Environmental stresses in plants result from changes in growth condition(s), within the plant's

natural habitat, that alter or disrupt its metabolic homeostasis (Shulaev, 2008). Such change(s) in growth condition requires an adjustment of biological pathways, intended at achieving a new status of homeostasis, in a progression that is usually referred to as acclimation (Mittler 2006; Suzuki and Mittler, 2006). It also has long been established that the plausible explanation of the ways in which a plant species or community interacts with its environment in nature can most beneficially be investigated by various experimental methods *in vitro* (Clapham, 1956).

Plants encounter a wide range of environmental hurdles during a typical life cycle and have evolved mechanisms by which to increase their tolerance to these through both physical adaptations and interactive molecular and cellular changes that begin after the onset of stress (Bohnert *et al.*, 1995). The earliest step in switching on such molecular responses is to perceive the stress as it occurs and to relay information about it through a signal transduction pathway (Shinozaki and Yamaguchi-Shinozaki, 1997). Plants also utilize diverse environmental signals, such as light, temperature and water availability, to regulate the normal processes of growth and development (Shinozaki and Yamaguchi-Shinozaki, 1996). Plants counter environmental stresses at cellular and molecular levels, as well as at physiological levels, so as to confer tolerance of the stress and ensure survival (Shinozaki and Dennis, 2003). The expression levels of many plant genes change in response to environmental signals. Molecules including proteins, hormones and second messengers function in signal transduction pathways during these responses (Figure 1.1; Shinozaki and Dennis, 2003).

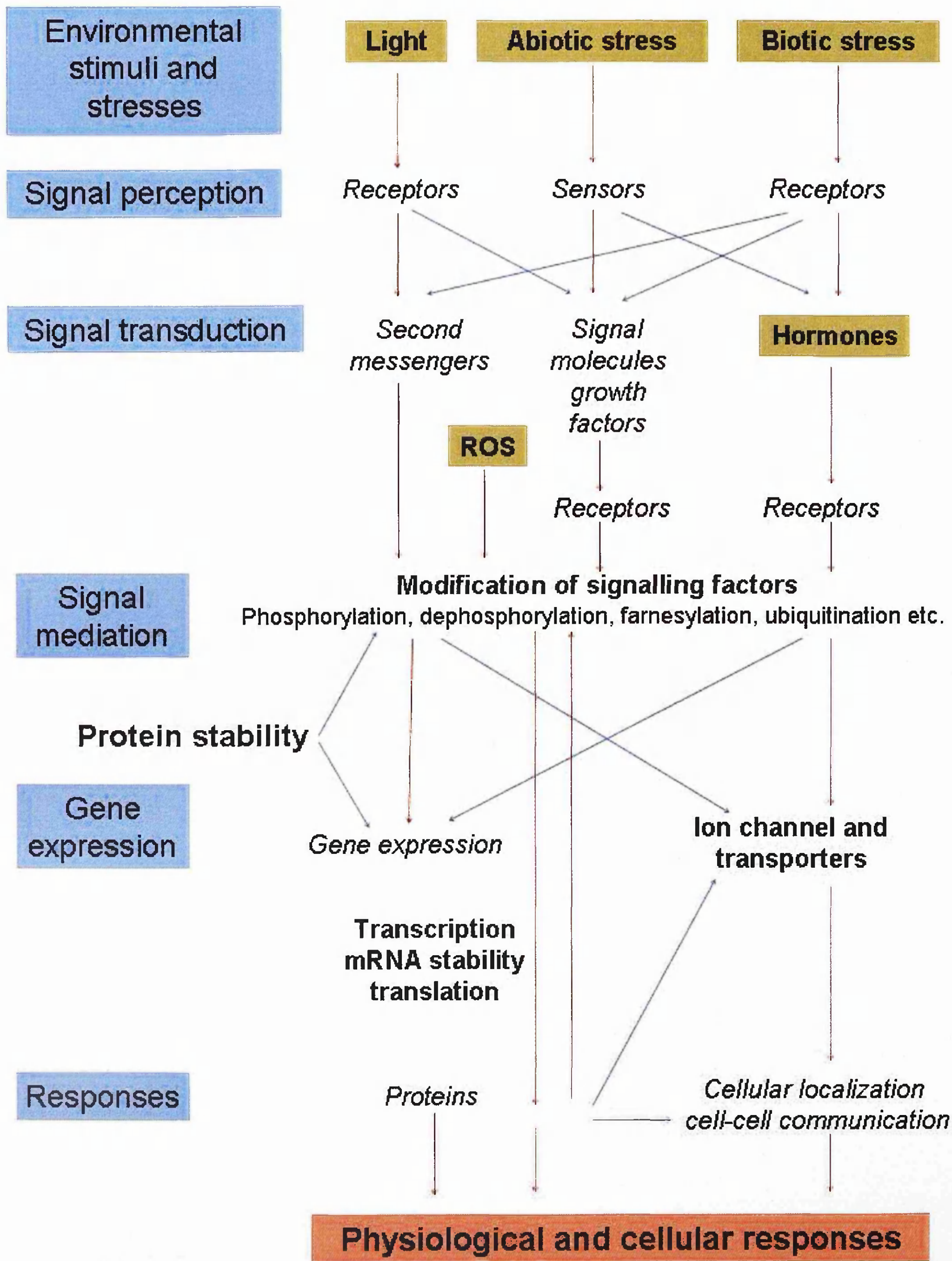


Figure 1.1: Schematic description of signal transduction pathways in plants in response to allied stresses that begin with the initial process of signal perception and end with terminal events, such as the alteration of gene expression and physiological responses (Adapted from Shinozaki and Dennis, 2003).

Signaling processes use linear pathways that begin with the initial process of signal perception and end with terminal events, such as the alteration of gene expression and physiological responses (Figure 1.1; Shinozaki and Dennis, 2003). Modification of protein factors by phosphorylation, dephosphorylation, farnesylation and ubiquitination are also important events in signaling pathways. It also has also been recognised that the stability of both signaling factors and transcription factors (TFs) is important for the regulation of signal transduction, gene expression and phase transition during development. Important regulatory roles for proteosomal destabilisation have been found in hormone, light and stress signaling processes (Figure 1; Shinozaki and Dennis, 2003). Moreover, posttranscriptional regulation at the RNA level plays an important role in various signaling pathways, such as abscisic acid (ABA) and waterstress signalling (Shinozaki *et al.*, 2003). These pathways eventually lead to physiological changes, such as guard cell closure, or to the expression of genes and resultant modification of molecular and cellular processes (Knight and Knight, 2001). Plants have to cope with a continuously changing environment, which challenges their ability to adapt to a variety of stress conditions. Adaptation to poor growing conditions can be achieved by a pre-existing anatomical trait or physiological behaviour (Loretti *et al.*, 2005).

Alternatively, a rapid change in gene expression triggered by the stressful episode leads to an adaptive response (Loretti *et al.*, 2005). The development or appearance of tolerance to stress triggers a complex set of cellular interactions that ultimately result in altered expression patterns of numerous genes involved in the adaptive responses (Ruiz, 2001). It has been put forward that such morphogenic responses by plants are part of an

acclimation strategy that constitutes the 'flight' response of plants analogous to the 'flight-or-fight' response displayed by animals to defend stressful situations (Potters *et al.*, 2007). Also, genetic effects of environmental stress have been comprehensively studied from the perspective of cellular physiology (Chen and Zhu, 2004) and increasingly, molecular biology (Denby and Gehring, 2005; Vinocur and Altman, 2005; Pellegrineschi *et al.*, 2004).

Plants are known to respond by initiating adaptive responses that are necessary to alleviate primary and secondary effects caused by abiotic stresses for their regular growth and development (Zhu, 2003, 2002; Hasegawa *et al.*, 2000; Thomashow, 1999). Though the precise stress sensory mechanisms have yet to be determined it has been known that expression levels of many plant genes change in response to environmental signals and it is apparent that stress perception leads to activation of signal transduction pathways that manage adaptive responses (Liu *et al.*, 2005, 1998; Zhu, 2003, 2002, 2001; Xiong and Zhu, 2001; Stockinger *et al.*, 1997). These signaling pathways and their target genes have been at the forefront of intense research (Koiwa *et al.*, 2006), but it has been a daunting task to dissect individual gene functions due to the overlapping etiologies of abiotic stress responses and thus, the involvement of numerous signaling systems (Costa *et al.*, 2007; Seki *et al.*, 2003, 2002; Xiong and Zhu, 2001). It is likely that abiotic stress-responsive signal pathways are connected to growth and development pathways and are modulated by hormonal signals that specify precise regulatory cues in these complex biological systems (Koiwa *et al.*, 2006). Also, dynamic transcriptome changes are assumed to play an important role in a beneficial management of different molecular and cellular

responses responsible for homeostasis in adaptation to multiple stressful conditions encountered by plants (Koiwa *et al*., 2006). Therefore, the susceptibility of plants to continuous stress from the environment has stimulated the evolution of a wide range of stress-resistance and tolerance mechanisms (Bohnert *et al*., 2006; Chen and Zhu, 2004; Mahalingam *et al*., 2003; Singh *et al*., 2002) the physiological basis of which has been pursued by the scientific community with the eventual goal of elucidating the biochemical pathways involved in stress perception, signal transduction, and adaptive responses (Swindell *et al*., 2006; Liu *et al*., 2005; Takashi *et al*., 2004). Though various stress responses are specific to the type of stress, some stress responses are general and potentially confer tolerance to multiple stresses (Chinnusamy *et al*., 2004; Kim *et al*., 2004). The genes involved in these responses are thought to provide clues as to which biochemical networks underlie stress resistance (Swindell *et al*., 2006).

Until very recently the strategy of molecular genetic approaches to stress tolerance studies in plants has been termed the 'candidate gene-by-gene' approach, aiming to dissect single genes in various pathways with the aim of determining their position in stress-response cascades and to determine their contribution to stress tolerance (Vinocur and Altman, 2005). However, another approach through functional genomics has enabled integrated analysis of stress-dependant behaviour by the whole plant (Bohnert *et al*., 2006). This strategy has been very useful in linking whole-plant physiology and phenotypic studies with information on gene complexes, transcription and transcript regulation, the behaviour of proteins, protein

complexes and pathways, evolutionary adaptive diversification, and mutated or engineered phenotypic characters (Bohnert *et al.*, 2006).

Resistance to abiotic stress is a multigenic trait; depending on the amalgamation of many genes, proteins and metabolic pathways all working in parallel (Wang *et al.*, 2003). Tolerance to abiotic stress has been accomplished both by genetic engineering and by conventional plant breeding combined with the use of molecular markers and quantitative trait loci (QTLs) (Vinocur and Altman, 2005). Figure 1.2 illustrates the probable factors involved in attained plant stress tolerance in plants that can be enhanced by manipulating stress-associated genes and proteins and by overexpression of stress-associated metabolites. Plant resistance to abiotic stress is a multigenic trait, depending on the combination of many genes, proteins and metabolic pathways all playing in tandem. Acquired plant tolerance to abiotic stress can be achieved both by genetic engineering and by conventional plant breeding combined with the use of molecular markers and quantitative trait loci (QTLs) (Vinocur and Altman, 2005).

Primary stresses, such as drought, salinity, cold, heat and chemical pollution, are interrelated and cause cellular damage and secondary stresses, such as osmotic and oxidative stress (Wang *et al.*, 2003; Serrano *et al.*, 1999; Smirnoff, 1998). The early stress signals (e.g. osmotic and ionic effects or changes in temperature or membrane fluidity) activate the downstream signaling process and transcription controls, which in turn activate stress-responsive mechanisms to re-establish homeostasis and to defend and repair damaged proteins and membranes (Knight and Knight, 2001; Cushman and Bohnert, 2000).

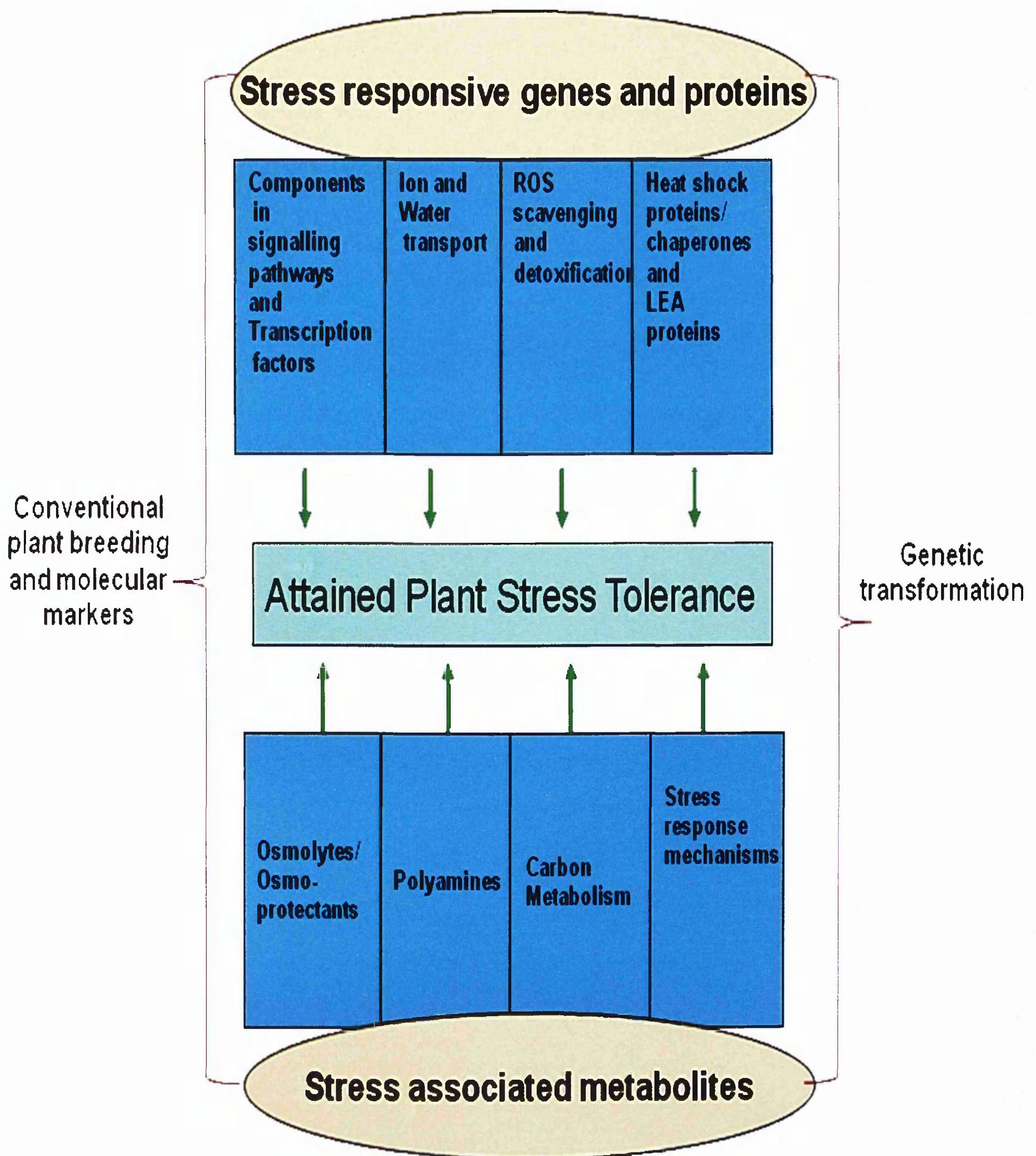


Figure 1.2: Acquired plant stress tolerance can be enhanced by manipulating stress-associated genes and proteins and by over expression of stress-associated metabolites (Adapted from Vinocur and Altman, 2005). LEA, late embryogenesis abundant; ROS, reactive oxygen species.

Responses at one or more steps in the signaling and gene activation process might ultimately result in irreversible changes in cellular homeostasis and in the destruction of functional and structural proteins and membranes, leading to cell death (Figure 1.3; Li *et al.*, 2007; Van Breusegem and Dat, 2006; Kuriyama and Fukuda, 2002). Figure 1.3 illustrates the cascades of molecular networks that control a plants adaptive strategy to combat environmental stresses.

Because of all these complexities involved in perceiving stress, a robust molecular genetic model plant system is required for functional categorization of adaptive response coordination. *Arabidopsis thaliana* has been proposed as an ideal plant system to study adaptive responses to abiotic stresses as, like all plants, it senses and responds adaptively to a multitude of stresses (Koiwa *et al.*, 2006; Zhu, 2002, 2003; Hasegawa *et al.*, 2000;), thus facilitating the use of its vast molecular and genetic attributes in a better understanding of the biological paradigm.

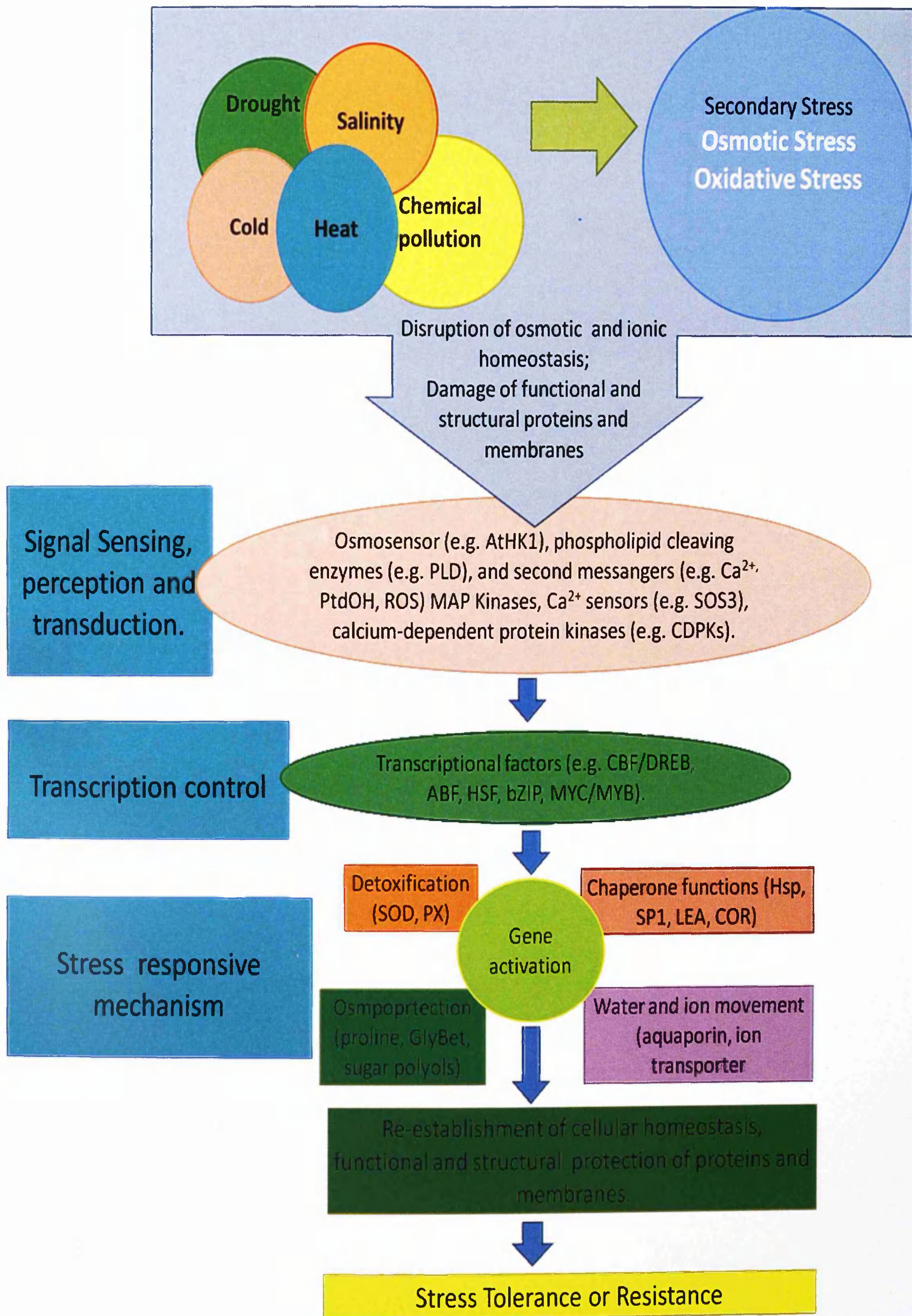


Figure 1.3: The complexity of the plant response to abiotic stress (Adapted from Wang *et al.*, 2003).

1.2 Calcium conundrum

1.2.1 Calcium in the cell

Calcium (Ca^{2+}) is an essential plant nutrient and an essential second messenger in both animals and plants that controls a variety of cellular functions (Hepler, 2005; Harper *et al.*, 2004; Hetherington and Brownlee, 2004; Reddy and Reddy, 2004; Berridge *et al.*, 2003; White and Broadley, 2003; Sanders *et al.*, 2002, 1999; Bush, 1995). It also has been established that Ca^{2+} plays a role in cellular regulation of plant cell signaling that closely compares with that in animal cells (Hepler, 2005). Broadly, Ca^{2+} has four main functions within plant cells; involvement in cell structure, electrical activity across membranes, intracellular signaling and as an enzyme cofactor (Gilroy *et al.*, 1987). Plants have evolved to rely on the unique properties of Ca^{2+} for a range of structural, enzymatic, and signaling functions (Hirschi, 2004). As the divalent cation (Ca^{2+}), it is essential for structural roles in the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol (Marschner, 1995). Ca^{2+} plays an essential role in both plant and animal growth and is involved in multiple signal transduction pathways (Wyatt *et al.*, 2002). It is also implicated in regulating a number of fundamental cellular processes that are involved in cytoplasmic streaming (Curt and West, 2007; Shimmen and Yokota, 1994), thigmotropism (Gow, 2004; Grabski *et al.*, 1998), gravitropism (Sinclair and Trewavas, 1997; Poovaiah and Reddy, 1993; Perdue *et al.*, 1988), cell division (Baran, 1996; Hepler, 1994), cell elongation (Cho and Hong, 1996), cell differentiation (Webb, 1999), cell polarity (Wymer *et al.*, 1997),

photomorphogenesis (Johnson *et al.*, 1995; Shacklock *et al.*, 1992), plant defence and stress responses (Lecourieux *et al.*, 2006). Ca^{2+} is also a required micronutrient and lack of Ca^{2+} can be detrimental to plant growth and development (Marshner, 1995). It is stored in organelles, e.g. endoplasmic reticulum (ER), vacuole, mitochondria and chloroplasts, and the cell wall (Sanders *et al.*, 2002).

As lack of Ca^{2+} leads to changes in organelle structure, alterations in membrane permeability and decrease in cell elongation, plants need Ca^{2+} for proper functioning (Hepler, 2005). Acting as a second messenger in plants, Ca^{2+} also mediates responses to a wide range of environmental stresses, like; salt, drought (Knight *et al.*, 1997), cold (Knight *et al.*, 1996), heat, light (Shacklock *et al.*, 1992) touch (Knight *et al.*, 1991), pathogenesis (Knight *et al.*, 1991) and oxidative stress (Evans *et al.*, 2005; Rentel and Knight, 2004; Clayton *et al.*, 1999; McAinsh *et al.*, 1996; Price *et al.*, 1994) and hormonal stimuli, like ABA (McAinsh *et al.*,1990). Rapid increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) are observed in response to these stimuli and this increase can operate over a very wide time domain (e.g. microseconds to hours) to regulate many different cellular processes (Lecourieux *et al.*, 2006). Vitally, $[Ca^{2+}]_{cyt}$ elevations can vary markedly, in magnitude, duration, and also location of the elevation and the Ca^{2+} source (Lecourieux *et al.*, 2002; Blume *et al.*, 2000; Irving 1992). However, this process is likely to be far more complex due to a multitude of Ca^{2+}-binding proteins that can act as effectors, the numerous Ca^{2+} stores from which this signal can be derived and the potential sites of interaction with other signaling molecules (Hetherington and Brownlee, 2004). Thus, it has been implied that Ca^{2+} signaling is part of an

integrated network of transduction events (Hetherington and Woodward, 2003; Trewasvas and Malhó, 1998). It is this Ca^{2+} 'signature' which is thought to encode information about the stimulus applied thereby initiating a stimulus-specific change in Ca^{2+} (McAinsh and Hetherington, 1998). Ca^{2+}-signaling stimuli act by generating Ca^{2+}-mobilizing signals that act on various 'ON' mechanisms to trigger an increase in the $[Ca^{2+}]_{cyt}$ (Berridge *et al.*, 2000; Putney, 1998). These signaling mechanisms fall into two groups depending on how they are activated, where most of them are activated by external stimuli and transfer information from the cell surface to internal effector systems, the other signaling systems respond to information generated within the cell in the form of metabolic messengers (Berridge *et al.*, 2006). Information is conveyed either through protein-protein interactions or is transmitted by diffusible factors (referred to as secondary messengers) for all of these signaling pathways (Berridge *et al.*, 2006). The response is terminated by 'OFF' mechanisms that remove these signals allowing cells to recover from stimulation. In the case of Ca^{2+} signaling, Ca^{2+} pumps and exchangers remove Ca^{2+} from the cytoplasm to reinstate Ca^{2+} to resting levels (Figure 1.4; Berridge *et al.*, 2006). For example, in the guard cells surrounding the stomatal pore on the leaf surface, elevations in the $[Ca^{2+}]_{cyt}$ of less than 1 μM from a resting level of about 200 nM regulates the turgor-driven cellular movements that cause closing of open stomata in response to ABA, which in turn, controls uptake of CO_2 during photosynthesis and prevents loss of water during transpiration via the stomatal pore (McAinsh *et al.*, 1990).

Ca^{2+} signaling involves extremely precise regulation of $[Ca^{2+}]_{cyt}$ levels. Cellular Ca^{2+} homeostasis is maintained by both the regulated influx into the

cytosol of Ca^{2+} from the apoplast or from intracellular stores mediated by Ca^{2+} permeable channels, and the active efflux of Ca^{2+} from the cytosol mediated by pumps or carriers (Ca^{2+} ATPases and H^+/Ca^{2+} antiporters) (review by Sanders *et al*, 2002). A submicromolar $[Ca^{2+}]_{cyt}$ is sustained in unstimulated cells by H^+/Ca^{2+} antiporters and Ca^{2+} ATPases as high $[Ca^{2+}]_{cyt}$ is cytotoxic (Hirschi, 2001; Sze *et al.*, 2000). These enzymes eliminate cytosolic Ca^{2+} to either the apoplast or the lumen of intracellular organelles, such as the vacuole or ER.

The H^+/Ca^{2+}-antiporters present in the plasma membrane and tonoplast have been categorised biochemically and were found to have a lower affinity for Ca^{2+} than the Ca^{2+}-ATPases (Sanders *et al.*, 2002; Evans and Williams, 1998). The reaction stoichiometry of the dominant H^+/Ca^{2+}-antiporter in the tonoplast is $3H^+/1Ca^{2+}$ (Blackford *et al.*, 1990). The *A. thaliana* genome contains eleven genes encoding putative H^+/Ca^{2+}-antiporters (*AtCax*) (Mäser *et al.*, 2001; Hirschi, 2001). The AtCAX1 antiporter demonstrates both a high affinity and specificity for Ca^{2+} and, contrastingly, the AtCAX2 transporter is a high affinity, high capacity H^+/heavy metal cation antiporter. However, the cation specificities of other AtCAXs are unknown (Shigaki *et al.*, 2002). Though the transporters AtCAX1, AtCAX2 and AtCAX4 were found to be located in the tonoplast (Cheng *et al.*, 2003, 2002; Hirschi, 2001), particulars of operation of this regulatory mechanism *in vivo* are yet to be understood. However, AtCAXs have homologues in Cyanobacteria and other plant species and their physiological functions have been studied

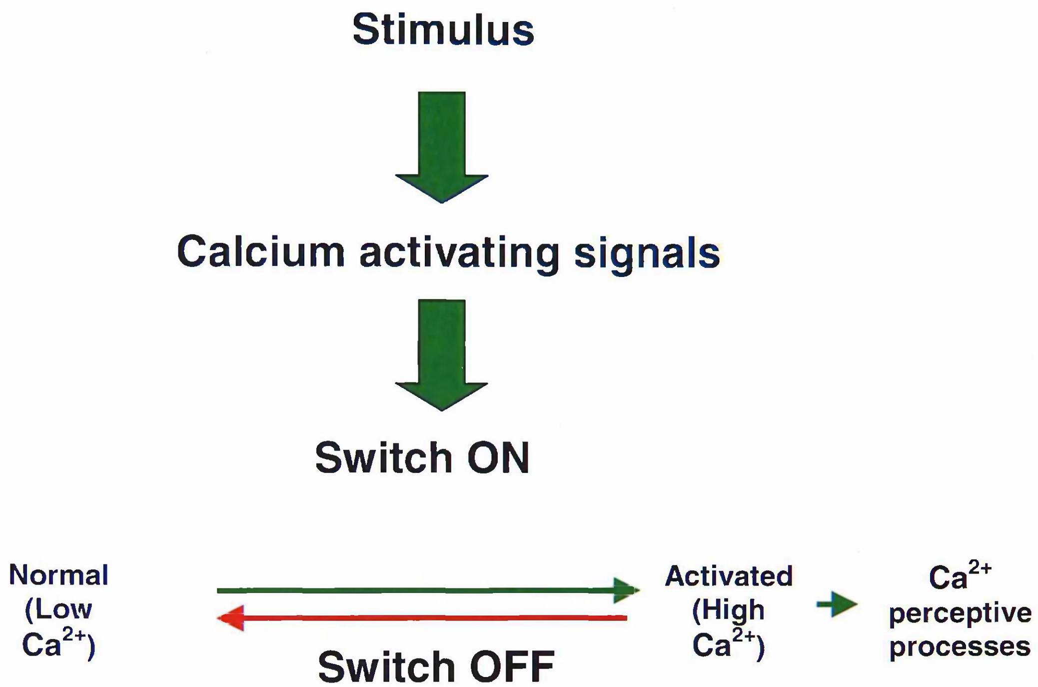


Figure 1.4: The four units of the Ca^{2+}-signaling network (adapted from Berridge *et al.*, 2006). Stimuli act by generating Ca^{2+} activating signals that act on various ON mechanisms to trigger an enhancement in the $[Ca^{2+}]_{cyt}$. The response is terminated by OFF mechanisms that restore $[Ca^{2+}]_{cyt}$ to normal levels.

(Waditee *et al.*, 2004, Hirschi, 2001). Tobacco mutants overexpressing *AtCAX1* exhibited Ca^{2+}-deficiency disorders, such as tip burn, susceptibility to chilling and metal hypersensitivity which was reversed by Ca^{2+} supply (Hirschi, 2001). It was proposed that this phenotype resulted from low $[Ca^{2+}]_{cyt}$ and that the primary role of AtCAX1 was to maintain $[Ca^{2+}]_{cyt}$ homeostasis by removing surplus $[Ca^{2+}]_{cy}$ to the vacuole (Hirschi, 2001). Specifically, the expression of *AtCAX1* and *AtCAX3* was found to be increased by raising Ca^{2+} supply (Cheng *et al.*, 2002; Hirschi, 2001; Shigaki and Hirschi, 2000). Unlike *Arabidopsis* with its 11 putative CAX genes, cyanobacteria have only a single CAX gene (Nakamura *et al.*, 2002; Kaneko *et al.*, 2001, 1996), which was considered to be more suitable for studying the physiological function of CAX. Waditee *et al.* (2004) isolated the putative Ca^{2+}/H^+ antiporter gene from *Synechocystis* sp. PCC 6803 (*synCAX*) as well as a homologous gene from a halotolerant cyanobacterium *Aphanothece halophytica* (*apCAX*) and observed that, in contrast to plant vacuolar CAXs, the full-length *apCAX* and *synCAX* genes complemented the $Ca2^+$sensitive phenotype of an antiporter-deficient *E. coli* TO114 mutant. Also, overexpression of ApCAX and SynCAX enhanced the salt tolerance of *Synechococcus* sp. PCC 7942 cells and so they concluded that cyanobacteria contain a Ca^{2+}/H^+ antiporter in their plasma membranes which plays an important role in salt tolerance.

In animals, fungi as well as in higher plants, Ca^{2+}-ATPases are thought to function mainly in a fine-regulation of low $[Ca^{2+}]_{cyt}$ (Bush, 1995). Plant cells contain several types of Ca^{2+}-ATPases, including those possessing a calmodulin-binding autoinhibitory domain and those lacking such a domain (Evans and Williams, 1998). Genomic data from *A. thaliana* reveal the

existence of at least 11 Ca^{2+}-ATPase genes, and an analysis of expressed sequence tags suggests that the number of Ca^{2+} pumps in this plant might be even higher (Geisler *et al.*, 2000). Low $[Ca^{2+}]_{cyt}$ levels in plants (typically <100 nM) – a prerequisite for second messenger function – are achieved by the function of high-affinity Ca^{2+}-ATPases and low-affinity H^{+}/Ca^{2+} antiporters (Evans and Williams, 1998; Bush, 1995). Because of the higher affinity of the Ca^{2+}-ATPases toward Ca^{2+} (Evans and Williams, 1998; Liand and Sze, 1998; Bush, 1995) they were considered to be responsible for the fine tuning of Ca^{2+} concentrations (Hwang *et al.*, 1997). Enhanced Ca^{2+}-ATPase gene expression and enhanced Ca^{2+} levels in plant cells exposed to high-salt and drought conditions have postulated that these phenomena represent an adaptive response to these environments (Perez-Prat *et al.*, 1992; Wimmers *et al.*, 1992). Geisler *et al.* (2000a; 2000b) characterized a calmodulin-regulated Ca^{2+} pump, ACA4 (*Arabidopsis* auto-inhibited Ca^{2+}-ATPase, isoform 4) from *Arabidopsis* that was associated with a vacuolar compartment and identified as a vacuolar pump. At least two other isoforms, ACA8 and ACA2, have also been found functioning in the plasma membrane and ER, respectively (Bonza *et al.*, 2000; Hong *et al.*, 1999). The activities of Ca^{2+}-ATPases that clean up $[Ca^{2+}]_{cyt}$ at a constant rate after any Ca2+ release, was viewed as a “house-keeping” function (Sanders *et al.*, 2002).

Ca^{2+} permeable channels are present in both the plasma membrane and the vacuolar membrane (White, 2000; MacRobbie, 1998). At the plasma membrane, identified Ca^{2+} permeable channels include non-selective cation channels (Demidchik *et al.*, 2002), depolarisation activated channels (review by White, 2000), and hyperpolarisation activated channels (Gelli *et al.*, 1997),

none of which have to date been characterised at the molecular level. Importantly, Chan *et al.*, (2003) showed that cyclic nucleotide gated channel (CNGC2) is a key determinant for the plant response to Ca^{2+} in the growth environment. In *Arabidopsis*, 20 CNGCs have been identified (Mäser *et al.*, 2001). Molecular genetic studies in plants have established that CNGCs can function in altering plant development as well as a plant's response to pathogens and abiotic stresses, such as heavy metals and high levels of Ca^{2+} or Na^{+} (Ali *et al.*, 2007; Borsics *et al.*, 2007; Gobert *et al.*, 2006; Yoshioka *et al.*, 2006; Li *et al.*, 2005; Chan *et al.*, 2003; Talke *et al.*, 2003).

There is very little information about the physiological functions of CNGCs in plants. However, studies have implicated some members in uptake and homeostasis of heavy metals such as Ni^{2+} and Pb^{2+} (Sunkar *et al.*, 2000; Arazi *et al.*, 1999). On the other hand, genetic studies in *Arabidopsis* have shown that a mutation in *CNGC2* (also called *DND1*; Yu *et al.*, 1998) resulted in a near-complete loss of hypersensitive response (HR), a type of programmed cell death directly associated with disease resistance (Heath, 2000). In animals, CNGCs were found to be important for sensory transduction with the best characterized from the visual and olfactory systems. This class of channels does not differentiate well between alkaline metals and will tolerate a substantial Ca^{2+} flow. Na^{+}, K^{+}, and Ca^{2+} are all physiologically relevant permeant ions, but in both visual and olfactory transduction, Ca^{2+} influx is important for desensitization and adaptation (Kaupp and Seifert, 2002; Broillet and Firestein, 1999; Zagotta and Siegelbaum, 1996). CNGC2 can form an ion channel in heterologous expression systems that mediate Ca^{2+} and K^{+} influxes but do not allow substantial Na^{+} influx (Leng *et al.*, 2002; Leng *et al.*,

1999). Although the mechanism by which this ion channel arbitrates hypersensitive responses (HR) and defence responses is not completely understood, Ca^{2+} influx and the subsequent rise in cytoplasmic Ca^{2+} have been linked with the commencement of many defence responses and the inception of HR (Blume *et al.*, 2000; Grant *et al.*, 2000; Xu and Heath, 1998; Jabs *et al.*, 1997). Also, Chan *et al.* (2003) reported that *CNGC2* mutant plants were affected in vegetative and reproductive growth, programmed cell death, and adaptive responses to biotic and abiotic stimuli and were specifically hypersensitive when grown under physiological calcified conditions, leading to a hypothesis that there is a causal link between CNGC2 function and the adaptive response of plants to overcome Ca^{2+} stress (Chan *et al.*, 2003).

Glutamate receptors (GLRs) are a further class of ion channels that might provide a route for Ca^{2+} across the plasma membrane. Studies by Kim *et al.*, (2001) suggest that an *Arabidopsis* GLR, *At*GLR2 might be involved in unloading Ca^{2+} from the xylem vessels. The location of GLRs in the plasma membrane has yet to be identified. However, many classes of Ca^{2+} current have been characterized electro-physiologically in plant membranes, which can be divided into depolarisation-activated, hyperpolarisation-activated and voltage-insensitive channels (White, 2000). Depolarisation-activated Ca^{2+}-permeable channels (DACC) have been identified in the plasma membrane of all plant root cells (White, 2000; Thion 1998), leaf mesophyll cells (Thion 1998) and suspension-cultured cells (Thuleau, 1994). Hyperpolarisation-activated Ca^{2+} channels have been identified in protoplasts of various cell types, where they are proposed to maintain physiological equilibrium (White,

2000). Two elicitor-activated or voltage-insensitive plasma-membrane Ca^{2+}-permeable channels have been identified in tomato (*Lycopersicon esculentum* L.) (Gelli and Blumwald, 1997; Gelli *et al.*, 1997) and parsley (*Petroselinum crispum*) (Zimmermann *et al.*, 1997) suspension-cultured cells and Ca^{2+} influx through these channels was proposed to be an early event in the initiation of defence responses to pathogens.

Though the vacuole is the principal intracellular Ca^{2+} store in plants, ER (Klusener *et al.*, 1995), chloroplast (Malhó *et al.*, 1998) and mitochondria (Babcock, *et al.*, 1997) all have the capacity to sequester Ca^{2+} (Sanders *et al.*, 2002). Several Ca^{2+} release channels at the vacuolar membrane have been identified using biochemical and electrophysiological methods (Sanders *et al.*, 2002, 1999). Two are voltage-gated channels, the slowly activating vacuolar (SV) and vacuolar voltage-gated Ca^{2+} channel (VVCa) (Pottosin and Schönknecht, 2007). Two further channels are ligand-gated and are activated by cyclic ADP ribose (cADPR) and inositol triphosphate (IP_3). Under physiological conditions the SV channels are known to be permeating monovalent and divalent cations and catalyse a small Ca^{2+} influx to the cytoplasm (Pottosin *et al.*, 2001, 1997; White, 2000). As they are synchronized by many effectors, they could be crucial in effecting the coincidence control of $[Ca^{2+}]_{cyt}$ signaling (Sanders *et al.*, 2002,1999; White, 2000). Cytoplasmic alkalinization or vacuolar acidification and increasing $[Ca^{2+}]_{cyt}$ or decreasing vacuolar Ca^{2+} concentration ($[Ca^{2+}]_{vac}$) promotes their opening at physiological trans-tonoplast voltages and their response to $[Ca^{2+}]_{cyt}$ is sensitized both by calmodulin (CaM) and by cytosolic Mg^{2+} (White and Broadley, 2003). It has been anticipated that $[Ca^{2+}]_{cyt}$-dependent

regulation of SV channels might avert a disproportionate rise in $[Ca^{2+}]_{cyt}$ or amend the kinetics of changes in $[Ca^{2+}]_{cyt}$ by playing an important role in Ca^{2+}-induced Ca^{2+} release (CICR) from the vacuole during stomatal closure. (Sanders *et al.*, 1999; Ward and Schroeder, 1994). The activities of SV channels are known to be reduced by 14-3-3 proteins (van den Wijngaard *et al.*, 2001). SV channels are ubiquitous in plant vacuoles, where they form the principal conductance at micromolar $[Ca^{2+}]_{cyt}$. However, it has been shown that the *TPC1* ('two-pore channel 1') gene of *A. thaliana* encodes a class of Ca^{2+}-dependent Ca^{2+}-release channel (Peiter *et* al., 2005) that is known from numerous electrophysiological studies as the slow vacuolar channel (Allen and Sanders, 1997; Ward and Schroeder, 1994; Hedrich and Neher, 1987). Also, Peiter *et al.* (2005) demonstrated that a *TPC1* knockout mutant lacked the functional slow vacuolar channel activity and was defective in the reaction of stomata to extracellular Ca^{2+}. However, in mesophyll protoplasts of *kco1* mutant *Arabidopsis*, SV-channel currents were smaller than those in wild type plants implicating double pore K^+ channel (KCO1) in SV channel formation (Schönknecht *et al.*, 2002). Ranf *et al.* (2008) showed that AtTPC1 co-localizes with the K^+-selective channel AtTPK1 in the vascuolar membrane and loss of *AtTPC1* abolishes Ca^{2+}-activated SV currents, which were increased in *AtTPC1*- over-expressing *Arabidopsis* when compared to wild-type. However, stimulus- and Ca^{2+}-dependent gene activation was not affected by alterations in *TPC1* expression and so they concluded that the loss of *TPC1* did not affect the activity of hyperpolarization-activated Ca^{2+}-permeable channels in the plasma membrane and that TPC1 functions as a

vacuolar cation channel without a major impact on cytolosic Ca^{2+} homeostasis under physiological conditions (Ranf *et al.* 2008).

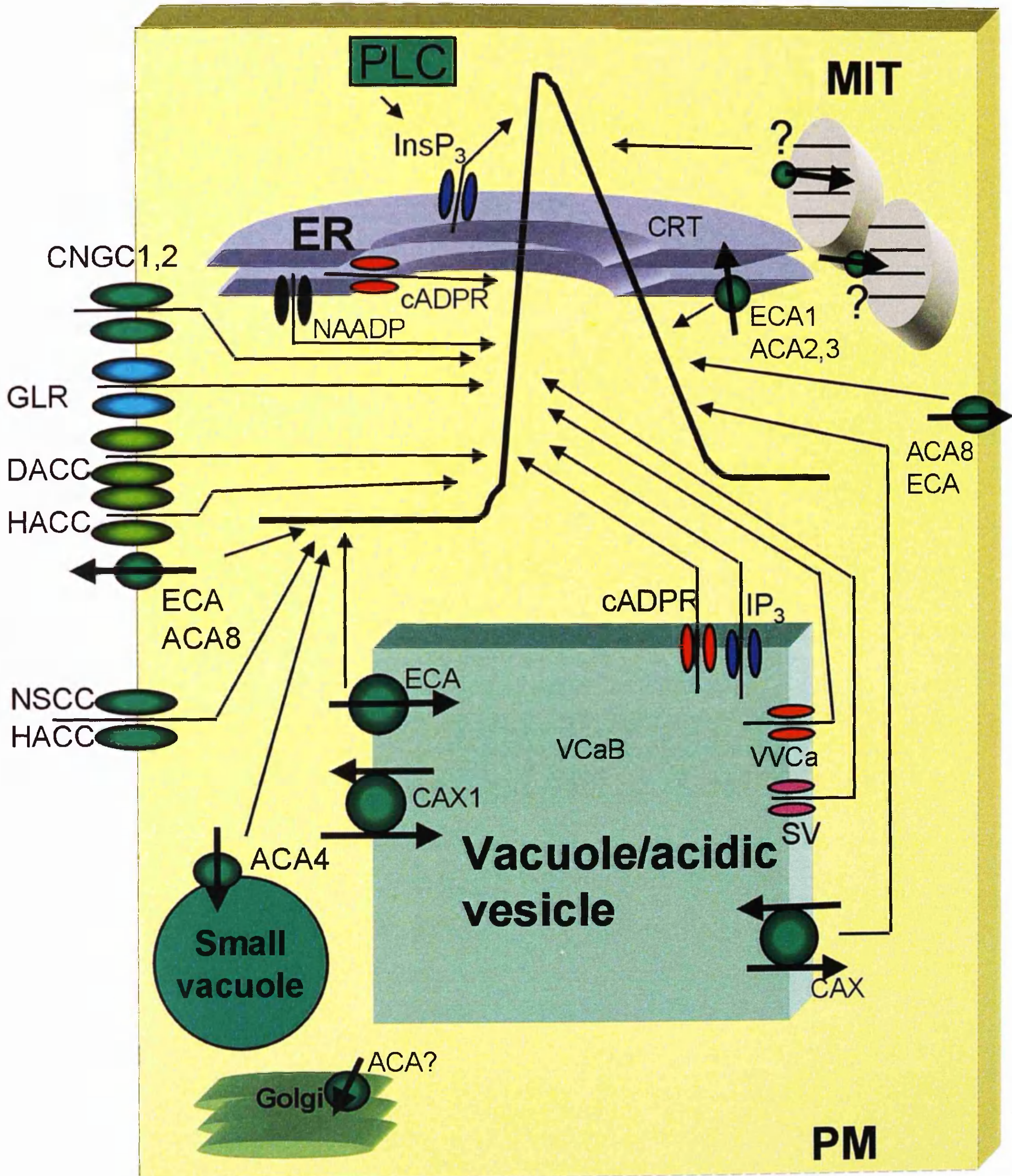
As mentioned above, highly selective Ca^{2+} channels activated by cytosolic second messengers (IP_3, inositol hexakisphosphate, IP_6, or cADPR) are also present in the tonoplast (Sanders *et al.*, 2002; White, 2000; Allen and Sanders, 1997). All arbitrate Ca^{2+} influx to the cytoplasm. The IP_3-dependent Ca^{2+} channels are activated half-maximally by IP_3 concentrations as low as 200 nM. and are considered to have a role in numerous responses including turgor regulation in response to salt and hyper-osmotic stresses (Xiong *et al.*, 2002; DeWald *et al.*, 2001; Drøbak and Watkins, 2000), in nastic movements (Kim *et al.*, 1996), in gravitropic movements of roots (Fasano *et al.*, 2002) and pulvini (Perera *et al.*, 1999), in stomatal closure (Klüsener *et al.*, 2002; Ng *et al.*, 2001; Schroeder *et al.*, 2001) and in pollen tube elongation (Rudd and Franklin-Tong, 2001). They are also involved in plant defence responses (Blume *et al.*, 2000; Mithöfer *et al.*, 1999). The cADPR-dependent Ca^{2+} channels are inhibited by $[Ca^{2+}]_{cyt}$ greater than 600 nM though they open at physiological trans-tonoplast voltages (Leckie *et al.*, 1998). The cADPR-dependent Ca^{2+} channels have been implicated in ABA-signaling pathways leading to temperature acclimation, drought tolerance (Wu *et al.*, 1997) and stomatal closure (Klüsener *et al.*, 2002; Leckie *et al.*, 1998), in circadian $[Ca^{2+}]_{cyt}$ rhythms (Dodd *et al.*, 2007) and in initiation of plant defence responses (Klessig *et al.*, 2000; Durner *et al.*, 1998). Evidence for Ca^{2+} channels activated by sub-micromolar concentrations of IP_6 and having a role in stomatal closure is incidental but influential (Lemtiri-Chlieh *et al.*, 2000). To

date, no genes encoding plant genes homologous to the IP_3-receptors or cADPR-receptors have been identified.

The role of Ca^{2+} transporters and binding proteins in modulating cytosolic Ca^{2+} has been highlighted by Pittman and Hirschi (2003). As discussed earlier, efflux of Ca^{2+} from the cytosol is achieved via two major routes. Firstly, through H^+/Ca^{2+} antiporters (e.g CAX1) probably localised to the plant vacuole (Hirschi, 1999; Hirschi *et al* 1996) and secondly, via Ca^{2+} ATPase pumps that use ATP directly to drive ion translocation. These pumps fall into two groups; autoinhibited Ca^{2+} ATPases (ACAs) and ER-type Ca^{2+} ATPases (ECAs). Figure 1.5, adapted from Sanders *et al.* (2002), shows the location of channels, pumps, and carriers involved in Ca^{2+} transport for a generalized *Arabidopsis* cell described in detail in earlier sections.

Examples of the developmental processes and responses to abiotic and biotic challenges initiated by a perturbation in $[Ca^{2+}]_{cyt}$ include pollen tube elongation (Rudd and Franklin-Tong, 2001), pollen tube self-incompatibility response (Franklin-Tong *et al.*, 2002), cell polarity after fertilization (Antoine *et al.*, 2001), cell division (Bush, 1995), seed germination (giberellins) (Anil and Sankara Rao, 2001), apoptosis (Levine *et al.*, 1996), responses to red, and blue light (Malhó *et al.*, 1998), circadian rhythms (Wood *et al.*, 2001), stomatal closure (ABA, sphingosine-1-phosphate) (Klüsener *et al.*, 2002; Schroeder *et al.*, 2001), CO_2 (Webb *et al.*, 1996), increasing apoplastic Ca^{2+} (Allen *et al.*, 2000; McAinsh *et al.*, 1995), auxin responses (Plieth and Trewavas, 2002), xylem K^+ loading (De Boer, 1999), exocytosis (Camacho and Malhó, 2003), root cell elongation (Demidchik *et al.*, 2002), root hair elongation (Bibikova *et al.*, 1999), inhibition of cyclosis (Ayling and Clarkson, 1996), nodulation (*nod*

factors) (Shaw and Long, 2003), senescence (Huang *et al.*, 1997), responses to UV-B (Frohnmeyer *et al.*, 1999), heat-shock (Malhó *et al.*, 1998), cold-shock (Plieth, 2001), slow cooling (Moore *et al.*, 2002), oxidative stress (paraquat, superoxide, H_2O_2, ozone) (Lecourieux *et al.*, 2002), anoxia (Plieth, 2001), drought/ hyper-osmotic stress (mannitol) (Plieth, 2001), salinity (NaCl) (Halperin *et al.*, 2003), hypo-osmotic stress (Plieth, 2001), mechanical stimulation (motion, touch, wind) (Fasano *et al.*, 2002), aluminium stress (Zhang and Rengel, 1999) and pathogens (elicitors) (Lecourieux *et al.*, 2002) (complete review by White and Broadley, 2003).


PLC
MIT
InsP3
?
ER
CRT
CNGC1,2
cADPR
NAADP
ECA1
ACA2,3
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GLR
ACA8
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DACC
HACC
ECA
ACA8
cADPR
IP3
NSCC
HACC
ECA
VCaB
VVCa
CAX1
SV
ACA4
Vacuole/acidic vesicle
Small vacuole
CAX
ACA?
Golgi
PM

Figure 1.5: Schematic representation of major identified Ca^{2+} transport pathways and interaction with Ca^{2+}-binding proteins in *Arabidopsis* cell membranes (Adapted from Hetherington and Brownlee, 2004). Light arrows indicate hypothetical actions in raising or lowering $[Ca^{2+}]_{cyt}$ at particular points in a Ca^{2+} transient. The assignment of Ca^{2+} pumps and transporters to different membranes is suggestive rather than comprehensive. The combined activity of channels and pumps contribute to resting $[Ca^{2+}]_{cyt}$. NSCCs and HACCs may be mainly involved in maintaining steady Ca^{2+} influx. The chloroplast and nucleus are not shown. ACA,ECA, Ca^{2+}-ATPAses; CAX, H^{+}/Ca^{2+} exchangers; NSCC, nonselective cation channels; DACC, depolarization-activated Ca^{2+} channels; HACC, hyperpolarization-activated Ca^{2+} channels; GLR, glutamate receptor channels; CNGC, cyclic nucleotide gated channels; PM, plasma membrane; mit, mitochondria; ER, endoplasmic reticulum; CRT, calreticulin; VCaB, vacuolar Ca^{2+}-binding protein.

1.2.2 Calcium in soils and plant communities on these soils

Calcium (Ca^{2+}) deficiency is rare in nature, but excessive Ca^{2+} restricts plant communities on calcareous soils (White and Broadley, 2003). Ecologists have classified plant species into calcifuges, which occur on acid soils (with low effective cation exchange capability and so low Ca^{2+}) and do not grow well in lime-rich (calcareous) soils due to limitations in mineral nutrition (Zohlen and Tyler, 1997), whereas calcicoles occur on calcareous soils (soils with high Ca^{2+} content) (Lee, 1999). Also, the Ca^{2+} concentrations in calcifuge and calcicole plants growing in their natural habitat differ markedly. An interest in particular edaphic constraints of calcicole and calcifuge plants extends back to studies in the 18th century (Rorison, 1960). Calcifuges generally grow well at low rhizospheric Ca^{2+} concentrations and, conversely, the mechanisms that enable calcicole plants to maintain low $[Ca^{2+}]_{cyt}$ in their natural habitat are believed to restrict their growth at low rhizospheric Ca^{2+} by inducing Ca^{2+} deficiency (Jefferies and Willis, 1964; Lee, 1999). De Silva (1934) carried out the initial studies to understand the distribution of a few plant species on both calcareous as well as acidic soils and determined that the amount of exchangeable Ca^{2+} in the soils correlated with the distribution of calcicoles and soil condition being the chief factor for calcifuges.

Soils normally have large amounts of exchangeable Ca^{2+} in the range of 300-5000 ppm (Kelling and Schulte, 1998). A broad variety of studies report variable Ca^{2+} concentrations in soils from regions across the UK. Total Ca^{2+} levels in dried (80°C) soil from four sites including two sites in Gordano Valley (N. Somerset), Cadbury Camp (N. Somerset) and in Braunton Burrows (N.

Devon) ranged from 6,650 ppm to 12,520 ppm (Jefferies and Willis, 1964). The mean extractable Ca^{2+} concentration of soil collected from Royston, Hertfordshire was 5840 ppm (approx.) (Wilson *et al.*, 1995). Total soil Ca^{2+} from soils collected from eight sites within the Westerleigh-Yate area (northeast of Bristol, England) ranged from 1,250 ppm to 5,540 ppm (Morgan *et al.*, 2001).

Calcareous grasslands in the UK and Europe are sites of conservation importance that support a diverse and often specialised flora and fauna, including many rare or threatened species (Van Helsdingen *et al.*, 1996; Rodwell, 1992; Wallis DeVries *et al.*, 2002; Bobbink and Willems, 1987). Also, the most species-rich plant populations are found among the calcareous grasslands around Europe (Hillier *et al.*, 1999; Rodwell, 1991). The question of why some soil substrates host a greater diversity of plant species than others is old, and generations of plant ecologists have put emphasis on these studies (Wohlgemuth and Gigon, 2003). The adaptations shown by plants that grow in calcareous soils (calcicoles) to the distinctive habitats have long fascinated ecologists (Lee, 1999), yet our understanding of the physiological basis of the calcicolous habit remains limited. The derived question of whether plant species richness differs depending on calcareous or acidic substrate has thus far been of trivial scientific interest in Europe (Kinzel, 1983; Wohlgemuth and Gigon, 2003). Huge losses among these grasslands and their continuing vulnerability to either agricultural improvement or neglect have focussed attention on the need for conservation of their biodiversity (DETR, 2000).

De Silva *et al.* (1994a) established that transpiration rate was unchanged with increasing concentrations of rhizospheric Ca^{2+} up to 20 mM

in three calcicole species, *Campanula glomerata*, *Centaurea scabiosa* and *Leontodon hispidus* grown at high concentrations of rhizospheric Ca^{2+}, although concentrations of Ca^{2+} in the xylem sap were very close to those in the rhizosphere. These results were in marked contrast to those observed for *Lupinus luteus*, a known calcifuge, subjected to similar experimental conditions (De Silva *et al.*, 1994b). In this species xylem concentrations were maintained at 2 mM even when rhizospheric Ca^{2+} concentrations were 15 mM. Further to this, they observed that high rhizospheric Ca^{2+} disrupted stomatal behaviour in *L. luteus*, resulting in a significant reduction of leaf conductance, transpiration rate and net assimilation rate which was again in contrast with that observed in calcioles (De Silva *et al.*, 1994a), where the stomatal opening was unperturbed by high rhizospheric Ca^{2+}. These studies suggested that a strategy has been developed in calcicoles for protecting the guard cells from deleterious concentrations of this cation by regulating the distribution of Ca^{2+} reaching the epidermal tissues. X-ray microanalysis in the leaves of *Centaurea scabiosa* and *Leontodon hispidus* showed that the bulk of the Ca^{2+} entering the epidermis is limited within the trichomes, most likely as insoluble Ca^{2+} oxalate (De Silva *et al.*, 2001, 1996). The Ca^{2+} concentration in the region of stomata in these species thus remains minimal, allowing efficient intracellular signaling to continue in the guard cells without any hindrance. This study also provides evidence of a very specific regulatory mechanism operating in calcicoles where the trichomes act as Ca^{2+} sequestering structures. A similar mechanism was reported in plants with an induced expression of Ca^{2+}-transporters demonstrating Ca^{2+}-deficiency

symptoms at low rhizospheric Ca^{2+}, where the Ca^{2+} is removed from the cytoplasm to the vacuole (Hirschi, 2001).

It is likely that part of the adaptation of plants to survival on high Ca^{2+} soils would be echoed at the molecular level by changes in the expression of various genes underlying the many biochemical pathways, including Ca^{2+} regulation and Ca^{2+} signaling, that are operating in these plants. For example, the plant H^+/Ca^{2+} antiporter (CAX1) was identified by its ability to re-establish growth on high Ca^{2+} media of a yeast mutant defective in vacuolar Ca^{2+} transport (Hirschi *et al.,* 1996). Additionally, tobacco plants over-expressing CAX1 showed an alleviated response to Ca^{2+} deficiency by increasing Ca^{2+} uptake from the media (Hirschi, 2001). Also, Chan *et al* (2003) demonstrated a hypersensitive response by CNGC2 mutants of *Arabidopsis* in response to increased rhizospheric Ca^{2+} which they ascribed to Ca^{2+} toxicity, perhaps due to accumulation of Ca^{2+} or to a defect in signaling pathways that facilitate normal growth on high Ca^{2+}.

1.3 Arabidopsis Experience

Arabidopsis belongs to the *Brassicaceae* or *Cruciferae* family, which includes the related genera *Arabis*, *Brassica* and *Cardamine* (Yang *et al.*, 1999; Koch, *et al.*, 2000, 2001; Hall *et al.*, 2002). Also referred to as mouse-ear cress, it was a reasonable choice for genome sequencing with its low chromosome number and, due to the availability of mutants and ecotypes, it was being promoted as a model organism as early as 1943 (Meyerowitz, 2001). The ubiquitous embracing of *Arabidopsis* as a model plant, followed by

the ongoing revolution in plant genetics, physiology, and molecular genetics, occurred in the 1980s (Meyerowitz, 2001).

Initial considerable interest in *Arabidopsis* as a model organism arose in Germany and the United States in the 1940s, primarily through the work of Friedrich Laibach (based in Frankfurt) and George Redei (at the University of Missouri in Columbia). It was the unusual existing natural variation in the phenotype of *Arabidopsis* that drew Laibach to the species, and in 1937 he commenced the first systematic collection and classification of *Arabidopsis* wild ecotypes. This interest was initiated by a belief that *Arabidopsis* could develop into a suitable model organism to study and understand the mechanisms responsible for this surprising diversity (Leonelli, 2007).

The plant kingdom represents highly divergent life forms. In addition to ancestral characteristics conserved between plants and animals, flowering plants have unique organizational and physiological properties. The genome sequence of a plant provides a definite source for understanding the genetic basis of differences between plants and other eukaryotes, and provides the basis for extensive functional characterization of plant genes (The Arabidopsis Genome Initiative, 2000). The advantages of *Arabidopsis thaliana*, such as a short generation time, small size, large number of offspring, and a relatively small nuclear genome prompted molecular and genetic studies which culminated in the publication of the complete genome sequence of 125-megabase pairs comprising ~25,498 genes encoding proteins from ~11,000 families, analogous to the functional diversity of *Drosophila* and *Caenorhabditis elegans*, two other sequenced multicellular eukaryotes (Schlesier *et al.,* 2003; The Arabidopsis Genome Initiative, 2000).

Consequently, it is also one of the most studied plant systems. *Arabidopsis* accessions have been collected from an extensive range of habitats and the genetic differences between the local populations are thought to be associated with adaptation to the existing environmental conditions and the subsequent phenotypic variations reflect the genetic variation that might be important for adaptation to specific environmental conditions (Beemster *et al.,* 2002). Also, extensive variation has been reported in *Arabidopsis* ecotypes for tolerance to abiotic stress parameters, such as drought, freezing, temperature, heavy metals, carbon dioxide and ozone (Rao and Davis, 1999; Murphy and Taiz, 1995; Zhang and Lechowicz, 1995; Sharma *et al.*, 1979; Langridge and Griffing, 1959).

There are vast data from field studies on many plant species in varied environments and, with the advancement of molecular methods it has become increasingly feasible to study the functional evolution of genes that are significant in ecology and those that control responses to various biotic and abiotic stresses and environmental challenges (Mitchell-Olds, 2001). Plants are ideal for ecologically pertinent molecular studies as they are sessile and confronted by considerably varied environmental challenges (Kroemer *et al.,* 2004; Shinozaki and Dennis, 2003). They also have the ability to considerably vary their gene expression patterns in response to environmental changes such as temperature, water availability or the presence of deleterious levels of ions (Hazen *et al.,* 2003; Zhu, 2003; Thomashow, 2001; Xiong and Zhu, 2001; Shinozaki and Yamaguchi-Shinozaki, 2000, 1997). Though numerous identified genes function in response to these environmental challenges, whose loci might be influenced by natural selection, quite a few ecologically

significant polymorphisms have already been identified at known loci in *A. thaliana* (Johanson *et al.*, 2000; Stahl *et al.*, 1999) allowing genes to be isolated readily from wild relatives or ecotypes growing in natural populations (Mitchell-Olds, 2001).

In addition, it has become apparent that natural variation between plant accessions is an unexploited valuable resource for identifying gene function (Rensink and Buell, 2004). Naturally occurring intra-species ecotypes of *A.thaliana* vary in morphological form, physiology and environmental responses and their natural variation supplies the genetic material for constructive natural selection and breeding (Maloof, 2003). Analysis of existing natural variation among ecotypes facilitates the detection of gene function for which no mutants can be isolated due to either a restrained phenotype or lethality of the mutation (Alonso-Blanco and Koornneef, 2000). It has therefore been proposed that *A. thaliana* and its wild relatives offer a powerful model system to elucidate quantitative genetic variation and provide insights into the molecular basis of adaptation to environmental stresses (Maloof, 2003; Mitchell-Olds, 2001). As new high throughput genomic technologies become easily available, polymorphisms between accessions can be detected on a genome-wide scale and increase the amount of fundamental information available on plant molecular based ecological adaptations (Borevitz and Nordborg, 2003). Results on inter- and intra-species genetic diversity in stress responses have become ever more useful in understanding how species, ecotypes, or breeding lines in a plant species can either mount a successful defence or succumb to abiotic stress (Bohnert *et al.*, 2006).

1.4 Microarray studies

In the near past there has been an extensive advancement in molecular biological techniques and, therefore, a subsequent growth in the knowledge of intricate molecular processes. In addition to the completion of many genome sequencing projects, the development of technologies amenable to system-wide analysis has been the steering force behind this progress.

Whole genomes have been sequenced in plants, including *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000) and rice (Yuan *et al.*, 2005; Yu *et al.*, 2002; Goff *et al.*, 2002). Draft genome sequence is available for poplar (http://genome.jgi-psf.org/Poptr1/) and lotus (http://www.kazusa.or.jp/lotus/), and sequencing efforts are in progress for several others including tomato, maize, *Medicago truncatula,* sorghum (Bedell *et al.*, 2005) and close relatives of *A. thaliana*. Expressed sequence tags (ESTs) generated from many plants including lotus, beet, soybean, cotton, wheat, and sorghum are accessible at http://www.ncbi.nlm.nih.gov/dbEST/.

As discussed above, regulation of growth and development of plants is often associated with radical changes in gene expression. The ability to assess extensive changes in gene expression in a comprehensive and unbiased way has been made possible with the introduction of 'microarray' technology (Schena *et al.*, 1995). This has heralded a major shift from the conventional single gene approach to monitoring alterations in expression of thousands of genes at once (Kathiresan *et al.*, 2006). A microarray, or genechip, is an assemblage of microscopic DNA spots or oligonucleotide sequences placed on a solid surface that is used for expression profiling or

transcriptional analysis in which expression patterns of thousands of genes can be simultaneously monitored (vanBaarlen *et al.*, 2008).

Three types of biological experiments can be performed using gene expression microarrays (Richmond and Somerville, 2000). A 'biology discovery' or exploratory experiment is used to measure an experimental transcriptome against a reference transcriptome to identify altered gene expression under the given experimental conditions (Cates, 2007). These experiments generate vast amounts of data which require sophisticated bioinformatic tools to organise the information efficiently. In 'marker discovery' experiments, a small number of specific marker genes for a given treatment or tissue type are identified. This type of experiment is commonly used in cancer studies to identify a signature for different types of cancers (Russo *et al.*, 2003). 'Marker discovery' experiments can also be used to identify marker genes for environmental stresses, which can then be used for specific smaller scale microarrays (Matsuyama *et al.*, 2002). The third type of microarray experiment is the 'gene-function discovery' experiment, which is on a much wider scale and uses information from multiple experiments to imply gene function on the basis of where and when the gene is expressed. Openly available microarray public databases like 'GENEVESTIGATOR' (Zimmermann *et al.*, 2004) provide access to experiments from smaller databases and are typically employed for 'gene-function discovery' experiments. This type of experiment is increasingly important with the high proportion of unknown genes within newly sequenced genomes (Wanchana *et al.*, 2008; The Genome Sequencing Consortium, 2001; The Arabidopsis Genome Initiative, 2000; Goffeau *et al.*, 1996). Very recently, The Generation

Challenge Programme (GCP; www.generationcp.org) has developed an online resource documenting stress-responsive genes comparatively across plant species. This open resource is a compendium of protein families, phylogenetic trees, multiple sequence alignments (MSA) and associated experimental evidence with the prime objective to elucidate orthologous and paralogous relationships between plant genes that may be involved in response to environmental stress, mainly abiotic stresses (Wanchana *et al.*, 2008).

In recent years, microarray analysis has become a key experimental tool, enabling the analysis of genome-wide patterns of gene expression (Brewster *et al.*, 2004). A PubMed query of microarray literature for the years 2005, 2006 and 2007 yields 4537, 5340 and 5693 hits respectively, with 3731 articles already published up to July, 2008 (Figure 1.6). In plant biology, microarrays have been used for studying plant development and function, regulation of metabolic pathways, and the effects of biotic and abiotic stresses. Several cDNA microarrays are available for *Arabidopsis* (Schaffer *et al.*, 2001; Seki *et al.*, 2001; Girke *et al.*, 2000; Maleck *et al.*, 2000; Reymond *et al.*, 2000; Schenk *et al.*, 2000; Wang *et al.*, 2000) to study gene expression patterns. Also, *Arabidopsis* microarrays have been successful in obtaining expression patterns of genes under a range of abiotic and biotic stress conditions (e.g., Liu *et al.*, 2005; Seki *et al.*, 2004; Takahashi *et al.*, 2004; Hazen *et al.*, 2003; Cheong *et al.*, 2002, Kreps *et al.*, 2002; Rishi *et al.*, 2002;

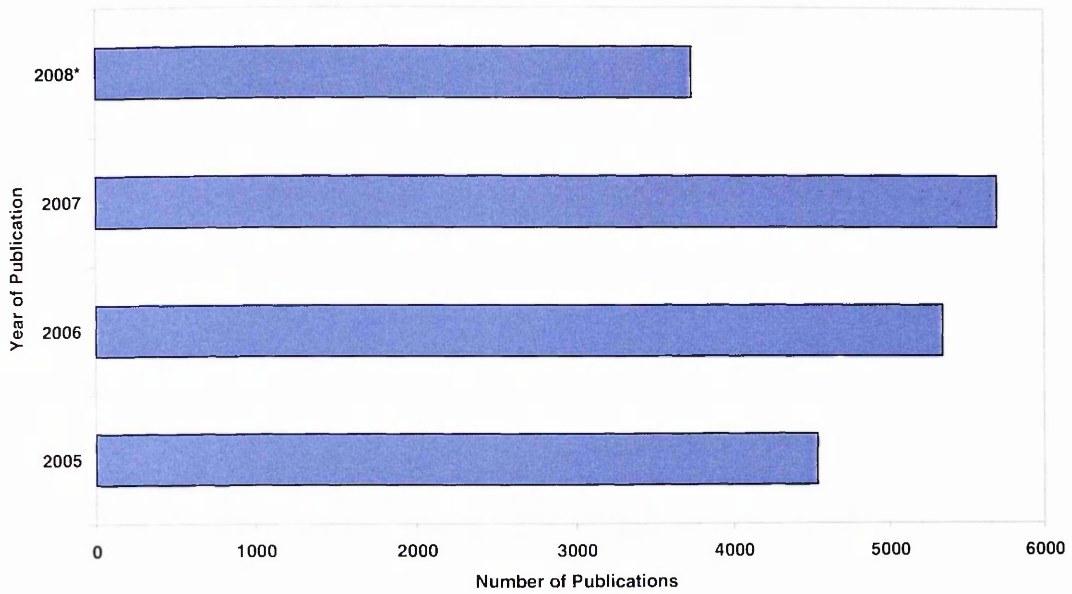


Figure 1.6: Number of Microarray related publications in years 2005-2008. '*' - Data available up to July, 2008. (Source: Pubmed)

Seki *et al.*, 2002; Seki *et al.*, 2001). These studies helped in identifying gene sets that are strongly up or down-regulated in expression under stress conditions. For example, the study of Seki *et al.*, (2002), identified five-fold gene expression change in 351 genes among 7,000 Arabidopsis cDNAs under cold, drought or salinity stress conditions. Of these 351 genes, only 22 were found to be induced by five-fold under all three stress scenarios. Kreps *et al.*, (2002) observed that 2409 of 8100 Arabidopsis cDNAs were induced greater than twofold under salt, osmotic or cold stress. Of these, under all three stress conditions, they observed twofold induction in only 118 (4.9%) after three hours of stress and only 12 genes (0.5%) after 27 hours.

As plant defence reactions are complex and consist of both general stress responses and stimulus-specific responses, data analysis tools have been used to group genes involved in these responses to show a high degree of co-ordination of signaling pathways (Swindell, 2006; Kreps *et al.*, 2002; Schenk *et al.*, 2000). Also, genome wide quantification of transcript levels by DNA microarrays provided a potential approach towards the identification and functional analysis of genes underlying multiple-stress tolerance (Clarke and Zhu, 2006).

As well as gene expression analysis, microarray technology can be used to address other biologically pertinent questions that rely on the principle of complementary base-pairing. For example, microarrays are also commonly used to investigate genotype variation in the form of single nucleotide polymorphisms (SNPs) particularly in relation to human diseases (Roses, 2000). Recent applications of microarray technologies by plant physiologists include the identification of DNA-binding proteins (Iyer *et al.*, 2001; Ren *et al.*,

2000) and utilization of protein arrays (Schweitzer and Kingsmore, 2002; Templin *et al.*, 2002) in the characterization and identification of individual genes and gene families with potential application in the fields of agriculture, horticulture and forestry (Wullschleger and Difazio, 2003). As the avalanche of genome data grows daily, the new challenge will be to use this vast reservoir of data to explore how DNA and proteins work with each other and the environment to create complex, dynamic living systems.

Perceiving a plant's response to stress will require a comprehensive evaluation of stress-induced changes in gene expression. A compiled list of genes connected to abiotic stress responses in *Arabidopsis* and other plants can be accessed at http://stress-genomics.org. Complete *Arabidopsis* genome microarrays were used by Armengaud *et al.* (2004) to assess transcriptional responses of *Arabidopsis* seedlings to altered external supply of the essential macronutrient potassium (K^+). Bioinformatics to assess transcriptional responses led to the discovery of four main super-categories of K^+-responsive genes namely; genes related to the plant hormone JA signaling pathway, genes encoding cell wall proteins (e.g. extensins and arabinogalactans), genes with a putative function in ion transport (e.g. the high-affinity K^+ transporter HAK5 and the nitrate transporter NRT2.1), and genes encoding Ca^{2+}-binding proteins (e.g. calmodulins). Also, to understand the gene network regulating cold stress, Lee *et al.*, (2005) performed an *A. thaliana* genome transcript expression profile using Affymetrix GeneChips (containing ~24,000 genes) and statistically determined 939 cold-regulated genes (655 upregulated and 284 downregulated). A number of genes important for the biosynthesis of signaling of plant hormones, such as ABA, gibberellic acid,

and auxin, were found to be regulated by cold stress, which was proposed to be of potential importance in coordinating cold tolerance with growth and development (Lee *et al.*, 2005).

Transcription profiling of roots of *A. thaliana* and closely related *Thlaspi caerulescens* J. & C. Presl (*Brassicaceae*) plants grown under deficient, sufficient, and excess supply of zinc revealed a total of 608 zinc-responsive genes with at least a 3-fold difference in expression level in *A. thaliana*, and 352 in *T. caerulescens* in response to changes in zinc supply (van de Mortel *et al.*, 2006). A large number of these genes are of yet unknown function and many genes with a differential expression between *A. thaliana* and *T. caerulescens* had putative functions in metal homeostasis, in abiotic stress response, and in lignin biosynthesis. The high expression of lignin biosynthesis genes corresponded to the deposition of lignin in the endodermis, of which there are two layers in *T. caerulescens* roots and only one in *A. thaliana* (Van de Mortel *et al.*, 2006). Also, transcript profiling of *A. thaliana*, revealed the influence of the two factors independently and the interactions between phosphate (P) and sugar (sucrose; Suc) -dependent gene regulation (Müller, 2007). In response to P starvation, 171 genes were induced and 16 repressed, whereas Suc incubation resulted in 337 induced and 307 repressed genes. Nearly 150 genes were synergistically or antagonistically regulated by the two factors. Also, a number of new candidate genes involved in P acquisition were identified. In addition, several putative transcription factors and signaling proteins of P sensing were disclosed (Müller, 2007).

Ever since the studies of Turesson (1922), local adaptations have been extensively demonstrated in *Arabidopsis* plants (Banta *et. al.*, 2007; Riihimäki and Savolainen, 2004) as well as in other plant communities such as, *Potamogeton pectinatus*, a common aquatic plant (Santamaria, *et. al.*, 2003), *Cakile edentula* var. *lacustris*, a member of the family *Brassicaceae* (Dudley, 1996) and in an alpine *Polemonium viscosum* (Galen *et. al.*, 1991). *Arabidopsis* has been collected from a wide range of habitats and the genetic differences between the local populations have been studied to understand the adaptations to their prevailing environmental conditions (Beemster *et. al.*, 2002). Several *Arabidopsis* ecotypes collected over time have since been used to investigate a wide range of physiological processes, through evaluations between ecotypes and by genetic mapping using recombinant inbred lines (Alonso-Blanco and Koornneef, 2000). Even in early stages of A*rabidopsis* research, phenotypic characterization of *Arabidopsis* plants collected from different geographical regions in relation to ecologically important traits for adaptation to varied environments revealed extensive genetic variation (Rédei, 1970; Langridge and Griffing, 1959). However, with some important exceptions reported by Li *et al.*, 1998, that plants from high latitudes tend to have smaller plant size in terms of seed size, cotyledon width, rosette size, number of rosette leaves, size (leaf area) of the largest leaves, total leaf area, and total dry weight per plant than those from low latitudes under green house conditions, little is known about differences in the growth parameters of *Arabidopsis* ecotypes compared under standardized laboratory conditions.

1.5 Aims of the Project

Based on the existing work carried out by De Silva *et al.*, (1996; 1994) and De Silva and McAinsh (Personal Communication) at Lancaster University on the regulation of apoplastic Ca^{2+} in *A. thaliana* and calcicole-calcifuge physiology, it is apparent that a detailed understanding of the genetic basis of the mechanisms that enable calcicoles to thrive on calcareous soils is essential. The hypothesis is that adaptation to calcareous environment will be reflected in altered gene expression at varied rhizospheric Ca^{2+} conditions.

The aim of the present study is to 1) identify and determine the role and function, if any, of differentially expressing Ca^{2+} responsive genes in putative calcicoles and calcifuges under altered rhizospheric Ca^{2+} levels in soil and 2) to assess the relative importance of differentially expressed genes in adaptation to a calcareous environment.

This will be done by:

A) Physiological studies of plant growth at altered levels of rhizospheric Ca^{2+} in sand as well as on agar medium supplemented with 0-30 mM concentrations of $CaCl_2$ to understand phenotypic differences in growth and adaptation to altered levels of rhizospheric Ca^{2+}.

B) Analysis of pooled microarray data from 24 chips from experiments on *A. thaliana* "lab" ecotypes Col-4 (Putative calcifuge), Cal-0 (Putative calcicole) and "wild" ecotypes Elland (Putative calcicole) and Penicuik (Putative calcifuge) grown on altered levels of rhizospheric Ca^{2+}.

C) Quantitative real time expression analysis of differentially expressing rhizospheric Ca^{2+}-responsive genes in two "lab" ecotypes: Col-4, Cal-0

and four *A. thaliana* “wild” ecotypes: Elland, Halifax (Putative calcicoles), Penicuik and Glenisla (Putative calcifuges).

D) Phenotypic characterisation of T-DNA insertion lines for a subset of high Ca^{2+}-responsive genes.

Chapter 2

Materials and Methods

2.1 Chemicals

All chemicals were obtained from Sigma Company Ltd. (UK) unless otherwise stated.

2.2 Plant Material

The plant species selected for this study is *Arabidopsis thaliana* (thale cress) of the mustard family (*Brassicacea*) (TAIR database; Anderson and Wilson, 2000). The material for the investigation comprised 6 ecotypes. The ecotypes included the putative non-calcicole Columbia (Col-4) and the putative calcicole ecotype Cal-0, which is a genetically uniform line from an original population collected by Ratcliffe from a rocky limestone slope in 1954. These seed stocks were acquired from the Nottingham Arabidopsis Seed Stock Centre (NASC) (Nottingham, UK) initially and were bulked up for subsequent experiments. Also, four wild accessions were collected for the study from various regions across the UK (Table 2.1).

No.	Name	Site of collection	Map reference
1	Elland	Elland, West Yorkshire, England.	SE105190
2	Glenisla	Glenisla, Angus, Scotland	NO225607
3	Halifax	Halifax, West Yorkshire, England.	OS088235
4	Penicuik	Penicuik, Midlothian, Scotland	NT215595

Table 2.1: List of wild *A. thaliana* accessions collected from sites across UK.

2.3 Ca^{2+} content and pH estimation

Total soil Ca^{2+} concentration was calculated following the protocol of Bosnak and Grosser (1996). Atomic Absorbtion Spectrophotometer Analyst 200 (Perkin Elmer Corp., USA) was used for the same.

Soil samples were mixed with distilled water in 1:25 ratio, vortexed and pH values were recorded after 24 hours using a pH meter. Prior to the testing, the pH meter was calibrated using pH 4.0 and 7.0 buffers.

2.4 Growth conditions

Seeds were surface-sterilised in 1 ml 75% (v/v) ethanol for 10 minutes, and then dried for 1 hour. Seeds were then sown into half strength (2.2 g/l) Murashige and Skoog basal medium (MS) salts including 1% (w/v) sucrose and 0.8% (w/v) plant cell tissue culture grade agar. Petri plates were prepared in a sterile laminar hood (Envair Ltd., UK) with 25 ml of the above medium. Plates were then sealed with a microporous tape (Micropore®, 3M Health Care). Around 20-25 seeds were sown in each plate, and after 48 h of cold treatment in the dark at 4-5°C, plates were transferred to a controlled environment cabinet (Percival, USA) with a 10 h photoperiod, 160 $\mu mol\ m^{-2}\ s^{-1}$ photon flux density at an optimum temperature of 23 ± 1°C and 34% humidity, for 10 days. Illumination was provided by 740 TL warm white lamps (Philips, UK) and 25 W incandescent lamps (GE, USA).

2.5 Ca^{2+} treatments

10 day old seedlings usually having 2 cotyledons and 2 true leaves were transplanted into 2.5 cm^2 plug trays (Teku, UK) filled with sterile nutrient-

free sand (Silver Sand; Joseph Metcalf Ltd., Accrington, UK). All the seedlings were returned to the controlled environment cabinet and grown under similar conditions as described in section 2.3 and supplied daily with 1 mM $CaCl_2$ in half-strength Long Ashton nutrient solution (Table 2.2) for a further 3 days. The seedlings were then divided into groups of at least 5 plants and supplied daily with 1, 12.5 or 15 mM $CaCl_2$ {a concentration that is a little lower than the maximum quoted for calcareous soils (Fitter and Hay, 2002; De Silva *et al.*, 1994)} in half-strength Long Ashton nutrient solution, at least 4 groups per ecotype/mutant per Ca^{2+} treatment. The growth and development of the individual plants were monitored for 30 days following the imposition of the differential Ca^{2+} treatments.

Ten random individual plants of each ecotype/Ca^{2+} treatment were used to carry out various physiological measurements *viz.*, rosette length, root length and number of leaves. After the physiological measurements plants were harvested to obtain the average fresh weight. Then all the plant samples were dried at 70° C in a drying oven (Sanyo Gallenkamp, UK) for 3 days and weighed for an average dry weight for each ecotype/treatment. Also, 10 healthy plants were selected and washed three times with distilled water and pooled before snap-freezing in liquid nitrogen for RNA extraction from whole plants (see section 2.10) and subsequent assays. The experiment was repeated to obtain biological replicates for each ecotype and treatment.

Salt	Stock Solution (g/l)	Volume (ml) of stock sol. for 10 l working solution (1 X)
0.5 M Mg $SO_4.7H_2O$	184	20
2 M KNO_3	202	20
2 M Ca $(NO_3)_2.4H_2O$	472	20
0.75 M $NaH_2PO_4.4H_2O$	208	10
0.1 M FeNaEDTA	37.3	5
0.1 M $MnSO_4.4H_2O$	22.3	1
0.01 M $CuSO_4.5H_2O$	2.5	1
0.01 M $ZnSO_4.7H_2O$	2.9	1
0.5 M H_3BO_3	31	1
1 M NaCl	58.5	1
0.05 M $NaMoO_3.2H_2O$	1.2	1

Table 2.2: Recipe for Long Ashton Solution (Hewitt, 1966)

2.6 Physiological Measurements

The number of leaves on each individual plant was hand counted pre-harvest. The length between the two largest leaves of each plant was individually measured using a standard measuring ruler and considered as the rosette diameter.Individual plants were harvested and the roots were washed with distilled water and measured as the length of the primary root from the base of the cotyledon to the tip of the root with a standard measuring ruler. A weighing balance (Ohaus, Switzerland) was used to measure the fresh and dry weights.

2.7 Root Growth Studies

Twenty seeds on each petri dish (plated as described in Section 2.3) containing modified B5 medium (Zhang and Forde, 1998) (Table 2.3) were given a 2 day cold treatment in the dark at 4-5°C and then transferred to a controlled environment cabinet and grown vertically for 3 days. Two seedlings were then transferred to a fresh set of petri dishes (vertical) containing the modified B5 medium and a specific $CaCl_2$ concentration (0 mM/ 1 mM/ 15 mM/ 30 mM) and grown for a maximum of 10 days. In subsequent studies and discussion, '0 mM' refers to no added $CaCl_2$ in the medium. The medium in these plates was cut out from the top using a scalpel to facilitate shoot growth in air (Figure 2.1).

One litre of 20X Basal medium was prepared by mixing 40 ml 10X Macronutrients, 4 ml 100X Micronutrients, 0.8 ml 500X Fe-EDTA and dH_2O to make up 1 litre. pH was calibrated to 5.7 using KOH. One litre of plating

medium was prepared using 50 ml 20X Basal medium, 5 g Sucrose (0.5%), 10 g Agar (1%), 2.5 ml KNO_3 (of 2 M stock solution) and dH_2O to make up 1 litre. Each petri dish was supplied with 25 ml of the plating medium.

The plates were scanned using a Canon (CanoScan 4200F) Scanner. The plates were placed horizontally on the glass surface of the scanner for the scan. The room was darkened and the lid of the scanner was left open for optimum scans. Root lengths were measured using Optimas™ version 6.1 image analysis software (Optimas Corporation, USA). Physiological measurements *viz.*, shoot and root weights were recorded as specified in section(s) 2.4 and 2.5.

Modified B5 stock solutions				
• 10X Macronutrients				
Chemical	Concn. of 1x*	MW	Concn. of 1x (mg/l)	Concn. of 10x (mg/l)
KCl	5 mM	74.55	373	3730
$MgSO_4.7H_2O$	2 mM	246.46	500	5000
NaH_2PO_4	1.1 mM	119.98	132	1320
$MnSO_4.4\ H_2O$	45 μM	223.06	10	100

• 100X Micronutrients

Chemical	Concn. of 1x*	MW	Concn. of 1x (mg/l)	Stock	100x
KI	4.5 μM	166	0.75	0.5	9 ml
H_3BO_3	48.5 μM	61.83	3.0		300 mg/l
$ZnSO_4.\ H_2O$	7 μM	287.54	2.0		200 mg/l
$CuSO_4$	0.1 μM	249.70	0.025	50 mM	0.2 ml
$Na_2MoO_4.2\ H_2O$	1.03 μM	241.95	0.25	10 mM	10.3 ml
$CoCl_2.6H_2O$	0.105 μM	237.90	0.025	10 mM	0.95 ml

***Note**: The final concentrations in the medium are 1/50th of these.

- **500 x Fe-EDTA**

33 g/l (MW 367.1, concentration of 500x = 90 mM, final concentration in medium = 3.6 μM)

Table 2.3: Modified B5 Growth Medium

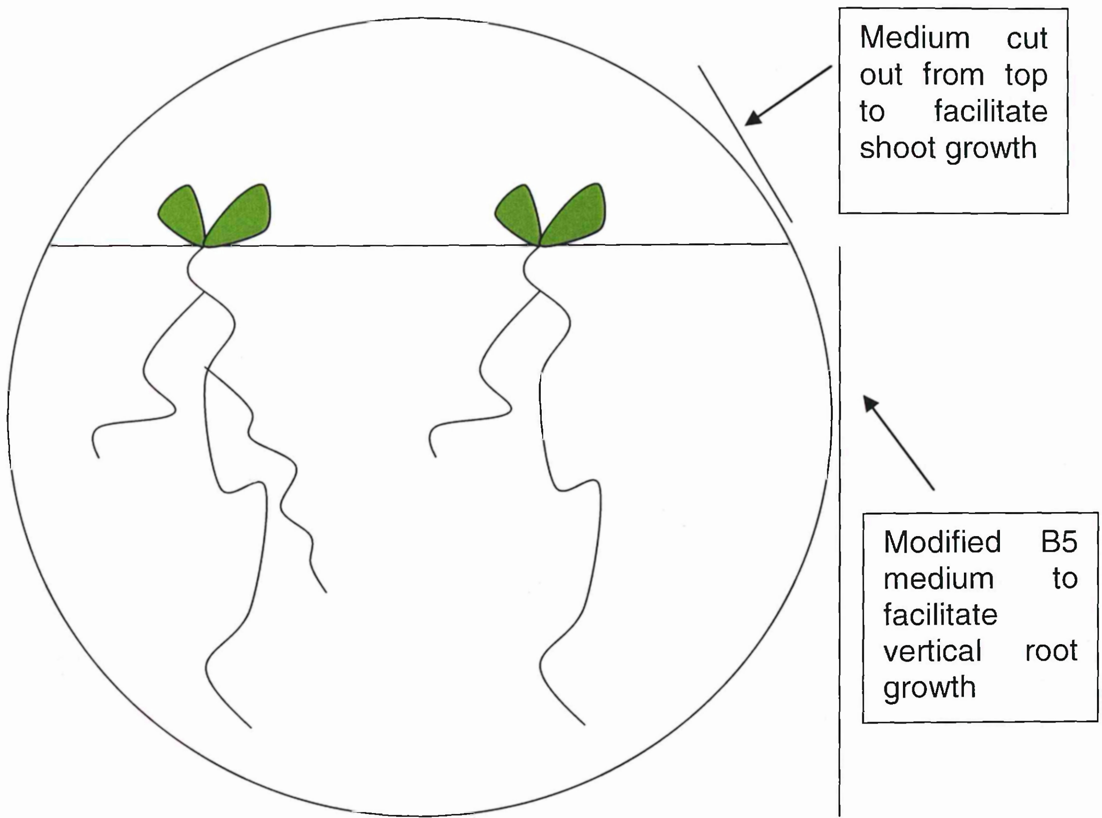


Figure 2.1: Diagrammatic representation of a vertical petri dish.

2.8 TUNEL assay

Apoptosis was assayed using the DeadEnd™ Flourometric TUNEL System (Promega, USA) for the specific detection of apoptotic cells in the roots grown on vertical petri dishes with varied Ca^{2+} concentrations. The protocol of the manufacturer was followed with slight modifications. The composition of buffers and solutions is given in Table 2.4. Whole intact seedlings grown in vertical petri dishes (prepared and grown as per section 2.6) with half strength MS medium (as per section 2.3) were used for the assay. The whole seedlings were initially fixed by immersing in 4% (w/v) paraformaldehyde dissolved in Phosphate Buffer Saline (PBS) in coplin jars for 25 min at room temperature. Each seedling was then placed on a glass slide and treated directly on the glass slides in the subsequent steps. Cover slips were used to cover the seedlings on the glass slides to facilitate the even spreading of solutions. Care was taken that the solutions were pipetted out near the root end on the glass slide and a cover slip was used to help the spreading of solution to the residual parts of the seedling. After washing with PBS (pH 7.4) for 5 min at room temperature to remove the fixative, the seedlings were permeabilized by treating with 0.2% (v/v) Triton® X-100 solution in PBS for 5 min. The seedlings were then washed using fresh PBS for 5 min at room temp and equilibrated at room temp for 10 min using the 100 µl equilibration buffer from the kit. The seedlings were then treated with 50 µl rTdT incubation buffer to catalytically incorporate flourescein-12-dUTP to the 3'-OH ends of fragmented DNA in apoptotic cells. The slides were then incubated in a light protected humid chamber at 37°C for 60 min. The reaction was then terminated by treating the seedlings with 2X Standard Saline Citrate

(SSC) solution for 15 min at room temp. Unincorporated fluorescein-12-dUTP was removed by three repeated washes with PBS for 5 min at room temperature. Propidium Iodide (1 µg/ ml in PBS) staining was performed at the end of the procedure as it stains both apoptotic and non-apoptotic cell walls red and stains the nuclei of dead cells only, whereas flourescein-12-dUTP incorporates localized green fluorescence within the nucleus of apoptotic cells only. Calibrator samples were prepared without any flourescein-12-dUTP staining to detect false positive dead nuclei by Propidium Iodide staining. All the steps previous to the Propidium Iodide staining were carried out as described above. The samples were immediately analysed by a confocal microscope (TCS SP2; Leica Microsystems AG, Wetzlar, Germany).

1X Phosphate Buffer Saline (PBS)	Equilibration Buffer
137 mM NaCl	200 mM Potassium cacodylate
2.68 mM KCl	25 mM Tris-HCl
1.47 mM KH_2PO_4	0.2 mM DTT
8.1 mM Na_2HPO_4	0.25 mg/ml BSA
	25 mM $CoCl_2$
rTdT incubation buffer (for 2 reactions)	**Nucleotide Mix**
90 µl Equilibration Buffer	50 µM fluorescein-12-dUTP
10 µl Nucleotide Mix	100 µM dATP
2 µl rTdT enzyme	10 mM Tris-HCl
	1 mM EDTA
20X Standard Saline Citrate	
87.7g NaCl	
44.1g Sodium Citrate	

Table 2.4: Composition of Buffers and Solutions for TUNEL assay

2.9 Confocal microscopy

Fixed, treated and stained roots were imaged *in situ* on slides using a laser- scanning confocal microscope (TCS SP2; Leica Microsystems AG, Wetzlar, Germany). Imaging was performed using an excitation wavelength of 500 nm and emission window of 750 nm. Root samples were analyzed using a standard fluorescein filter set to view the green fluorescence of fluorescein at 520 ± 20 nm and view red fluorescence of propidium iodide at >620 nm.

2.10 RNA Extraction

Individual pools consisting of ten plants, each grown as per section 2.3 were collected and used for subsequent RNA extraction. An RNeasy® plant mini kit (Qiagen) was used for extractions. This kit was designed to isolate up to 100 μg total RNA from plant cells from small quantities of starting material. A maximum of 100 mg plant material frozen in liquid nitrogen was used with RNeasy® mini columns for each extraction. The plant material was ground to a fine powder in liquid nitrogen (-196°C) in a pre-chilled pestle and mortar and the RNA extracted as recommended by the manufacturer. The RNA was placed into quartz cuvettes and quantified, using a spectrophotometer (Ultrospec 2100 pro; Biochrom Ltd., Cambridge, UK). Quality was ensured by A260/280 ratio (>1.9) and visualization after electrophoresis on a 1.4% (w/v) agarose TAE gel under an UV illuminator. The samples were stored at -20°C for further use.

2.11 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Synthesis of cDNA from total RNA was carried out as recommended by the manufacturer of the TaqMan® reverse transcription reagents (Applied Biosystems, US). Using this protocol a 100 μl RT reaction efficiently converts a maximum of 2 μg total RNA to cDNA. RNase-free water, Random hexamers, 10X TaqMan RT Buffer, $MgCl_2$, deoxyNTPs, RNase Inhibitor and MultiScribe Reverse Transcriptase constituted the RT reaction mix (Table 2.5).

RT-PCR was performed with the thermal steps: 25°C for 10 min (incubation step to maximize hexamer-RNA template binding), 48°C for 30 min (Reverse Transcription step) and 95 °C for 5 min (Reverse Transcriptase inactivation step) in an Applied Biosystems, Inc. Mastercycler® Personal, a programmable thermal controller. Products were visualised after electrophoresis on a 1% (w/v) agarose TAE gel under an UV illuminator.

Component	Volume (μl) Per sample	Final Concentration
Rnase-free water	See below [a]	_
10X TaqMan RT Buffer	10.0	1X
25 mM $MgCl_2$	22.0	5.5 mM
DeoxyNTPs Mixture	20.0	500 μM per dNTP
Random Hexamers	5.0	2.5 μM
RNase Inhibitor	2.0	0.4 U/μl
MultiScribe Reverse Transcriptase (50/μl)	2.5	1.25 U/μl
Total	61.5	_

a. The volume of Rnase-free water (μl) is 38.5-RNA (2 μg) sample volume in a 100 μl reaction

Table 2.5: RT Reaction Mix

2.12 Microarray Data Analysis

GeneSpring® software (Agilent Technologies, US) was initially used in Chapter 5 to identify differentially expressed genes from microarray experiments in Col-4 and Cal-0 *Arabidopsis* treated with 1 mM and 12.5 mM rhizospheric Ca^{2+}. Reliable statistical tests including t-tests, 2-way ANOVA tests and 1-way post-hoc tests were applied to filter the microarray data. In addition, GeneSpring's class prediction tools were used to identify genes capable of discriminating between one or more experimental parameters or sample phenotypes. Clustering techniques were also used to reduce the complexity of data and discover genes that are primarily responsible for the variation. Normalization steps were applied and with the pathway viewer, genes and their expression patterns were visually characterized. Using GeneSpring, the expression profile of a given sample was compared to all of the other samples using various parameters from the microarray experiments carried out earlier in the lab on Cal-0 and Col-4 treated at low and high Ca^{2+} concentrations. Different groups of genes were generated based on their expression patterns at a specific experimental scenario.

Detailed analysis of 24 microarray chips for Chapter 5 was carried out using DNA-Chip Analyzer (dChip) software version 1.0.0.1 (Zhong *et al.*, 2003; Li and Wong 2003, 2001a, 2001b). Genes with $\log_2$ expression values less than 6.0 in greater than 50 per cent of the arrays and genes with a $\log_2$ standard deviation less than 0.5 across all arrays were excluded from further analysis.

Functional classification of the differentially regulated genes was performed by the identification of functional classes over-represented in the

induced and suppressed gene clusters by dChip software and manual inspection. The probability that the number of genes within each Gene Ontology classification present in each cluster was calculated (*i.e.* that there are '*n*' Ca^{2+} transport genes in the cluster). Functional categories with a $P \leq 0.005$ were considered significant.

2.13 Quantitative Reverse Transcriptase PCR (qRT-PCR)

2.13.1 Designing Custom Target Sequences for Quantitation

Primer Express™ version 2.0 software (Applied Biosysytems, US) was used to design primers to amplify short segments of the target cDNA for real time quantitation assays. These short segments of cDNA are called amplicons. Shorter amplicons work most efficiently, with the most consistent results obtained for amplicon sizes in the 50 to 150 bp range. Gene sequence information was obtained from GenBank and primers were designed for the gene(s) of Interest (GOI) as follows:

- Primers were designed as close as possible to each other so that they do not overlap.
- The Guanine-Cytosine (GC) content was in the range 20 to 80%.
- Runs of an identical nucleotide were avoided. This is especially crucial for guanine, where runs of four or more Gs were avoided.
- The melting temperature of the primer(s) (Tm) was in the range of 58 to 60°C.
- The five nucleotides at the 3' end didn't have any more than two G and/ or C bases.

After identifying a set of genes of interest using the GeneSpring® software, real time PCR was carried out using the specific designed primers (Appendix 1).

2.13.2 qRT-PCR

Quantitative Reverse Transcriptase PCR (qRT-PCR) or real time PCR facilitates the use of a fluorescent dye (SYBR Green) to detect the absolute or relative amount of amplified product. The quantity of cDNA is measured in real time throughout the reaction and the cycle number at which the amplicon reaches a fixed threshold is recorded. This recorded C_T (cycle number) value was used to calculate a relative amount of gene expression by comparison to a control. In this study Col-4 watered with 1 mM Ca^{2+} was considered as control.

A qRT-PCR reaction mixture comprising of 12.5 µl SYBR Green master mix (Applied Biosystems, UK), 1 µl cDNA (prepared from experimental samples), 1.5 or 4.5 µl of each primer and dH_2O were used to make up 25 µl. The PCR run was carried out using the thermal profile: 96°C for 10 min and then, 40 cycles of: 96°C for 20 s, 50°C for 20 s and 60°C for 4 min in an Applied Biosystems 7000 Sequence Detection System. The relative gene expression was calculated by the ΔC_T method using the formula $2^{-\Delta\Delta CT}$ (as recommended by Applied Biosystems), where:

ΔC_T is the difference in threshold cycles for target (Gene of Interest) and reference genes (Endogenous Control *i.e.*, Housekeeping genes).

$\Delta\Delta C_T$ is the difference in ΔC_T for sample and calibrator (Col-4 treated with 1 mM Ca^{2+}).

Ubiquitin (house keeping gene) was chosen as an endogenous control gene, for its stability under various sets of experimental controls (Brunner *et al.*, 2004) after trying out actin and tubulin as well. For an example of $2^{-\Delta\Delta CT}$ (Relative Gene expression) calculation refer to Appendix 3.

2.14 T-DNA insertion lines

2.14.1 Identification of homozygous lines

Arabidopsis T-DNA insertion mutants (Chapter 6) were obtained from Nottingham *Arabidopsis* Seed Stock Centre (NASC) (Nottingham, UK). Insertion mutant information was obtained from http://signal.salk.edu. The T3 seeds obtained from NASC have a Col-0 background and were sown straight to soil in plug trays containing 3: 1 ratio of sterilised compost (William Sinclair Horticulture, UK) and fine horticultural grit (William Sinclair Horticulture, UK) and watered with distilled water daily. These trays were placed in growth cabinets with a PFD of 100 µmol m^{-2} s^{-1} and a 16/ 8 h (light/dark) photo period with an optimum day/ night temperature of 25 ± 2°C/ 18 ± 2°C. These plants were transferred to Aracon baskets in Aracon Trays (Araysystems, Betatech, Belgium) with the same soil mix, after a further 2 weeks and grown till harvest of seeds.

T4 seeds were surface sterilised in 1 ml of 75 % (v/v) ethanol for 10 min and then dried for 1 h. Subsequently, these seeds were grown on a full-strength MS medium with 0.8 % (w/v) Agar and 50 µg/ ml kanamycin. After 48 h in the dark at 4°C the sealed plates were transferred to a controlled environment cabinet as in section 2.3. After 2 weeks kanamycin (marker

gene) resistant seedlings were transferred to plug trays as above and T5 seeds bulk harvested.

2.14.2 PCR confirmation of homozygous T-DNA lines

To identify the homozygous lines a conventional PCR was carried out on the DNA of each individual plant. DNA was extracted using DNeasy® plant mini kits (Qiagen) from the leaves ground to a fine powder in liquid N_2. PCR was carried out using the T-DNA left border primer (LBb1) along with the left border (LP) and right border genomic primer (RP) for the specific Gene of Interest to identify the homozygous lines. The left and right border specific genomic primers were designed using the Primer Express™ version 2.0 software (Applied Biosysytems, US) for the gene of interest with the same parameters as above (section 2.13.1). The primers for the left and right flanking regions of the genomic DNA are presented in Appendix 2. DNA amplification was carried out in a Thermal Cycler (Applied Biosystems, Inc. Mastercycler® Personal), programmed for 5 min at 94°C for initial denaturation and 35 cycles consisting of: 45 seconds at 94°C, 45 s at 58°C and 1 min at 72°C with a final 5 min extension at 72°C using fastest ramp time between the temperature transitions.

2.14.3 Physiological treatments

All the T-DNA insertion lines were germinated on petri dishes as per section 2.3 and transferred to plug trays for Ca^{2+} treatments as per section 2.4. The insertion lines were grown at 1 mM or 15 mM Ca^{2+} for a maximum of 30 days. Then, 10 healthy individual plant representatives of each ecotype

under the respective experimental conditions were used to carry out various physiological measurements *viz.*, rosette length, root length and number of leaves (See section 2.6). Also, 10 healthy plants were selected for RNA extraction as per section 2.10.

2.14.4 qRT-PCR of insertion lines

Quantitative real time PCR was carried out similar to section 2.11.2 on all the insertion lines grown at 1 mM or 15 mM concentrations of external added Ca^{2+}. Clustering analysis of representative ecotypes based on their relative gene expression was carried out using DNA-chip Analyzer (dChip) software version 1.0.0.1.

2.15 Statistical analysis

Statistical analysis of results was performed using SigmaStat software version 2.03 (SPSS Inc., USA) or Microsoft Excel where appropriate. Physiological measurements were subjected to ANOVA and Fisher's Least Significance Difference (LSD) test was applied to assess the statistical significance among the means.

Chapter 3

Effect of rhizospheric Ca^{2+} on the growth of laboratory and wild calcicole and calcifuge ecotypes of *Arabidopsis*

3.1 Introduction

Plants exhibit a wide range of morphogenic and adaptive responses when exposed to abiotic stress conditions (Potters *et al.,* 2007). The adaptation shown by plants to survive on calcareous soils has interested researchers for many years (Lee, 1999; Clarkson, 1965; HopeSimpson, 1938; Salisbury, 1920). *A. thaliana* is reported to exist widely across Europe, continental Asia and Northern Africa (Hiffmann *et al.,* 2003; Al-Shehbaz and O'Kane, 2002; Vander Zwan *et al.,* 2000; Ratcliffe, 1965). Ratcliffe (1961) reported the existence of *A. thaliana* in a wide spread of open natural habitats which were divergently calcareous. Also, according to Ratcliffe (1965) it is clear that *Arabidopsis* occurs in areas with widely different climates, and if it was introduced to these regions, it has adapted to the local conditions and survived.

A. thaliana is an excellent 'model' dicot plant species to investigate natural variation (Maloof, 2003) in tolerance to varied rhizospheric Ca^{2+} concentrations. Also, according to Boyes *et al.* (2001), altered environmental conditions may affect any number of traits in *Arabidopsis* ecotypes resulting in morphological changes.

Plant researchers tend to grow plants in pure sand for mineral nutrition experiments and in many experiments various crops have been grown in pure

glass sand, with the sand providing only mechanical anchorage for the plant (Throckmorton, 2007). Over the years, *Arabidopsis* plants have been grown on various solid media as well as hydroponic solutions for various experimental procedures (Wright, 1992). But, if we need to analyze roots, or want roots free of debris, a soilless culture has to be established. Also, by growing plants on soilless culture the influx of nutrients available to the plant can be controlled by adding a suitable nutrient solution. The advantages of sand culture are that the plants are provided with made to measure fresh nutrient solution at every irrigation cycle, the excellent capillary action of sand helps in even distribution of nutrients over the whole root zone (Wright, 1992) and easy harvest of whole plants with an intact root system after the experimental cycle.

For the current investigation, suitable culture conditions for growth of *Arabidopsis* ecotypes were established and then physiological studies of the responses to varied edaphic Ca^{2+} conditions were carried out. The work presented in this chapter is a study of physiological differences exhibited between populations of *A. thaliana*, collected from a variety of distinct soils (here referred to as "wild' ecotypes) as well as established accessions held in seed collections (here referred to as "lab" ecotypes). The recorded parameters such as: number of leaves, rosette diameter, and root length, fresh and dry weight, on altered levels of rhizospheric Ca^{2+} will give an initial insight into how the ecotypes respond to these altered edaphic conditions. This study will help in furthering the understanding of the calcicole-calcifuge phenotype in lab ecotypes used commonly in stress response studies as well as wild ecotypes collected from natural areas varyingly calcareous. Further,

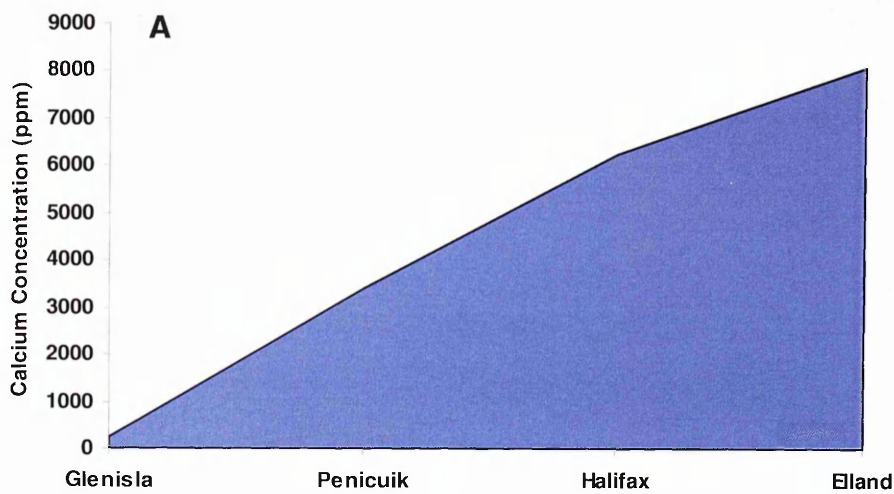
the data obtained will give a fundamental basis to understand further the calcicole-calcifuge variation and adaptive approaches of plants to varyingly calcareous soils.

3.2 Results

3.2.1 Selection of a suitable plant growth medium to perform physiological studies

A. thaliana natural habitats across the U.K. showed diverse amounts of soil Ca^{2+} and pH (Figure 3.1). Soil Ca^{2+} concentrations from the sampled sites ranged from 260 ppm Ca^{2+} in soil from Glenisla to 8140 ppm Ca^{2+} in soil from Elland (Figure 3.1). Also, the pH of the soil differed varyingly across the sampled sites (Figure 3.1). In the present study, *A. thaliana* ecotypes collected from very low Ca^{2+} regions were classified as putative calcifuges (Glenisla and Penicuik) and those from highly calcareous regions as putative calcicoles (Halifax and Elland).

In order to manipulate Ca^{2+} concentrations over a wide range, a stable plant growth medium with a minimal amount of inherent Ca^{2+} was necessary. Ca^{2+} content analysis of untreated soil, diatomaceous earth and sand by atomic absorption spectrophotometry revealed high levels of total Ca^{2+} even in untreated soil (4700 ppm) compared to diatomaceous earth (260 ppm) and sand (60 ppm) (Figure 3.2). A good plant growth medium should also retain sufficient moisture, provide adequate aeration and be firm enough to support the plant (Saupe, 2005). Sand was therefore chosen as the growth medium for further physiological studies. 1 mM $CaCl_2$ (~40 ppm concentration of free Ca^{2+}) and 12.5 mM $CaCl_2$ (~500 ppm of free Ca^{2+}) solutions were used to alter the rhizospheric Ca^{2+} concentrations in sand. The plants were watered daily with these $CaCl_2$ solutions.



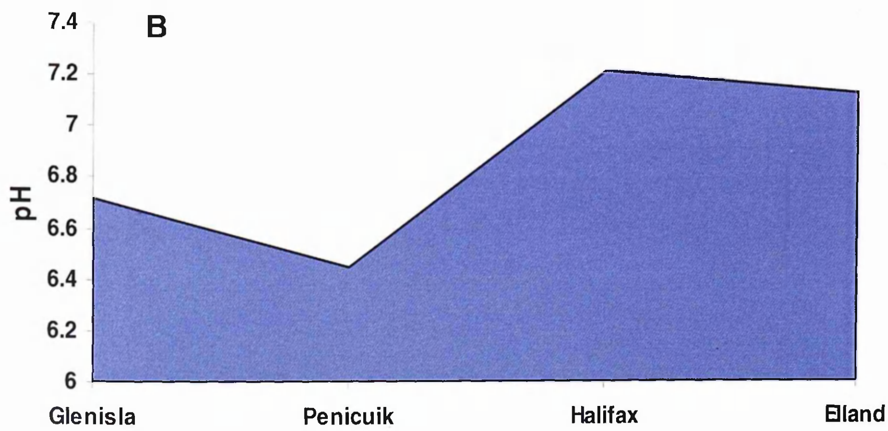


Figure 3.1: A- Total soil Ca^{2+} concentration (ppm) and **B-** pH of 4 sites where wild *Arabidopsis* were collected.

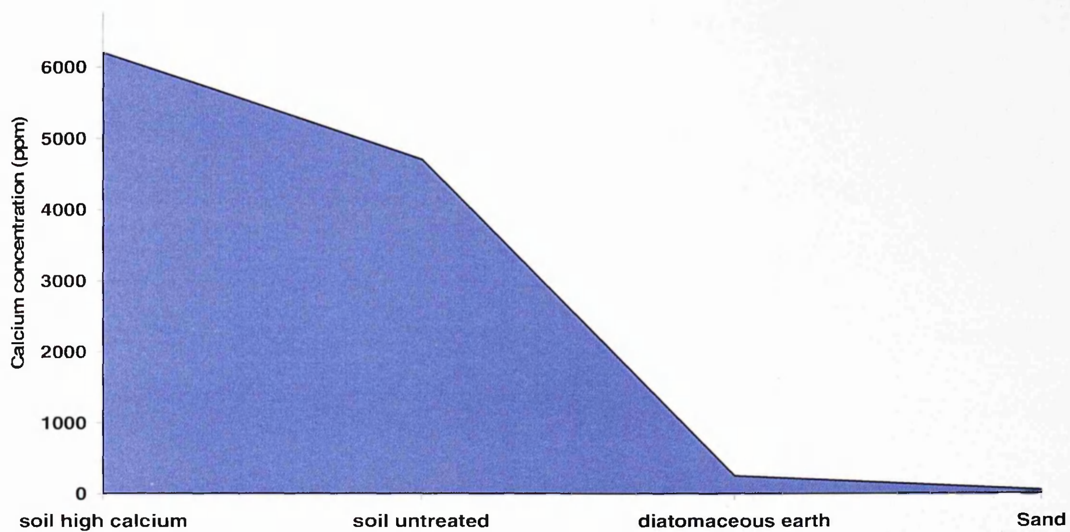


Figure 3.2: Rhizospheric Ca^{2+} concentration (ppm) in various growth media. High Ca^{2+} treatment is 12.5 mM of $CaCl_2$. Soil high Ca^{2+} was treated with 12.5 mM $CaCl_2$ for 30 days. Diatomaceous earth and sand are shown untreated.

3.2.2 Differences in growth of *Arabidopsis* ecotypes in response to altered rhizospheric calcium

To characterise initially the morphogenic responses of the 6 ecotypes under study, in response to 1 mM and 12.5 mM rhizospheric Ca^{2+}, number of leaves, root length, rosette diameter, fresh weight and dry weight were recorded over a 30 day period at 10th, 20th and 30th day time points (Harvest 1, 2 and 3 respectively). All the measurements were subjected to ANOVA using SigmaStat® (SPSS Inc., USA). Fisher's least significance difference (LSD) test was applied to assess the statistical significance among the means.

Number of Leaves

The number of leaves of the 6 ecotypes grown on sand with altered rhizospheric Ca^{2+} concentrations (1 mM and 12.5 mM) differed significantly ($P \leq 0.05$) at different harvest points among the ecotypes (Figure 3.3; Table 3.1). After 30 days, Cal-0 plants grown on 1 mM Ca^{2+} concentration had the highest average number of leaves (24.4 ± 0.50) with Glenisla plants grown on 12.5 mM Ca^{2+} concentration the least (13.7 ± 0.37) (Figure 3.3). ANOVA analysis illustrated that all the 6 ecotypes grown on either 1 mM or 12.5 mM Ca^{2+} concentration, respectively, had a significant difference ($P \leq 0.05$) in their mean number of leaves between the two treatments after 20 days, which was evident after 30 days as well. Multiple comparison procedure showed that Glenisla (putative calcifuge) plants differed significantly ($P \leq 0.05$) in the number of leaves with all other ecotypes, excluding Penicuik (putative calcifuge) when grown on 12.5 mM rhizospheric Ca^{2+}, at harvest point(s) 2

and 3 (Table 3.1). Also, after 20 days of Ca^{2+} exposure, there were significant differences ($P \leq 0.05$) in the number of leaves among the ecotypes for either (1 mM or 12.5 mM) of the Ca^{2+} treatments, with the exception of Col-4 and Halifax and between Elland and Penicuik, respectively, at 1 mM Ca^{2+}, and between Penicuik and Glenisla at 12.5 mM Ca^{2+} treatments (Table 3.1).

Multiple comparisons for the mean number of leaves among all ecotypes treated with 1 mM Ca^{2+} concentration revealed significant differences ($P \leq 0.05$) at 30 days, excluding between Cal-0 (24.2 ± 0.41) and Halifax (23.7 ± 0.33) (Table 3.1). Comparing all the ecotypes treated with 12.5 mM concentration of Ca^{2+}, revealed a significant difference ($P \leq 0.05$) in the mean number of leaves for harvest 3 (30 days), with the exception of Penicuik (14.2 ± 0.36) and Glenisla (13.7 ± 0.37).

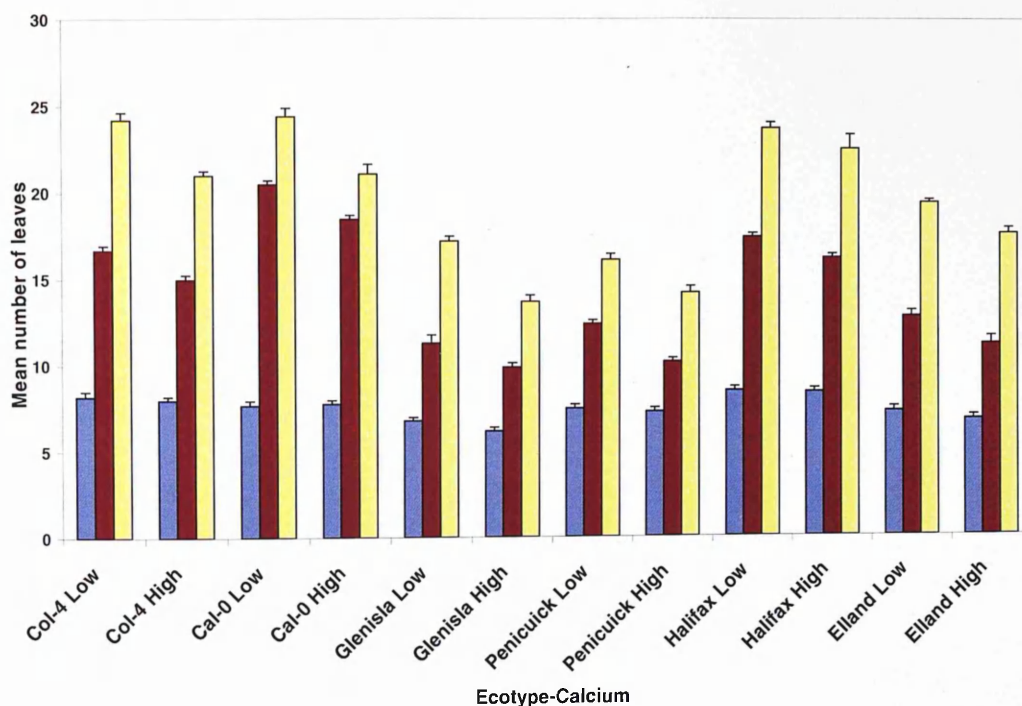


Figure 3.3: The effect of growth on low (1 mM) and high (12.5 mM) rhizospheric Ca^{2+} for 30 days on the number of leaves in six ecotypes of *Arabidopsis.* Bars (mean of 10 plants) in Blue = 10th Day, Brown = 20th Day and Yellow = 30th Day harvest points. Bars represent mean ± SEM.

	Ca^{2+} conc.	Col-4 1 mM	Col-4 12.5 mM	Cal-0 1 mM	Cal-0 12.5 mM	Glenisla 1 mM	Glenisla 12.5 mM	Penicuik 1 mM	Penicuik 12.5 mM	Halifax 1 mM	Halifax 12.5 mM	Elland 1 mM	Elland 12.5 mM
Col-4	1 mM	-	•▲		▲	*•▲	*•▲	•▲	•▲		▲	•▲	*•▲
	12.5 mM		-			*•▲	*•▲	▲	•▲	•	•	•▲	*•▲
Cal-0	1 mM	•	•▲	-	•▲	•▲	*•▲	•▲	•▲	•	•▲	•▲	•▲
	12.5 mM	•	•▲		-	*•▲	*•▲	•▲	•▲			•▲	*•▲
Glenisla	1 mM					-	•▲	▲	•▲				
	12.5 mM						-						
Penicuik	1 mM					•	*•▲	-	•▲				•
	12.5 mM						*		-				
Halifax	1 mM		•▲		▲	*•▲	*▲	*•▲	*•▲	-	•▲	*•▲	*•▲
	12.5 mM		•▲		▲	*•▲	*•▲	•▲	*•▲		-	*•▲	*•▲
Elland	1 mM					•▲	*▲	▲	•▲		•	-	•▲
	12.5 mM						•▲	▲	•▲				-

Table 3.1: Multiple comparisons among ecotypes for number of leaves. Symbols *, •, ▲ indicate a significant difference ($P \leq 0.05$) in mean number of leaves at harvests on 10th (1), 20th (2) and 30th (3) day respectively, grown on 1 mM or high 12.5 mM Ca^{2+} concentrations.

Rosette diameter

All the 6 ecotypes were treated with either 1 mM or 12.5 mM rhizospheric Ca^{2+} as specified in Section 2.4 and the rosette diameter was measured after 10, 20 and 30 days of rhizospheric Ca^{2+} exposure (Figure 3.4). After only 10 days of Ca^{2+} exposure, significant differences ($P \leq 0.05$) in rosette diameter were observed between putative wild calcifuges Glenisla compared to Penicuik as well as Glenisla compared to Cal-0 treated with 1 mM Ca^{2+}, and between Elland compared to Cal-0, and Penicuik treated with 12.5 mM rhizospheric Ca^{2+} (Table 3.2). Twenty days of exposure to higher Ca^{2+} concentration (12.5 mM) significantly decreased ($P \leq 0.05$) the rosette diameters of all six ecotypes in comparison to them treated with 1 mM rhizospheric Ca^{2+}, and a similar decreased trend was evident after 30 days as well (Figure 3.4; Table 3.2).

Exposure of the ecotypes to 1 mM Ca^{2+} for 20 days revealed a significantly larger ($P \leq 0.05$) rosette diameter of putative wild calcicole Elland in comparison to the other three wild ecotypes *viz.*, Glenisla, Penicuik (putative wild calcifuges) and Halifax (putative wild calcicole) and Cal-0 (putative lab calcicole). However, by 30 days of exposure only Glenisla, Penicuik and Cal-0 had significantly smaller ($P \leq 0.05$) rosette diameters. Also, 12.5 mM rhizospheric Ca^{2+} exposure resulted in a significantly large ($P \leq 0.05$) rosette diameter of Elland, in comparison to all the other five ecotypes which was evident after 20 days as well as 30 days of treatment (Figure 3.4; Table 3.2).

Harvest 3, after 30 days of exposure to either Ca^{2+} treatment (1mM or 12.5 mM) of the six ecotypes showed that both the putative calcicoles, Elland

and Halifax had a significantly larger ($P\leq0.05$) rosette diameter in comparison to the other four ecotypes comprising Col-4, Cal-0, Glenisla and Penicuik when exposed to 12.5 mM rhizospheric Ca^{2+} (Table 3.2). Also, the former both had a significantly large rosette diameter ($P\leq0.05$) in comparison to Cal-0, Glenisla and Penicuik when exposed to 1 mM rhizospheric Ca^{2+} for 30 days (Table 3.2).

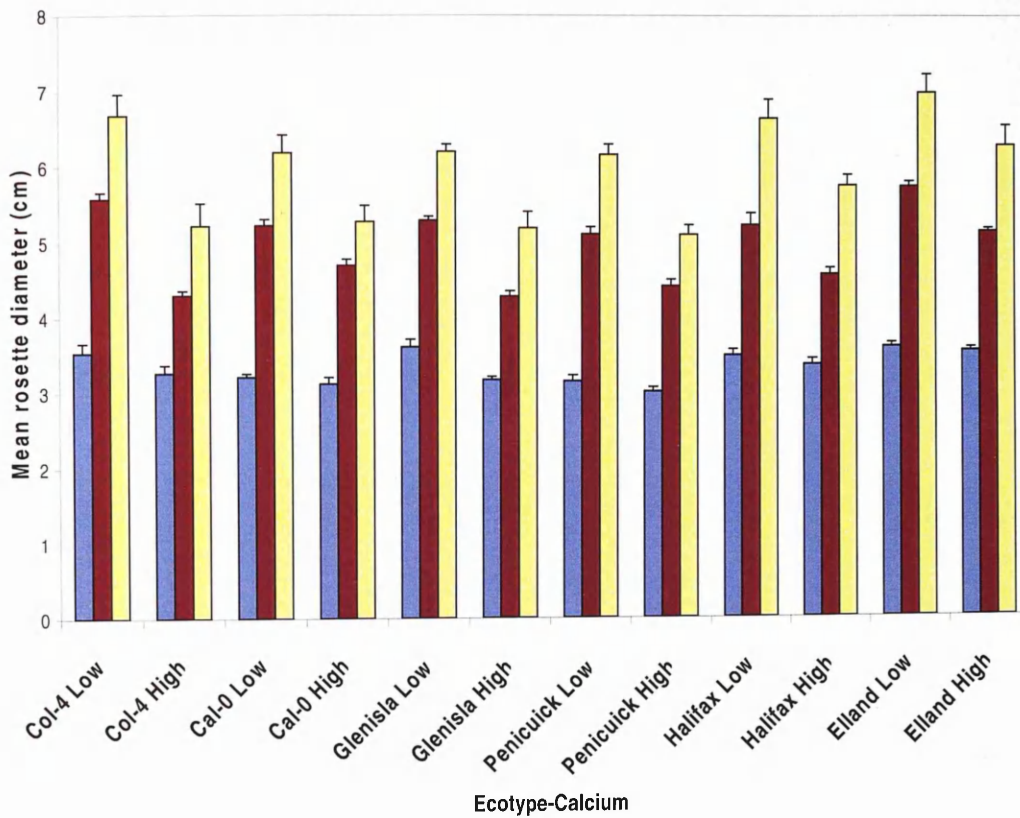


Figure 3.4: The effect of growth on low (1 mM) and high (12.5 mM) rhizospheric Ca^{2+} for 30 days on the rosette diameter (cm) in six ecotypes of *Arabidopsis*. Bars (mean of 10 plants) in Blue = 10th Day, Brown = 20th Day and Yellow = 30th Day harvest points. Bars represent ± SEM.

		Col-4		Cal-0		Glenisla		Penicuik		Halifax		Elland	
	Ca^{2+} conc.	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM
Col-4	1 mM	-	•▲	▲	*•▲	▲	•▲	•▲	*•▲		•▲		•▲
	12.5 mM		-										
Cal-0	1 mM		•▲	-	•▲		•▲		•▲		•▲		
	12.5 mM		•		-		•						
Glenisla	1 mM		•▲	*	*•▲	-	*•▲	*	*•▲		•▲		
	12.5 mM						-						
Penicuik	1 mM		•▲		•▲		•▲	-	•▲		•▲		
	12.5 mM								-				
Halifax	1 mM		•▲	▲	•▲	▲	•▲	▲	*•▲	-	•▲		
	12.5 mM		▲		▲		▲		▲		-		
Elland	1 mM		•▲	•▲	*•▲	•▲	*•▲	•▲	*•▲	•	•▲	-	•▲
	12.5 mM		•▲		*•▲		•▲		*•▲		•▲		-

Table 3.2: Multiple comparison among six ecotypes for rosette diameter (cm). Symbols *, •, ▲ indicate a significant difference ($P \leq 0.05$) in mean rosette diameter at harvests on 10th (1), 20th (2) and 30th (3) day respectively, grown on 1 mM or 12.5 mM Ca^{2+} concentrations

Primary root Length

Primary root length (Figure 3.5) was measured for ecotypes grown under low/high Ca^{2+} concentrations (1 mM and 12.5 mM) after 10, 20 and 30 days. Among the six ecotypes, only Glenisla root length significantly varied ($P \leq 0.05$) between Ca^{2+} treatments (12.5 mM compared to 1 mM) at all the three harvest points, whereas, Col-4 had a significant root length difference after 20 days as well as 30 days and Cal-0 had a significantly different root length after 20 days between 1 mM and 12.5 mM Ca^{2+} treatments (Table 3.3).

There were no significant differences ($P > 0.05$) in the root lengths of the ecotypes for 1 mM Ca^{2+} treatment after 10 days. However, for the 12.5 mM Ca^{2+} treatment, a significantly longer ($P \leq 0.05$) root length was observed in all ecotypes in comparison to Glenisla (2.86 ± 0.07 cm), as well as a significant difference ($P \leq 0.05$) between Elland (4.20 ± 0.04 cm) and both Cal-0 (3.53 ± 0.05 cm) and Penicuik (3.6 ± 0.03 cm).

After 20 days, 1 mM Ca^{2+}-exposed plants of both Penicuik (4.78 ± 0.08 cm) and Halifax (4.82 ± 0.05) revealed a significantly shorter ($P \leq 0.05$) root length in comparison to both Col-4 (5.74 ± 0.10 cm) and Cal-0 (5.54 ± 0.1 cm) ecotypes, respectively (Table 3.3). Elland (5.88 ± 0.06 cm) and Glenisla (3.51 ± 0.06 cm) at 20 days, 12.5 mM rhizospheric Ca^{2+} had a significant difference in the mean root length when compared to all the other five ecotypes (Table 3.3).

One mM rhizospheric Ca^{2+} treatment for 30 days revealed a significant ($P \leq 0.05$) root length difference between Col-4 and all the other 5 ecotypes, with Col-4 having the longest mean root length (7.96 ± 0.31 cm). 12.5 mM Ca^{2+} exposure for 30 days revealed a significant difference ($P \leq 0.05$) between

the root length of Glenisla and all the other 5 ecotypes, with, putative wild calcifuge Glenisla having the smallest roots (5.45 ± 0.21 cm) (Figure 3.5; Table 3.3). Also, putative wild calcicole Elland (6.92 ± 0.27 cm) had a significantly longer ($P \leq 0.05$) root length in comparison to both lab ecotypes, Col-4 (6.23 ± 0.17 cm) and Cal-0 (6.32 ± 0.10 cm) after 30 days of exposure to 12.5 mM rhizospheric Ca^{2+} (Figure 3.5; Table 3.3).

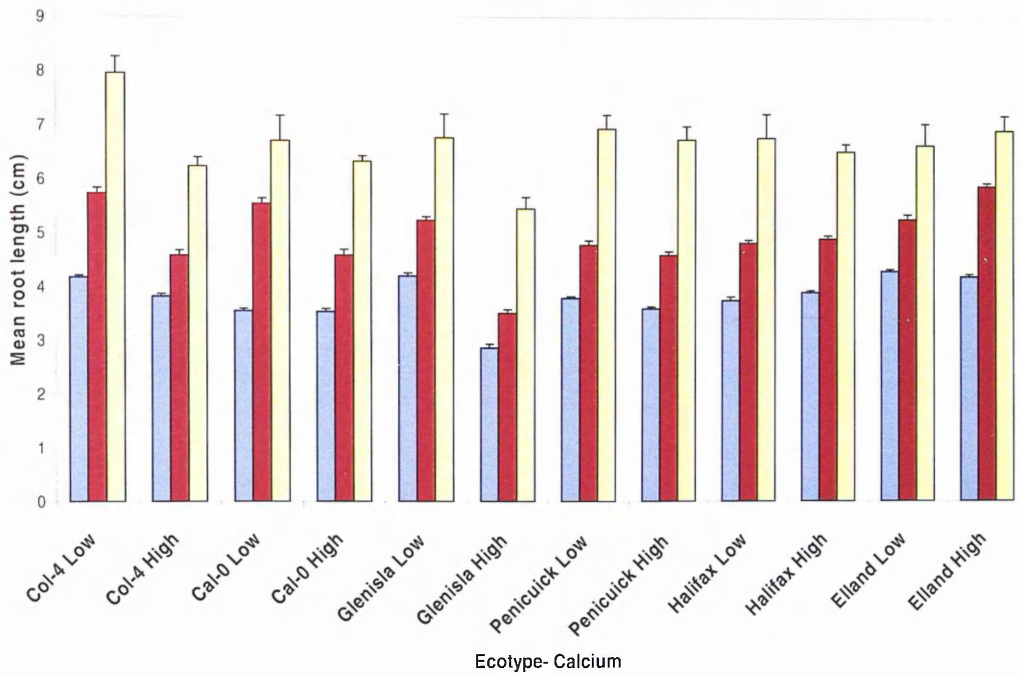


Figure 3.5: The effect of growth on low (1 mM) and high (12.5 mM) rhizospheric Ca^{2+} for 30 days on the root length (cm) in six ecotypes of *Arabidopsis*. Bars (mean of 10 plants) in Blue = 10th Day, Brown = 20th Day and Yellow = 30th Day harvest points. Bars represent ± SEM.

	Ca^{2+} conc.	Col-4		Cal-0		Glenisla		Penicuik		Halifax		Elland	
		1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM
Col-4	1 mM	-	● ▲	*▲	*● ▲	▲	*●▲	●▲	*●▲	●▲	●▲	▲	▲
	12.5 mM		-				*●▲						
Cal-0	1 mM		●	-	●		*●▲	●	●	●	●		
	12.5 mM				-		*●▲						
Glenisla	1 mM		● ▲	*	*●	-	*●▲		*●				
	12.5 mM						-						
Penicuik	1 mM		▲		▲		*●▲	-					
	12.5 mM						*●▲		-				
Halifax	1 mM		▲				*●▲			-			
	12.5 mM						*●▲				-		
Elland	1 mM		●	*	*●		*●▲		*●	*		-	
	12.5 mM		● ▲	*	*● ▲	●	*●▲	●	*●	●	●	●	-

Table 3.3: Multiple comparisons among six ecotypes for root length (cm). Symbols *, •, ▲ indicate a significant difference ($P \leq 0.05$) in mean root length at harvests on 10th (1), 20th (2) and 30th (3) day respectively, grown on 1 mM or 12.5 mM Ca^{2+} concentrations.

Fresh and Dry Weight Measurements

The plants of the 6 ecotypes under experimental rhizospheric Ca^{2+} treatments were harvested after 10, 20 and 30 days. A total of ten plants per ecotype and treatment were weighed for their fresh weight (Figure 3.6) and the subsequent dry weights measured (Figure 3.7) after oven drying as specified in Section 2.4.

All six ecotypes had no significant difference (P>0.05) in their respective fresh weights between the Ca^{2+} treatments (1 mM compared to 12.5 mM) after 10 days of exposure (Table 3.4). But, Col-4 (putative lab calcifuge) and Halifax (putative wild calcicole) exposed to 1 mM rhizospheric Ca^{2+} had a significant fresh weight difference (P≤0.05) after 10 days which was evident after 20 as well as 30 days, respectively (Table 3.4). Also, only Cal-0 had a significant difference (P≤0.05) in fresh weight after 20 days between the Ca^{2+} treatments (1mM compared to 12.5 mM), which wasn't evident (P>0.05) after 30 days of exposure (Table 3.4). However, Cal-0 (putative lab calcicole) had a significantly higher (P≤0.05) fresh weight than both putative wild calcifuges, Glenisla and Penicuik after 20 days of exposure to 12.5 mM rhizospheric Ca^{2+}, but the same was observed in respect to Penicuik only after 30 days of exposure (Table 3.4).

After 30 days of exposure to either 1mM or 30 mM rhizospheric Ca^{2+} only four ecotypes (Col-4, Glenisla, Elland and Halifax) had a significant difference (P≤0.05) in their respective fresh weights between 1 mM Ca^{2+} treated plants and 12.5 mM Ca^{2+} treated ones. Also, after 30 days, both Col-4 and Glenisla (putative calcifuges) had significantly higher (P≤0.05) fresh weights in comparison to the other four ecotypes *viz.*, Cal-0, Penicuik, Halifax

and Elland for 1 mM rhizospheric Ca^{2+} exposure (Figure 3.6; Table 3.4). Also, Col-4 and Glenisla (putative calcifuges) had a significantly different ($P \leq 0.05$) fresh weight to the putative wild calcifuge Penicuik, after 30 days of exposure to high rhizospheric Ca^{2+} (12.5 mM) (Table 3.4). Putative wild calcicoles, Halifax and Elland had a significantly higher ($P \leq 0.05$) fresh weight compared to the putative wild calcifuge Penicuik after 30 days of exposure to 12.5 mM rhizospheric Ca^{2+} (Table 3.4). Also, both Elland and Halifax had a significantly higher ($P \leq 0.05$) fresh weight at 1 mM than 12.5 mM rhizospheric Ca^{2+} exposure for 30 days (Table 3.4).

The dry weight measurements after oven drying of the pooled plants did not show such marked differences as the fresh weights among the ecotypes, neither for Ca^{2+} treatments nor the duration of exposure (Figure 3.7). However, a significant difference ($P \leq 0.05$) was observed in the dry weight of Col-4 plants compared to the other 5 ecotypes *viz.*, Cal-0, Glenisla, Penicuik, Halifax and Elland, when exposed to 1 mM rhizospheric Ca^{2+} for 20 days, which was evident after 30 days as well (Table 3.5). Also, only Col-4 plants significantly differed ($P \leq 0.05$) in their respective dry weights between 1 mM and 12.5 mM rhizospheric Ca^{2+} exposure, evident after 20 as well as 30 days of treatment (Table 3.5). However, putative wild calcifuge, Glenisla had a significantly ($P \leq 0.05$) higher dry weight than Cal-0, Penicuik, Halifax or Elland after 30 days of 1 mM rhizospheric Ca^{2+} exposure.

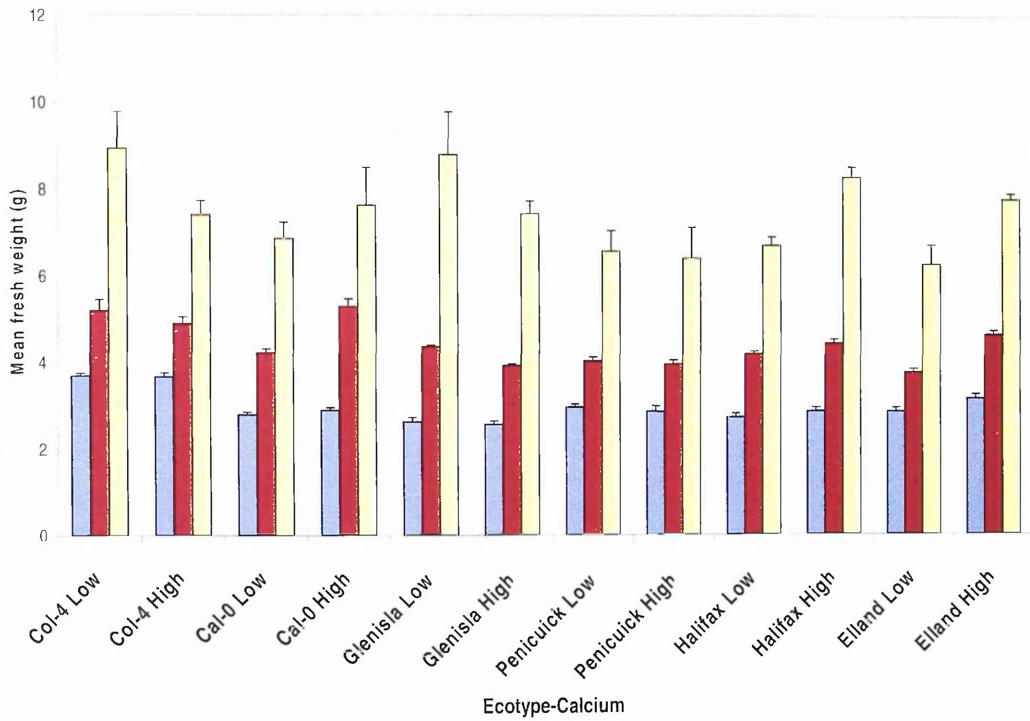


Figure 3.6: The effect of growth on low (1 mM) and high (12.5 mM) rhizospheric Ca^{2+} for 30 days on the fresh weight (g) in six ecotypes of *Arabidopsis*. Bars (mean of 10 plants) in Blue = 10th Day, Brown = 20th Day and Yellow = 30th Day harvest points. Error bars represent ± SEM.

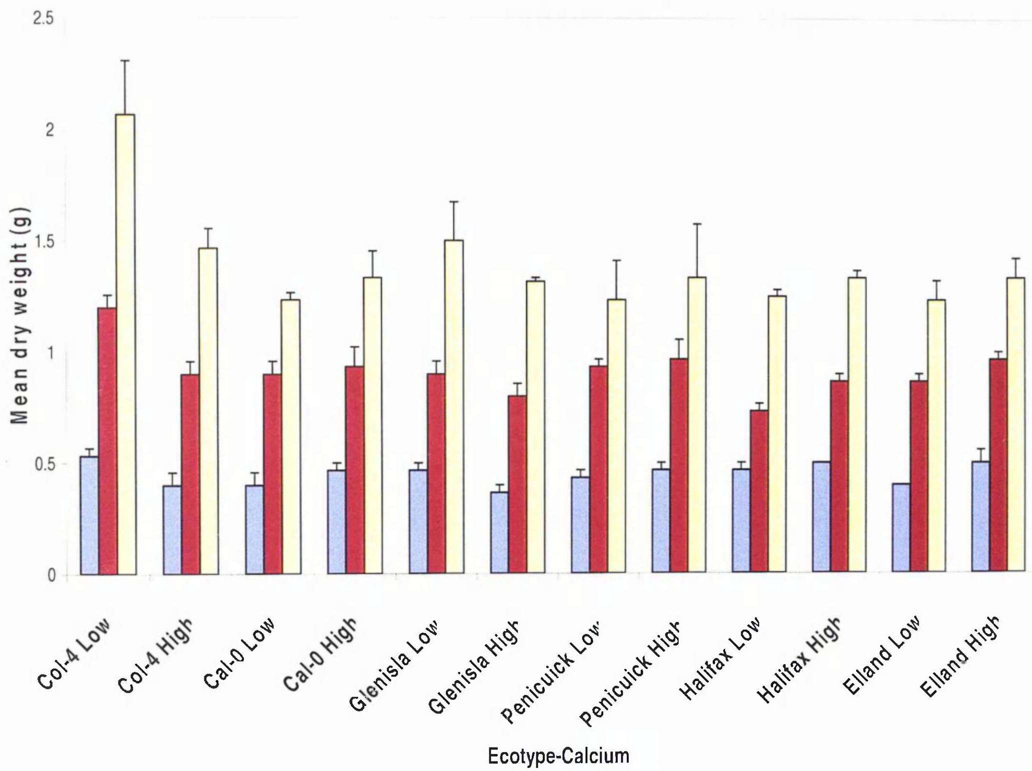


Figure 3.7: The effect of growth on low (1 mM) and high (12.5 mM) rhizospheric Ca^{2+} for 30 days on the dry weight (g) in six ecotypes of *Arabidopsis*. Bars (mean of 10 plants) in Blue = 10th Day, Brown = 20th Day and Yellow = 30th Day harvest points. Error bars represent ± SEM.

	Ca^{2+} conc.	Col-4 1 mM	Col-4 12.5 mM	Cal-0 1 mM	Cal-0 12.5 mM	Glenisla 1 mM	Glenisla 12.5 mM	Penicuik 1 mM	Penicuik 12.5 mM	Halifax 1 mM	Halifax 12.5 mM	Elland 1 mM	Elland 12.5 mM
Col-4	1 mM	-	▲	●▲	▲	*	*● ▲	●▲	●▲	*●▲		●▲	▲
	12.5 mM		-			*	*●		▲			●▲	
Cal-0	1 mM			-									
	12.5 mM			●	-		●	●▲	●▲	●		●▲	
Glenisla	1 mM		▲	▲	▲	-	▲	▲	▲	▲		▲	▲
	12.5 mM						-		▲			▲	
Penicuik	1 mM							-					
	12.5 mM								-				
Halifax	1 mM									-			
	12.5 mM			▲				▲	▲	▲	-	▲	
Elland	1 mM											-	
	12.5 mM							▲	▲	▲		▲	-

Table 3.4: Multiple comparisons among ecotypes for fresh weight (g). Symbols *, ●, ▲ indicate a significant difference ($P \leq 0.05$) in mean fresh weight at harvests on 10th (1), 20th (2) and 30th (3) day respectively, grown on 1 mM or high 12.5 mM Ca^{2+} concentrations.

		Col-4		Cal-0		Glenisla		Penicuik		Halifax		Elland	
	Ca^{2+} conc.	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM	1 mM	12.5 mM
Col-4	**1 mM**	-	● ▲	●▲	● ▲	●▲	●▲	●▲	▲	●▲	●▲	●▲	▲
	12.5 mM		-										
Cal-0	**1 mM**			-									
	12.5 mM				-								
Glenisla	**1 mM**			▲		-		▲		▲		▲	
	12.5 mM						-						
Penicuik	**1 mM**							-					
	12.5 mM								-				
Halifax	**1 mM**									-			
	12.5 mM										-		
Elland	**1 mM**											-	
	12.5 mM												-

Table 3.5: Multiple comparisons among ecotypes for dry weight (g). Symbols *, ●, ▲ indicate a significant difference ($P \leq 0.05$) in mean dry weight at harvests on 10th (1), 20th (2) and 30th (3) day respectively, grown on low (1 mM) or high (12.5 mM) Ca^{2+} concentrations.

3.3 Discussion

Environmental constraints on plant development, survivability and reproduction have been a broad area of interest for researchers for a very long time now (Chapin *et al.,* 1993; Chapin, 1991). An early study on adaptability of similar plant species to various environments by Turesson (1925, 1922), revealed morphological differences within plant species associating them with their habitats, suggesting that the differences are the innate outcome of evolutionary processes occurring within the species. Levitt (1972) was one of the early scientists to study environmental stress phenomena (responses to salinity, drought and temperature) and propose a framework for the analysis of the response of vegetation to environmental stress.

In this study, six ecotypes of *A. thaliana*, grown on two levels of rhizospheric Ca^{2+} (high, 12.5 mM; low, 1 mM) on sand *in vitro* showed differential responses of various growth parameters. Fitter and Hay (2002) have reported that the mean soil solution concentration of Ca^{2+} expressed in a population of nine natural soils is 8.9 mM. They also opine that uncultivated soils will have lower concentrations, but few data are available (Fitter and Hay, 2002). It has already been demonstrated in crop plants that Ca^{2+} toxicity results in overall poor growth (Raza *et al.,* 2001). Raza *et al.,* (2001) used white lupin (*Lupinus albus* L.), for which calcareous soil is a major growth constraint in Europe (Duthion, 1992; Siddons *et al.,* 1994) and Australia (Tang *et al.,* 1995), to study its growth properties in Egyptian soils which were very high in Ca^{2+} content. They found ecotypes of white lupin tolerant to calcareous soils (20-26% $CaCO_3$) and concluded that these ecotypes would

be highly desirable in studies on Ca^{2+} tolerance and in further breeding programs. De Silva *et al.* (1994) also explained the adverse effect of 12.5 mM soil Ca^{2+} on *Lupinus luteus* as a result of unrestrained Ca^{2+} uptake resulting in poor growth, and it is known that a similar mechanism exists in *A. thaliana* (De Silva and McAinsh, personal communication) .

Initial studies at Lancaster University carried out by De Silva and McAinsh (personal communication) compared the growth responses of two *A. thaliana* ecotypes, Cal-0 and Col-4 to rhizospheric Ca^{2+}. Their results show that Cal-0 (putative calcicole), exhibits a strikingly higher tolerance to growth on high rhizospheric Ca^{2+} compared to the non-calcicole ecotype, Col-4. Low concentrations of rhizospheric Ca^{2+} (1 and 5 mM) had little effect on the survival of either ecotype. However, increased concentrations of rhizospheric Ca^{2+} (>5 mM) cause a marked reduction in the survival of both Col-4 and Cal-0. They observed that at 15 mM rhizospheric Ca^{2+} exposure, the survival rate of Cal-0 was significantly higher ($P \leq 0.01$) than that of Col-4 after 30 days. At 10 mM rhizospheric Ca^{2+} exposure Col-4 exhibited a reduction in survival within 8 days whereas Cal-0 only showed a decrease after 15 days, though the overall survivability of both the ecotypes was >70%. Based on these results, concentrations of 1 mM as low and 12.5 mM rhizospheric Ca^{2+} as high were chosen to carry out this current study.

To characterise the individual responses of *Arabidopsis* ecotypes to low and high Ca^{2+}, rosette diameter, number of leaves, root length, fresh and dry weight(s) were recorded for 3 harvests over a period of 30 days. The ecotypes comprised putative calcicoles Cal-0, Elland and Halifax as well as putative calcifuges Col-4, Penicuik and Glenisla. Disparities were visually

evident in the ecotypes exposed to high rhizospheric Ca^{2+} (12.5 mM) when compared to low Ca^{2+} (1 mM).

Experiments described in this chapter revealed a significant difference ($P \leq 0.05$) in the number of leaves between each of the 3 putative calcicole ecotypes (Cal-0, Elland and Halifax) and the 3 putative calcifuges (Col-4, Penicuik and Glenisla) when treated with high concentration (12.5 mM) of rhizospheric Ca^{2+}. Also, there was no significant difference between the number of leaves of Glenisla and Penicuik (putative wild calcifuges) at high rhizospheric Ca^{2+} indicating that both putative calcifuges were responding in a similar manner. Treatment with 1 mM Ca^{2+}, however, revealed a significant difference ($P \leq 0.05$) in number of leaves between Glenisla and Penicuik with no significant difference ($P > 0.05$) in mean number of leaves between calcicoles Cal-0 and Halifax. This might be an indication that the various *Arabidopsis* ecotypes under study with varied origins responded diversely with altered rhizospheric Ca^{2+} levels.

Chan *et al.* (2003), carried out their studies on Ca^{2+} stress in *Arabidopsis* mutants and wild type plants (Col-0), and observed a significant decrease in rosette diameters in both, when watered with additional 10 mM $CaCl_2$ compared to distilled water treatment. The present investigation also revealed an overall reduction in mean rosette diameter of all six ecotypes when treated with 12.5 mM Ca^{2+} (supplied as $CaCl_2$) compared to plants treated with 1 mM Ca^{2+}. Also, a significant increase ($P \leq 0.05$) in rosette diameter was observed in putative wild calcicoles Elland and Halifax in comparison to putative wild calcifuges Glenisla and Penicuik as well as with lab ecotypes Col-4 and Cal-0 when exposed to 12.5 mM rhizospheric Ca^{2+}.

However, rosette diameter(s) of Glenisla and Penicuik did not differ ($P>0.05$) to the ecotypes Cal-0 (putative calcicole) and Col-4 (putative calcifuge). Treatment with 1 mM Ca^{2+} also revealed a significant ($P\leq0.05$) mean rosette diameter difference between putative 'wild' calcicoles, Elland and Halifax and putative 'wild' calcifuges, Glenisla and Penicuik. However, both 'lab' ecotypes Col-4 and Cal-0 had no significant ($P>0.05$) rosette diameter difference with the respectively similar edaphic tolerant wild ecotypes under study, but were significantly ($P\leq0.05$) different to each other. These observations and results again might be an indication that both naturally occurring calcicoles and calcifuges are more sensitive to rhizospheric Ca^{2+} than the 'lab' ecotypes and so great care must be taken in interpreting stress adaptations in the later.

The effect of environmental limitations on plant growth can either be direct, via physical conditions on primary growth processes, or indirect, due to developmental adaptation (Walter and Schurr, 2005). Plant roots exposed to uneven distribution of nutrients in soil have to respond adequately to optimize their resource acquisition and utilization (Göttlein and Stanjek, 1996) Root length measurements in this investigation revealed that the calcifuge Glenisla had a significantly ($P\leq0.05$) reduced root system when treated with 12.5 mM (high) rhizospheric Ca^{2+}, in comparison to the mean root length of other ecotypes. Also, Elland a putative calcicole had the longest mean root length which was significantly longer ($P\leq0.05$) to both the lab ecotypes Col-4 and Cal-0 at high rhizospheric Ca^{2+} exposure. At the low Ca^{2+} (1 mM) treatment Col-4, a 'lab' putative calcifuge, had the longest roots. However, all other ecotypes had a non significant difference ($P>0.05$) between their mean root length(s). There is, therefore evidence that at least some ecotypes respond to

different rhizospheric Ca^{2+} by altering root morphology or length. However, in this study there was no direct correlation between soil Ca^{2+} and growth parameters at 1 mM and 12.5 mM Ca^{2+}, where one putative calcifuge (Col-4) grew normally in a 1 mM Ca^{2+} environment in comparison to other putative calcifuges (Glenisla and Penicuik) which had a significant reduction ($P \leq 0.05$) to their root growth. Another important finding is that Penicuik (putative wild calcifuge) did not have any significant difference ($P > 0.05$) with Elland (putative wild calcicole) when exposed to 12.5 mM rhizospheric Ca^{2+}, which might suggest that, of the ecotypes studied, only Glenisla severely modifies its root phenotype in response to increased rhizospheric Ca^{2+}. It also has to be noted that the putative wild calcicole ecotypes did not radically respond to the altered Ca^{2+} concentrations. Moreover, both the 'lab' ecotypes had altered root phenotypes, different to the wild ecotypes, but without displaying a common decrease or increase in root length in response to altered rhizospheric Ca^{2+} levels. Further detailed studies on the root growth of these ecotypes would pave a way to dissect their respective adaptive mechanisms to altered rhizospheric Ca^{2+} conditions. Root morphology of the ecotypes at low and high Ca^{2+} treatments is studied in detail in Chapter 4.

Plants have intricate stress responses designed to deal with varied environmental fluxes, and the pertaining modifications or adaptations by them can be inferred by measuring their fresh and dry weights (Silk, 2006). So, inhibition of growth in *Arabidopsis* due to stress can be scored by monitoring fresh weight gain (Weigel and Glazebrook, 2002). For some plants, high rhizospheric Ca^{2+} can have a negative impact on plant growth (Lee, 1999), though experiments with *Arabidopsis* grown on agar media suggested no

significant toxicity at concentrations up to 30 mM (Chan *et al.,* 2003). Fresh weight measurements described in this chapter showed a weight increase in all putative calcicoles with the 12.5 mM $CaCl_2$ treatment, but the contrary for the calcifuges. The same trend also persisted in the dry weight measurements. From the fresh weight measurements, Glenisla (putative calcifuge) plants had a significant reduction ($P \leq 0.05$) between 1 mM and 12.5 mM Ca^{2+} treatments but not significantly different ($P > 0.05$) to most of the ecotypes apart from Penicuik (putative calcifuge).

In summary, based on the physiological responses of the six ecotypes to high and low Ca^{2+} treatments, putative wild calcicoles Halifax and Elland showed responses as true calcicoles without any disparaging responses to high Ca^{2+} and analogously putative wild calcifuge Glenisla exhibited responses as a true calcifuge. However, putative wild calcifuge, Penicuik behaved more like a calcicole than a putative calcifuge in its physiological responses, which might be due to its natural environment which was not as acid as that of Glenisla. Also, the physiological responses of the lab ecotypes Cal-0 and Col-4 did not put them exactly onto either being a true 'calcicole' or 'calcifuge' which may be the result of bulking the seed samples continuously in the lab conditions.

Chapter 4

Effect of rhizospheric Ca^{2+} on the root growth of wild *A. thaliana* ecotypes collected from soils that are varyingly calcareous

4.1 Introduction

Plant roots encounter continual challenges from both biotic and abiotic stresses. Consequently, they have evolved some unique and intricate growth responses for dealing with multiple environmental challenges (Eapen *et al.*, 2005). Maloof (2004) commented that plant roots are hidden underground and receive less attention in research than they deserve, though they are critical to plants' survivability as well as anchorage, absorption, storage and conduction. Roots of higher plants alter their growth pattern in response to varying environmental stimuli such as gravity, light, moisture, nutrients, ions and chemicals, temperature, and obstacles, to adapt to their environment and gain maximum advantage for growth (Mochizuki *et al.*, 2005; Okada and Shimura, 1990). Root system development is also an important agronomic trait and roots show a remarkable variation in architecture in response to environmental constraints (Malamy, 2005; Lynch and Brown, 2001). Morphogenesis of the root system is highly plastic and extremely responsive to changes in environmental conditions, as well as the accessibility of nutrients for growth, which in turn might result in extreme changes in root architecture (Malamy and Ryan, 2001; Zhang and Forde, 1998). Root systems moreover provide an optimal system for studying developmental plasticity, a characteristic feature of plant growth (Malamy, 2005). The impact of environmental constraints (biotic or abiotic factors) on root growth can

either be direct, via physical conditions on primary growth processes, or indirect, due to developmental adaptation through acclimatization processes that adjust their performance to the environmental conditions (Walter and Schurr, 2005).

Studies have been carried out to understand the variation in root architecture both between species and between accessions of *Arabidopsis* (Mouchel *et al.,* 2004; Beemster *et al.,* 2002). Mouchel *et al.* (2004) compared 44 *Arabidopsis* accessions for naturally occurring genetic variations in root growth and found an overall 2-3-fold variation in primary root length between the accessions. Also, Beemster *et al.* (2002) observed a 4-fold difference in root elongation rate among 18 ecotypes of *Arabidopsis* which comprised some of the most commonly used laboratory strains as well as a few from contrasting growth habitats. Ecological theories addressing the interaction of suites of traits with nutrient supply and plant potential growth rate have concentrated primarily on leaf structure and physiology and much less are known about interspecific patterns of variation in root structure and physiology (Eissenstat, 2000). The same applies to information on variation in root architecture and morphology in varied rhizospheric Ca^{2+} concentrations.

In this chapter, the six *Arabidopsis* ecotypes under study were grown on petri dishes with altered levels of Ca^{2+} to study the adaptation in terms of root morphology in detail. Subsequently, two ecotypes exhibiting extreme differences in root morphology were selected for in-depth analysis.

4.2 Results

4.2.1 Primary root length measurements

Differences in root architecture resulting from growth on varying concentrations of rhizospheric Ca^{2+} were studied by growing seedlings on modified agar medium containing 0 mM, 1 mM, 15 mM or 30 mM $CaCl_2$ (See section 2.7), where 0 mM indicates that $CaCl_2$ was not added to the agar medium. This was used to grow 6 ecotypes of *Arabidopsis*, and the primary root length was measured using Optimas™ software on day 5 and 8 after transfer onto varyingly $CaCl_2$ supplemented modified B5 medium (refer Section 2.7; Figure 4.1). Statistical analysis of mean root length measurements was carried out using the ANOVA tool of Fisher's Least Significant Difference (LSD) and the ecotypes were compared for their performance on respective concentrations of supplemental Ca^{2+} (Table 4.1).

On day 5 of root length measurement, there was no significant difference ($P>0.05$) in the root length of individual ecotypes with altered rhizospheric Ca^{2+} treatment, apart from Col-4 plants which had a significant ($P\leq0.05$) mean root length difference between 0 mM Ca^{2+} and all the other three (1 mM, 15 mM and 30 mM) Ca^{2+} treatments (Table 4.1). All the ecotypes had no significant ($P>0.05$) mean root length difference at day 5 when grown on 0 mM Ca^{2+} but the mean root length of putative calcifuge Col-4 plants grown on 1 mM Ca^{2+} (2.98 ± 0.20 cm), 15 mM (2.80 ± 0.10 cm) and 30 mM (2.63 ± 0.12 cm) was significantly ($P\leq0.05$) different to all the other ecotypes grown on similar Ca^{2+} concentrations (Table 4.1).

On day 8, longest primary roots (Figure 4.1) were observed in plants of putative calcicole Halifax grown with 0 mM Ca^{2+} (5.47 ± 0.08 cm) or 1 mM added Ca^{2+} (5.47 ± 0.14 cm), whereas, the shortest roots were in plants of the putative calcifuge Glenisla grown at 30 mM Ca^{2+} (2.49 ± 0.17cm). Multiple comparisons revealed a significant difference ($P \leq 0.05$) between the above mentioned root lengths of Halifax and Glenisla and all other ecotypes grown at similar Ca^{2+} concentrations (Table 4.1), respectively. However, Halifax plants had no significant mean root length difference ($P > 0.05$) between 0 mM Ca^{2+} and 1 mM Ca^{2+} treatment, or between those grown on 15 mM Ca^{2+} (4.25 ± 0.46 cm) or 30 mM Ca^{2+} (3.99 ± 0.26 cm), on day 8. Also, on day 8, both Cal-0 and Elland plants had no significant difference ($P > 0.05$) in the mean root length of plants grown on respective varied levels of rhizospheric Ca^{2+} (0 mM, 1mM, 15mM and 30 mM) (Table 4.1).

The two wild putative calcifuges, Penicuik and Glenisla significantly differed ($P \leq 0.05$) in mean root length, when grown on 30 mM added Ca^{2+} at day 5 but had no significant difference ($P > 0.05$) on day 8 of measurement (Penicuik - day 5, 1.76 ± 0.06 cm; day 8 - 2.86 ± 0.09 cm and Glenisla - day 5- 1.12 ± 0.11 cm, day 8- 2.49 ± 0.17 cm). Observation of roots revealed that Glenisla plants had short and thick roots whereas the roots of the other ecotypes were branched (Figure 4.2).

Comparison of plants grown on low and high Ca^{2+} revealed a definite developmental difference in mean root length between Glenisla and Halifax (Figure 4.2). There was no significant difference ($P > 0.05$) in mean root lengths on day 5 of measurement at any of the varied rhizospheric Ca^{2+} treatments, whereas by day 8 there was a significant difference ($P \leq 0.05$) in

mean root length over all the Ca^{2+} treatments (Table 4.1). All the other ecotypes had differences in the mean root length at high rhizospheric Ca^{2+} (30 mM), but without the short and stumpy roots, characteristic of Glenisla. This observation might be an indication that the putative calcifuge Glenisla is showing a specific root phenotype in response to high rhizospheric Ca^{2+}. These observations led to further in depth root studies on the putative calcicole Halifax and the putative calcifuge Glenisla (See Section 4.2.3).

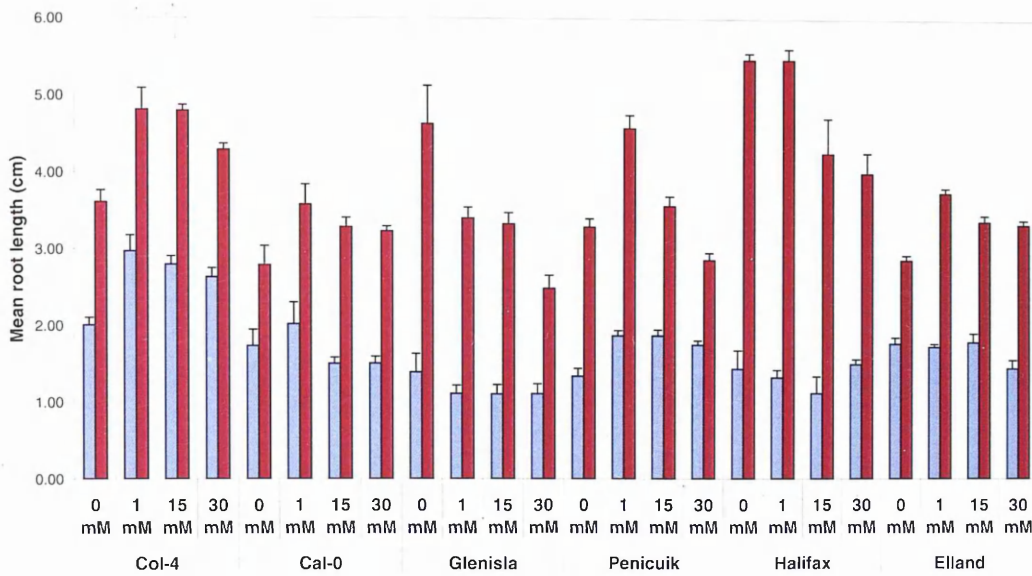


Figure 4.1: Mean primary root length of six *Arabidopsis* ecotypes on day 5 (Blue bars) and 8 (Brown bars) grown on agar medium supplemented with 0-30 mM $CaCl_2$ concentrations. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants on respective days.

	Rhizospheric Ca^{2+} concentration (mM)	Mean root length ± SEM (cm) on day of measurement	
		Day 5	Day 8
Col-4	0 mM	2.01±0.10	3.61±0.15
	1 mM	2.98±0.20	4.81±0.28
	15 mM	2.80±0.10	4.80±0.07
	30 mM	2.63±0.12	4.29±0.08
Cal-0	0 mM	1.74±0.21	2.79±0.24
	1 mM	2.02±0.28	3.58±0.26
	15 mM	1.51±0.08	3.28±0.12
	30 mM	1.51±0.09	3.23±0.07
Penicuik	0 mM	1.35±0.10	3.29±0.11
	1 mM	1.88±0.06	4.57±0.17
	15 mM	1.88±0.07	3.56±0.12
	30 mM	1.76±0.06	2.86±0.09
Glenisla	0 mM	1.40±0.24	4.63±0.50
	1 mM	1.12±0.11	3.40±0.14
	15 mM	1.11±0.12	3.33±0.15
	30 mM	1.12±0.12	2.49±0.17
Halifax	0 mM	1.44±0.24	5.47±0.08
	1 mM	1.33±0.09	5.47±0.14
	15 mM	1.12±0.22	4.25±0.45
	30 mM	1.50±0.06	3.99±0.26
Elland	0 mM	1.77±0.08	2.86±0.06
	1 mM	1.73±0.04	3.74±0.06
	15 mM	1.79±0.12	3.36±0.08
	30 mM	1.45±0.11	3.32±0.06

Table 4.1 (a): Mean root length ± SEM of 10 plants on day 5 and 8 of measurement in ecotypes on plant growth medium in vertical petri plates. '0 mM' refers to no added $CaCl_2$ to the agar medium.

	Ca^{2+} conc.	Col-4				Cal-0				Glenisla				Penicuik				Halifax				Elland			
		0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM
Col-4	0 mM	-				•		*	*		*	*	*•				•		*	*	*	•			*
	1 mM	*•	-		•	*•	*•	*•	*•	*	*•	*•	*•	*•	*	*•	*•	*	*	*•	*•	*•	*•	*•	*•
	15 mM	*•		-	•	*•	*•	*•	*•	*	*•	*•	*•	*•	*	*•	*•	*	*	*•	*•	*•	*•	*•	*•
	30 mM	•			-	*•	*•	*•	*•	*	*•	*•	*•	*•	*	*•	*•	*	*	*	*	*•	*•	*•	*•
Cal-0	0 mM					-					*	*	*							*					
	1 mM					•	-	*	*	*	*	*	*•	*			•	*	*	*	*	•			*
	15 mM					•		-					•												
	30 mM								-				•												
Glenisla	0 mM					•	•	•	•	-	•	•	•		•	•	•				•	•	•	•	•
	1 mM					•					-		•				•					•			
	15 mM					•						-	•												
	30 mM												-												
Penicuik	0 mM					•							•	-											
	1 mM	•				•	•	•	•		*•	*•	*•	*•	-	•	•		*	*	•	•	•	•	•
	15 mM					•					*	*	*•	*		-	•		*	*		•			
	30 mM								•		*	*	*				-			*					
Halifax	0 mM	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	-		•	•	•	•	•	•
	1 mM	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•		-	•	•	•	•	•	•
	15 mM	•				•	•	•	•		•	•	•	•		•	•			-		•	•	•	•
	30 mM					•		•	•		•	•	•	•			•				-	•		•	•
Elland	0 mM										*	*	*							*		-			
	1 mM					•			•		*	*	*•				•			*		•	-		
	15 mM					•					*	*	*•				•			*		•		-	
	30 mM					•							*•												-

Table 4.1 (b): Multiple comparison among ecotypes for mean root length (cm) grown on vertical petri dishes where symbols *, • indicate a significant difference (P≤0.05) in mean root length at 5th and 8th day respectively. '0 mM' refers to no added $CaCl_2$ to the agar medium.

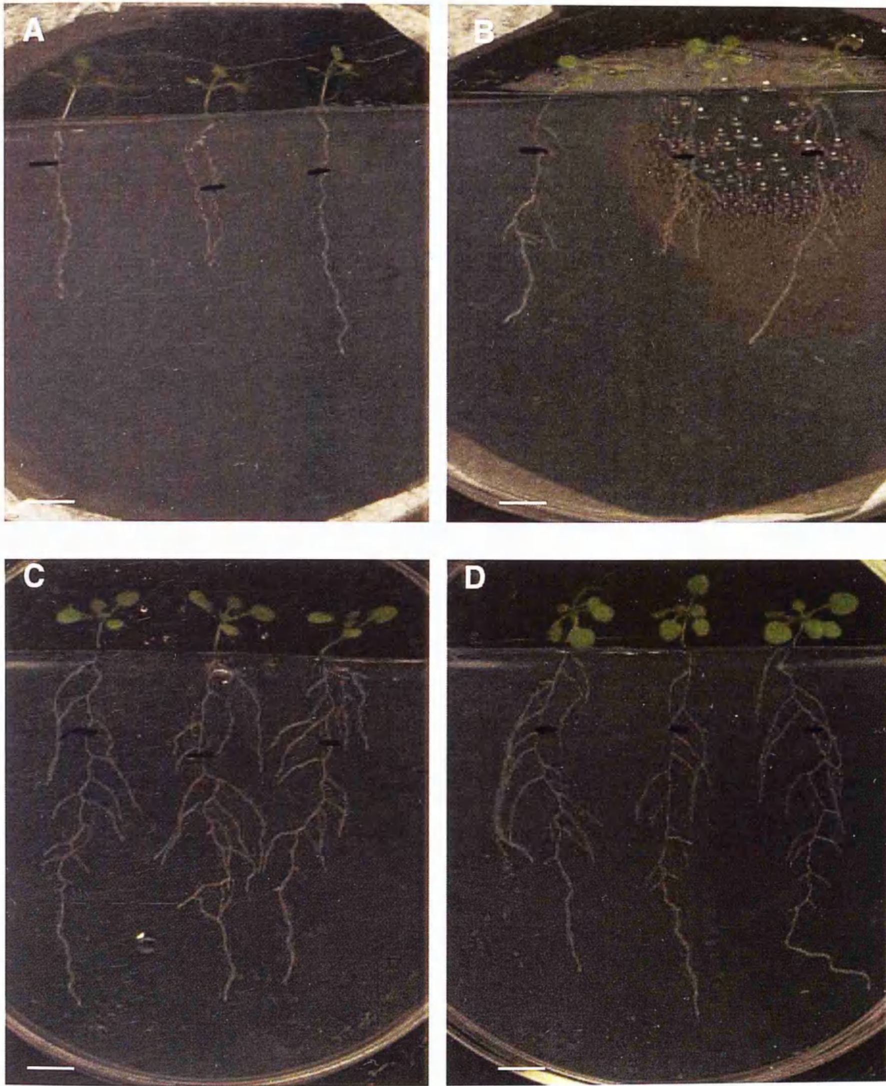


Figure 4.2: 8 day old *Arabidopsis* plants grown on agar supplemented with 30 mM $CaCl_2$. A - Glenisla, B - Penicuik, C - Col-4 and D – Halifax ecotypes, respectively. Bars = 1 cm.

4.2.2 Fresh and dry weights of roots and shoots

4.2.2.1 Fresh weight measurements

Fresh shoot and root weights were recorded at the end of experiments on day 8 from 10 plants of each of the six ecotypes grown on altered levels of rhizospheric Ca^{2+} (0 mM, 1 mM, 15 mM and 30 mM; '0 mM' refers to no added $CaCl_2$ to the agar medium). Mean fresh shoot weight was highest in Elland plants grown with 1 mM added Ca^{2+} (16.20 ± 1.87 mg) whereas, Cal-0 plants grown at 15 mM Ca^{2+} had the least shoot weight (5.30 ± 1.32 mg) (Figure 4.3). ANOVA analysis using Fisher's LSD revealed that there was no significant differences ($P>0.05$) among the ecotypes when there was no external Ca^{2+} added to the medium (Table 4.2). However, at 1mM added Ca^{2+} treatment significant mean shoot weight difference ($P\leq0.05$) was observed between putative calcicole Elland and all ecotypes except Halifax (putative calcicole). Also, Glenisla shoot weight was significantly different ($P\leq0.05$) to that of Halifax for the same treatment. At 15 mM external rhizospheric Ca^{2+} treatment, there was no significant shoot weight difference ($P>0.05$) among the three putative calcifuges Col-4, Glenisla and Penicuik as well as between Elland and both Glenisla and Col-4. However, 30 mM external Ca^{2+} treatment revealed significant shoot weight difference ($P\leq0.05$) between Halifax and all ecotypes except Elland, as well as between Elland and both Penicuik and Cal-0 (Table 4.2). These data revealed that, with increased external rhizospheric Ca^{2+} (30 mM), there was a general decrease in the mean fresh shoot weight of all the ecotypes but only Halifax plants (putative calcicole) significantly differed ($P\leq0.05$) in shoot weight from all the putative calcifuge

ecotypes (Col-4, Glenisla and Penicuik) as well as the 'lab' ecotype Cal-0 (putative calcicole).

The fresh root weight was observed to be highest (20.50 ± 1.55 mg) in Cal-0 plants treated with 1 mM Ca^{2+} whereas the least was in Penicuik plants grown with 30 mM rhizospheric Ca^{2+} (3.60 ± 0.98 mg). All the ecotypes had a decrease in mean root weight with an increase in the rhizospheric Ca^{2+} (Figure 4.4). Only the putative calcifuges Col-4 and Penicuik had a significant difference ($P \leq 0.05$) in the mean root weight for the plants treated with 30 mM rhizospheric Ca^{2+} when compared to those treated with other Ca^{2+} concentrations (0 mM, 1 mM and 15 mM) (Table 4.2). However, though both Penicuik and Glenisla had decreased root lengths at 30 mM Ca^{2+} treatment (See Section 4.2.1) compared to Halifax plants, only Penicuik had a significantly different mean root weight on day 8 (Table 4.2). Also, the mean shoot: root weight ratio revealed that there was no significant difference between Glenisla and Halifax plants treated with 1 mM or 15 mM or 30 mM rhizospheric Ca^{2+} (Table 4.2 and Figure 4.5).

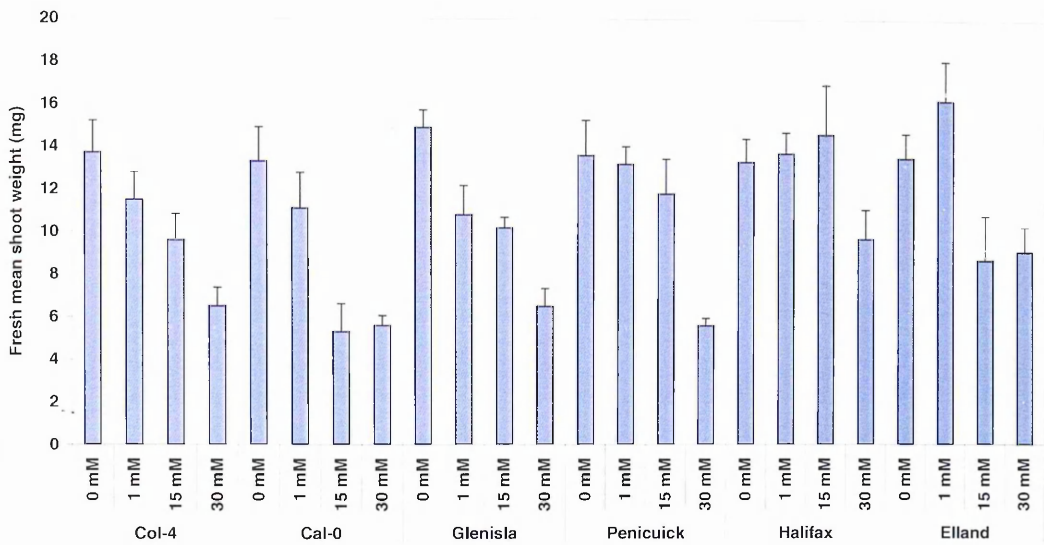


Figure 4.3: Fresh shoot weight of six *Arabidopsis* ecotypes on day 8 of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants.

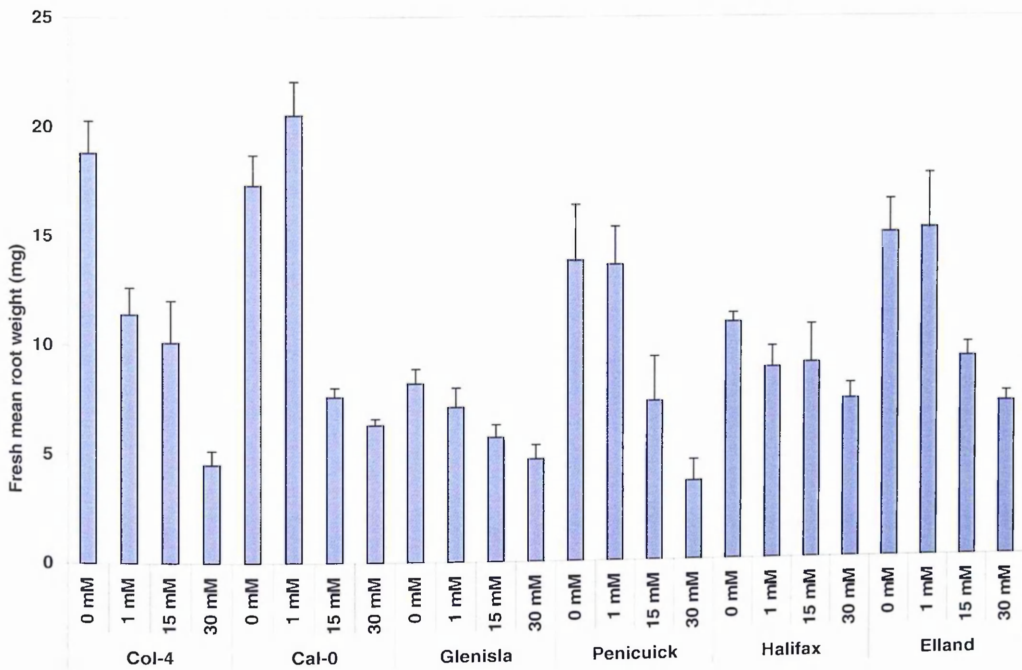


Figure 4.4: Fresh root weight of six *Arabidopsis* ecotypes on day 8 of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants.

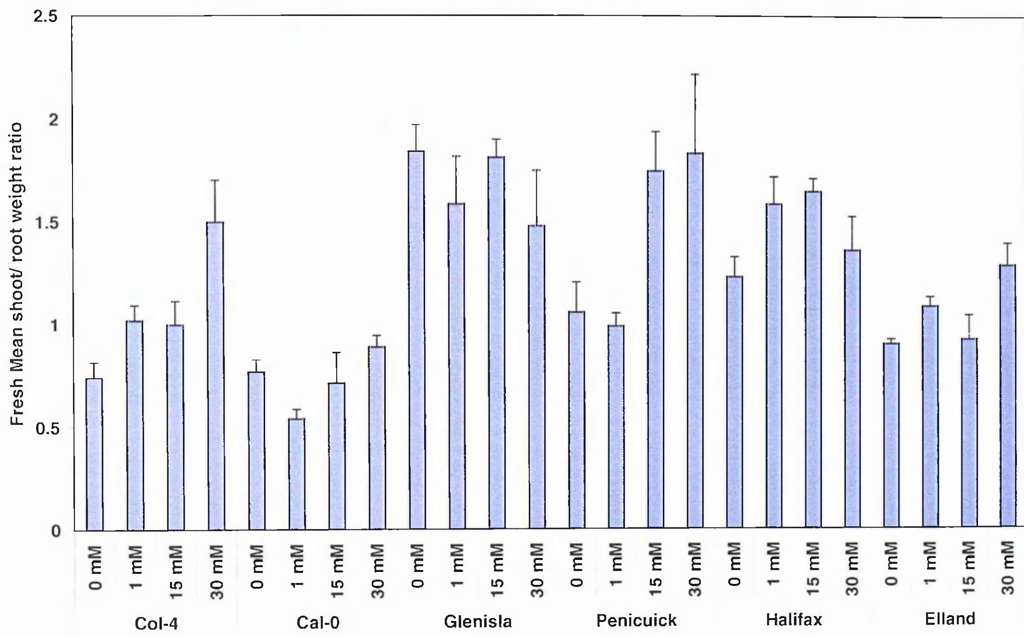


Figure 4.5: Mean fresh shoot: root weight ratio of six *Arabidopsis* ecotypes on day 8 of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants.

	Rhizospheric Ca^{2+} concentration (mM)	Mean fresh shoot weight ± SEM (mg) of 10 plants	Mean fresh root weight ± SEM (mg) of 10 plants	Mean fresh shoot: root weight ratio for 10 plants
		Day 8	Day 8	Day 8
Col-4	0 mM	13.70±1.49	18.80±1.46	0.74±0.07
	1 mM	11.50±1.26	11.40±1.21	1.02±0.08
	15 mM	9.60±1.21	10.10±1.92	1.00±0.12
	30 mM	6.50±0.87	4.50±0.63	1.50±0.20
Cal-0	0 mM	13.30±1.59	17.30±1.37	0.77±0.06
	1 mM	11.10±1.66	20.50±1.55	0.54±0.05
	15 mM	5.30±1.32	7.60±0.41	0.72±0.15
	30 mM	5.60±0.47	6.30±0.28	0.89±0.06
Glenisla	0 mM	14.90±0.82	8.20±0.66	1.84±0.13
	1 mM	10.80±1.37	7.10±0.88	1.59±0.23
	15 mM	10.20±0.48	5.70±0.57	1.81±0.09
	30 mM	6.50±0.84	4.70±0.66	1.48±0.27
Penicuik	0 mM	13.60±1.65	13.80±2.56	1.06±0.15
	1 mM	13.20±0.82	13.60±1.74	0.99±0.06
	15 mM	11.80±0.48	7.30±2.06	1.75±0.19
	30 mM	5.60±0.84	3.60±0.98	1.83±0.38
Halifax	0 mM	13.30±1.65	10.90±0.41	1.23±0.10
	1 mM	13.70±0.82	8.80±0.96	1.59±0.13
	15 mM	14.60±2.32	9.00±1.76	1.65±0.06
	30 mM	9.70±1.35	7.30±0.73	1.36±0.16
Elland	0 mM	13.50±1.14	15.00±1.52	0.90±0.02
	1 mM	16.20±1.87	15.20±2.51	1.09±0.05
	15 mM	8.70±2.08	9.20±0.66	0.93±0.12
	30 mM	9.10±1.17	7.10±0.47	1.29±0.11

Table 4.2 (a): Mean fresh shoot weight, fresh root weight and fresh shoot: root weight ratio ± SEM of 10 plants on day 8 of 6 *Arabidopsis* ecotypes growth on medium containing 0-30 mM $CaCl_2$ in vertical petri dishes. '0 mM' refers to no added $CaCl_2$ to the agar medium.

	Ca^{2+} conc.	Col-4				Cal-0				Glenisla				Penicuik				Halifax				Elland			
		0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM
Col-4	0 mM	-	•	*•	*•					•				•				•				•			
	1 mM		-	*	*•						•														
	15 mM			-	*•			*				•												•	
	30 mM				-																				
Cal-0	0 mM					-		*•	*•	•				•				•							
	1 mM		•			•	-	*•	*•		•				•				•				•		
	15 mM							-																	
	30 mM								-																
Glenisla	0 mM									-	*	*	*•												
	1 mM										-		*												
	15 mM							*				-	*												
	30 mM												-												
Penicuik	0 mM									•				-		•	*•	•							
	1 mM										•				-	•	*•		•						
	15 mM							*								-	*•							*	
	30 mM																-								
Halifax	0 mM																	-			*•				
	1 mM										*								-		*				
	15 mM		*					*				*•				*				-	*			*	
	30 mM				*				*				*				*•				-				
Elland	0 mM									•								•				-		*•	*•
	1 mM		*•				*				*•				*				•			*	-	*•	*•
	15 mM							*																-	
	30 mM								*								*								-

Table 4.2 (b): Multiple comparisons among ecotypes grown on vertical petri dishes. *, • indicate a significant difference (P≤0.05) in mean fresh shoot weight and mean fresh root weight on 8th day respectively. '0 mM' refers to no added $CaCl_2$ to the agar medium.

	Ca^{2+} conc.	Col-4				Cal-0				Glenisla				Penicuik				Halifax				Elland			
		0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM
Col-4	0 mM	-																							
	1 mM		-				*																		
	15 mM			-																					
	30 mM	*	*	*	-				*																
Cal-0	0 mM					-																			
	1 mM						-																		
	15 mM							-																	
	30 mM								-																
Glenisla	0 mM	*				*				-				*				*				*			
	1 mM		*				*				-				*								*		
	15 mM			*				*				-												*	
	30 mM												-												
Penicuik	0 mM													-											
	1 mM						*								-										
	15 mM			*				*						*	*	-								*	
	30 mM								*					*	*		-				*				*
Halifax	0 mM	*				*												-							
	1 mM		*				*								*				-				*		
	15 mM			*				*												-				*	
	30 mM																				-				
Elland	0 mM																					-			
	1 mM						*																-		
	15 mM																							-	
	30 mM																								-

Table 4.2 (c): Multiple comparisons among ecotypes grown on vertical petri dishes. '*' indicates a significant difference ($P \leq 0.05$) in mean fresh shoot/ root weight on 8th day. '0 mM' refers to no added $CaCl_2$ to the agar medium.

4.2.2.2 Dry weight measurements

Dry shoot and root weight was recorded after recording the wet weight (Section 4.2.2.1) and 3 days oven drying the samples as specified in Section 2.5 (Figure 4.6; Figure 4.7). Elland plants grown on 1 mM Ca^{2+} had the highest dry shoot weight (3.24 ± 0.26 mg) and were significantly different ($P \leq 0.05$) to all other ecotypes grown on 1 mM Ca^{2+} as well as Elland plants grown on 0, 15 or 30 mM Ca^{2+} (Table 4.3). Putative lab calcifuge, Col-4 plants grown on 30 mM Ca^{2+} had the lowest dry shoot weight (1.30 ± 0.12 mg) and was significantly different ($P \leq 0.05$) to Penicuik, Halifax and Elland grown on 30 mM Ca^{2+} as well as Col-4 plants grown on 0, 1 or 15 mM Ca^{2+} (Table 4.3). Also, both Col-4 and Elland plants grown on 30 mM Ca^{2+} had a significantly lower ($P \leq 0.05$) dry shoot and root weight to those grown on 0, 1 or 15 mM Ca^{2+} concentrations (Table 4.3). However, there was no difference ($P > 0.05$) in the dry shoot: root weight ratio of Col-4 plants (putative calcifuge) on either of the Ca^{2+} concentrations, though a significant difference ($P \leq 0.05$) in dry shoot: root weight ratio was observed in Elland plants (putative calcicole) grown on 0 mM or 30 mM Ca^{2+} (Table 4.3c). Putative lab calcicole, Cal-0 plants grown on either 0 or 1 mM Ca^{2+}, significantly differed ($P \leq 0.05$) in both dry shoot and root weights to those grown on either 15 or 30 mM Ca^{2+} (Table 4.3b).

Putative calcifuge, Glenisla with a dissimilar (short and stumpy) root phenotype to other ecotypes at 30 mM Ca^{2+}, did not have a significant difference ($P > 0.05$) in the mean dry shoot or root weight to other ecotypes grown on 30 mM Ca^{2+}. However, Glenisla plants grown on 30 mM Ca^{2+} had a significantly lower ($P \leq 0.05$) dry shoot weight and dry shoot: root weight ratio

to those grown on 0, 1 or 15 mM rhizospheric Ca^{2+} with no difference ($P>0.05$) in the dry root weights between these three concentrations.

All six ecotypes, when grown on 30 mM rhizospheric Ca^{2+} had no significant difference ($P>0.05$) in their dry root weight with only Halifax and Col-4 plants having a significant difference ($P\leq0.05$) in their dry shoot weights (Table 4.3b). However, only Penicuik (putative calcifuge) had a significantly different ($P\leq0.05$) dry shoot: root weight ratio to Col-4 and Glenisla (putative calcifuges) as well as Cal-0 (putative lab Calcicole) at 30 mM Ca^{2+} treatment (Table 4.3c).

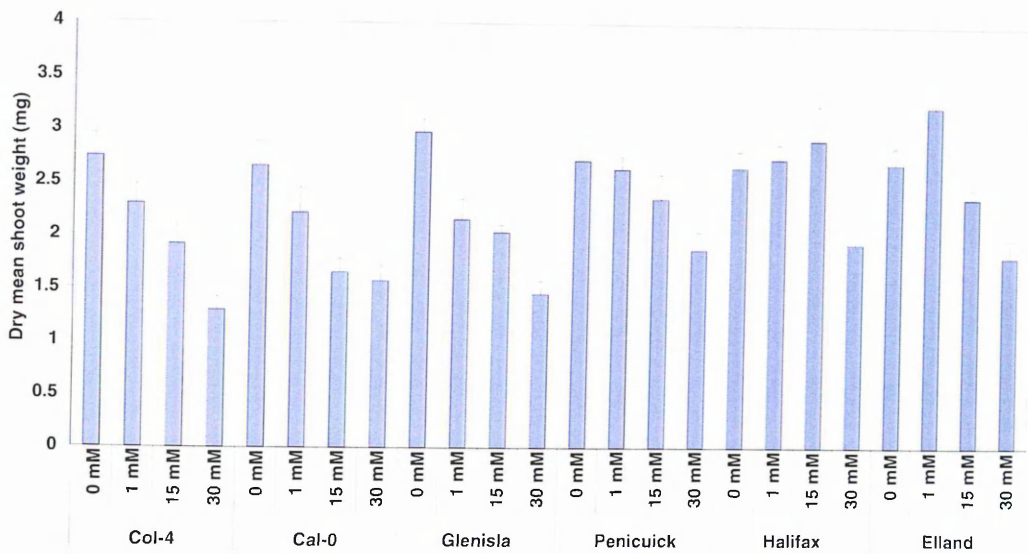


Figure 4.6: Mean dry shoot weight of six *Arabidopsis* ecotypes on day 8 of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants.

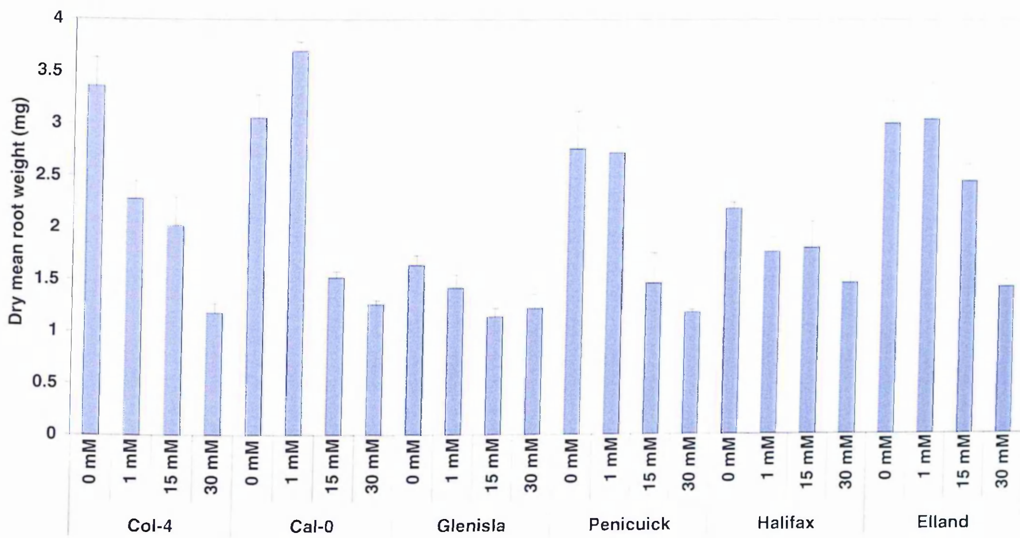


Figure 4.7: Mean dry root weight of six *Arabidopsis* ecotypes on day 8 of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants.

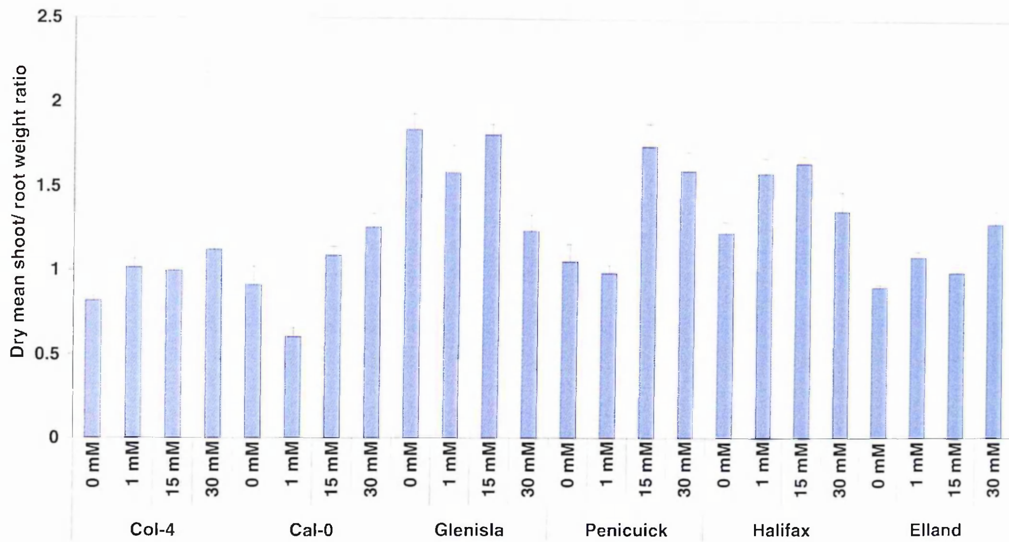


Figure 4.8: Mean dry shoot: root weight ratio of six *Arabidopsis* ecotypes on day 8 of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants.

	Rhizospheric Ca^{2+} concentration (mM)	Mean dry shoot weight ± SEM (mg) of 10 plants	Mean dry root weight ± SEM (mg) of 10 plants	Mean dry shoot/ root weight ratio for 10 plants
		Day 8	Day 8	Day 8
Col-4	0 mM	2.74±0.21	3.36±0.27	0.82±0.03
	1 mM	2.3±0.18	2.28±0.17	1.02±0.05
	15 mM	1.92±0.17	2.02±0.27	1.00±0.08
	30 mM	1.3±0.12	1.18±0.09	1.12±0.10
Cal-0	0 mM	2.66±0.22	3.06±0.22	0.91±0.11
	1 mM	2.22±0.24	3.70±0.09	0.61±0.05
	15 mM	1.66±0.13	1.52±0.06	1.09±0.05
	30 mM	1.58±0.14	1.26±0.04	1.26±0.08
Glenisla	0 mM	2.98±0.12	1.64±0.09	1.84±0.09
	1 mM	2.16±0.19	1.42±0.12	1.59±0.16
	15 mM	2.04±0.07	1.14±0.08	1.81±0.06
	30 mM	1.46±0.12	1.22±0.14	1.24±0.10
Penicuik	0 mM	2.72±0.23	2.76±0.36	1.06±0.10
	1 mM	2.64±0.12	2.72±0.25	0.99±0.04
	15 mM	2.36±0.23	1.46±0.29	1.75±0.14
	30 mM	1.88±0.17	1.18±0.02	1.60±0.12
Halifax	0 mM	2.66±0.15	2.18±0.06	1.23±0.07
	1 mM	2.74±0.14	1.76±0.14	1.59±0.09
	15 mM	2.92±0.33	1.80±0.25	1.65±0.04
	30 mM	1.94±0.19	1.46±0.10	1.36±0.12
Elland	0 mM	2.7±0.16	3.00±0.21	0.90±0.02
	1 mM	3.24±0.26	3.04±0.35	1.09±0.03
	15 mM	2.38±0.10	2.44±0.16	0.99±0.06
	30 mM	1.82±0.17	1.42±0.07	1.29±0.08

Table 4.3 (a): Mean dry shoot weight, dry root weight and dry shoot: root weight ratio ± SEM of 10 plants on day 8 of ecotypes on plant growth medium in vertical petri dishes. '0 mM' refers to no added $CaCl_2$ to the agar medium.

	Ca^{2+} conc.	Col-4				Cal-0				Glenisla				Penicuik				Halifax				Elland			
		0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM
Col-4	0 mM	-	•	* •	* •					•				•				•							
	1 mM		-		* •						•														
	15 mM			-	* •							•				•									
	30 mM				-																				
Cal-0	0 mM					-		* •	* •	•								•							
	1 mM		•			•	-	* •	* •		•				•				•				•		
	15 mM							-																	
	30 mM								-																
Glenisla	0 mM									-	*	*	*												
	1 mM										-		*												
	15 mM											-	*												
	30 mM												-												
Penicuik	0 mM									•				-		•	* •	•							
	1 mM										•				-	•	* •		•						
	15 mM							*								-									
	30 mM				*												-								
Halifax	0 mM									•								-			* •				
	1 mM						*				*								-		*				
	15 mM			*				*				* •				*				-	*			*	
	30 mM				*																-				
Elland	0 mM									•								•				-		•	* •
	1 mM		* •				*				* •				*				•			*	-	* •	* •
	15 mM							* •				•				•				•				-	* •
	30 mM				*																				-

Table 4.3 (b): Multiple comparisons among ecotypes grown on vertical petri dishes. *, • indicate a significant difference ($P \leq 0.05$) in mean dry shoot weight and mean dry root weight on 8th day respectively. '0 mM' refers to no added $CaCl_2$ to the agar medium.

	Ca^{2+} conc.	Col-4				Cal-0				Glenisla				Penicuik				Halifax				Elland			
		0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM
Col-4	0 mM	-																							
	1 mM		-																						
	15 mM			-																					
	30 mM				-																				
Cal-0	0 mM					-																			
	1 mM						-																		
	15 mM						*	-																	
	30 mM					*	*		-																
Glenisla	0 mM	*				*				-			*	*				*				*			
	1 mM		*				*				-		*		*								*		
	15 mM			*				*				-	*											*	
	30 mM												-												
Penicuik	0 mM													-											
	1 mM						*								-										
	15 mM			*				*						*	*	-								*	
	30 mM				*				*				*	*	*		-								
Halifax	0 mM	*																-							
	1 mM		*				*								*			*	-				*		
	15 mM			*				*										*		-				*	
	30 mM																				-				
Elland	0 mM																					-			
	1 mM						*																-		
	15 mM																							-	
	30 mM																					*			-

Table 4.3(c): Multiple comparisons among ecotypes grown on vertical petri dishes. '*' indicates a significant difference ($P \leq 0.05$) in mean dry shoot: root weight on 8th day. '0 mM' refers to no added $CaCl_2$ to the agar medium.

4.2.3 Experiments on Glenisla and Halifax

Initial experiments (4.2.1 and 4.2.2) on the six *Arabidopsis* ecotypes examining the effects of rhizospheric Ca^{2+} on root growth showed a specific short and stumpy root phenotype in plants of the 'wild' putative calcifuge Glenisla treated with 30 mM Ca^{2+} and no significant difference ($P>0.05$) in mean root length of 'wild' putative calcicole, Halifax plants grown at elevated levels of Ca^{2+} (15 mM and 30 mM). This prompted a more comprehensive analysis of the impact of rhizospheric Ca^{2+} on the root lengths of these two 'wild' *Arabidopsis* ecotypes. Accordingly, Glenisla and Halifax plants were grown on agar medium containing 0 mM, 1 mM, 15 mM and 30 mM $CaCl_2$ in vertical petri dishes for a period of 12 days, and the root length measured on days 3, 6 and 12, where, '0 mM' refers to no added $CaCl_2$ to the agar medium. The mean root lengths (Figure 4.9) of Glenisla and Halifax were subjected to statistical analysis using the ANOVA tool of Fisher's Least Significant Difference (LSD).

As observed in the previous experiment the shortest roots were in Glenisla plants grown at 30 mM rhizospheric Ca^{2+} (3.20 ± 0.14 cm) which, along with Glenisla plants grown on 15 mM Ca^{2+}, had a significantly ($P\leq0.05$) shorter mean root length than all Glenisla and Halifax plants grown on different concentrations of Ca^{2+} (0 mM, 1 mM, 15 mM and 30 mM) (Figure 4.9). At day 12, Halifax plants grown on 0 mM rhizospheric Ca^{2+} had the longest primary root length (6.53 ± 0.06 cm) (Figure 4.9) which was significantly different ($P\leq0.05$) to the mean primary root length of Glenisla plants grown on all concentrations of Ca^{2+} (0 mM, 1 mM, 15 mM and 30 mM)

(Table 4.4) as well as Halifax plants grown on 15 mM and 30 mM rhizospheric Ca^{2+}.

With an increase in rhizospheric Ca^{2+} concentration (0-30 mM), there was a decrease in the mean root length of both Glenisla and Halifax plants after 12 days of treatment (Table 4.4) and also, as observed in the previous experiments (Section 4.2.1), Glenisla plants grown on 30 mM Ca^{2+} developed a very distinctive short and stumpy root phenotype (Figure 4.10) when compared to those grown at other rhizospheric Ca^{2+} concentrations (0 mM, 1 mM and 15 mM) and to Halifax ecotype roots. To ascertain the reasons for the observed short root phenotype, a cellular imaging approach was used to observe the roots of these ecotypes.

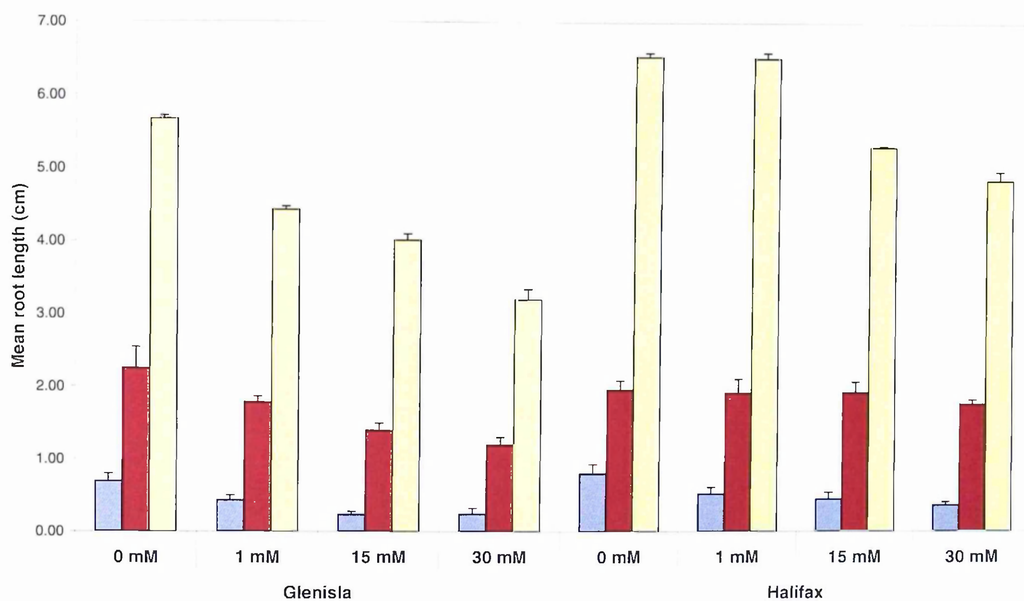


Figure 4.9: Mean primary root length of Glenisla and Halifax on day 3 (blue bars), day 6 (brown bars) and day 12 (yellow bars) of treatment with 0-30 mM $CaCl_2$. '0 mM' refers to no added $CaCl_2$ to the agar medium. Bars represent mean ± SEM of 10 plants on respective days.

	Rhizospheric Ca^{2+} concentration (mM)	Mean root length ± SEM (cm) on day of measurement		
		Day 3	Day 6	Day 12
Glenisla	0 mM	0.69±0.10	2.25±0.29	5.67±0.04
	1 mM	0.43±0.07	1.78±0.07	4.42±0.05
	15 mM	0.23±0.04	1.39±0.09	4.01±0.09
	30 mM	0.24±0.08	1.19±0.11	3.20±0.14
Halifax	0 mM	0.80±0.13	1.96±0.12	6.53±0.06
	1 mM	0.52±0.09	1.91±0.19	6.51±0.08
	15 mM	0.45±0.09	1.92±0.14	5.29±0.02
	30 mM	0.36±0.05	1.76±0.06	4.83±0.12

Table 4.4 (a): Mean root length ± SEM of Glenisla and Halifax plants on day 3, 6 and 12 of growth on agar medium containing 0-30 mM $CaCl_2$ in vertical petri dishes. Values are means of 10 plants ± SEM. '0 mM' refers to no added $CaCl_2$ to the agar medium.

	Rhizospheric Ca^{2+} concentration (mM)	Glenisla				Halifax			
		0 mM	1 mM	15 mM	30 mM	0 mM	1 mM	15 mM	30 mM
Glenisla	0 mM	-	● ▲	*● ▲	*● ▲		●	● ▲	*● ▲
	1 mM		-	● ▲	● ▲				
	15 mM			-	▲				
	30 mM				-				
Halifax	0 mM	▲	* ▲	*● ▲	*● ▲	-		* ▲	* ▲
	1 mM	▲	▲	● ▲	● ▲		-	▲	▲
	15 mM			● ▲	● ▲			-	▲
	30 mM		▲	● ▲	● ▲				-

Table 4.4 (b): Multiple comparison among Glenisla and Halifax plants for mean root length, grown on medium containing 0-30 mM $CaCl_2$ in vertical petri dishes. '0 mM' refers to no added $CaCl_2$ to the agar medium. *, •, ▲ indicate a significant difference ($P \leq 0.05$) in mean root length at 3rd, 6th and 12th day respectively.

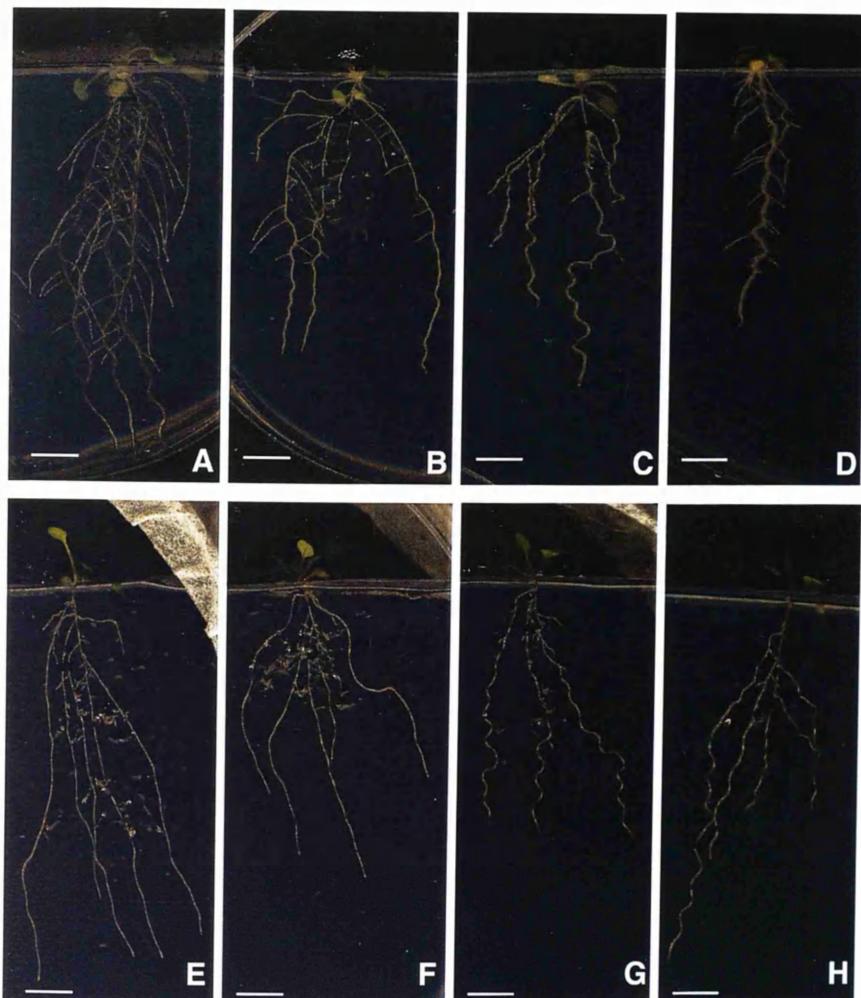


Figure 4.10: Representative Glenisla (A, B, C and D) and Halifax (E, F, G and H) plants grown at 0-30 mM $CaCl_2$ supplemented modified B5 medium for 12 days. '0 mM' refers to no added $CaCl_2$ to the agar medium. A & E, 0 mM; B & F, 1 mM; C & G, 15 mM and D & H, 30 mM. Short and stumpy root phenotype of Glenisla plants grown on 30 mM calcium can be seen in image D. Bars = 1 cm.

4.2.4 Imaging of roots of Glenisla and Halifax

Root imaging studies were performed to investigate the cellular basis for the short and stumpy root phenotype observed in Glenisla plants grown at 30 mM rhizospheric Ca^{2+}. Preliminary confocal imaging studies were carried out using Propidium Iodide (PI) to stain the intact roots of both Glenisla and Halifax grown on 0 mM (no added $CaCl_2$ to the medium) or 30 mM rhizospheric Ca^{2+} for 3 or 6 days. PI stains intact cell walls red, whereas it percolates into dead or damaged cells. This study revealed that, by day 3 of the 30 mM Ca^{2+} treatment, the cells in the Glenisla roots were damaged or dead, with PI percolation (Figure 4.11). However, Halifax roots were not affected by the increased rhizospheric Ca^{2+} as PI was excluded, even at 6 days of treatment (Figure 4.11). Root cells of both ecotypes were intact at 0 mM rhizospheric Ca^{2+} at both 3 and 6 days of growth on the medium (Figure 4.11).

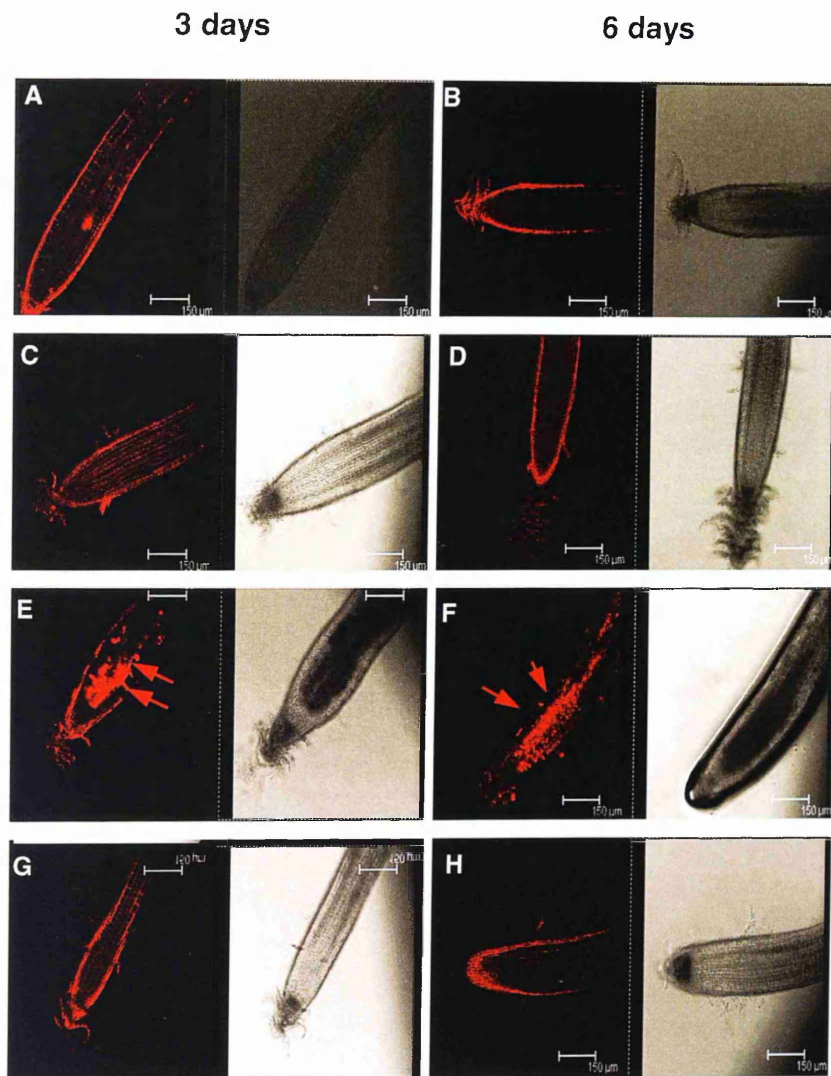


Figure 4.11: Confocal images of Propidium Iodide stained roots grown on 0 mM (no $CaCl_2$ added to the agar medium) and 30 mM rhizospheric Ca^{2+} for 3 and 6 days. A, B, E and F – Glenisla; C, D, G and H – Halifax. A-D - 0 mM; E-H - 30 mM rhizospheric Ca^{2+} treatment. A, C, E and G - after 3 days; B, D, F and H - after 6 days. Arrows represent dead cells with PI percolation. Panel on the left shows PI excited (600 ± 65 nm) images and bright field image on the right. Bars = 150 µm.

To determine whether the cell death observed by PI staining in Glenisla plants grown at 30 mM rhizospheric Ca^{2+} was caused by apoptosis, a TUNEL assay was performed to compare Glenisla plants grown on 30 mM Ca^{2+} with 0 mM Ca^{2+} as well as Halifax plants grown on both 0 mM Ca^{2+} and 30 mM rhizospheric Ca^{2+} for 12 days. A DeadEnd™ Flourometric TUNEL System (Promega, USA) kit was used as specified in Section 2.8 to carry out the assay. This assay was designed to specifically stain only apoptotic nuclei in the cells in comparison to healthy nuclei or necrotic nuclei. The whole intact roots of both Glenisla and Halifax plants grown at varied rhizospheric Ca^{2+} concentrations (0 mM and 30 mM) were stained with flourescein-12-dUTP from the kit as well as PI. To assess background levels of fluorescence both Glenisla and Halifax roots grown on 30 mM rhizospheric Ca^{2+} were calibrated by imaging roots without adding flourescein-12-dUTP to the protocol (Figure 4.12).

Confocal imaging of the stained roots revealed that the root cells in Glenisla plants grown at 30 mM rhizospheric Ca^{2+} had undergone severe apoptosis (Figure 4.13), with a large number of green fluorescent nuclei as well as PI percolation. Also, Halifax plants grown on 30 mM Ca^{2+} showed slight signs of apoptosis with PI percolation and a few apoptotic cells in the root tip region along the border cells with green fluorescent nuclei. However, as observed earlier (Figure 4.11), Glenisla roots grown on 0 mM Ca^{2+} showed no signs of apoptosis (Figure 4.13). Halifax roots grown on 0 mM Ca^{2+} also illustrated the presence of a very few apoptotic cells with green fluorescent nuclei.

These assays demonstrated that the short and stumpy root phenotype in the Glenisla plants grown at 30 mM Ca^{2+} correlated with a large number of cells undergoing apoptosis and suggest its part in the observed phenotype.

12 days

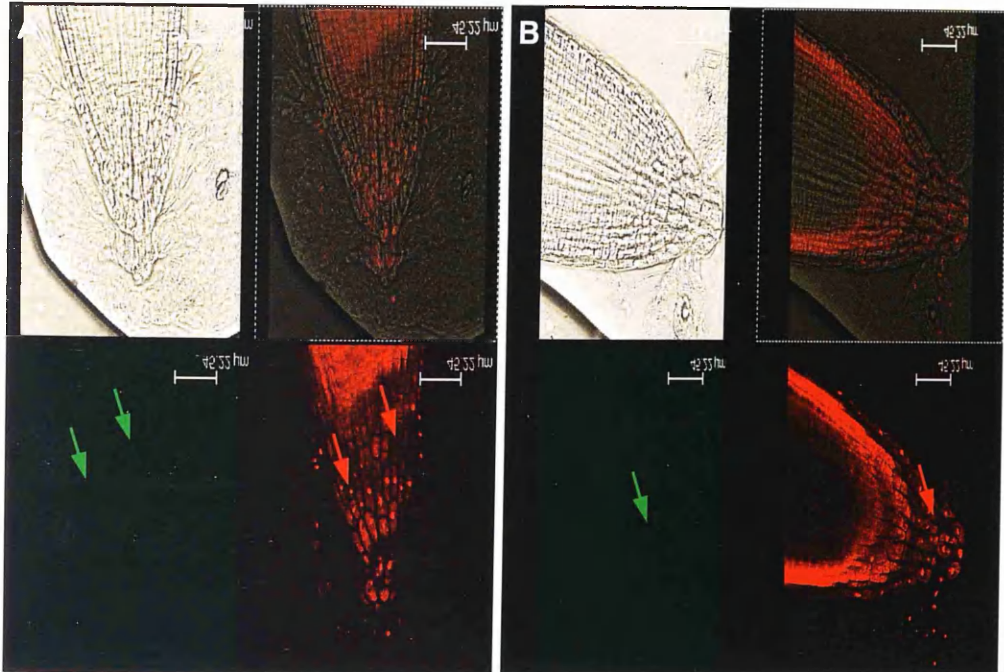


Figure 4.12: Images of Glenisla (A) and Halifax (B) roots grown on 30 mM Ca^{2+} for 12 days without fluorescein 12-dUTP labelling. Panels: Top left - Bright Field Channel (800 ± 50 nm); Top right - Overlay of red and green channels; Bottom left - Fluorescein (Green) Channel (520 ± 20 nm); Bottom right - PI (Red) Channel (600 ± 60 nm). Red arrows show dead cells with PI staining which do not appear in the green channel (Green arrows). Bars = 45.22 μm

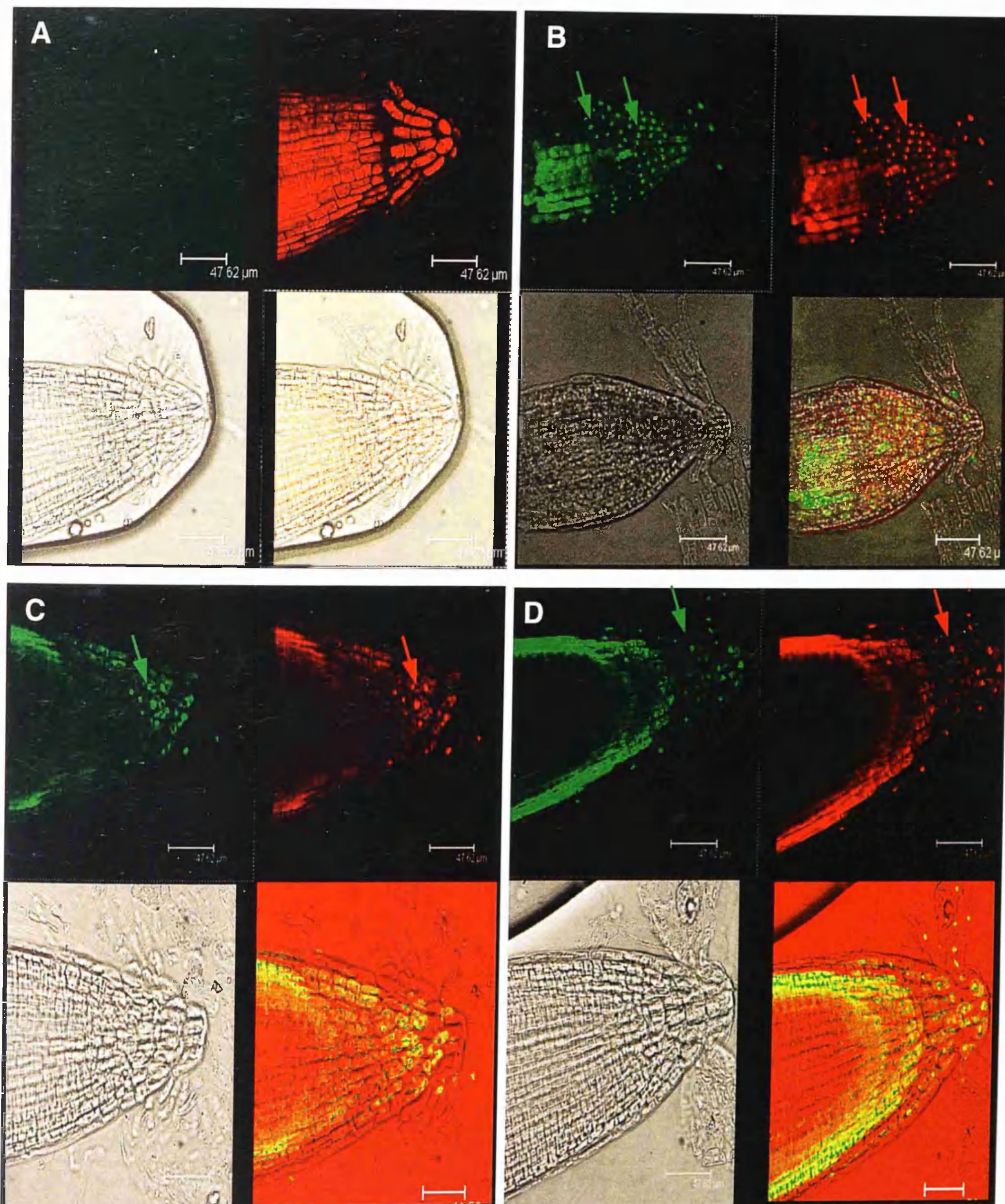


Figure 4.13: Confocal imaging of TUNEL stained roots of Glenisla (A and B) and Halifax (C and D) plants grown on 0 mM (no $CaCl_2$ added to the agar medium) or 30 mM rhizospheric Ca^{2+} for 12 days. A & C - 0 mM and B & D - 30 mM rhizospheric Ca^{2+} treatment. Panels: Top left - Fluorescein (Green) Channel (520 ± 20 nm); Top right - PI (Red) Channel (600 ± 60 nm); Bottom left - Bright Field Channel (800 ± 50 nm); Bottom right - Overlay of red and green channels. Red arrows indicate dead cells and green arrows indicate apoptotic nuclei. Bars = 47.62 µm

4.3 Discussion

Rhizospheric Ca^{2+} had a markedly different effect on the root growth of putative calcicole and calcifuge ecotypes of *Arabidopsis.* Differential root growth was also observed in the experiments on solidified agar medium in petri dishes with altered Ca^{2+} concentrations. Gottlein and Stanjek (1996) demonstrated that forest tree roots encountering uneven distribution of nutrients in soil tend to respond in order to optimize the resource acquisition. The altered root growth in these experiments might be an indication of the response of roots to the varied rhizospheric Ca^{2+}, which is in accord with Walter and Schurr's (2005) theory that roots respond directly to changes in their environment. Walter and Schurr (2005) proposed that root growth, control mechanisms react immediately to environmental parameters like nutrient concentration to optimize attributes such as nutrient use efficiency in soils and concluded that roots respond strongly and directly to changes in their environment.

Notably, it also has been observed in wild *Arabidopsis* ecotypes that there is an existing variation in root growth parameters (Mouchel *et al.,* 2004; Beemster *et al.,* 2002). Likewise, mean root lengths of the 4 natural accessions viz., Glenisla, Penicuik, Halifax and Elland were significantly ($P \leq 0.05$) different even when they were grown on 0 mM Ca^{2+}. However, at 30 mM rhizospheric Ca^{2+} concentration, the mean root length varied significantly ($P \leq 0.05$) only in the putative calcifuge Glenisla when compared to all three putative calcicoles Cal-0, Halifax and Elland. Penicuik, another 'wild' putative calcifuge had a significantly different ($P \leq 0.05$) mean root length to Halifax, but not to Elland when grown on 30 mM rhizospheric Ca^{2+}. The two 'lab'

ecotypes, Col-4 a putative calcifuge and Cal-0 a putative calcicole, exhibited a contrasting difference in the mean root length with Col-4 significantly ($P \leq 0.05$) differing to both putative calcifuges Glenisla and Penicuik as well as putative calcicoles Cal-0 and Elland when compared at 30 mM Ca^{2+}. However, Cal-0 significantly ($P \leq 0.05$) differed in mean root length with putative calcifuges Col-4, Glenisla and Penicuik as well as putative calcifuge Halifax. These data suggest that Glenisla and Halifax, which are a putative calcifuge and a putative calcicole respectively, show the most contrast in their root growth response to high rhizospheric Ca^{2+}, and might yield further information regarding the mechanisms underlying the observed changes in root morphology.

It has previously been noted that environmental stresses seriously limit the growth and development of plants (Bohnert *et al.*, 1995), but plants also may have evolved to adapt to the dynamic stressful environments due to their immobility (Liu *et al.,* 2007). Plant roots are interesting structures, crucial for the plants in anchorage, absorption, storage and conduction (Maloof, 2004). Also, studies on root architecture in crop plants including *Medicago sativa* (Vaughan *et al.,* 2002) and *Phaseolus vulgaris* (Lynch and vanBeem, 1993) have shown substantial variations within species in response to edaphic stress. Morphological and physiological characteristics of 36 *Arabidopsis* accessions differing in their phosphate acquisition efficiencies (PAEs) have been analyzed and detailed analysis of the five most divergent accessions, revealed significant differences in root morphology and the ability of roots to penetrate substrates (Narang *et al.,* 2000). All the *A. thaliana* ecotypes in the current investigation varied in their root architecture which was reflected in

their mean root lengths and weight variations, even with 0 mM rhizospheric Ca^{2+} (no added $CaCl_2$).

Differences in response to Ca^{2+} have been demonstrated between natural populations in *Festuca ovina* L. (Snaydon and Bradshaw 1961), *Euphorbia thymifolia* L. (Ramakrishnan 1961) and by Kruckeberg (1954) in chapparal vegetation (4-12-foot tall evergreen woody shrubs and/or dwarf trees) of California, *Bodenvag* species and genus *Streptanthus*. These differences can be related to the differences in the Ca^{2+} concentrations in the native soils of the populations. Snaydon and Bradshaw (1961) investigated population differentiation within the species *Festuca ovania* in response to varied Ca^{2+} in culture solutions and observed that populations from acid, low Ca^{2+} soils showed a negative growth response to increased Ca^{2+} concentrations, whilst the ones from calcareous soils showed a strong response to the same in their overall growth pattern. Also, Bradshaw *et al.* (1958) reported similar results in seven grass species comprising, *Lolium perenne*, *Agrostis stolonifera*, *Cynosurus cristatus*, *Agrostis canina*, *Nardus stricta* and a normal and a lead-resistant ecotype of *Agrostis tenuis*. The fresh mean shoot and root weight measurements in the *A. thaliana* ecotypes under study exposed significant differences among their response to altered levels of rhizospheric Ca^{2+}. A decrease in both fresh and dry, shoot as well as root weights of putative calcifuge ecotypes (Col-4, Glenisla and Penicuik) was observed with an increase in the rhizospheric Ca^{2+} concentration from 0 mM (no added $CaCl_2$) to 30 mM, which was not evident in the putative calcicoles (Cal-0, Halifax and Elland). However, 'wild' putative calcifuges, Glenisla and Penicuik had a significantly different ($P \leq 0.05$) dry shoot: root weight ratio for

30 mM Ca^{2+} treatment, whereas, there was no significant difference (P>0.05) in the dry root: shoot ratio at 30 mM Ca^{2+} between Glenisla and other ecotypes. A possible explanation for these results is that Halifax and Elland, being putative calcicoles, were not affected by the increased levels of rhizospheric Ca^{2+}, unlike the putative calcifuges (Col-4, Glenisla and Penicuik) as well as Cal-0.

It has been reported that *A. thaliana* seedlings exposed to copper (Pasternak *et al.,* 2005) or phosphate deficiency (López-Bucio *et al.,* 2002) exhibited decreased root elongation. Similarly, the current investigation illustrated a decrease in primary root elongation in putative calcifuge Glenisla plants exposed to elevated levels of rhizospheric Ca^{2+}. Where elevated levels of rhizospheric Ca^{2+} (~30 mM) have been used to ameliorate the effects of heavy metal exposure in *Arabidopsis* (Suzuki, 2005) it has been reported that prolonged exposure to increased Ca^{2+} might be harmful, as increased Ca^{2+} is harmful to cells because it leads to activation of particular Ca^{2+}-dependent enzymes, having a potentially adverse effects (Hirschi, 2004).

Duchen (2000) reported that surplus Ca^{2+} caused a loss of mitochondrial potential and when the exposure was continued, it eventually led to cell death in mammalian cells. The host of adverse stimuli that impact on plant growth can bring about very specific responses at the cellular level *viz.,* osmotic challenge, oxidative damage, changes in membrane chemistry and ion transport, alterations in cellular redox potential and protein denaturation (Hare *et al.,* 1997; 1996). Of these numerous responses, Pennell and Lamb (1997) indicated that cell death is an important part of plant responses to environmental stresses such as water logging and hypoxia,

though it still remains inadequately understood regarding where and how cell death occurs in plant organs in response to different abiotic stresses (Liu *et al.,* 2007). Cell death, being a genetically defined program, plays a vital part of plant life, leading to the death of some cells in response to developmental and, biotic as well as abiotic environmental signals thereby contributing to survival of the whole organism (Grennberg, 1996; Havel and Durzan, 1996; Jones and Dangl, 1996). Programmed cell death (PCD) is also an essential process during plant growth and development (Shiskova and Dubrovsky, 2005). Although the defined mechanisms of PCD are still far from clear, it is often mediated by two important molecular signals involving reactive oxygen species (ROS) and Ca^{2+} ions (Brookes *et al.*, 2004). Apoptosis is a genetically controlled PCD which is an evolutionarily conserved physiological cell death in eukaryotes that functions in developmental and cellular responses to stress (Wang *et al.,* 2006). Apoptosis in plants has been reported to be induced by multiple stimuli, including pathogen invasion, inoculation by avirulent pathogens, various types of abiotic stress, developmental signals and environmental cues (Greenberg *et al.,* 1994; Katsuhara and Kawasaki, 1996; Rao and Davis, 1999; Huh *et al.,* 2002; Greenberg and Yao, 2004). In this study, initially using Propidium Iodide staining, it was demonstrated that putative calcifuge Glenisla roots had damaged or dead cells when exposed to elevated (30 mM) rhizospheric Ca^{2+} and TUNEL staining of the roots confirmed the occurrence of apoptosis in the same. However, the putative calcicole Halifax roots had no marked morphological effect or cellular defects with elevated levels of Ca^{2+}. So, Halifax ecotype is more tolerant to rhizospheric Ca^{2+} as root cells don't undergo apoptotic death, whereas,

Glenisla (putative calcifuge), develops short and stumpy roots, on exposure to high concentration of rhizospheric Ca^{2+}, possibly due to Ca^{2+}-induced apoptosis making them less rhizospheric Ca^{2+}-tolerant. However, along with severe apoptosis observed in Glenisla roots a few apoptotic cells were seen in the roots of Halifax which presumably might be constantly dying cells that are sloughed off as the root grows. Also, as Glenisla and Halifax plant root morphologies differed in different growth mediums (sand and agar), the effect of calcium on the plants may be different in the different media.

Although environmental factors have an important influence on root system architecture, it is plausible that root growth is limited by inherent genetic boundaries (Beemster *et al.,* 2003). Such boundaries might, for instance, be controlled by a framework of genes involved in stress adaptation and tolerance. The next chapter investigates the possibility by examining genes potentially involved in rhizospheric Ca^{2+} tolerance showing an induced or repressive response in the *Arabidopsis* ecotypes under study.

Chapter 5

A Transcriptomics study of Ca^{2+}-responsive genes in *Arabidopsis*

5.1 Introduction

The advent of microarrays has made it possible to profile alterations in transcriptional activity to definite stimuli at a genome-wide level and provide an insight into comprehensive cell biology (van Baarlen *et al.,* 2008). The utilization of microarray-based transcriptome analyses in abiotic stress studies has subsequently resulted in a much needed database of gene expression profiles in *Arabidopsis*. Examples include salt stress (Kreps *et al.,* 2002; Seki *et al.,* 2002), oxidative stress (Desikan *et al.,* 2001; Klok *et al.,* 2002), drought stress (Seki *et al.,* 2001, 2002), cold stress (Kreps *et al.,* 2002, Fowler and Thomashow, 2002; Seki *et al.,* 2001, 2002), osmotic stress (Kreps *et al.,* 2002), ozone stress (Short, 2005), as well as other plant abiotic stresses (Ozturk *et al.,* 2002; Rizhsky *et al.,* 2002; Kawasaki *et al.,* 2001). However, linking expression profiles to biological or biochemical pathways as they happen in a cell is still a major challenge.

To date only two transcriptome studies of calcicole-calcifuge environmental physiology have been performed {McAinsh, Shirras, Abram, Gene Expression Omnibus series GSE5728 (Col-4 and Cal-0) and GSE5523 (Elland and Penicuik)}. In the first of these studies, a microarray analysis was carried out on duplicate samples of two lab ecotypes of *Arabidopsis*, Cal-0 a "calcicole" and Col-4 a "calcifuge" grown at 1 mM and 12.5 mM rhizospheric Ca^{2+} on sand with a hypothesis that adaptation to a calcareous environment will be reflected in altered gene expression patterns. The second of these

studies was carried out by performing triplicate arrays on two wild *A. thaliana* ecotypes Elland (calcicole) and Penicuik (calcifuge) grown at the same rhizospheric Ca^{2+} concentrations as in the previous experiment. Taken together, this whole analysis comprises 24 microarrays with triplicate chips for each of the four ecotypes grown at either 1 mM or 12.5 mM rhizospheric Ca^{2+}.

The aim of this chapter was to identify genes that respond to the rhizospheric Ca^{2+} from these data using Genespring® software. The identified set of genes with altered expression profiles was further assayed using quantitative real time PCR in both the lab ecotypes (Col-4 and Cal-0) as well as the wild Arabidopsis ecotypes under study comprising the putative calcifuge Glenisla and Penicuik and putative calcicoles Elland and Halifax over three harvest points (day 10, 20 and 30 of varied rhizospheric Ca^{2+} exposure). Additional analysis of microarray data from all four ecotypes was carried out using dChip® software.

5.2 Results

5.2.1 Microarray analysis

To obtain a group of genes whose expression altered with rhizospheric Ca^{2+}, Abram *et al.* (2005) grew *Arabidopsis* plants (ecotypes Col-4, Cal-0, Penicuik and Elland) on sterile, half-strength MS media with either 1mM or 12.5 mM $CaCl_2$ added to the growth media/ nutrient solution from day 0 in a controlled growth cabinet (23$\pm$ 1°C, 275 µmol m^{-2} s^{-1} photon flux density) for 10 days after which they were transferred to sterile, nutrient –free sand. Plants were watered daily with 1mM or 12.5 mM $CaCl_2$ in half-strength Long Ashton nutrient solution. At least 20 plants were grown per ecotype, per treatment. When plants reached growth stage 3.90 (Boyes *et al.,* 2001), whole seedlings were harvested and total RNA was extracted from pooled plants of Cal-0 and Col-4 grown on both high (12.5 mM) and low (1 mM) $CaCl_2$ using Qiagen RNEasy Extraction kits. Each experiment was repeated 3 times so that biological replicate samples could be compared by microarray analysis. Total RNA (from 24 samples in total) was sent to the Genomic Arabidopsis Resource Network (GARnet) facility at the Nottingham Arabidopsis Seed Centre (NASC) for microarray analysis using Affymetrix microarray chips. They used the Affymetrix ATH1 Arabidopsis Genome Array to perform the microarray analysis to identify genes which were differentially expressed between "calcioles" and "non-calcicoles". A grand total of 24 Affymetrix chips were used to carry out the microarray experiments (3 replicates of each Ca^{2+} treatment for each ecotype).The publicly available Cal-0, Col-4 microarray data (NCBI GEO dataset GSE5728) was initially analysed with Genespring

software to identify genes with decreased expression in plants treated with 12.5 mM rhizospheric Ca^{2+} compared to 1 mM Ca^{2+}. In addition, to avoid false positives, genes with signal values below 150 and less than 50 per cent presence in the arrays used, were excluded from the analysis. Using these parameters, seventeen genes in Cal-0 (from now on referred as Ca^{2+} Responsive Genes in Cal-0, Cal-CRG) and twenty three genes in Col-4 (from now on referred to as Ca^{2+} Responsive Genes in Col-4, Col-CRG) were identified (Tables 5.1 and 5.2).

Abram *et al.* (2005) analyzed genes with increased expression levels in ecotypes treated with 12.5 mM rhizospheric Ca^{2+}. Therefore this study focuses on genes with repressed expression at 12.5 mM rhizospheric Ca^{2+}, in other words, relatively increased expression at 1 mM Ca^{2+}. For putative calcicoles, this low level of Ca^{2+} might be expected to be stressful and to induce compensatory mechanisms.

Ca^{2+} Responsive Genes in Cal-0 (Cal-CRG)	Putative Function	Fold change relative to normalized intensity (Linear scale)			
		Cal-0 1 mM	Cal-0 12.5 mM	Col-4 1 mM	Col-4 12.5 mM
At4g11850 (Cal-CRG1)	membrane, phospholipase D activity, embryonic development ending in seed dormancy	2.243	0.868	1.191	0.938
At5g18820 (Cal-CRG2)	chloroplast, ATP binding, protein binding, embryonic development ending in seed dormancy, unfolded protein binding	2.411	1.399	0.801	0.739
At2g37880 (Cal-CRG3)	cellular component unknown, molecular function unknown, biological process unknown	2.331	0.942	1.543	0.887
At2g40080 (Cal-CRG4)	cellular component unknown, molecular function unknown, biological process unknown	2.287	1.423	0.661	0.801
At5g18470 (Cal-CRG5)	cellulose and pectin-containing cell wall, sugar binding, biological process unknown	4.588	2.116	0.968	0.621
At3g19080 (Cal-CRG6)	nucleus, biological process unknown	2.872	1.803	0.823	0.853
At5g03720 (Cal-CRG7)+	member of Heat Stress Transcription Factor (Hsf) family	2.364	0.959	1.474	0.705
At3g20370 (Cal-CRG8)	cellular component unknown, molecular function unknown, biological process unknown	2.026	0.93	1.146	0.907
Orf 215b (Cal-CRG9)	hypothetical protein	2.32	0.824	1.45	0.789
At4g29690 (Cal-CRG10)	hydrolase activity, nucleotide metabolic process, endomembrane system	2.048	1.012	0.955	0.91
At1g69930 (Cal-CRG11)*	glutathione transferase activity	2.334	0.608	0.975	0.577
At1g26240 (Cal-CRG12)*	structural constituent of cell wall, cellulose and pectin-containing cell wall organization and biogenesis, endomembrane system	2.136	1.567	0.408	0.473
At5g28080 (Cal-CRG13)+	mitogen activated protein kinase – like	1.965	1.772	0.501	0.412
At1g75040 (Cal-CRG14)	Thaumatin-like protein involved in response to pathogens.	1.943	1.572	0.742	0.478
At1g14220 (Cal-CRG15)+	ribonuclease T2 family protein	1.846	1.477	0.581	0.462
At5g15360 (Cal-CRG16)*	molecular function unknown, biological process unknown	3.223	1.824	0.297	0.262
At1g31680 (Cal-CRG17)	copper ion binding, biological process unknown	2.616	1.957	0.287	0.491

Table 5.1: List of genes showing increased expression levels in Cal-0 grown at 1 mM rhizospheric Ca^{2+} and the expression of the same genes in Col-4 Highlighted Cal-CRGs have been selected for further studies. *- SALK-TDNA mutant lines for these genes were not available, so '+' genes mutant lines were selected for studies in Chapter 6.

Calcium Responsive Genes in Col-4 (Col-CRG)	Putative Function	Fold change relative to normalized intensity (Linear scale)			
		Col-4 1 mM	Col-4 12.5 mM	Cal-0 1 mM	Cal-0 12.5 mM
At5g06640 (Col-CRG1)	putative protein	1.507	0.335	1.183	0.55
At1g61566 (Col-CRG2)	Expressed protein	3.88	1.266	1.00	0.64
At1g36130 (Col-CRG3)	athila ORF 1, putative	1.579	0.468	1.187	0.997
At5g53830 (Col-CRG4)	putative protein	1.002	0.405	1.121	0.92
At3g30210 (Col-CRG5)	myb-like transcription factor, putative	1.00	0.352	1.308	0.838
At1g05250 (Col-CRG6)	peroxidase activity, response to oxidative stress, endomembrane system	1.00	0.271	1.505	0.541
At1g78840 (Col-CRG7)	hypothetical protein	1.228	0.447	1.189	0.78
At2g20870 (Col-CRG8)	expressed protein	20.69	2.197	0.876	0.968
At5g22430 (Col-CRG9)	expressed protein	12.26	1.547	0.851	0.625
At3g54590 (Col-CRG10)	extensin precursor -like protein	1.099	0.435	1.374	0.649
At1g62490 (Col-CRG11)	hypothetical protein	1.266	0.466	1.38	0.619
At1g69120 (Col-CRG12)	floral homeotic gene APETALA1	2.971	0.907	1.172	0.81
At5g33370 (Col-CRG13)	putative protein	4.201	0.864	0.994	0.787
At1g32020 (Col-CRG14)	F-box protein family	3.147	0.622	1.223	0.802
At1g57750 (Col-CRG15)	Cytochrome P450, putative	3.483	0.407	0.999	0.822
At2g24210 (Col-CRG16)	myrcene/ocimene synthase	3.302	0.562	1.441	0.956
At1g59930(Col-CRG17)	hypothetical protein	0.706	0.434	0.993	1.325
At2g04500 (Col-CRG18)	CHP-rich zinc finger protein, putative	2.444	0.323	1.053	0.965
At5g02540 (Col-CRG19)	putative protein	2.095	0.787	1.106	0.951
At2g38060 (Col-CRG20)	putative Na+-dependent inorganic phosphate cotransporter	4.579	0.782	1.568	0.762
At1g53140 (Col-CRG21)	hypothetical protein	2.359	0.549	0.996	0.771
At4g12960 (Col-CRG22)	putative protein	2.462	0.6	0.886	0.69
At1g30795 (Col-CRG23)	Expressed protein	2.143	0.694	1.319	0.632

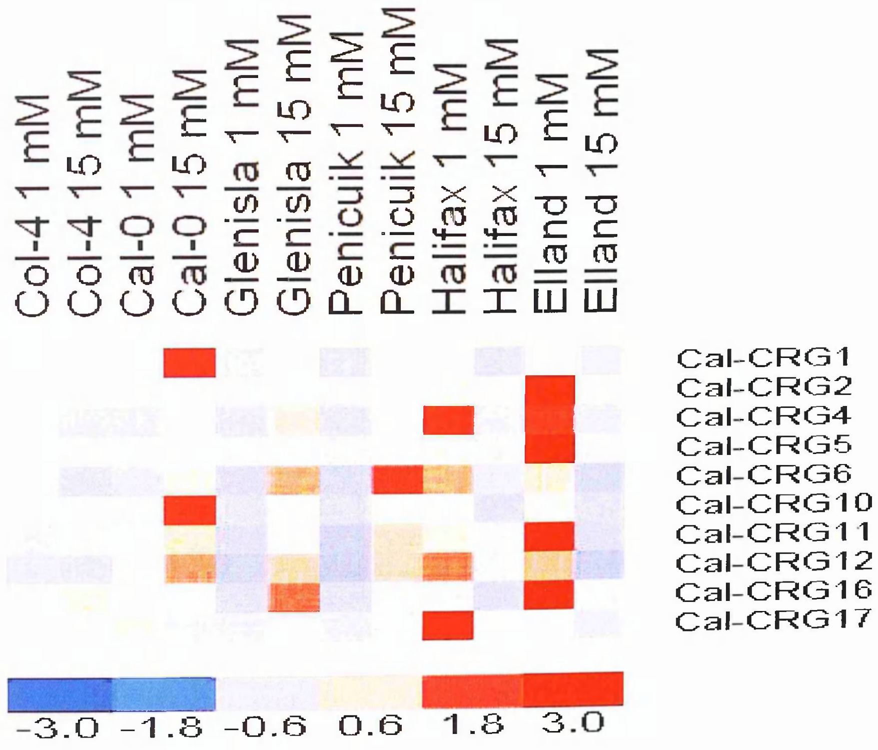
Table 5.2: List of genes showing increased expression levels in Col-4 grown at 1 mM rhizospheric Ca^{2+} compared to those grown at 12.5 mM rhizospheric Ca^{2+} and the expression of the same genes in Cal-0.

5.2.2 Real time analysis of Calcium Responsive Genes

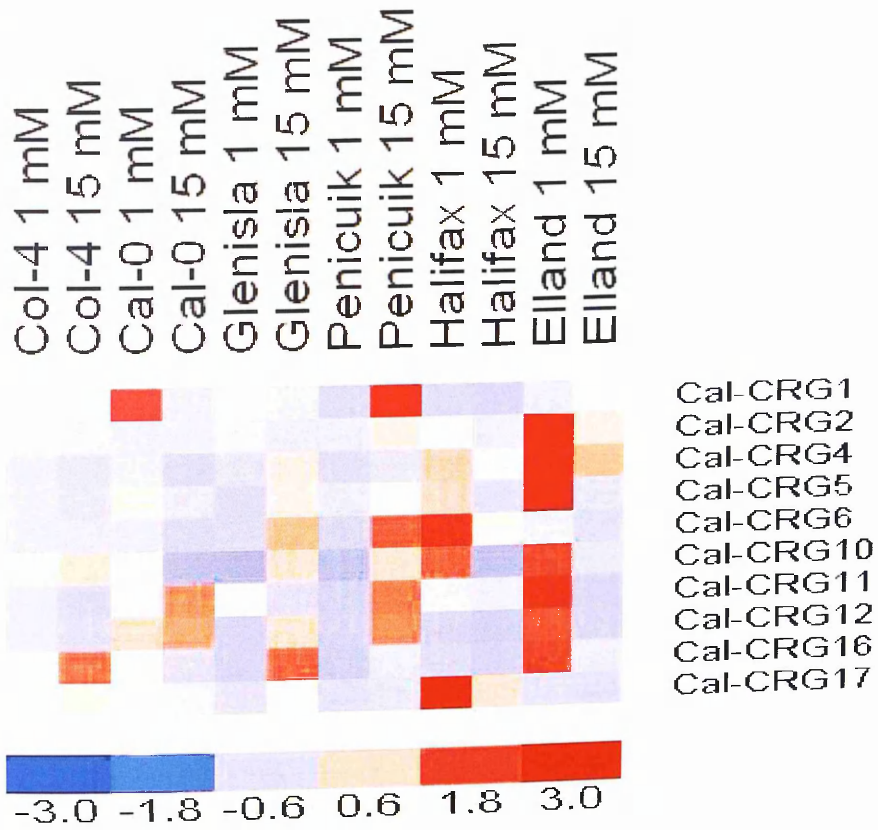
Real-time quantitative RT-PCR (qRT-PCR) was used to quantify the expression of ten Cal-CRGs in the six ecotypes under study *viz.*, Col-4, Cal-0, Glenisla, Penicuik, Halifax and Elland on day 10, 20 and 30 of treatments with 1 mM or 15 mM rhizospheric Ca^{2+}, respectively. The plants were grown as specified in Section 2.5 and RNA extracted from whole plant tissue (as per Section 2.10) for subsequent RT-PCR (Section 2.11) and qRT-PCR analysis (Section 2.13). Of the Seventeen Cal-CRGs only ten were selected for carrying out real time gene quantification of which only seven had SALK-TDNA insertion mutant lines available for the Cal-CRGs (see Chapter 6). Clustering analysis of the real time PCR data was carried out using the dChip® clustering tool (Figure 5.1). All the ecotype samples were compared to Col-4 samples treated with 1 mM rhizospheric Ca^{2+} which was used as a calibrator or reference sample for the whole experiment.

Ten repressed Cal-CRGs highlighted in Table 5.1 were picked for the quantitative real time PCR analysis.

A



B



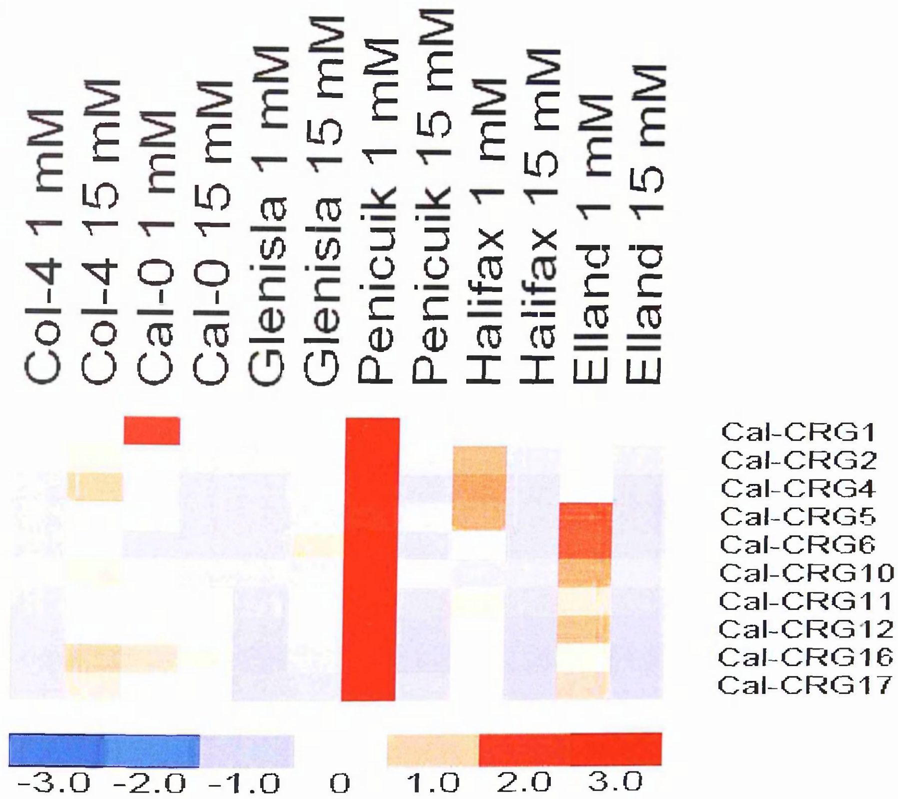


Figure 5.1: Quantitative real time PCR clustering analysis of 10 Cal-CRGs in the 6 ecotypes under study. A. Plants harvested on the 10th day of treatment; B. Plants harvested on the 20th day of treatment; C. Plants harvested on the 30th day of treatment. Numbers at the bottom correspond to relative gene expression and the colours correspond to numbers (red - induced, blue – repressed, white - no change in expression of specific Gene of Interest (Cal-CRGs). Genes and ecotypes were clustered using dChip® clustering and enrichment tool based on their real time gene expression values for similarity of expression between the 6 ecotypes and 10 Cal-CRGs.

Six Cal-CRGs (1, 4, 6, 10, 11 and 12) were induced in expression in Cal-0 at 15 mM rhizospheric Ca^{2+} (Figure 5.1a), but by 20 days only Cal-CRG 11 and Cal-CRG12 had similar induced expression and on 30th day all the ten Cal-CRGs had a repressed expression at the high Ca^{2+} treatment which is similar to that observed in the microarray data (Table 5.1). Also, all ten Cal-CRGs were severely repressed in the other 2 wild putative calcicoles Elland and Halifax after 30 days of Ca^{2+} treatment (Figure 5.1c).

After 10 days of Ca^{2+} treatment, wild putative calcifuges Penicuik and Glenisla treated with 15 mM Ca^{2+} showed similar expression pattern, whereas, both treated with 1 mM Ca^{2+} and Col-4 (putative lab calcicole) treated with 1 mM Ca^{2+} had similar expression pattern of 10 Cal-CRGs (Figure 5.1a). But by day 30, gene expression pattern of Penicuik was similar to the two wild putative calcicoles, Elland and Halifax, when treated with either 1 mM or 15 mM rhizospheric Ca^{2+} (Figure 5.1c). Also, these three (Penicuik, Elland and Halifax) treated with 15 mM Ca^{2+} were in a cluster together with Col-4 and Glenisla treated with 1 mM rhizospheric Ca^{2+} (Figure 5.1c).

5.2.3 Complete set of Microarray analysis

The whole set of twenty four microarray chip data was used to carry out the subsequent analysis of the differentially expressed Ca^{2+} responsive genes in ecotypes Col-4, Cal-0, Penicuik and Elland (NCBI GEO datasets GSE5728 and GSE5523) using dChip software. Of the 22392 unique genes on the chips, only 1508 (6.6%) genes satisfied the filtering criteria of $\geq$6.0 $\log_2$ gene expression in $\geq$50% of the arrays and SD across all arrays >0.5. (see Section 2.11). Therefore 94% of the genes were excluded from the analysis.

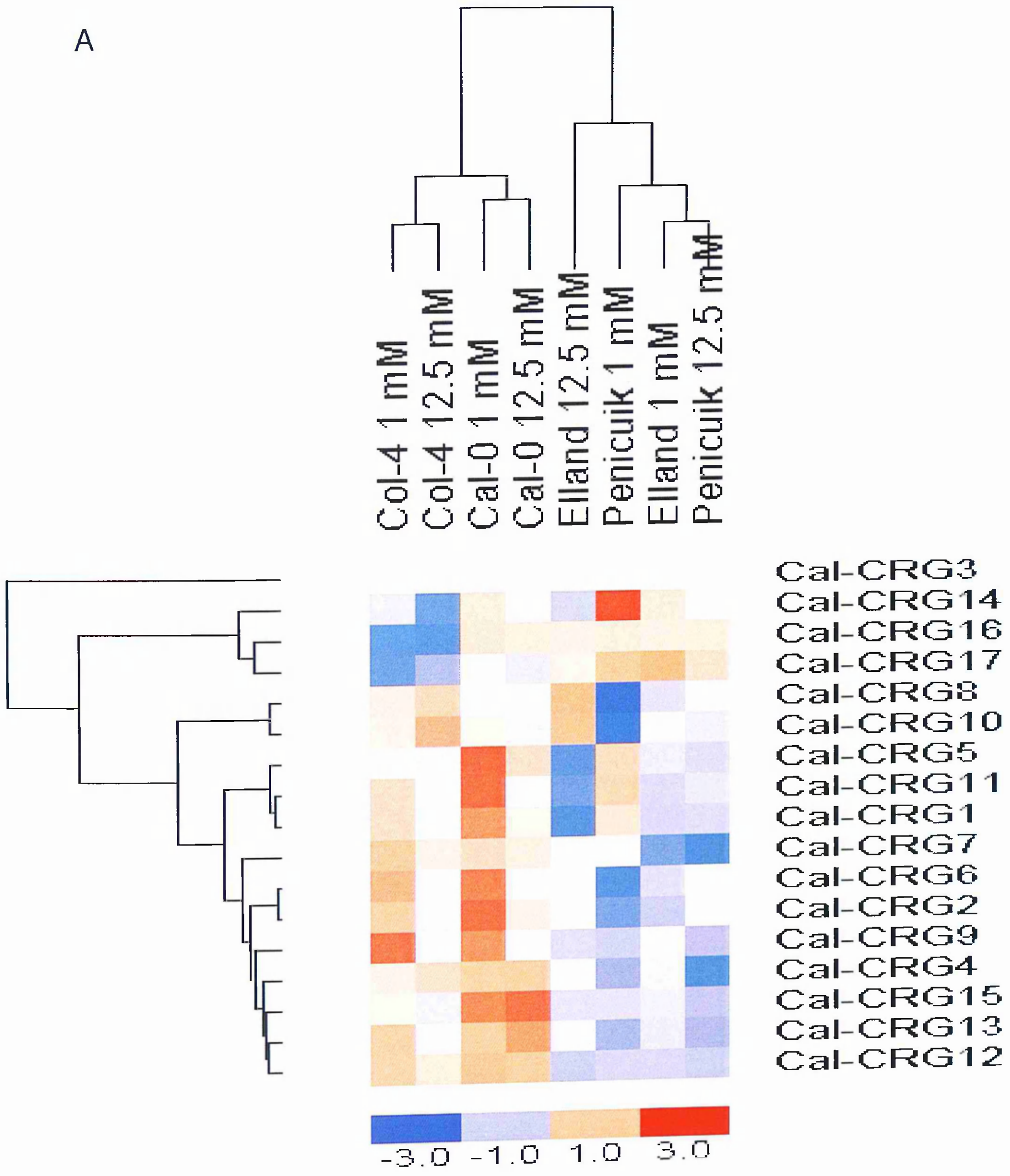
These genes were either unchanging or their expression was too low to give reliable expression values. Of the 1508 filtered genes, none displayed a statistically significant difference (paired t-test, $P \leq 0.05$) in expression between 1 mM and 12.5 mM rhizospheric Ca^{2+} treatments in all 4 ecotypes. The absence of filtered genes that respond to varying rhizospheric Ca^{2+} treatments across all the 4 ecotypes in the study signifies that an individual ecotype is responding to the altered rhizospheric Ca^{2+} in a differential manner to the other ecotypes.

Clustering analysis of Cal-CRGs and Col-CRGs identified in Section 5.2.1 (Table 5.1 and 5.2) confirms this (Figure 5.2). For Cal-CRGs, both the lab ecotypes (Col-4 and Cal-0) clustered together with the other cluster comprising of the wild ecotypes (Elland and Penicuik) independent of rhizospheric Ca^{2+} treatments. Also, Elland and Penicuik treated with either low (1 mM) or high (12.5 mM) rhizospheric Ca^{2+}, clustered together, showing contrasting Ca^{2+}-responsive gene expression when exposed to altered rhizospheric Ca^{2+} (Figure 5.2a).

Col-CRGs (Figure 5.2 b) expression broadly had similar clusters as Cal-CRGs, where the wild ecotypes Elland and Penicuik clustered together. However, Col-4 treated with 1 mM Ca^{2+} clustered with the wild ecotypes rather than with Cal-0 or Col-4 treated with 12.5 mM rhizospheric Ca^{2+}. However Cal-0 treated with either of the Ca^{2+} concentration (1 mM or 12.5 mM) formed an individual close cluster for both Cal-CRG and Col-CRG expression. This analysis showed that regardless of their calcicole-calcifuge nature, the wild ecotypes have a completely different gene expression pattern

to the lab ecotypes. Also, as expected, the lab ecotypes Col-4 and Cal-0 had a differential response to rhizospheric Ca^{2+}.

An analysis was then performed on individual ecotypes to unravel significantly responding Ca^{2+}-responsive genes and enriched Gene Ontology (GO) functional annotations of the responsive genes were identified.


A
Col-4 1 mM
Col-4 12.5 mM
Cal-0 1 mM
Cal-0 12.5 mM
Elland 12.5 mM
Penicuik 1 mM
Elland 1 mM
Penicuik 12.5 mM
Cal-CRG3
Cal-CRG14
Cal-CRG16
Cal-CRG17
Cal-CRG8
Cal-CRG10
Cal-CRG5
Cal-CRG11
Cal-CRG1
Cal-CRG7
Cal-CRG6
Cal-CRG2
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-3.0
-1.0
1.0
3.0

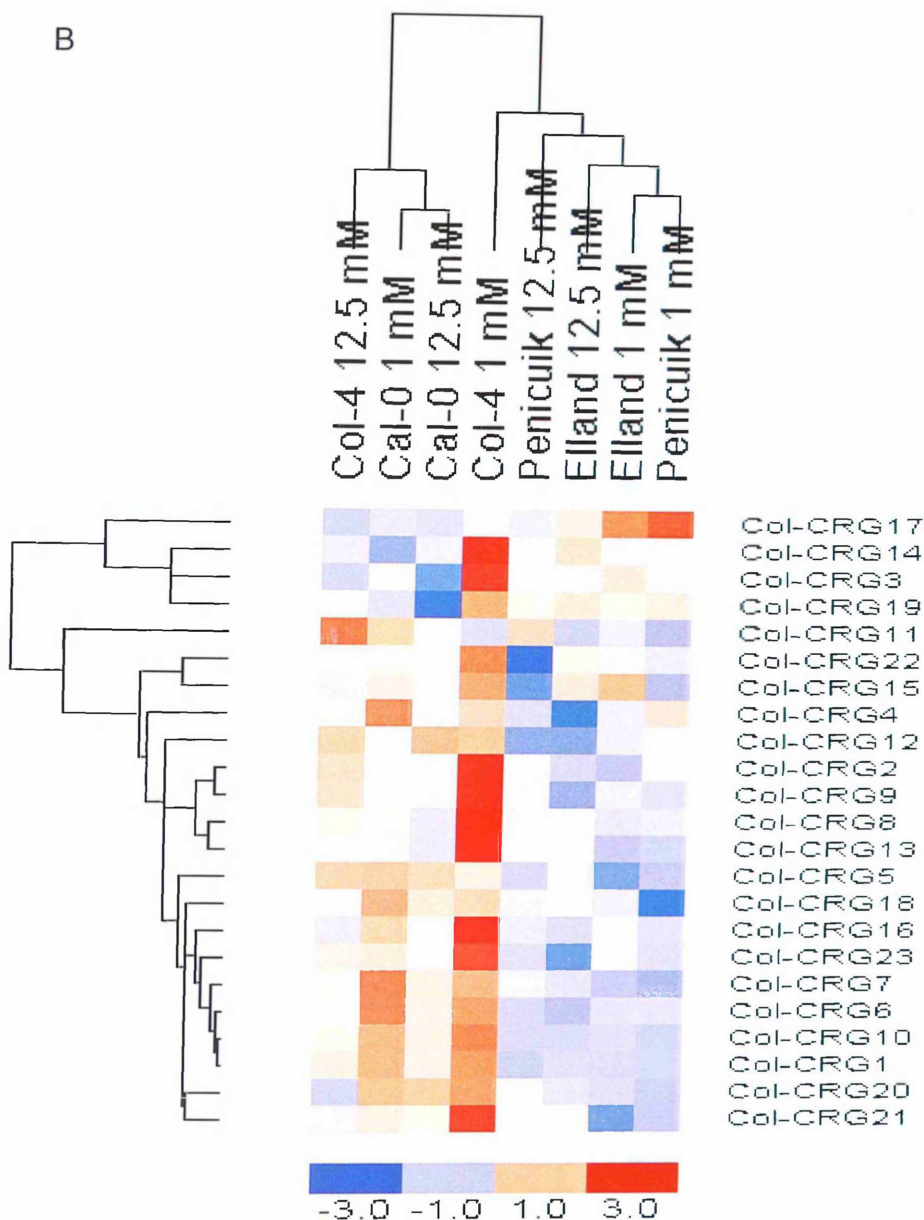

Figure 5.2: Clustering analysis of Cal-CRGs (A) and Col-CRGs (B) in Col-4, Cal-0, Elland and Penicuik exposed to either 1 mM or 12.5 mM rhizospheric Ca^{2+} for 30 days. Numbers at the bottom correspond to relative gene expression and the colours correspond to numbers (red - induced, blue – repressed, white - no change in expression of specific Gene of Interest (Cal-CRGs).

Col-4 (Putative Calcifuge)

Comparing 1 mM rhizospheric Ca^{2+} treated Col-4 plants with 12.5 mM treated ones revealed 3936 (17.3%) genes which satisfied the filtering criteria. Of these, only 53 genes displayed a statistically significant difference (paired t-test, $P \leq 0.05$) in their expression levels between the treatments. Of the 53 significantly differentially expressed genes in Col-4, 15 were repressed with 38 genes being up-regulated in plants treated with 12.5 mM rhizospheric Ca^{2+}. Clustering and functional enrichment analysis of these genes identified GO molecular function group(s) 'catalytic activity' and 'cation binding' classes and GO biological process group(s) 'carbohydrate metabolic process' and 'response to oxidative stress' groups. Individual breakdown and the number of genes involved in specific GO enriched ($P \leq 0.001$) functions are presented in Table 5.3.

Cal-0 (Putative Calcicole)

3997 (17.6%) genes satisfied the initial filtering criteria in comparing Cal-0 plants treated with 12.5 mM rhizospheric Ca^{2+} to those treated with 1 mM rhizospheric Ca^{2+}. Of these, only 46 genes revealed a significant difference (paired t-test, $P \leq 0.05$) in expression between the treatments. Only 9 of the 46 significantly differentially expressed genes showed a repression in their expression at 12.5 mM rhizospheric Ca^{2+} treatment. Clustering of these genes did not place the genes into any major clusters. However, 8 enriched functional groups were significant ($P \leq 0.001$) and are presented with annotations (Table 5.4).

Putative Gene Ontology	No. of Genes
acid-ammonia (or amide) ligase activity	2
ammonia ligase activity	2
amylase activity	2
aromatic amino acid family catabolic process	2
cellular polysaccharide metabolic process	5 (1)
glucan metabolic process	5 (1)
glycogen biosynthetic process	2
glycogen metabolic process	2
L-phenylalanine metabolic process	2
polysaccharide metabolic process	5 (1)
starch metabolic process	3

Table 5.3: Gene Ontology functions of genes significantly induced/repressed by altered rhizospheric Ca^{2+} in Col-4. Functional classification of genes with GO annotations. Statistically significant ($P \leq 0.001$) enriched groups identified by dChip® software. Number of genes repressed at 12.5 mM Ca^{2+} treatment is shown in brackets.

Putative Gene Ontology	No. of Genes
amino acid and derivative metabolic process	7
amino acid derivative biosynthetic process	4
amino acid derivative metabolic process	5
aromatic compound biosynthetic process	4
oxidoreductase activity	3
phenylpropanoid biosynthetic process	4
phenylpropanoid metabolic process	5
starch biosynthetic process	2

Table 5.4: Gene Ontology functions of genes significantly induced/repressed by altered rhizospheric Ca^{2+} in Cal-0. Functional classification of genes with GO annotations. Statistically significant ($P \leq 0.001$) enriched groups identified by dChip® software. All the genes in this list were induced in expression at 12.5 mM Ca^{2+} treatment.

Penicuik (Putative calcifuge)

1963 (8.6%) genes passed the filtering criteria comparing Penicuik plants treated with 1 mM rhizospheric Ca^{2+} to those treated with 12.5 mM Ca^{2+}. 246 genes from the above filtered list were significantly differentially expressed (paired t-test, $P \leq 0.05$) between the rhizospheric Ca^{2+} treatments. 199 of these 246 genes were down regulated in expression levels when plants treated with 1 mM rhizospheric Ca^{2+} were compared to plants treated with 12.5 mM rhizospheric Ca^{2+} (Figure 5.6) (or up-regulated at low Ca^{2+} concentration). Gene annotation enrichment analysis clustered genes into different groups, *viz.*, cellulose and pectin-containing cell wall, defence response, defence response to fungus, DNA binding ethylene mediated signaling pathway, nucleus, regulation of transcription, regulation of transcription, DNA-dependent, response to abscisic acid stimulus, response to cadmium ion, response to cold, response to ethylene stimulus, response to gibberellin stimulus, response to hydrogen peroxide, response to jasmonic acid stimulus, response to salicylic acid stimulus, response to salt stress, transcription, transcription factor activity, and transcriptional activator activity. The above annotated genes were placed into 55 significantly enriched ($P \leq 0.001$) GO groups (Table 5.5).

Elland (Putative Calcicole)

1071 (4.7%) genes satisfied the filtering criteria for Elland plants treated with either 1 mM or 12.5 mM rhizospheric Ca^{2+}. Only 183 genes from the filtered genes list significantly differed (paired t-test, $P \leq 0.05$) between the two rhizospheric Ca^{2+} treatments. Of these 183 genes, 154 genes were down

regulated in expression in Elland plants treated with 12.5 mM rhizospheric Ca^{2+} (Figure 5.7). These genes were clustered into the following GO groups: defence response, defence response to bacterium, defence response to fungus, ethylene mediated signaling pathway, glucan metabolic process, nucleus, regulation of transcription, DNA-dependent, response to abscisic acid stimulus, response to cold, response to ethylene stimulus, response to gibberellin stimulus, response to jasmonic acid stimulus, response to salicylic acid stimulus, response to wounding, transcription factor activity and xyloglucan:xyloglucosyl transferase activity. The annotated genes were placed in 45 significantly enriched functional groups ($P \leq 0.001$) by performing the gene enrichment analysis using dChip software (Table 5.6).

Putative Gene Ontology	No. of Genes
biological regulation	54 (46)
biopolymer metabolic process	66 (55)
cell motility	3 (3)
cell projection	3 (3)
cell wall	11 (10)
cellulose and pectin-containing cell wall	8 (7)
ciliary or flagellar motility	3 (3)
defence response	22 (22)
DNA binding	45 (39)
ethylene mediated signaling pathway	7 (6)
external encapsulating structure	11 (10)
flagellin-based flagellum	3 (3)
Flagellum	3 (3)
hormone-mediated signaling	11 (10)
immune response	7 (7)
immune system process	7 (7)
localization of cell	3 (3)
Nucleus	46 (42)
programmed cell death	5 (5)
regulation of biological process	48 (42)
regulation of cellular metabolic process	47 (41)
regulation of cellular process	48 (42)
regulation of metabolic process	47 (41)

Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	47 (41)
regulation of transcription	47 (41)
regulation of transcription, DNA-dependent	35 (33)
response to abiotic stimulus	23 (18)
response to abscisic acid stimulus	11 (8)
response to bacterium	6 (6)
response to biotic stimulus	14 (11)
response to cadmium ion	5 (5)
response to chemical stimulus	35 (29)
response to chitin genes	3 (3)
response to cold	10 (10)
response to endogenous stimulus	30 (26)
response to ethylene stimulus	12 (11)
response to gibberellin stimulus	6 (5)
response to hormone stimulus	25 (21)
response to inorganic substance	7 (7)
response to jasmonic acid stimulus	8 (8)
response to metal ion	7 (7)
response to other organism	14 (11)
response to salicylic acid stimulus	7 (7)
response to stimulus	56 (47)
response to stress	22 (19)
response to temperature stimulus	14 (11)

RNA biosynthetic process	35 (34)
RNA metabolic process	37 (35)
Transcription	47 (41)
transcription factor activity	41 (35)
transcription regulator activity	44 (38)
transcription, DNA-dependent	35 (33)
transcriptional activator activity	7 (5)
Transferase activity	3 (3)
two-component signal transduction system	7 (6)

Table 5.5: Gene Ontology functional groups significantly induced/repressed by altered rhizospheric Ca^{2+} in Penicuik. Functional classification of genes with GO annotations. Statistically significant ($P \leq 0.001$) enriched groups identified by dChip® software. Number of genes induced at 1 mM Ca^{2+} treatment is shown in brackets.

Putative Gene Ontology	No. of Genes
Aging	5 (4)
cell death	8 (8)
cell motility	3 (3)
cell projection	3 (3)
cell wall	8 (7)
ciliary or flagellar motility	3 (3)
Death	8 (8)
defence response	23 (21)
defence response to bacterium	6 (6)
defence response to fungus	5 (4)
external encapsulating structure	8 (7)
flagellin-based flagellum	3 (3)
Flagellum	3 (3)
innate immune system process response	7 (7)
localization of cell	3 (3)
programmed cell death	7 (7)
regulation of cellular metabolic process	29 (27)
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	29 (27)
regulation of transcription	29 (27)
regulation of transcription, DNA-dependent	23 (22)
response to abscisic acid stimulus	9 (8)
response to bacterium	6 (6)

response to biotic stimulus	11 (9)
response to chemical stimulus	27 (24)
response to endogenous stimulus	26 (23)
response to ethylene stimulus	8 (8)
response to external stimulus	11 (8)
response to fungus	5 (4)
response to gibberellin	6 (6)
response to hormone stimulus	19 (17)
response to jasmonic acid stimulus	10 (8)
response to osmotic stress	8 (7)
response to other organism	11 (9)
response to salicylic acid stimulus	8 (7)
response to stimulus	46 (41)
response to stress	20 (15)
response to wounding	10 (6)
RNA biosynthetic process	23 (22)
Transcription	29 (27)
transcription factor activity	26 (25)
transcription regulator activity	28 (26)
transcription, DNA-dependent	23 (22)
xyloglucan:xyloglucosyl transferase activity	4 (3)

Table 5.6: Gene Ontology functional groups of genes significantly induced/repressed by altered rhizospheric Ca^{2+} in Elland. Functional classification of genes with GO annotations. Statistically significant ($P \leq 0.001$) enriched groups identified by dChip® software. Number of genes induced at 1 mM Ca^{2+} treatment is shown in brackets.

Response of "calcium" genes

High Affinity K^+ transporter HAK5 was significantly up-regulated during transcriptome studies on K^+ stress (Armengaud *et al.,* 2004). By analogy, it may be hypothesised that differential regulation of genes involving Ca^{2+} transport or Ca^{2+} binding or those involved in calcium-dependant proteins or calcium transport might be observed in plants grown at different levels of rhizospheric Ca^{2+}. Using dChips 'genelist by key word' function, 'Calcium' generated 113 genes involved in Ca^{2+}-related processes. Of these 113 genes 9 satisfied the filtering criteria (paired t-test, $P \leq 0.05$) (Table 5.7) of which 6 were Ca^{2+}-binding proteins, 2 Ca^{2+}-dependant (At3g57530 and At5g66210) and a Ca^{2+}-transporting ATPase (ACA1 which encodes a Ca^{2+} pump).

All the 9 'calcium genes' were induced in expression in 'wild' *A. thaliana* ecotypes Elland and Penicuik at 1mM Ca^{2+} whereas the expression was mixed in lab ecotypes Col-4 and Cal-0. Cal-0 had 5 induced calcium genes at low rhizospheric Ca^{2+} whereas Col-4 had 3. Of the 5 Ca^{2+}-binding EF hand family proteins in the list, 2 (At3g47480, At5g39670) were induced in all four ecotypes at 1 mM rhizospheric Ca^{2+}.

Gene	AGI number	log₂ ratio gene expression high/low Ca^{2+} Cal-0	Col-4	Elland	Penicuik
calmodulin-like calcium-binding protein, 22 kDa (CaBP-22)	At2g41090	-0.05	0.48	-0.85	-0.57
ACA1/PEA1: calcium-transporting ATPase 1, plasma membrane-type / $Ca^{(2+)}$-ATPase isoform 1 (ACA1) / plastid envelope ATPase 1 (PEA1)	At1g27770	0.31	0.67	-0.79	-1.35
calcium-binding EF hand family protein	At1g76650	0.09	-0.48	-1.47	-3.07
calcium-binding EF hand family protein	At4g27280	0.22	1.45	-3.13	-2.09
calcium-binding EF hand family protein	At3g47480	-0.73	-0.34	-0.81	-1.92
calcium-dependent protein kinase, putative / CDPK, putative	At3g57530	0.18	0.48	-0.96	-1.29
calcium-binding EF hand family protein	At5g39670	-0.77	-0.53	-1.51	-1.95
calcium-binding EF-hand protein, putative	At5g54490	-0.62	0.38	-2.18	-2
calcium-dependent protein kinase family protein / CDPK family protein	At5g66210	-0.18	0.25	-1.04	-1.65

Table 5.7: The effect of altered rhizospheric Ca^{2+} exposure on the expression of 'calcium genes'. Difference in gene expression of the ecotypes was generated by comparing 12.5 mM rhizospheric treatment to 1 mM treatment.

Transcription factor genes

Transcription factors are DNA-binding proteins that are capable of regulating transcription (Singh, 1998; Ptashne and Gann, 1997). The *A. thaliana* genome encodes ~1500 transcription factors comprising more than 20 families (Reichmann *et al.,* 2000). There are 602 transcription factor genes represented on the ATH-1 chip. However, only 82 genes passed the filtering criteria for variation on standard deviation across the samples and an expression level of ≥6 in ≥ 50% of the samples which grouped into 19 functional clusters (Table 5.8). Transcription factor families that are represented include those regulating biosynthetic and catalytic activities, those controlling metabolic processes, defence pathways and biotic and abiotic stress responses.

GO cluster	No. of genes
defence response to bacterium	4
defence response to fungus	4
DNA binding	75
Nucleus	75
protein dimerization activity	5
regulation of defence response	2
regulation of transcription	47
regulation of transcription, DNA-dependent	66
response to abscisic acid stimulus	15
response to auxin stimulus	14
response to cadmium ion	13
response to ethylene stimulus	14
response to gibberellin stimulus	13
response to jasmonic acid stimulus	17
response to salicylic acid stimulus	15
response to salt stress	17
sequence-specific DNA binding	25
Transcription	51
transcription factor activity	70

Table 5.8: Gene Ontology clusters of the genes encoding transcription factors from the filtered gene list. No genes were found to be either uniformly induced or repressed in all four ecotypes at 12.5 mM rhizospheric Ca^{2+} treatment compared to 1 mM treatment.

Comparison of GeneSpring® and dChip® analysis of Col-4 and Cal-0 microarray data

Initial GeneSpring analysis was carried out on data from eight microarrays of two ecotypes Col-4 (putative calcifuge) and Cal-0 (putative calcicole) grown at 1 mM and 12. 5 mM rhizospheric calcium, whereas dChip analysis was carried out as more microarray data (3 replicates for each ecotype at specific rhizospheric calcium concentration, totalling 24) from two more ecotypes (Penicuik and Elland) grown under similar conditions facilitated the altered gene expression studies for tolerance to high rhizospheric calcium. It was interesting to observe that the genes identified from Col-4 (Col-CRGs) and Cal-0 (Cal-CRGs) comparisons in the initial study did not pass the filtering criteria when comparisons were carried out with inclusion of the third replicate. This was due to the stringent statistical filtering of the genes and non-significant expression of the genes across all the twelve arrays (3 replicates each for Col-4 and Cal-0 grown at 1 mM and 12.5 mM rhizospheric calcium).

Table 5.9 and 5.10 emphasis the mean difference in the gene expression values (difference between expression of a gene in an individual ecotype treated with 12.5 mM or 1 mM rhizospheric calcium) analysed using Genespring compared to the analysis done using dChip on the initial eight microarray data from Cal-0 (Cal-CRGs) and Col-4 (Col-CRGs), respectively. Highlighted values show similar expression in both analyses. Although 12 of 17 Cal-CRGs in Cal-0 from the initial data were also repressed in the dChip analysis (Table 5.11), these were non-significant on the dChip filtering criteria. In the 'wild' putative calcicole Elland, nine Cal-CRGs showed a repressed

gene expression patterns similar to those in putative lab calcicole Cal-0 (Table 5.11).

A similar analysis of the difference of gene expression values in Col-4 treated with 12.5 mM or 1 mM rhizospheric calcium showed 20 of 23 Col-CRGs being induced in expression at low calcium in the dChip analysis (Table 5.12). Also, only 10 Col-CRGs were induced at low calcium in the wild putative calcifuge Penicuik compared to Col-4. This comparison of the initial data (8 chips) using different software and the inclusion of an extra replicate as well as data from two other ecotypes (Elland and Penicuik) for subsequent analysis highlights the complexity of changes in gene expression patterns and increases the robustness of understanding the expression profiles of calcium-responsive genes.

	Mean difference in gene expression (log_2 ratios high/low calcium) of Cal-0 using initial 8 arrays	
Cal-CRGs	**Genespring®**	**dChip®**
Cal-CRG1	-1.375	-0.86
Cal-CRG2	-1.012	-0.54
Cal-CRG3	-1.389	0
Cal-CRG4	-0.864	-0.56
Cal-CRG5	-2.472	-1.28
Cal-CRG6	-1.069	-0.58
Cal-CRG7	-1.405	-0.63
Cal-CRG8	**-1.096**	**-1.04**
Cal-CRG9	-1.496	-0.88
Cal-CRG10	-1.036	-0.79
Cal-CRG11	-1.726	-0.97
Cal-CRG12	-0.569	-0.08
Cal-CRG13	-0.193	0.09
Cal-CRG14	-0.371	-0.78
Cal-CRG15	**-0.369**	**-0.36**
Cal-CRG16	-1.399	-0.33
Cal-CRG17	-0.659	-0.43

Table 5.9: Comparison of difference in mean expression values (log2 ratio values) of Cal- CRGs in Cal-0 treated with either 1 mM or 12.5 mM rhizospheric Ca^{2+} from the initial eight microarray chips. Data were analysed initially using Genespring® and then repeated using dChip® software. Values in bold, show similar expression values using both packages.

Col-CRGs	Mean difference in gene expression (log_2 ratios high/low calcium) of Col-4 using initial 8 arrays	
	Genespring®	dChip®
Col-CRG1	-1.172	-1.37
Col-CRG2	-2.614	-1.91
Col-CRG3	-1.111	-0.86
Col-CRG4	-0.597	-0.72
Col-CRG5	-0.648	0.02
Col-CRG6	-0.729	-1.75
Col-CRG7	**-0.781**	**-0.77**
Col-CRG8	-18.493	-3.58
Col-CRG9	-10.713	-2.81
Col-CRG10	-0.664	-1.57
Col-CRG11	-0.8	0.16
Col-CRG12	-2.064	-0.48
Col-CRG13	-3.337	-2.71
Col-CRG14	-2.525	-1.05
Col-CRG15	-3.076	-1.98
Col-CRG16	-2.74	-1.75
Col-CRG17	-0.272	0.07
Col-CRG18	-2.121	-0.49
Col-CRG19	-1.308	-0.46
Col-CRG20	-3.797	-0.94
Col-CRG21	-1.81	-0.7
Col-CRG22	-1.862	-0.92
Col-CRG23	-1.449	-0.64

Table 5.10: Comparison of difference in mean expression values (log2 ratio high/low Ca^{2+}) of Col- CRGs in Col-4 treated with either 1 mM or 12.5 mM rhizospheric Ca^{2+} from the initial eight microarray chips. Data analysed initially using Genespring® and then repeated using dChip® software. Values in bold, show similar expression values using both packages.

	Mean difference in microarray log_2 gene expression from 24 chip experiment			
Cal-CRGs	**Cal-0**	**Col-4**	**Elland**	**Penicuik**
Cal-CRG1	**-1.09**	-0.77	**-0.58**	-1.42
Cal-CRG2	**-0.35**	-0.44	0.29	0.35
Cal-CRG3	0	-0.37	0.28	-0.57
Cal-CRG4	0.14	0.91	0.82	-0.47
Cal-CRG5	**-1.33**	-0.68	**-1.03**	-1.98
Cal-CRG6	**-0.46**	-0.58	0.23	0.55
Cal-CRG7	**-0.33**	-0.77	1.31	-1.52
Cal-CRG8	0.07	0.23	1.01	0.97
Cal-CRG9	**-0.54**	-0.66	**-0.24**	-0.03
Cal-CRG10	**-0.26**	0.24	0.42	0.42
Cal-CRG11	**-0.69**	-0.46	**-0.23**	-0.59
Cal-CRG12	**-0.1**	-0.61	**-0.17**	-0.11
Cal-CRG13	0.1	-0.4	0.06	0.01
Cal-CRG14	**-0.64**	-0.74	**-1.29**	-1.4
Cal-CRG15	0.12	-0.68	**-0.18**	-0.27
Cal-CRG16	**-0.26**	-0.08	**-0.02**	-0.17
Cal-CRG17	**-0.51**	0.2	**-0.52**	-0.23

Table 5.11: Comparison of mean expression values (log_2 ratio high/low Ca^{2+}) of Cal- CRGs in Cal-0, Col-4, Elland and Penicuik from all twenty four microarrays analysed using dChip. Highlighted values in Cal-0 (putative lab calcicole) show repressed expression observed in the initial 8 microarray chip data as well. Highlighted values in Elland (putative wild calcicole) show repressed expression of Cal-CRGs.

	Mean difference in microarray log_2 gene expression from 24 chip experiment			
Col-CRGs	**Cal-0**	**Col-4**	**Elland**	**Penicuik**
Col-CRG1	-0.95	**-1.03**	0.05	**-0.08**
Col-CRG2	0.16	**-1.05**	0.11	**-0.02**
Col-CRG3	-0.47	**-0.79**	-0.24	0.08
Col-CRG4	-0.76	**-0.4**	-0.58	**-0.77**
Col-CRG5	-0.06	0.16	0.37	0.04
Col-CRG6	-1.39	**-1.6**	-0.43	**-0.27**
Col-CRG7	-0.4	**-0.46**	0.15	0.31
Col-CRG8	-0.27	**-2.67**	0.38	0.18
Col-CRG9	0.07	**-1.62**	-0.67	0.27
Col-CRG10	-0.84	**-1.27**	-0.13	0.08
Col-CRG11	-0.17	0.59	-0.11	0.47
Col-CRG12	0.57	0	-0.39	**-0.56**
Col-CRG13	-0.54	**-2.35**	1.13	1.16
Col-CRG14	0.2	**-0.95**	0.37	**-0.03**
Col-CRG15	-0.68	**-1.4**	-0.35	**-0.21**
Col-CRG16	-0.46	**-1.5**	-0.25	0.06
Col-CRG17	-0.1	**-0.55**	-0.8	**-1.9**
Col-CRG18	-0.31	**-0.41**	0.24	0.7
Col-CRG19	-0.41	**-0.52**	0.1	**-0.07**
Col-CRG20	-0.03	**-0.72**	0.05	0.3
Col-CRG21	-0.07	**-0.75**	0.53	0.26
Col-CRG22	0.01	**-0.49**	0.18	**-0.56**
Col-CRG23	-0.22	**-0.66**	-0.58	**-0.02**

Table 5.12: Comparison of mean expression values (log_2 ratio) of Col- CRGs in Cal-0, Col-4, Elland and Penicuik from all the twenty four microarrays analysed using dChip. Highlighted values in Col-4 (putative lab calcifuge) show repressed expression observed in the initial 8 microarray chip data as well. Highlighted values in Penicuik (putative wild calcifuge) show repressed expression of Col-CRGs.

5.3 Discussion

A transcriptomics approach was adopted to investigate plant response to altering levels of rhizospheric Ca^{2+}. This might help in understanding the molecular mechanisms that enable calcicoles to thrive on calcareous soils and calcifuges to tolerate low Ca^{2+} environments. Abram *et. al.* (2005) performed microarray experiments on four *A. thaliana* ecotypes to test the hypothesis that adaptation to a calcareous environment will be reflected in altered patterns of gene expression. Currently, this is the only available microarray data to understand the genomics of calcicole-calcifuge physiology. However, this study is comprehensive in including the expression patterns of genes in both established accessions and wild putative calcicole and calcifuges ecotypes of *A. thaliana*. A total of 22,810 genes represented on twenty four microarray chips were studied simultaneously.

This study on six different *A. thaliana* ecotypes from different regions across UK will help improve the understanding of the underlying molecular mechanisms of plants growing on calcareous soils.

Functional classification of reliable genes with altered expression profile

Completion of whole genome sequences in *Arabidopsis and* Rice (*Oryza sativa*) is one of the enormous achievements of modern plant biology (Saito *et al.,* 2008). However, of the ~27,000 genes forecast in *Arabidopsis* based on nucleotide sequence information, only half have been functionally annotated with sequence similarities to acknowledged genes and among these, the function of only 11% (approx) of genes has been correlated with direct experimental confirmation (The Multinational *Arabidopsis* Steering

Committee, 2007). The classification of genes with unknown function is a major challenge for plant biologists currently. Though gene expression patterns change with niche alterations in the environment of the plant, a clear morphological phenotype is not always observed (Eisenreich *et al.,* 2004). Environmental stress invokes a response in plants and the transduced signals bring about a cascade in changes in the expression of stress tolerance genes (Nakashima and Yamaguchi-Shinozaki, 2005). Analyses of plant lines with specific genes being induced or repressed are therefore very useful in the process of elucidating the function of these genes. The current study was carried out to study the genome-wide expression of rhizospheric Ca^{2+} responsive genes in four ecotypes of *A. thaliana*.

Overall patterns of Ca^{2+}-responsive gene expression in individual ecotypes showed a larger number of responsive genes in wild *A. thaliana* ecotypes Elland (183) and Penicuik (246) compared to the lab ecotypes Col-4 (53) and Cal-0 (46). Also, most of these filtered genes were found to be induced at low Ca^{2+} in Elland (154) and Penicuik (199) compared to only 15 and 9 in Col-4 and Cal-0, respectively. Of these genes, expression of a number of stress responsive genes in the wild ecotypes (Elland and Penicuik) may indicate the requirement for higher rhizospheric Ca^{2+} concentration in these ecotypes. This can be related to the growth studies (Chapter 3 and Chapter 4) where Penicuik (putative wild calcifuge) wasn't as affected by the increase in rhizospheric Ca^{2+} concentrations as Glenisla which brings into perspective if the former ecotype is a true calcifuge or not.

The expression of numerous genes with regulatory functions, such as Ca^{2+}-binding proteins, kinases, phosphatases RNA-binding proteins

transcription factors is known to be altered by stress and play a significant role in stress perception and signaling (Kant *et al.,* 2007; Yamaguchi-Shinozaki and Shinozaki, 2006; Bartels and Sunkar, 2005; Shinozaki *et al.,* 2003; Xiong *et al.,* 2002 and Zhu, 2002). In this study, analysis of microarray data helped in identifying a number of genes with putative functions involved in biotic and abiotic stress and defence responses, regulation of cellular processes, cellular components, intracellular signaling activity, and transcription factor activity, regulation of metabolic processes, immune responses, and cell death as well as programmed cell death.

A number of genes involved in plant adaptation to a variety of biotic and abiotic stresses have been and are being identified. The products of the stress-inducible genes can be classified into two groups: those that directly protect against environmental stresses, and those that regulate gene expression and signal transduction in the stress response (Hasegawa *et al.*, 2000; Thomashow, 1999; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Hundreds of genes are thought to be involved in abiotic stress responses (Seki *et al.*, 2002). Comparing all the four ecotypes under study at two different concentrations of rhizospheric Ca^{2+} revealed no genes with differential expression that satisfied the statistical measures significantly (paired t-test, $P \leq 0.05$, 1.0 $\log_2$ change in expression and at least 50% presence in all the arrays). This is a common problem in microarray analysis (Tamoki *et al.*, 2003). In order to ensure high confidence in the expression values observed and to formulate the data to comply very stringent criteria for overall reliability it is usually necessary to discard large numbers of genes, including those with potentially interesting expression profiles. For example,

Tamoki *et al.,* (2003) discarded two thirds of their EST data from their investigation.

Expression analyses of drought-, cold- and high-salinity-inducible genes have shown the existence of several regulatory systems of stress responsive gene expression. Some are dependent on ABA, while others are ABA-independent (Shinozaki and Yamaguchi-Shinozaki, 2000, 1996; Thomashow, 1999; Bray, 1997), which indicate the existence of complex regulatory mechanisms between perception of abiotic stress signals and gene expression (Zhu, 2001; Shinozaki and Yamaguchi-Shinozaki, 2000, 1997, 1996). The present investigation on rhizospheric Ca^{2+} tolerance revealed an extensive network of reliably changing genes involved in regulatory systems of stress response as well as regulation in transcription activity in individual ecotypes.

Ca^{2+} signaling has been implicated in plant responses to assorted stimuli, such as touch, light, pathogens, temperature, and hormones (Reddy, 2001). Proteins that bind Ca^{2+} can act as potential sensors and mediate appropriate cellular responses through interaction with target proteins (Delk *et al.,* 2005). The Arabidopsis *calmodulin-like* (CML) gene family encodes potential Ca^{2+} sensors that contain conserved Ca^{2+}-binding domains, called EF hands, and share sequence similarity with the essential, ubiquitous, and highly conserved Ca^{2+} receptor, calmodulin (CaM; McCormack and Braam, 2003). Though there are 50 CMLs in *Arabidopsis* (McCormack and Braam, 2003) and regardless of the implication of Ca^{2+} signaling in many fundamental cellular processes, the understanding of the regulation and function(s) of this potentially critical family of proteins is very limited. The expression of

calmodulin-like Ca^{2+}-binding protein, 22 kDa (CaBP-22) was induced in Col-4 at high Ca^{2+} and repressed in Cal-0, Penicuik and Elland. However, five other Ca^{2+}-binding EF hand family proteins were differentially expressed in the four ecotypes with only one gene (At3g47480) being repressed at high Ca^{2+} in all four ecotypes and only one induced (At4g27280) in lab ecotypes (Col-4 and Cal-0). This might suggest that the CAM and CML gene expression might be particularly interesting to study in the wild ecotypes where all the Ca^{2+}-related genes have repressed expression pattern at high Ca^{2+}. Alternatively, as these genes are induced at low Ca^{2+} concentration (1 mM), in wild ecotypes, this Ca^{2+} concentration might be optimum in nature for them to function optimally.

Cell death might be caused by exogenous factors like stress, trauma, and disease or due to endogenous factors like apoptosis, PCD or cell suicide. Programmed Cell Death (PCD) is a genetically controlled process that has a vital role in the development and homeostasis of tissues in multicellular organisms (Noodén, 2004). Heath (2000) investigated how PCD of infected cells prevents pathogen infection and examples of PCD during normal plant development include abscission, aerenchyma formation and embryogenesis (Morgan and Dew, 2004). The current microarray analysis identified 5 putative PCD genes (all repressed at high Ca^{2+}) in Penicuik and 7 putative PCD genes (all repressed at high Ca^{2+}) in Elland. *ACS6* (At4g11280; 1-aminocyclopropane-1-carboxylate synthase 6 / ACC synthase 6), reported to induce apoptosis by oxidative stress (Miller *et al.*, 1999) as well as encoding the rate limiting enzyme of ethylene biosynthesis (Liu and Zhang, 2004), was strongly repressed at high Ca^{2+} in Elland, the putative wild calcicole (~16 fold) whereas the same gene was repressed by only ~1 fold in Penicuik. This gene

profiling might hold a clue to the occurrence of PCD in wild ecotype Glenisla, a putative calcifuge (Chapter 4).

One of the important steps in control of stresses by plants appears to be the transcriptional activation or repression of genes (Chen *et al.,* 2002). A total of 402 potential stress-related genes that encode known or putative transcription factors were selected by Chen *et al.* (2002) from ~8300 genes covered by *Arabidopsis* GeneChip®. They observed that a majority of the transcription factor genes analyzed are expressed differentially after various stress treatments which include both biotic (viral infection, bacterial attack, fungal attack, oomycetic attack) and abiotic (cold, salt, osmoticum, wounding, jasmonic acid) stresses depending on the age of the plants used for the treatments and the type of the stresses and the duration applied. Penicuik revealed significantly ($P \leq 0.001$) altered expression of 47 genes (6 induced and 41 repressed) involved in regulation of transcription in plants treated with 12.5 mM rhizospheric Ca^{2+} compared to those treated with 1 mM rhizospheric Ca^{2+}. A similar comparison of Elland for 'regulation of transcription' revealed only 1 induced gene with 28 repressed genes at high Ca^{2+}. However, no genes involved in transcription passed the filtering criteria in comparisons made on Col-4 and Cal-0. Most of the represented transcription factor genes in this study were involved in defence responses to abiotic stimuli as well as in defence responses. The induction of most of the transcription factor genes at low (1 mM) rhizospheric Ca^{2+} concentration implies that these genes would play different roles in the regulation of plant responses to rhizospheric Ca^{2+} stress. Also, it can be implied that these genes are responding to stress induced by low Ca^{2+} in wild type ecotypes.

Quantitative real time PCR (qRT-PCR) is mostly employed to confirm the data from microarray experiments as well as to perform a multitude of other studies (Gachon *et al.,* 2004). The robustness of microarray experiments may sometimes be an issue, since plants display a high number of multigene families and cross-hybridization may lead to false interpretations (Ingham *et al.,* 2001). However, microarray experiments can analyse thousands of genes in one step, whereas real-time PCR is limited to a selected few genes. Since relative quantification is the objective for most real-time PCR experimentation, several data analysis procedures have been developed, of which the most commonly used are; the efficiency calibrated model (Pfaffl *et al.*, 2002; Pfaffl, 2001) and the ΔΔCt model (Livak and Schmittgen, 2001). Calcium responsive genes observed in putative calcicole Cal-0 (Cal-CRGs) identified from the initial microarray analysis were studied for expression profiles in the six ecotypes grown on either 1 mM or 15 mM rhizospheric Ca^{2+} at three harvest points. The majority of genes which were induced at low Ca^{2+} from the microarray experiments were induced in Cal-0 as well as the other two wild putative calcicoles Elland and Halifax in the realtime PCR experiments. Interestingly, putative wild calcifuge Penicuik had a similar expression pattern of Cal-CRGs to the two wild putative calcicoles Elland and Halifax, whereas the expression in Glenisla (putative wild calcifuge) at 1 mM Ca^{2+} was similar to Col-4 (putative lab calcifuge) and at 15 mM rhizospheric Ca^{2+} Glenisla was not similar to any ecotypes in the study. It can be inferred from this analysis that Penicuik is genetically more a calcicole than a calcifuge and Glenisla a true calcifuge. Also, the expression patterns of Penicuik combined with the microarray analysis for Ca^{2+}-responsive genes

and physiological growth studies may indicate that Penicuik is physiologically closer to calcicoles than calcifuges.

Microarrays combined with transcriptomics are a powerful tool for generating vast amounts of data for parallel gene expression analysis from a single- or combined sets of experiments. DNA microarray technology has made it possible to monitor gene expression levels on a genomic scale and as microarray gene experiments result in enormous amount of gene expression data, it needs careful statistical considerations for managing the quality of interpreted data (Chen *et al.,* 2002; Park *et al.,* 2008). In this study, Affymetrix Genechip® data obtained from experiments performed under altered rhizospheric Ca^{2+} concentrations were analysed using Genespring® (initially) and dChip® (full data set) to identify Ca^{2+} responsive genes. Similarities in the expression patterns of both Cal-CRGs as well as Col-CRGs were observed with both software packages increasing the validity of the experiments and the analysis. However, as whole plant tissue was used for RNA extraction and subsequent microarray and gene expression analysis, root specific transcripts might have been non-significant in comparison to the microarray data that has been procured using only tissue (shoot or root) specific samples.

In conclusion, this chapter provides a comprehensive analysis of gene expression in response to altered rhizospheric Ca^{2+} concentrations in four *A. thaliana* ecotypes using an *A. thaliana* genome array consisting of 22,810 genes. This analysis revealed a total of 1508 potential Ca^{2+}-responsive genes which have not previously been studied in this context. As no genes were found to satisfy the filtering criteria over all 4 ecotypes for altered rhizospheric Ca^{2+} an individual ecotype approach was used for identifying the Ca^{2+}-

responsive genes. The key findings take account of the induction at low Ca^{2+} of most genes involved in Ca^{2+} responses and transcription factor regulation. In addition genes with a role in PCD were shown to be differentially expressed in the wild ecotypes. These were repressed at high Ca^{2+} in the putative calcicole Elland as well as Penicuik (putative wild calcifuge; but with more calcicole characteristics). This may have significance for the PCD occurrence in the putative calcifuge Glenisla ecotype at 12.5 mM (Chapter 4) rhizospheric Ca^{2+} treatment. A few (10) genes from the initial data analysis were selected for further characterisation based on the availability of the SALK-TDNA "knockout" for the genes to allow the importance of these Ca^{2+} responsive genes to be assessed. To prevail over the technical limitations of the microarrays (such as cross-hybridization between closely related genes) as well as biological variance (Park *et al.,* 2008), this approach would also help in a better understanding of the Ca^{2+}-responsive genes as well as improve the understanding of Ca^{2+}-tolerance and calcicole-calcifuge physiology.

Chapter 6

Physiological studies and quantitative real time analysis of *Arabidopsis* SALK T-DNA insertion lines.

6.1 Introduction

Following completion of the sequencing of the ~120 MB *A. thaliana* genome the plant biology community faced the new challenge of assigning biological functions to all the genes. There remain a large number of genes for which no function is known or can be predicted. Accordingly, the ability to create loss-of-function mutations for all genes is an essential tool for the functional analysis of the *Arabidopsis* genome (Alonso *et al.,* 2003). *Agrobacterium*-mediated transfer DNA (T-DNA) transformation has been used for insertional mutagenesis in diverse plant species. This transformation by *Agrobacterium* results in the integration into the nuclear genome of a sequence called T-DNA, which is carried on a bacterial plasmid (Parinov and Sundaresan, 2000). T-DNA (Krysan *et al.*, 1999; Azpiroz-Leehan and Feldman, 1997) or transposons (Ramachandran and Sundaresan, 2001; Martienssen, 1998) insert randomly into plant chromosomes, and when this insertion event occurs within a gene or its regulatory region, its expression is impaired. By this means a large number of loss-of-function mutant genes in plants have been generated. The development of highly resourceful methods of T-DNA transformation for *Arabidopsis* (Krisan *et al.,* 1999; Clough and Bent, 1993) has made it realistic to generate thousands of transformants relatively swiftly, and as a result T-DNA is now a widely used insertional mutagen in *Arabidopsis*.

Of all the higher plants, *A. thaliana* has the best available resources for molecular studies and perhaps the most valuable is a collection of >250,000 sequence-indexed T-DNA inserts in the *Arabidopsis* genome (http://signal.salk.edu/cgi-bin/tdnaexpress). This corresponds to an average of ~10 T-DNA inserts per gene and for any particular gene there is >90% probability of a T-DNA insertion (http://*Arabidopsis*.info/info/MASC_2007.pdf). The T-DNA tag typically causes a loss of gene expression resulting in a monogenic recessive mutation of the gene. Mutants can then be used in phenotypic assessments and subsequent molecular analysis to determine gene function (Daxinger *et al.,* 2008).

To understand, the function of Ca^{2+} responsive genes identified from initial microarray analysis of Col-4 and Cal-0 (Col-CRGs and Cal-CRGs), SALK T-DNA insertion lines were obtained from NASC, Nottingham (UK). This chapter analyses the response of T-DNA insertion lines for ten Ca^{2+}-responsive genes to growth on low (1 mM) and high (15 mM) rhizospheric Ca^{2+}. In addition qRT-PCR was used to study the impact of T-DNA knockouts on expression of other Cal-CRGs to test the possibility that these genes form part of a network in the response of plants to rhizospheric Ca^{2+}.

6.2 Results

6.2.1 Isolation of homozygous T-DNA insertion lines of Cal-CRGs

Ten T-DNA insertion lines (Table 6.1) with a potential insert in a specific Cal-CRG (from now on referred as *Cal-CRG*'N' insertion lines), were obtained from NASC. The position of the insertion in these lines is specified in Table 6.1. Twelve T3 seeds of these lines were grown as described in section 2.14.1 and the resulting T4 seeds were grown on kanamycin to detect the presence of the T-DNA, which contains the *nptII* gene conferring kanamycin resistance. Figure 6.1 illustrates the germination performance of these seedlings on medium containing kanamycin. Figure 6.1B shows severely stressed necrotic leaves of the *Cal-CRG*15 line. After germination, PCR was performed on the DNA of leaf tissue extracted from T3 and T4 plants using combinations of a gene specific primer (LP & RP; Left & Right genomic primer) and a primer specific to the T-DNA (LBb1) to confirm the presence of T-DNA and to identify homozygous plants as described in section 2.14.2.

Using these primers (LBb1 + LP + RP), WT (Wild Type - no insertion) plants gave a PCR product of about 900~1100 bp, HM (Homozygous lines - insertions in both chromosomes) plants gave a band of 410+N bp (from Right Primer (RP) to insertion site - 300+N bases, where 'N' is the difference between the actual insertion site and the flanked sequence position i.e., 0~300 bp; plus 110 bases from LBb1 to the left border of the vector.), and HZ (Heterozygous lines - one of the paired chromosomes with insertion) plants gave both bands specified for WT and HM plants. The specific primers used are presented in Appendix 2.

Twelve plants (approx.) for each SALK insertion line were screened to identify homozygous lines. Figure 6.2 illustrates the PCR products of these plants. As can be seen, homozygous lines have a PCR product of ~410±300 bp depending on the insertion flanking sequence length and wild type plants produce a single band around the 900~1100 bp region, whereas, heterozygous plants have more than one PCR product bands. As observed in the agarose gel (Figure 6.2), wells 7-12 and 19-20 have a PCR product of ~800 bp which is characteristic to LBb1 primer that can sometimes produce an extra band of ~800 bp (http://signal.salk.edu/tdna_FAQs.html). Homozygous plants were identified in only five of the available ten Cal-CRG SALK T-DNA insertion lines (Table 6.1). The seeds from these homozygous plants were used to grow plants for subsequent physiological studies as well as quantitative real time PCR.

Gene of Interest	Gene Number	Putative Function	SALK Insertion line number	Location of T-DNA insertion	Insertion flanking sequence length	Homozygous lines identified
Cal-CRG1	At4g11850	membrane, phospholipase D activity, embryonic development ending in seed dormancy	SALK_075605	Exon	107	Yes
Cal-CRG2	At5g18820	chloroplast, ATP binding, protein binding, embryonic development ending in seed dormancy, unfolded protein binding	SALK_014823	Exon	428	No
Cal-CRG4	At2g40080	cellular component unknown, molecular function unknown, biological process unknown	SALK_134648	Exon	442	No
Cal-CRG5	At5g18470	cellulose and pectin-containing cell wall, sugar binding, biological process unknown	SALK_019318	Exon	190	Yes
Cal-CRG6	At3g19080	nucleus, biological process unknown	SALK_017640	Within 300 bases of the 3' end of At3g19080.	462	Yes
Cal-CRG7*	At5g03720	member of Heat Stress Transcription Factor (Hsf) family	SALK_036310	Intron	152	No
Cal-CRG10	At4g29690	hydrolase activity, nucleotide metabolic process, endomembrane system	SALK_024541	Exon	366	Yes
Cal-CRG13*	At5g28080	mitogen activated protein kinase - like ribonuclease T2 family protein	SALK_023094	Exon	183	No
Cal-CRG15*	At1g14220	ribonuclease T2 family protein	SALK_053974	Exon	354	No
Cal-CRG17	At1g31680	copper ion binding, biological process unknown	SALK_020480	Exon	392	Yes

Table 6.1: Cal-CRG SALK T-DNA insertion line numbers and the corresponding gene number. Location of T-DNA insertion and flanking sequence length was derived from TAIR database. '*' lines were randomly included on the basis of SALK lines availability to make the study more complete.

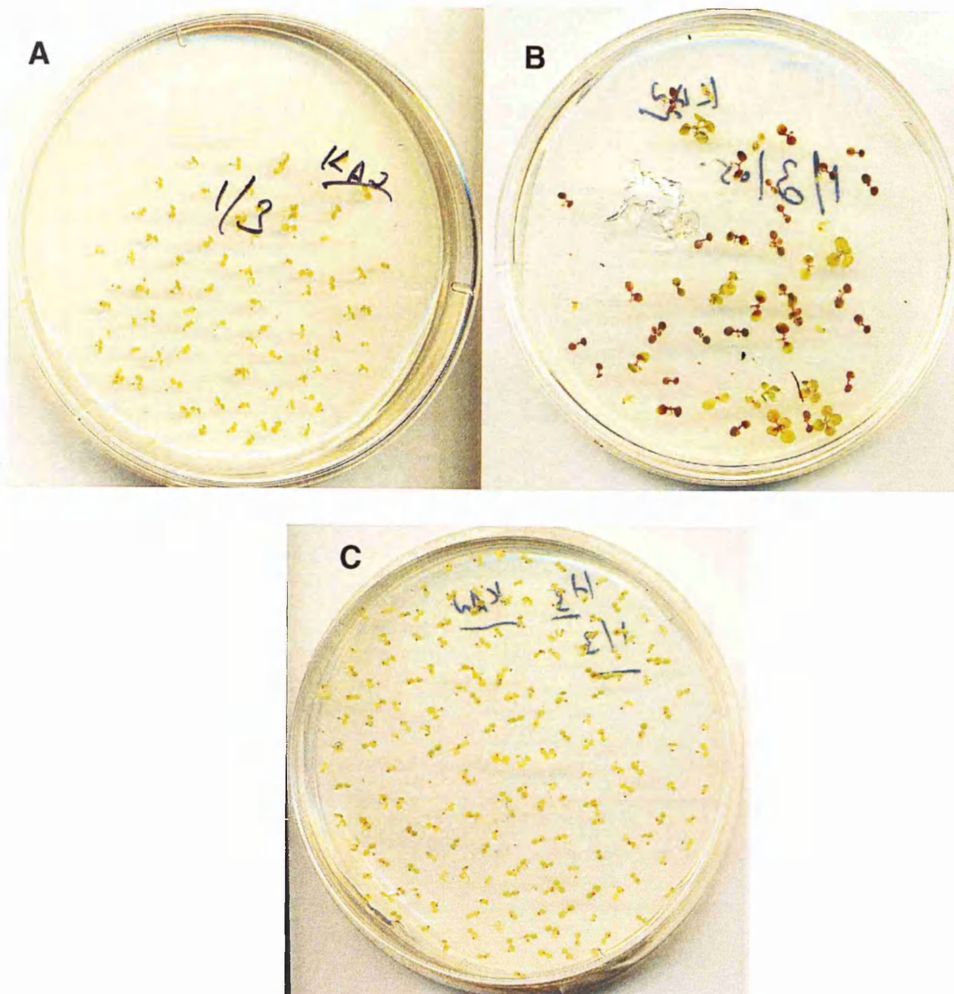

Figure 6.1: Germination of SALK T-DNA insertion line seedlings on petri dishes containing MS medium + kanamycin; A) *Cal-CRG*5, B) *Cal-CRG*15 (No homozygote's found) and C) *Cal-CRG*1 line seedlings, respectively.

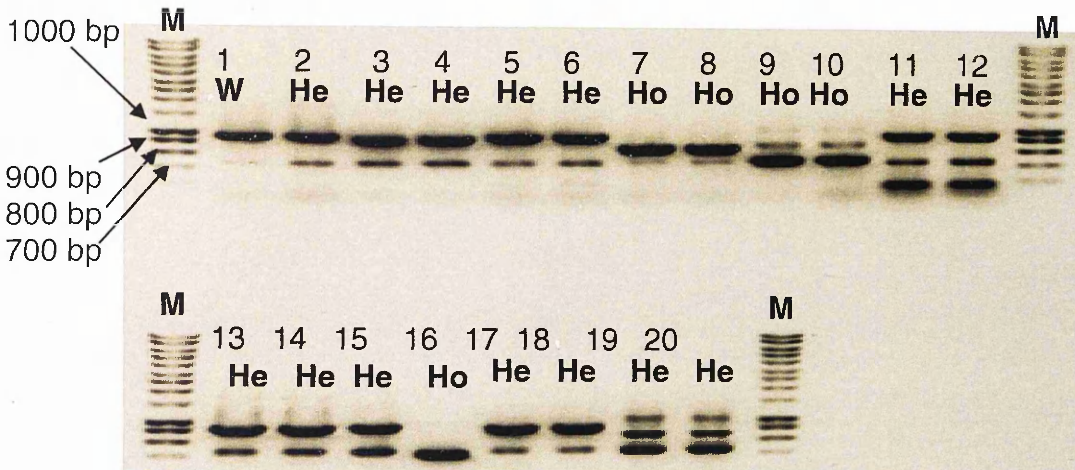

Figure 6.2: PCR of SALK T-DNA lines showing varied bands on an agarose gel: M - DNA Marker; W - Wild type lines; He - Heterozygous lines; Ho - Homozygous lines. Lines represented in this gel are *Cal-CRG1* (1-2), *Cal-CRG2* (3-4), *Cal-CRG5* (5-6), *Cal-CRG10* (7-8), *Cal-CRG15* (9-10), *Cal-CRG17* (11-12), *Cal-CRG4* (13-14), *Cal-CRG6* (15-16), *Cal-CRG13* (17-18) and *Cal-CRG7* (19-20).

6.2.2 Determination of true knockout in SALK T-DNA insertion lines

To determine the occurrence of a true knockout of respective Cal-CRGs in T-DNA insertion lines, relative quantification of gene expression was carried out on the insertion lines grown with 1 mM or 15 mM rhizospheric Ca^{2+} in comparison to Col-4 plants grown under similar conditions. As the insertion lines are from Col-0 background, Col-4 was used as the control for this comparative gene expression study. A true knockout line would theoretically show no or very little expression of the respective Cal-CRG in comparison to Col-4 plants.

Of the five homozygous SALK T-DNA insertion lines identified (Table 6.1), only two insertion lines, *viz.*, *Cal-CRG5* and *Cal-CRG17* had very low relative gene expression showing a true knockout effect of the T-DNA insertion (Figure 6.3B, 6.3E). So, only these two insertion lines were taken forward for subsequent physiological and relative gene quantification studies to understand the true function of Cal-CRGs.

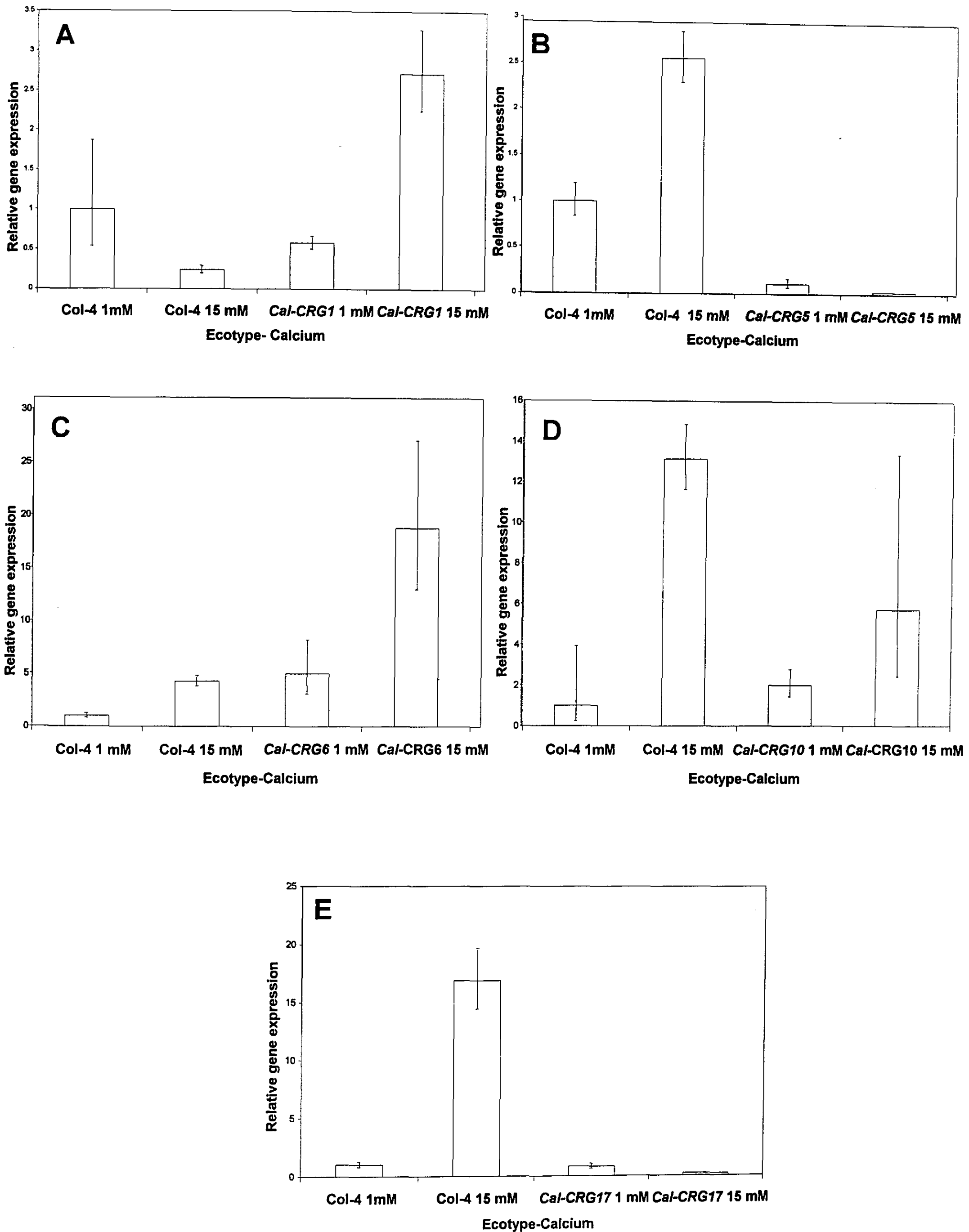

Figure 6.3: Relative gene expression of Cal-CRGs in Col-4 (control) and respective SALK_T-DNA insertion lines: A - Cal-CRG1; B - Cal-CRG5; C - Cal-CRG6; D - Cal-CRG10; E - Cal-CRG17, respectively. Bars represent mean relative gene expression of

respective Cal-CRG'N' ± SEM. Col-4 1 mM is the endogenous control.

6.2.3 Differences in growth of *Arabidopsis* SALK T-DNA insertion lines in response to altered rhizospheric calcium

The morphogenic responses of the two homozygous knock out SALK T-DNA insertion lines (*Cal-CRG5* and *Cal-CRG17*) were studied in comparison to Col-4 ecotype at 1 mM and 15 mM rhizospheric Ca^{2+} treatments. Number of leaves, root length, rosette diameter, fresh and dry weight was measured after 30 days of treatment. These studies would help in understanding the physiological responses of the plants with a specific Ca^{2+} responsive gene knocked out. All the measurements were subjected to statistical analysis as specified in Section 2.15.

Number of leaves

Col-4 plants had the highest number of leaves at both 1 mM (23.60 ± 0.68) and 15 mM (19.80 ± 0.37) rhizospheric Ca^{2+} in comparison to both *Cal-CRG5* and *Cal-CRG17* T-DNA knockout lines (Figure 6.4). The mean number of leaves in both *Cal-CRG5* and *Cal-CRG17* lines were significantly different ($P \leq 0.05$) to Col-4 at 1 mM rhizospheric Ca^{2+} treatment (Table 6.2). Also, *Cal-CRG5* had significantly ($P \leq 0.05$) fewer leaves than *Cal-CRG17* at both 1 mM and 15 mM rhizospheric Ca^{2+} treatments (Table 6.2). However, only *Cal-CRG5* exhibited a significantly lower ($P \leq 0.05$) mean number of leaves than Col-4 at 15 mM rhizospheric Ca^{2+}, showing that *Cal-CRG5* was very sensitive to the elevated rhizospheric Ca^{2+} conditions.

Also, all three genotypes had a significant difference ($P \leq 0.05$) in their respective mean number of leaves between the Ca^{2+} treatments with a reduction observed in Col-4 and, conversely, an increase in the number of leaves of both the insertion lines with 15 mM rhizospheric Ca^{2+} (Figure 6.4).

Rosette diameter

Comparing the two insertion lines with Col-4 at both 1mM and 15 mM rhizospheric Ca^{2+} treatment, Col-4 plants had the largest rosette diameter (6.76 ± 0.15) at 1 mM rhizospheric Ca^{2+} (Figure 6.5). This was significantly higher ($P \leq 0.05$) than the rosette diameters of both *Cal-CRG5* (5.38 ± 0.04) and *Cal-CRG17* (5.32 ± 0.04) at 1 mM rhizospheric Ca^{2+} (Table 6.3). The T-DNA insertion lines at 1 mM Ca^{2+} had similar non-significantly different ($P > 0.05$) rosette diameter(s), whereas at 15 mM rhizospheric Ca^{2+} exposure, there was a significant difference with *Cal-CRG17* knockouts having larger rosettes (Table 6.3).

The mean rosette diameter *of Cal-CRG5* plants was not affected ($P > 0.05$) by an increase in rhizospheric Ca^{2+}, whereas *Cal-CRG17* plants had a significant ($P \leq 0.05$) increase in rosette diameter with an increase in rhizospheric Ca^{2+} concentration (Figure 6.5, Table 6.3). Only *Cal-CRG17* mean rosette diameter was significantly different ($P \leq 0.05$) to the control Col-4 plants at 15 mM rhizospheric Ca^{2+}. An increase in rhizospheric Ca^{2+} concentration therefore had no effect on rosette diameter in the T-DNA insertion line *Cal-CRG5*, whereas that of *Cal-CRG17* was increased under increased rhizospheric Ca^{2+} conditions.

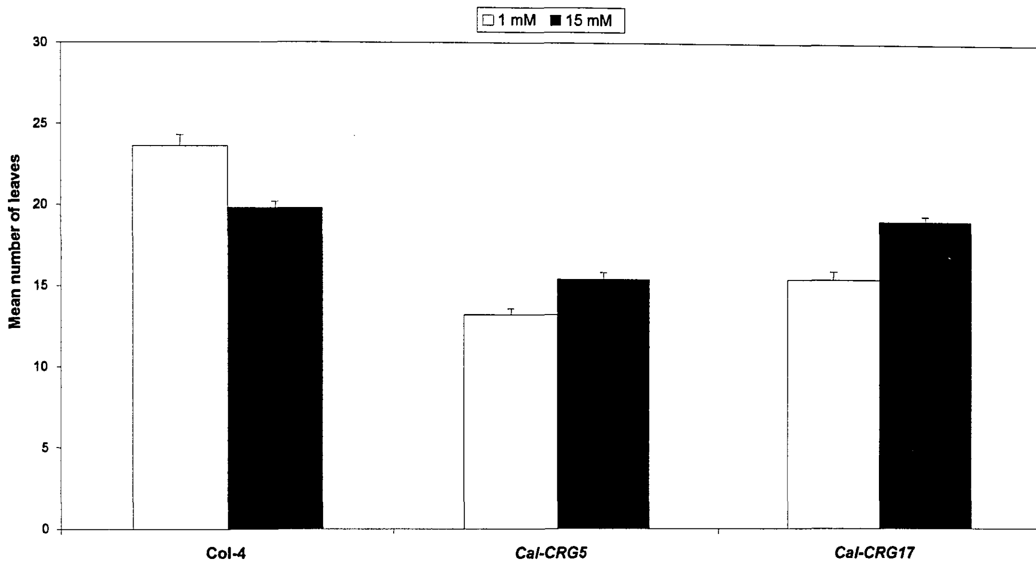

Figure 6.4: Number of leaves in Col-4 and two SALK T-DNA insertion lines treated with 1 mM or 15 mM rhizospheric Ca^{2+} and harvested after 30 days. Bars represent mean number of leaves ± SEM.

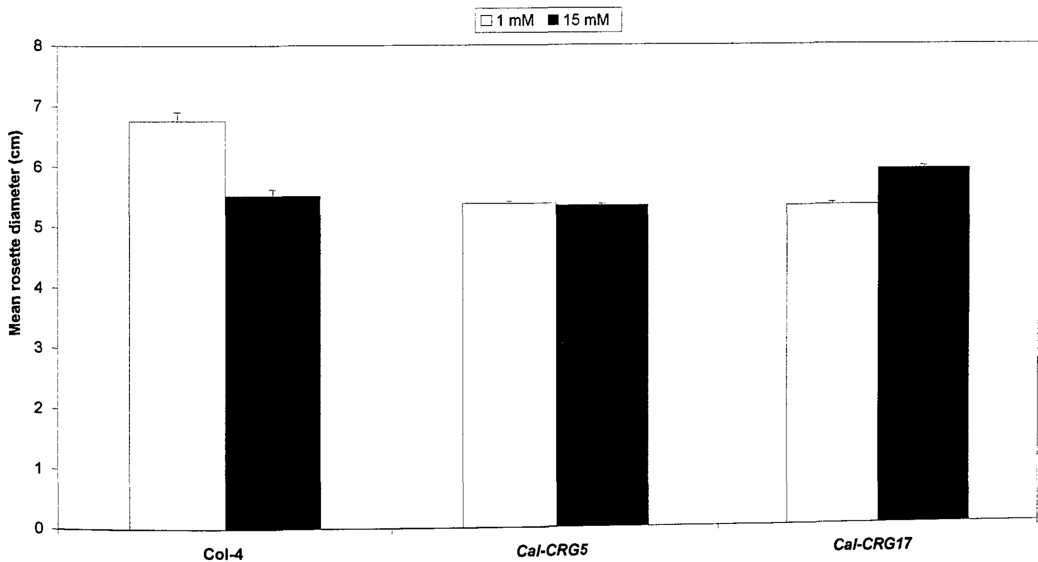

Figure 6.5: Rosette diameter in Col-4 and two SALK T-DNA insertion lines treated with 1 mM or 15 mM rhizospheric Ca^{2+} and harvested after 30 days. Bars represent mean rosette diameter ± SEM.

		Col-4		*Cal-CRG5*		*Cal-CRG17*	
	Ca^{2+} conc.	**1 mM**	**15 mM**	**1 mM**	**15 mM**	**1 mM**	**15 mM**
Col-4	**1 mM**	-	*	*		*	
	15 mM		-		*		
Cal-CRG5	**1 mM**			-	*	*	
	15 mM				-		*
Cal-CRG17	**1 mM**					-	*
	15 mM						-

Table 6.2: Multiple ANOVA comparison of Col-4 and SALK T-DNA insertion lines, where symbol '*' indicate a significant difference ($P \leq 0.05$) in mean number of leaves at harvest on 30th day. 1 mM and 15 mM indicate respective rhizospheric Ca^{2+} concentration.

		Col-4		*Cal-CRG5*		*Cal-CRG17*	
	Ca^{2+} conc.	**1 mM**	**15 mM**	**1 mM**	**15 mM**	**1 mM**	**15 mM**
Col-4	**1 mM**	-	*	*		*	
	15 mM		-				*
Cal-CRG5	**1 mM**			-			
	15 mM				-		*
Cal-CRG17	**1 mM**					-	*
	15 mM						-

Table 6.3: Multiple ANOVA comparison of Col-4 and SALK T-DNA insertion lines, where symbol '*' indicate a significant difference ($P \leq 0.05$) in mean rosette diameter at harvest on 30th day. 1 mM and 15 mM indicate respective rhizospheric Ca^{2+} concentration.

Primary root length

Only Col-4 plants had a significant difference ($P \leq 0.05$) in mean root length at 15 mM rhizospheric calcium compared to 1 mM rhizospheric calcium with smaller roots (Figure 6.6). Elevated rhizospheric calcium concentrations therefore had minimal effect on the mean root length of both the insertion lines. However, the mean root lengths of the all the three ecotypes significantly differed ($P \leq 0.05$) at 1 mM rhizospheric calcium treatment (Table 6.6). In contrast, at 15 mM rhizospheric calcium exposure *Cal-CRG17* plants had a significantly different ($P \leq 0.05$) mean root length to both Col-4 and *Cal-CRG5* with smaller roots.

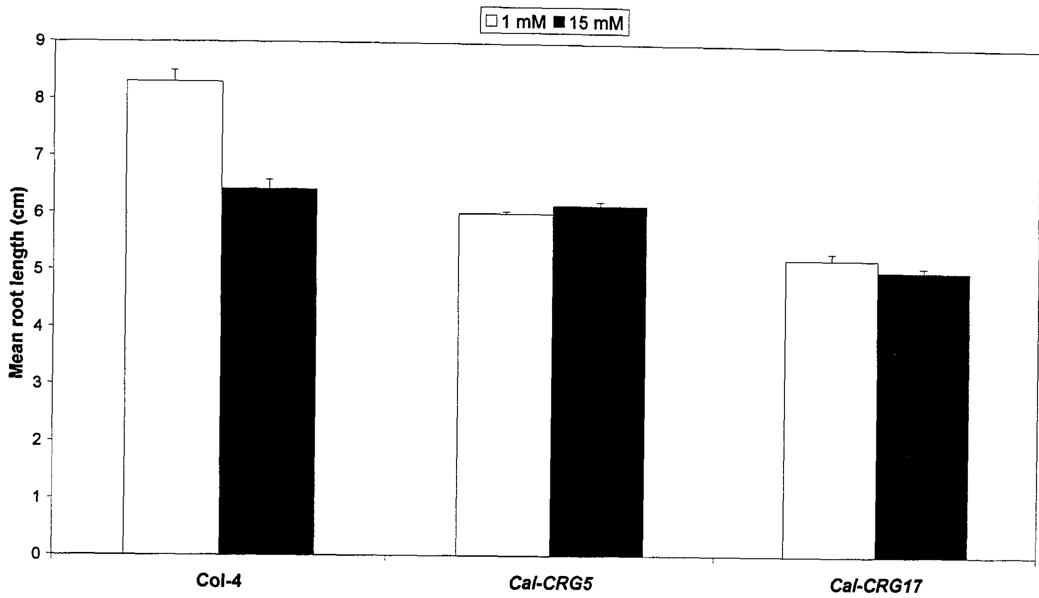

Figure 6.6: Primary root length of Col-4 and two SALK T-DNA insertion lines treated with 1 mM or 15 mM rhizospheric Ca^{2+} and harvested after 30 days. Bars represent mean root length ± SEM.

		Col-4		*Cal-CRG5*		*Cal-CRG17*	
	Ca^{2+} conc.	**1 mM**	**15 mM**	**1 mM**	**15 mM**	**1 mM**	**15 mM**
Col-4	**1 mM**	-	*	*		*	
	15 mM		-				*
Cal-CRG5	**1 mM**			-		*	
	15 mM				-		*
Cal-CRG17	**1 mM**					-	
	15 mM						-

Table 6.4: Multiple ANOVA comparison of Col-4 and SALK T-DNA insertion lines, where symbol '*' indicate a significant difference (P≤0.05) in mean root length at harvest on 30th day. 1 mM and 15 mM indicate respective rhizospheric Ca^{2+} concentration.

Fresh and dry weight measurements

Only Col-4 had a significant reduction ($P \leq 0.05$) in both fresh and dry weight(s) of whole plants at 15 mM rhizospheric Ca^{2+}, compared to 1 mM Ca^{2+} and *Cal-CRG5* had a significant reduction ($P \leq 0.05$) in dry weight at 15 mM rhizospheric Ca^{2+} exposure compared to 1 mM (Table 6.5 and 6.6). However, the three genotypes under study had a decrease ($P > 0.05$) in both their fresh and dry weight(s) at the elevated rhizospheric Ca^{2+} (15 mM) compared to 1 mM (Figure 6.7).

At 1 mM rhizospheric Ca^{2+} treatment, there was no significant difference ($P > 0.05$) in the fresh weights of Col-4, *Cal-CRG5* and *Cal-CRG17* insertion lines, and *Cal-CRG17* had a significantly higher ($P \leq 0.05$) dry weight than Col-4 (Table 6.5 and 6.6). However, both *Cal-CRG5* and *Cal-CRG17* insertion lines had a significantly higher fresh weight to Col-4 plants at 15 mM rhizospheric Ca^{2+} and *Cal-CRG17* had a significantly higher dry weight than Col-4 with *Cal-CRG5* but having a non significant ($P > 0.05$) higher dry weight (Table 6.5 and 6.6).

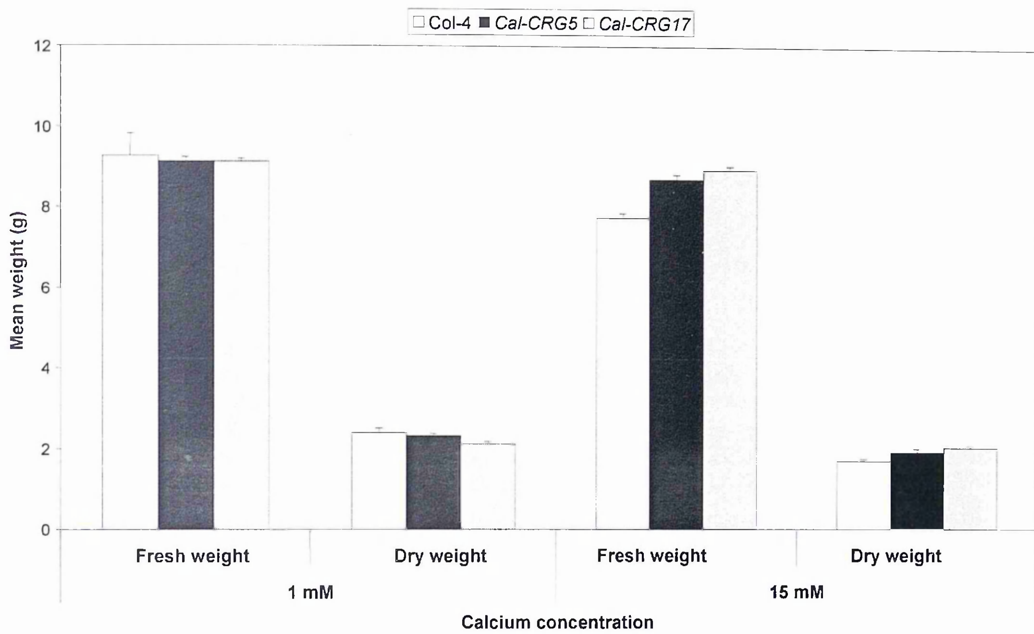

Figure 6.7: Mean fresh and dry weight of whole plants of Col-4 and two SALK T-DNA insertion lines treated with 1 mM or 15 mM rhizospheric Ca^{2+} and harvested after 30 days. White bars - Col-4; Black bars - *Cal-CRG5*; Grey bars - *Cal-CRG17*. Bars represent mean weight ± SEM.

	Ca^{2+} conc.	Col-4 1 mM	Col-4 15 mM	*Cal-CRG5* 1 mM	*Cal-CRG5* 15 mM	*Cal-CRG17* 1 mM	*Cal-CRG17* 15 mM
Col-4	1 mM	-	*				
Col-4	15 mM		-		*		*
Cal-CRG5	1 mM			-			
Cal-CRG5	15 mM				-		
Cal-CRG17	1 mM					-	
Cal-CRG17	15 mM						-

Table 6.5: Multiple ANOVA comparison of Col-4 and SALK T-DNA insertion lines, where symbol '*' indicates a significant difference ($P \leq 0.05$) in mean fresh weight at harvest on 30th day. 1 mM and 15 mM indicate respective rhizospheric Ca^{2+} concentration.

	Ca^{2+} conc.	Col-4 1 mM	Col-4 15 mM	*Cal-CRG5* 1 mM	*Cal-CRG5* 15 mM	*Cal-CRG17* 1 mM	*Cal-CRG17* 15 mM
Col-4	1 mM	-	*				*
Col-4	15 mM		-				*
Cal-CRG5	1 mM			-	*		
Cal-CRG5	15 mM				-		
Cal-CRG17	1 mM					-	
Cal-CRG17	15 mM						-

Table 6.6: Multiple ANOVA comparison of Col-4 and SALK T-DNA insertion lines, where symbol '*' indicates a significant difference ($P \leq 0.05$)

in mean dry weight at harvest on 30th day. 1 mM and 15 mM indicate respective rhizospheric Ca^{2+} concentration.

6.2.4 Quantitative real time analysis

Quantitative real time PCR (qRT-PCR) was carried out on these knockout lines, to assess if expression of other Cal-CRGs is dependent on Cal-CRG5 and Cal-CRG17 indicating that they might be involved in similar regulatory pathways or networks. qRT-PCR was performed to quantify the relative expression of ten Cal-CRGs (Table 5.1) in five homozygous SALK T-DNA insertion lines (Table 6.1) and Col-4 grown on 1 mM or 15 mM rhizospheric Ca^{2+}. The three insertion lines which were confirmed not to be true knockouts (Section 6.2.2) were also included in this study as they may nonetheless have subtle effects on other Cal-CRGs. The Cal-CRG expression was determined using UBIQUITIN10 (UBQ10) expression as an internal control. Clustering analysis of the real time PCR data was carried out using the dChip® clustering tool (Figure 6.8). All the insertion line samples were compared to Col-4 samples treated with 1 mM rhizospheric Ca^{2+} which was the calibrator or reference sample for the whole experiment.

Expression of ten Cal-CRGs in the five SALK T-DNA insertion lines and Col-4 clustered them into three different clusters with Cal-CRG1 and Cal-CRG17 in the smallest cluster, Cal-CRG12, Cal-CRG5 and Cal-CRG16 in the 2nd cluster and the remaining 5 Cal-CRGs in the third cluster (Figure 6.8), showing similarity in their expression patterns among the genotypes over altered levels of rhizospheric Ca^{2+}. Among the ecotypes, Col-4 plants grown on 1 mM Ca^{2+} were not a part of any major clusters and were distantly similar

in their expression pattern to the insertion lines. Also, *Cal-CRG17* insertion line plants treated with either 1 mM or 15 mM rhizospheric Ca^{2+} showed a relatively similar expression pattern but had a relatively dissimilar expression pattern to the other insertion lines. Another cluster comprised of *Cal-CRG1*, *Cal-CRG5*, *Cal-CRG6* and *Cal-CRG10* insertion lines treated with either 1 mM or 15 mM rhizospheric Ca^{2+} (Figure 6.8). This shows that these insertion lines are showing similar Cal-CRG expression patterns. However, it is important to remember that only *Cal-CRG5* of these four insertion lines was determined to be a true knockout (See Section 6.2.2). Both *Cal-CRG5* and *Cal-CRG17* knockout lines individually had very similar Cal-CRG expression patterns independent of the altered rhizospheric concentrations. Also, Cal-CRG17 expression in the *Cal-CRG5* knockout line and vice versa was very low, which indicates that the expression patterns of these genes are interlinked.

The *Cal-CRG5* insertion line treated with 15 mM rhizospheric Ca^{2+} also had a severe reduction in the expression of all the other nine Cal-CRGs in comparison to 1 mM rhizospheric Ca^{2+} treatment, which was similar to the way these genes behaved in the microarray data. The same wasn't the case for the *Cal-CRG17* line which showed a repression in only one of the other nine Cal-CRGs.

Cal-CRG expression in the insertion lines was also compared to the four wild and two lab ecotypes originating from varying rhizospheric Ca^{2+} regions (Chapter 5) by performing a clustering analysis (Figure 6.9). It revealed two major clusters: one containing the five insertion lines (excluding *Cal-CRG5* exposed to 15 mM rhizospheric Ca^{2+}), and the other all six ecotypes at both 1 mM and 15 mM rhizospheric Ca^{2+} treatments (Figure 6.9).

Cal-CRG5 and Halifax (putative wild calcicole) plants treated with 15 mM rhizospheric Ca^{2+} clustered very closely (Figure 6.9). Glenisla treated with 1 mM, Halifax treated with 15 mM rhizospheric Ca^{2+} and Col-4 at 1 mM rhizospheric Ca^{2+} treatment also grouped into a tight cluster (Figure 6.9).

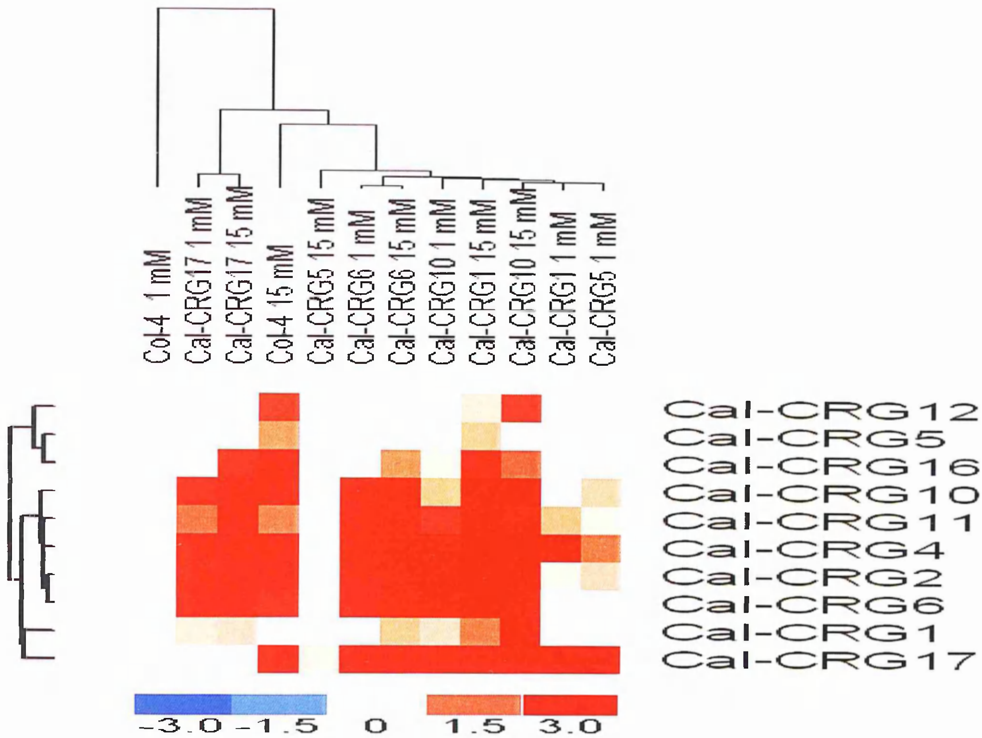

Figure 6.8: Quantitative real time PCR clustering analysis of 10 Cal-CRGs in five SALK T-DNA insertion lines grown for 30 days on 1 or 15 mM rhizospheric Ca^{2+}. The real time gene expression shown is the means of three biological replicates. Genes and SALK T-DNA lines were automatically clustered using dChip® clustering and enrichment tool based on their real time gene expression values for similarity of expression between the 6 ecotypes and 10 Cal-CRGs.

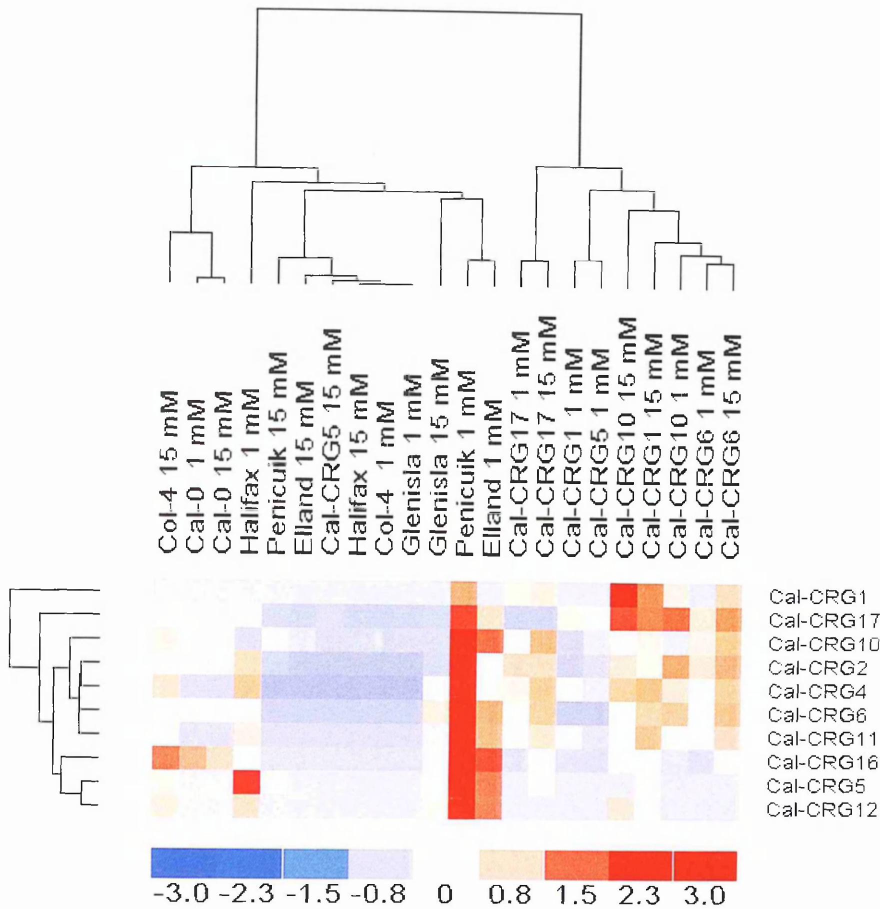

Figure 6.9: Quantitative real time PCR clustering analysis of 10 Cal-CRGs in seven SALK T-DNA insertion lines and six *Arabidopsis* ecotypes grown for 30 days on 1 or 15 mM rhizospheric Ca^{2+}. The real time gene expression shown is the means of three biological replicates. Genes, ecotypes and SALK T-DNA lines were automatically clustered using dChip® clustering and enrichment tool based on their real time gene expression values for similarity of expression between the 6 ecotypes and 10 Cal-CRGs.

6.3 Discussion

Variability in plants might be linked to the genomic plasticity resulting from the need to adapt to the prevailing conditions in which they are growing (Huber *et al.,* 1999). Consequently, novel adaptation mechanisms are expected. A powerful tool for functional analysis of a gene is to study the effects of loss-of-function alleles (reviewed by Bouche and Bouchez, 2001). In *Arabidopsis*, T-DNA insertion mutagenesis is the most fitting since lines with flanking sequence are accessible and other populations exist which can be assayed for insertions *via* PCR techniques (Young *et al.,* 2001). Mutations caused by T-DNA or transposon insertion result in principally loss of function mutagenesis (Weigel *et al.,* 2000). Of the ten available Cal-CRG'n' insertion SALK lines, only five genes (Cal-CRG1, Cal-CRG5, Cal-CRG6, Cal-CRG10 and Cal-CRG17) with homozygous mutant plants were identified. Similarly, of the available nine AtSWI3 SALK insertion lines, Sarnowski *et al.* (2005) indentified only five homozygous mutants. Subsequently, five T-DNA insertion lines comprising four Cal-CRGs with T-DNA insertions in exons and one in 3' upstream region were studied in an attempt to understand the phenotypic responses as well as changes in expression of other Cal-CRGs at altered levels of rhizospheric Ca^{2+}.

qRT-PCR was used in this current study to determine that T-DNA insertion resulted in true knockout of genes. Only Cal-CRG5 (At5g18470) and Cal-CRG17 (At1g31680) were identified as showing being true knockout lines. Therefore, subsequent physiological measurements at altered levels of rhizospheric Ca^{2+} were only carried out on these two SALK T-DNA insertion lines.

CNGCs are involved in the Ca^{2+} homeostasis of plant cells (Talke *et* al., 2003). Calcium hypersensitivity resulting in severe reduction of plant size and seed yield has been observed in two CNGC mutant *Arabidopsis* lines (*cngc2-1* and *cngc2-2)* (Chan *et al.,* 2003). When external Ca^{2+} was added to the medium (10-30 mM), a comparative decrease in rosette size, fresh weight and root weight was observed in these lines to that of their wild-type plants (Wassilewskija-WS) (Chan *et al.,* 2003). However, an increase in rosette diameter was observed in both T-DNA insertion lines investigated in this study (*Cal-CRG5*, $P>0.05$; *Cal-CRG17*, $P\leq0.05$) at 15 mM compared to that at 1 mM rhizospheric Ca^{2+}. Also, contrary to Col-4 controls, a significant ($P\leq0.05$) increase in mean number of leaves at 15 mM Ca^{2+} was observed in both the insertion lines compared to that at 1 mM Ca^{2+}. But, the number of leaves of *Cal-CRG17* and rosette diameter of *Cal-CRG5* at 15 mM Ca^{2+} were not significantly different ($P>0.05$) to the control Col-4 showing that the gene disruption didn't elicit a uniform range of physiological responses in the insertion lines at elevated rhizospheric Ca^{2+} exposure.

The root length of the two insertion lines differed ($P\leq0.05$) to that of Col-4 with altered rhizospheric Ca^{2+} concentration illustrating an altered root phenotype by knocking out the specific gene of interest. Interestingly, both insertion lines had a significant ($P\leq0.05$) reduction in root length at 1 mM Ca^{2+} compared to Col-4. This demonstrates that both the T-DNA insertion lines were showing a stronger loss of function phenotype at lower concentration of rhizospheric Ca^{2+} (1 mM). However, at 15 mM Ca^{2+}, only *Cal-CRG17* showed a significant difference ($P\leq0.05$) in root length compared to Col-4 with smaller roots.

Though, both insertion line *Cal-CRG5* and *Cal-CRG17* had no significant ($P>0.05$) wet weight difference to control ecotype Col-4 at 1 mM rhizospheric Ca^{2+} exposure, an increase in the rhizospheric Ca^{2+} concentration to 15 mM revealed a significant increase ($P\leq0.05$) in the fresh weight of the insertion lines compared to Col-4. This increase in wet weight was mirrored in the dry weight measurements of both the insertion lines though only *Cal-CRG17* showed a significant ($P\leq0.05$) increase, suggesting that the knockout lines have better adaptability to altered rhizospheric Ca^{2+} conditions than the control ecotype Col-4 plants. The fact that the exposure of the insertion lines to altered rhizospheric Ca^{2+} conditions for a period of 30 days resulted in a difference of growth in comparison to the control Col-4 plants implies the importance of these Cal-CRGs in putative calcicoles (Cal-0) to sustain growth in calcareous environments.

These parameters partially reveal the physiological effects of disrupting Cal-CRGs and the subsequent responses of plants to altered rhizospheric Ca^{2+} concentration. Disruption of Cal-CRG17 (At1g31680) with a putative molecular function of copper ion binding (TAIR) and involved in an unknown biological process (TAIR) and Cal-CRG5 (At5g18470) with a putative molecular function in sugar binding (TAIR) and involved in an unknown biological process (TAIR) demonstrated a markedly different phenotype compared to Col-4 at 1 mM rhizospheric Ca^{2+}. Though the two insertion lines had significantly fewer leaves, and reduced rosette diameter and root length compared to Col-4 at 1 mM rhizospheric Ca^{2+} exposure, no significant difference was observed in the fresh weights of the three. These findings are exactly opposite to that of Chan *et al.* (2003), who observed a reduction in

rosette diameter and average fresh weight(s) of two mutant homozygous *cngc2* lines at elevated levels of Ca^{2+} when compared to control Col-0 ecotype suggesting a strong link between CNGC2 function and the adaptive response of plants to biotic stresses such as Ca^{2+} stress.

Relative expression studies of Cal-CRGs on five insertion lines and six ecotypes at varied rhizospheric Ca^{2+} concentrations

In the present study, quantitative real time PCR was used to study ten genes, initially identified as having induced expression at low Ca^{2+} in Cal-0 (Cal-CRGs), in six *Arabidopsis* ecotypes comprising two lab ecotypes (Col-4 and Cal-0) and four wild ecotypes (Glenisla, Penicuik, Halifax and Elland) from varying rhizospheric Ca^{2+} regions across UK as well as five Cal-CRG SALK T-DNA insertion lines. Cal-CRG expression in *Arabidopsis* ecotypes and T-DNA insertion lines grown on altered levels of rhizospheric Ca^{2+} and harvested on 30th day of the treatment were compared with Col-4 (lab ecotype, putative calcifuge) treated with 1 mM Ca^{2+} as a calibrator.

Comparison of relative expression by Quantitative real time PCR of ten Cal-CRGs in six wild ecotypes and five Cal-CRG SALK-TDNA insertion lines clustered *Cal-CRG5* exposed to 15 mM rhizospheric Ca^{2+} with wild calcicole ecotypes Elland and Halifax treated with 15 mM Ca^{2+} and putative calcifuge Glenisla and Col-4 at 1 mM. As these Cal-CRGs were repressed in Cal-0 in the microarray study (see Chapter 5), it focuses attention on how four of the five insertion lines have similarity in Cal-CRG gene expression to Cal-0 plants. Such similarities suggest that, by knocking out these genes from the parent

Col-0 (a putative calcifuge) it might have been converted (at least partially) into a 'calcicole'.

The closeness of relative expression of Cal-CRGs in wild ecotypes Elland, Penicuik, Glenisla and Halifax at opposing concentrations of rhizospheric Ca^{2+} is an indication of their differential response to rhizospheric Ca^{2+}. The gene expression results suggest that the observed physiological difference in the wild calcicole-calcifuges is due to inherent genetic differences. Also, individual clustering of four (*Cal-CRG1*, *Cal-CRG6*, *Cal-CRG10* and *Cal-CRG17*) insertion lines exposed to either concentration of Ca^{2+} (1 mM or 15 mM) demonstrates that these Cal-CRGs do not respond to increased Ca^{2+} concentration in the same way as in the six ecotypes.

An insight into biochemical and molecular mechanisms by which plants tolerate environmental stresses is necessary for a better understanding of the environment (Bohnert *et al.,* 1995). This study was initiated with a hypothesis that tolerance to the edaphic condition of increased Ca^{2+} would be mirrored in altered expression patterns of genes corresponding to its exposure to rhizospheric Ca^{2+}. In Chapter 5, microarrays were used to study the whole transcriptome of *Arabidopsis* ecotypes at an elevated rhizospheric Ca^{2+} conditions compared to low concentrations. Subsequent analysis of the microarray data facilitated identification of a number of differentially expressing genes (Calcium Responsive Genes; CRGs), of which ten were selected for subsequent analysis in six *Arabidopsis* ecotypes and five *Arabidopsis* SALK T-DNA insertion lines. All ten selected Cal-CRGs were found to be repressed in putative wild calcicole ecotypes and induced in putative wild calcifuge ecotypes at high Ca^{2+}. A varied response was

observed in the lab ecotypes. Future investigations should be directed towards the evolutionary ecology of adaptations to elevated levels of rhizospheric Ca^{2+}. Further, it also has to be determined whether these CRGs are really indicative of Ca^{2+} tolerance over wide population sets or have been evolutionarily selected in local sub-set populations. It would also be interesting to see if these genes that display varied expression patterns at altered rhizospheric concentrations function similarly in other plant species, or whether they have attained novel functions in *Arabidopsis* in order to inhabit calcareous stressful environments?

Chapter 7

General Discussion

7.1 Background and aims of project

The dynamic environment involved in growth and development of plants means that they must be adaptable to withstand a wide range of environmental stresses. As a consequence, they have developed highly flexible survival mechanisms. Calcium is one such stress factor to which plants must respond in order to adapt edaphically and to maintain their continuous life cycle. As well as being an essential micronutrient, Ca^{2+} functions as a second messenger in multiple stress adaptations (see Chapter 1). An understanding of the molecular basis of the sensitivity of plants to either high or low rhizospheric Ca^{2+} will enhance the understanding of the importance of molecular variation as the basis of ecological fitness. Better understanding of the mechanisms underlying the response of vulnerable species-rich plant communities thriving on calcicolous grass land ecosystems to environmental change will accelerate the development of strategies for the conservation of their biodiversity. The adaptations shown by plants that grow in calcareous soils (calcicoles), and acidic soils (calcifuges), have intrigued ecologists for much of the 20th Century (Lee, 1999; Salisbury 1921). However, the understanding of the physiological characteristics of calcicoles and calcifuges remains inadequate. The distribution of plants on calcareous and acidic soils is influenced by several factors, including the abundance of Ca^{2+} ions, the levels of bicarbonate, and the availability of phosphate and iron (Kinzel, 1983).

Calcareous soils are typically saturated with Ca^{2+} carbonate, the predominant ions in the soil solution being Ca^{2+} and HCO^{3-}, whereas acidic soils tend to be deficient in Ca^{2+}. Different mechanisms have evolved to enable calcicoles to tolerate these conditions (Lee 1999). It has been demonstrated, that trichomes constitute an important system for regulating the concentration of Ca^{2+} in the apoplast of calcicoles, enabling plants to tolerate high concentrations of rhizospheric Ca^{2+}. It is sequestered in the cells of trichomes allowing a low-Ca^{2+} environment to be maintained around Ca^{2+}-sensitive cells such as stomatal guard cells (De Silva *et al.,* 2001, 1998, 1996). Interestingly, it has been demonstrated that this mechanism is conserved between species and operates in the calcicole (Cal-0) ecotype of *A. thaliana* (McAinsh *et al.,* Personal Communication). These findings at Lancaster University (Abram *et al.,* 2005; De Silva *et al.,* 2001, 1998, 1996; McAinsh *et al.,* unpublished data) and the availability of genomics resources for *Arabidopsis* implicated it as an ideal species to study the molecular basis of plant adaptations to high rhizospheric Ca^{2+}. To this end, the physiological response of ecotypes of *A. thaliana* that were collected from varyingly calcareous soils (Glenisla, Penicuik- Putative calcifuges; Elland, Halifax- Putative calcicoles), along with the lab ecotypes Col-4 (putative calcifuge) and Cal-0 (putative calcicole) to altered rhizospheric Ca^{2+} levels were studied. Physiological differences with respect to the Ca^{2+}-tolerance nature of the ecotypes were identified in the wild ecotypes. Also, a detailed microarray analysis was carried out on the publicly available chip data of putative calcifuges, Col-4 and Penicuik and putative calcicoles, Cal-0 and Elland grown at altered rhizospheric Ca^{2+} concentrations (1 mM, 12.5 mM).

Microarray analysis helped in identifying groups of Ca^{2+}-responsive genes specific to the ecotypes in the study. This project has assisted in furthering the understanding of adaptations of plants to varied edaphic conditions. Furthermore, the project has generated novel data regarding Ca^{2+} responsive genes and physiological characteristics of calcifuges in respect to high rhizospheric Ca^{2+} concentrations, which adds to the colossal international effort to elucidate the function of all *A. thaliana* genes by the year 2010 (Chory *et al.,* 2000; Somerville and Dangl, 2000).

7.2 Main Results and Conclusions

The main results and conclusions from this study are as follows:

1. Significant differences in the growth and root morphology were identified in putative wild calcifuges with respect to high rhizospheric Ca^{2+}. These observations imply that adaptation mechanisms are prevalent in the localised flora of specific soil types, where different ecotypes of the same species had varied growth patterns.
2. Detailed root morphology studies indicated the occurrence of apoptosis in the putative calcifuge Glenisla in response to high rhizospheric Ca^{2+}. Also, physiological factors and recordings indicated that Penicuik (putative wild calcifuge) might not be a true calcifuge. Apoptosis in Glenisla demonstrates that high rhizospheric Ca^{2+} conditions are lethal to calcifuges which bring on cellular changes in the organs (roots) that are in direct contact with the edaphic conditions. However, the indifferent response of the other wild calcifuge, Penicuik, compared to

Glenisla as well as molecular studies suggest that the former is not a true calcifuge and might have initially originated from a different edaphic region.

3. Microarray analysis using the Affymetrix® ATH1 full genome chip revealed the differential regulation of 1,508 genes ($P \leq 0.05$). This represents the first fully comprehensive microarray study to investigate the Ca^{2+}-response in *A. thaliana*. Functional classification of these genes demonstrated the many diverse processes that make up the response to Ca^{2+} and highlighted the intricacy of this response. Interestingly, a whole suite of genes were repressed at high Ca^{2+} (induced at low Ca^{2+}) in the wild ecotypes, many of which were involved in defence pathways, stress and stimulus response as well as PCD and cell wall regulation, indicating that low Ca^{2+} levels may have induced a stress response in these ecotypes. This analysis surely suggests that on its own 'Ca^{2+}' concentration in soils can alter the genetic regulation of its resident ecotypes as well as being involved with other stress responsive pathways as a secondary messenger.
4. Examination of T-DNA insertion mutants with disrupted Cal-CRGs revealed no growth-suppression effect of increased rhizospheric Ca^{2+} on the lines, indicating that down-regulation of Cal-CRGs may play an important role in Ca^{2+} tolerance. These observations surmise that the two true T-DNA knockouts for Cal-CRG5 and Cal-CRG17 genes were showing characteristics of a 'calcicole', where they weren't being affected by an increase in rhizospheric Ca^{2+} levels, which in turn

implies the importance of the suppression in expression of these two genes in adaptation to high rhizospheric Ca^{2+} conditions.

5. The importance of thorough data analyses of microarray data using multiple replicates was demonstrated in identifying true Ca^{2+}-responsive genes. Real-time PCR was used to establish the expression profiles of Cal-CRGs in ecotypes similar to those observed in the microarray experiments. The significance of understanding transcriptomic data from experimental samples grown on altered levels of rhizospheric Ca^{2+} and deducing conclusions from the same on a broad scale was accomplished by incorporating replicated samples as well as performing Real-time PCR to correlate the data in identifying 'true' Ca^{2+}-responsive genes.

7.3 Discussion

Plants have developed complex biochemical and regulatory pathways to acquire mineral nutrients from the soil environment and distribute them to appropriate tissues (Baxter *et al.,* 2008). Natural populations of *Arabidopsis* provide an excellent system to study adaptations in plants to mineral nutrient and trace element uptake pathways in order to thrive under different environmental conditions (Borevitz *et al.,* 2007; Nordborg *et al.,* 2005). Plant communities on calcareous soils (chalk and limestone) comprise many diverse species of ecological importance (Rodwell 1991; Hillier *et al.,* 1990). Impending impact of climate change and pollutants such as ozone on the constitution, composition and function of calcicolous ecosystems is a subject of apprehension (Grime *et al.,* 2000). An understanding of how these specific plant communities thrive in comparison to similar species ecotypes has been a subject of intense interest (Lee, 1999; Salisbury 1921). Also, analyses of natural variation in plants provide the potential to dissect the genetic networks controlling important biological processes (Benfey and Mitchell-Olds, 2008). In the present study, it has been shown that *A. thaliana* wild ecotypes collected from varying calcareous soils show varied physiological responses in respect to altered rhizospheric Ca^{2+}. Interestingly, Penicuik, an ecotype collected from acid soils, showed more similarity in response to elevated rhizospheric Ca^{2+} to putative wild calcicoles than to the other putative wild calcifuge, Glenisla. Glenisla plants in turn had a short root phenotype and the occurrence of apoptosis in roots in response to high rhizospheric Ca^{2+}.

Comparative biology across multiple species has been employed as a key strategy in identification of stress-responsive gene loci and their

corresponding alleles (Wanchana *et al.,* 2008). Although, regulation of gene expression in plants in relation to altered rhizospheric Ca^{2+} has not been studied previously, the involvement of Ca^{2+} signaling in various biotic and abiotic responses has been addressed (Sanders *et al.,* 2002). Accordingly, microarray data from four *A. thaliana* ecotypes (Col-4, Cal-0, Penicuik and Elland) grown on low (1 mM) and high (12.5 mM) rhizospheric Ca^{2+} were analysed to identify Ca^{2+}-responsive genes among the ecotypes. Though none of the 1,508 ($P \le 0.05$) differentially expressed genes from the 24 arrays showed a similar pattern of expression in the four ecotypes under study, individual ecotypes showed ecotype-specific Ca^{2+}-responsive genes (CRGs). 53 CRGs (15-repressed at high Ca^{2+}) were specific to putative lab calcifuge, Col-4, 46 CRGs (9 repressed at high Ca^{2+}) to putative lab calcicole, Cal-0, 246 CRGs (199 repressed at high Ca^{2+}) to Penicuik, a putative wild calcifuge and 183 CRGs (154 repressed at high Ca^{2+}) were identified in putative wild calcicole, Elland. This shows the enhanced response of wild ecotypes to altered rhizospheric Ca^{2+} concentration in respect to an altered gene expression pattern. Also, the majority of the CRGs in these ecotypes were repressed at high Ca^{2+}.

Programmed Cell Death (PCD) is an essential process in both plants and animals (Greenberg, 1996). A major pathway of PCD in animals is a greatly synchronized process of cell death that differs from autophagy (lysosomal degradation of organelles and certain proteins) and from necrosis (which results from acute tissue injury and incites an inflammatory response in animals) and is known as 'apoptosis' (Eckardt, 2006). The term 'apoptosis' was coined from Greek words (*apo* = from, and *ptosis* = falling) by Kerr *et. al.*

(1972) to describe plant leaf abscission, and they hypothesized that it plays a broad role in regular cell metabolism and that abnormalities in this process contribute to an assortment of diseases, including cancer. The demonstration of apoptosis in several plant hypersensitive responses indicates that this form of host cell deletion is not restricted to animals and is a widespread defence mechanism in eukaryotes (Levine *et al.,* 1996). PCD also plays a vital role in plant development pathways, including, formation of woody tissues, leaf abscission, self-incompatibility, and defence response to a wide variety of biotic factors and environmental stresses (Eckardt, 2006). In the present study, apoptosis was characterised using TUNEL staining in Glenisla (putative wild calcifuge) plants grown on high rhizospheric Ca^{2+} (15 mM). van Doorn and Woltering (2005) referred to PCD that occurs during normal differentiation and as a result of abiotic stress as, 'developmental'. Also, microarray analysis of the two wild *A. thaliana* ecotypes Elland and Penicuik showed the presence of genes involved in PCD in response to altered rhizospheric Ca^{2+} concentrations. Moreover, induction of apoptosis in mammalian cells often involves elevation of cytosolic Ca^{2+} (Martin *et al.,* 1994) which may directly activate cell death effectors (Martin *et al.,* 1994; Stewart, 1994). Also, Ca^{2+} influx has been implicated in bacterial induction of hypersensitive cell death in tobacco (Atkinson *et al.,* 1990), fungal rust induction of hypersensitive cell death in cowpea and the activation of a number of early events including K^+/H^+ exchange, the oxidative burst and defence gene transcription (Xu and Heath, 1998; Knight *et al.,* 1991; Atkinson *et al.,* 1990). Also, the involvement of Reactive Oxygen Species (ROS) in triggering PCD is well characterized in plants where inter- and intra-cellular generation of ROS (O_2^- and H_2O_2)

resulted in an oxidative burst along with an elevation in $[Ca^{2+}]_{cyt}$ that triggered PCD (Heath, 2000; Gilchrist, 1998). Although Ca^{2+} is a common trigger of apoptosis in animals and Ca^{2+}-mediated apoptosis in the plant hypersensitive response, and there are remarkable similarities with respect to DNA fragmentation (Hung *et al.,* 2007; Levine *et al.,* 1996), it remains to be established whether the underlying mechanisms are alike in plants and animals.

The transcriptomics approach of the present study has generated information on a large number of genes that could be used to identify potential targets for reverse genetic studies, with the aim to develop transgenic plants that are more resistant to fluctuating Ca^{2+} levels in the soils, to maximise productivity as well as produce robust plants. This approach has been used to produce numerous genetically modified plants for important traits such as those conferring insect resistance or desired nutrients (Aumaitre *et al.,* 2002; Hails, 2000). Agricultural productivity is severely affected by soil nutrient conditions, for example, damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations (Zhang and Blumwald, 2001). Much research has been aimed toward the breeding of crop cultivars with improved salt tolerance (Cuartero *et al.,* 1999; Yeo *et al.,* 1988). Also, a long list of salt stress-responsive genes seemed to support that salt tolerance is a complex trait (Zhu, 2000). However, the overexpression of a single gene was shown to improve salt tolerance in *Arabidopsis* (Apse *et al.,* 1999). Similarly, a transgenic tomato plant over-expressing a vacuolar Na^+/H^+ antiport improved the salinity tolerance of the plant (Zhang and Blumwald, 2001). In summary, this study provides a firm molecular basis upon which

future crop improvement and ecological studies can build, leading the way to preserve the biodiversity of calcicoles and calcifuges as well as a way to establishing Ca^{2+}-independent plants, thus saving huge revenues in liming agricultural soils.

7.4 Future Work

7.4.1 Functional genomics

The genome within all living systems acts through the transcriptome, proteome, metabolome, and ionome to route all aspects of an organism's diverse functions (Baxter *et al.,* 2007). The dynamic interaction of these biochemical 'omes' defines how a living system functions and its study, 'functional genomics', is one of the biggest challenges in life sciences research (Baxter *et al.,* 2007). Functional genomics comprises not only transcriptomics, but also the analysis of the whole complement of proteins or metabolites within a cell or tissue type, namely proteomics or metabolomics or ionomics (Saito *et al.,* 2008; Shualev *et al.,* 2008; Reddy and Reddy, 2004). Protein studies have been shown to be an important source of functional information, with whole protein analyses being carried out using the established technology of 2-dimensional (2D) gel electrophoresis followed by mass spectrometry (Arnott *et al.*, 1998). Although gene and protein expression are directly linked, gene expression data cannot be extrapolated to predict protein expression as protein and mRNA abundance show poor correlation (Gygi *et al.*, 1999).

In recent times, metabolomic analysis tool has been employed by functional genomics to elucidate biochemical function (Saito *et al.,* 2008;

Shualev *et al.,* 2008). As with proteins and mRNA, metabolites vary due to physiological, developmental and environmental conditions. Saito *et al.* (2008) proposed a framework for decoding genes with co expression networks using metabolomics.

To further extend this project, metabolomics and ionomics could be employed to investigate the biochemical properties of Ca^{2+}-responsive genes and compare them with the other 'ion-responsive' genes. This can be achieved by using the Purdue Ionomics Information Management System (PiiMS) database (Baxter *et al.*, 2007). PiiMS is set out as a 'World Wide Web-enabled' system, facilitating an open access to raw data for analysis by numerous laboratories. It currently hosts data on shoot concentrations of P, Ca, K, Mg, Cu, Fe, Zn, Mn, Co, Ni, B, Se, Mo, Na, As, and Cd in over 60,000 shoot tissue samples of Arabidopsis (*Arabidopsis thaliana*), including T-DNA mutants, and natural accession and populations of recombinant inbred lines from over 800 separate experiments, representing over 1,000,000 fully quantitative elemental concentrations (Baxter *et al.*, 2007).

7.4.2 Inclusion of more germplasm

Characterization of natural variation to abiotic stress responses in *Arabidopsis* is an important requirement to application of the compiled molecular genomic data associated with *Arabidopsis* to a broader understanding of the genetic control to these phenomena (Neinhuis *et al.*, 1994). This project employed four wild ecotypes of *Arabidopsis* with varying responses to Ca^{2+} to understand the molecular basis of rhizospheric Ca^{2+} tolerance. Inclusion of additional ecotypes of *Arabidopsis* as well as related

species like *Arabis* in the study would increase the spectrum of understanding of 'Ca^{2+}-stress' tolerance and mechanisms involved within.

7.4.3 Factors other than Ca^{2+}

Calcareous soils are low in soluble and easily exchangeable phosphate (Zohlen and Tyler, 2004). However, calcicoles are adapted to these conditions, whereas studies have demonstrated that calcifuge plants (which do not grow on calcareous soils) have a low capacity to solubilize forms of soil phosphate (Tyler, 1992). Short and stumpy root phenotype was observed in the wild putative calcifuge Glenisla at high rhizospheric Ca^{2+} concentration. It would be of importance to study the interactions of other ions such as phosphate, with Ca^{2+} in understanding the calcicole–calcifuge behaviour of plants.

7.4.4 Interaction of stresses and impact of climate change on calcicoles and calcifuges

Climate change will affect the conservation rate of limestone grasslands, which embrace a wide variety of species-rich plant communities (Hillier *et al.*, 1990; Perring, 1960). Also, climate-driven changes in grassland productivity could have grim consequences for the distribution and profitability of pastoral agriculture (Hossell *et al.*, 1996). The impact of climate change on the structure, composition, and function of grassland ecosystems has been a topic of concern and experiments were performed to assess the simulating climatic change and studying the total biomass statistics of plant communities (Grime *et al.*, 2000). However, in this project it was established that changes

in rhizospheric Ca^{2+} concentration were mirrored in different molecular responses of calcioles and calcifuge plants. So, a similar approach can be carried forward by simulating climatic changes in parallel to altered rhizospheric nutrient treatments. This study would help in understanding the basis of fitness of these plant communities as well as have immense implications for the sustainment of these grasslands and in turn for bio industry.

Appendix 1

List of Real time PCR primers

Gene of Interest	Gene Number	Forward primer	Reverse primer
CAL-CRG1	At4g11850	TCAAAACACAAGGAGTTATGAACACA	AACTTGCACCGACGAAATGC
CAL-CRG2	At5g18820	ATTAATCCAAGAGGTTGCGATTAAA	CCAAAATGATTGCAGTGGTTGT
CAL-CRG4	At2g40080	AGGCAGAGCAGGGAGAGGAT	CTGATTGCACTTGTCTGAAATTCC
CAL-CRG5	At5g18470	CCGCGCAGCTTTCATTG	CCACAAAGTTCCCGGTATCG
CAL-CRG6	At3g19080	CAAGCATATTTGGCCCTTAGGT	TGATGCCTCATCTTCATCTTCTTC
CAL-CRG10	At4g29690	TGTGGGTGACCGCAGTGA	CAGCGCCTGGCCAAAA
CAL-CRG11	At1g69930	CCATCCTTCCCTCTGATCCAT	TTCATCGATATAGACGTCCCAAAA
CAL-CRG12	At1g26240	CCTAATGGTTGGCCGTCTTTAC	TGGGCACTTGTATGAGCAGAAA
CAL-CRG16	At5g15360	TCGGTGAGGACGGTGATTGT	TGTCCACGAGCAATCTAGAGGAA
CAL-CRG17	At1g31680	AGACCGCAACGTCAAACCA	TCGGCTCATGCCCTTATCTC

Appendix 2

Primers used for identifying the homozygous SALK lines (LBb1- T-DNA left border primer, LP- Left border primer and RP-Right border primer)

Primer	Primer Sequence	Length (bp)
LBb1	AAATCCTGTTTGATGGTGGTTCCGAAAT CGGCAAAATCCCTTATAAATCAAAAGAA TAGCCCGAGATAGGGTTGAGTGTTGTT CCAGTTTGGAACAAGAGTCCACTATTAA AGAACGTGGACTCCAACGTCAAAGGGC GAAAAACCGTCTATCAGGGCGATGGCC CACTACGTGAACCAT	180
Cal-CRG1-LP	TTGTGCTGCAGAGGTTGTGAAA	22
Cal -CRG1-RP	ACTGCGGATGATGGACCAAGA	21
Cal -CRG2-LP	TGTTTCCATCACCTTAGGCCC	21
Cal -CRG2-RP	TGAAACTGCCATTGTGAAGCC	21
Cal-CRG4-LP	TCGGTGTCCGATTCTACTCAG	21
Cal-CRG4-RP	TGCAAAAGCTCTTTCGTCTTC	21
Cal -CRG5-LP	ACACGTGGAGGCATGGATGAA	21
Cal -CRG5-LP	GTTTTGAGTGGTGAAACGGCG	21
Cal-CRG6-LP	TTACATGTTCTGGGAGCCATC	21
Cal-CRG6-RP	AGCCACAATTTTCGTCAAATG	21
Cal –CRG7-LP	CTGACGAAGCTGGAGAAATTG	21
Cal –CRG7-RP	GAGCAGCTGGTAAATGCTGTC	21
Cal -CRG10-LP	TTTAATTTTGAAAGTTCCAACG	22
Cal -CRG10-RP	GCTGGGGCAAAGATGTCAAGA	21
Cal-CRG13-LP	TGTCTACACCAATTCTTTACCGCTC	25
Cal-CRG13-RP	GCTCTTTCTGATCTTTGTTGGGG	23
Cal -CRG15-LP	CATAACAACGAAACTGAAAACACG	25
Cal -CRG15-RP	TCTTTTCGTTTCACGTCCTCAAGA	24
Cal -CRG17-LP	ACTTGCCGGTATCTGTTCATG	21
Cal -CRG17-RP	TCGAACAAGATCCATGTAGGC	21

Appendix 3

Example Calculation for the $\Delta\Delta C_T$ Method of Relative Quantification for Real Time PCR

E.g.: 1 mM rhizospheric Ca^{2+} exposure for 30 days on ecotype, Cal-0

Treatment and Gene				Average C_T
1 mM Cal-0 Cal-CRG1	26.16	26.09	25.78	26.01
1 mM Cal-0 ubiquitin	18.84	18.91	18.54	18.76
1 mM Col-4 Cal-CRG1	25.68	25.23	24.00	24.97
1 mM Col-4 Ubiquitin	18.87	18.37	18.62	18.62

The above values give the three C_T for each cDNA and primer combination. Each reaction is set up in triplicate to nullify errors introduced by inaccurate pipetting.

The average value is then taken as the true C_T value.

The first calculation to be performed is ΔC_T for within each treatment and for each control gene:

ΔC_T (1 mM/ ubiquitin) = C_T of 1 mM Cal-0 Cal-CRG1 - C_T of 1 mM Ubiquitin

= 26.01-18.76

= 7.25

ΔC_T (1 mM/ ubiquitin) = C_T of 1 mM Col-4 Cal-CRG1 - C_T of 1 mM Col-4 Ubiquitin

$= \quad 24.97\text{-}18.62$

$= \quad 6.35$

The second set of calculations is $\Delta\Delta C_T$, i.e. the difference between the treatments for each gene and the mean of two is taken:

$\Delta\Delta C_T$ (1 mM Cal- 0 Cal-CRG1) $=$ ΔC_T (1 mM Cal-0/ ubiquitin) - ΔC_T(1 mM Col-4/ ubiquitin)

$= \quad 7.25\text{-}6.35$

$= \quad 0.9$

The relative transcript abundance is then calculated by the formula, $2^{-\Delta\Delta CT}$, which in this case would be: $2^{-(0.9)} = 0.54$, i.e. after exposure of Cal-0 to 1 mM rhizospheric Ca^{2+} for 30 days, Cal-CRG1 is repressed by 0.54-fold in comparison to Col-4. This whole process is repeated for three biological replicates and an average taken.

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