Isolation of *Arabidopsis* mutants resistant to root pattern disrupting signals from carrot embryogenic cultures.

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Anthony,

Mum and Dad.
Acknowledgements

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Abstract

During development, individual plant cells differentiate into specific cell types, creating characteristic cellular differentiation patterns. Plants use both positional information and cell-cell signalling in constructing these patterns. The nature of the cell-cell signals are unknown.

Cell to cell signalling is involved in the early stages of somatic embryogenesis with arabinogalactan-proteins (AGPs) being essential to the process. The type of AGPs expressed at the cell wall of cells undergoing embryogenesis are linked to the embryogenic potential of the cell and also the cell-cell signal secreted by a cell. AGPs are found exposed at the plasma membrane, in intravacuolar vesicles and in the extracellular matrix of plant cells. Monoclonal antibodies have been used to designate different type of AGPs. Using these, several AGPs have been found to be expressed in both embryogenic cultures and in the plant embryos and roots, with the expression patterns of some AGPs relating to, or predict cellular differentiation patterns.

The signals involved in the embryogenic process can be obtained from the culture media or by washing the cell walls with water. Extracts from somatic embryogenic cultures (CWSE) containing AGP like carbohydrates were shown to affect the root development of both Carrot and Arabidopsis seedlings. In response to CWSE, seedlings produced roots, which were shorter and fatter than controls with lateral root production also affected. There was an increase, in the number of cell layers, and in the number of cells in each cell layer of the root.
Abstract

Putative signals have been purified using a combination of the effect of the CWSE on *Arabidopsis* roots and separation columns. Purification was achieved by fractionation using a Sephadex G-10 column followed by molecular weight cut off membranes and partition chromatography using a Flash Biotage unit. 5 active fractions have been analysed for carbohydrate composition. Several unusual residues were found, including inositol O-methyl ethers. The similarity of the residues found in each of the fractions, raises the possibility that the compounds are part of a biochemical pathway and that some of the smaller molecules may be processed sections of the larger molecules.

Several putative mutants that had reduced sensitivity to the CWSE, were isolated from M2 populations of *Arabidopsis* derived from ethane methane sulphanate treated plants. The seedlines were retested for their resistance to CWSE against their control grown phenotype. The mutants isolated tended to have shorter roots than control grown *coll, gll* plants, and produce a reduced number of lateral roots.
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Chapter 1 – Introduction

Plants contain a number of tissues, which are arranged in specific patterns in highly organised organs. In order for organs to form, cells need to divide, commit developmentally and differentiate. When a cell divides it may undergo a symmetrical or asymmetrical division. Asymmetrical divisions produce cells that differ and can develop along different developmental pathways. Asymmetric divisions play an important role in plant development (Dolan, 1997). Cells that undergo symmetrical divisions can also develop along different pathways. These divergent developmental pathways are subjected to differing external signals, as each cell is placed in a separate environment and thereby develops according to its specific surroundings.

The creation of cell patterning in a plant may be controlled directly by the lineage of the cells or in response to positional information from other cells or the environment (Greenwald and Rubin, 1992, Horvitz and Herskowitz, 1992).

The *Arabidopsis* root has a very structured pattern of development (Dolan *et al.*, 1994) from the embryo through to the mature root. It can therefore be used to study the effects of signalling on pattern development. In this introduction, I discuss the development of the *Arabidopsis* embryo and seedling root including mutations that effect the developmental pattern. I then discuss the role of plant hormones, carbohydrate and/or protein signal molecules in influencing these developmental programmes.
1.1 Pattern development in the early Arabidopsis embryo

During embryo development in plants, the embryo passes through proembryonic, octant, globular, torpedo and cotyledonary stages. The embryogenic process has been particularly studied in Arabidopsis, Figure 1.1 (Lindsey and Topping, 1993).

After fertilisation the zygote undergoes an asymmetric division resulting in a small apical cell and a larger basal cell. After the initial asymmetric division, the apical cell divides horizontally, followed by each cell dividing twice, to form the octant embryo. Each cell then undergoes periclinal divisions to produce the protoderm. The protoderm eventually becomes the epidermis. The apical set of cells in total divide further to become the upper part of the embryo (embryo proper), which eventually become the cotyledons and apical meristem. The other cell formed by the initial asymmetric division, the basal cell, divides to form the suspensor. The top cell of the suspensor otherwise known as the hypophyseal cell, also divides producing the lower part of the embryo root.

Cells of the embryo continue to divide anticlinally and longitudinally to produce a globular embryo. Production of the heart shaped embryo requires the establishment of bilateral symmetry. Three layers of tissue, the epidermis, ground tissue and vascular tissue are established by the heart stage of development. The layers comprise cells that are not differentiated, but which will undergo further divisions to produce several cell types within the particular cell layer.
Figure 1.1 Embryogenesis in *Arabidopsis* and the embryonic origin of seedling tissue

Reproduced from Lindsey and Topping (1993)

A  initial asymmetric division / proembryo
D  octant
E / F globular stage
G  heart stage
H  torpedo stage
I  cotyledonary stage

(b) Reproduced form Laux and Jurgens (1997)

A  initial asymmetric division / proembryo, ac = apical cell, bc = basal cell
B  octant stage, ul = upper tier, lt = lower tier, hy = hypophysis, su = suspensor
C  globular stage, pd = protoderm
D  heart stage, cot = cotyledons, sm = shoot meristem, ult = upper lower tier, llt = lower lower tier
E  seedling, hc = hypocotyl, rt = root, crc = central root cap, qc = quiescent centre.
During embryogenesis the arrangement of the different cell types and thus the cell patterning of the embryo is established. Further divisions and cell shape changes follow which determine the final overall shape of the embryo. Several mutants that influence this process have been isolated. These can be grouped into four main types.

1. Apical basal mutants block the correct formation of cell pattern by removal of a cell type which would have gone on to form either the root, hypocotyl or cotyledons of the embryo.

2. Radial mutants fail to undergo the divisions that are involved in producing the correct radial cell pattern.

3. Shape mutants influence the ability of the cell produced to develop into the correct shape, this causes defects in the overall shape of the embryo.

4. Finally, mutants that effect the development of the embryo suspensor can also cause defects in the embryo proper.

1.1.1 Apical-basal development

Apical-basal mutants fail to complete one or more of the early vertical divisions. If this division failure involves the first asymmetric division of embryogenesis, large portions of the embryo do not develop. Mutants included in this group are *gurke*, *fackel*, *monopterous* and *gnom* (Mayer et al., 1991).

The *gnom* gene acts to promote the initial asymmetric division. The mutant can be recognised before the octant stages, with the asymmetric division resulting in two cells of almost equal size. The apical cells divide to produce an octant embryo with twice the usual number of cells, and the basal cell fails to develop the hypophysis.
The mutation can produce embryos with no visible apical-basal polarity. (Mayer et al., 1993).

Mutants in the *gurke* gene cause defects in the apical region of the embryo. Extreme phenotypes of this mutant can cause complete deletion of the apical region of embryo. Defects in *gurke* are first visible at the heart stage of the embryo development. The cotyledons are more sensitive to the lack of functional *gurke* expression than the apical meristem and the hypocotyl region (Laux and Jurgens, 1997).

*Gnom* acts epistatically to the *monopterous* mutant, which acts at the opposite pole of the embryo to *gurke*, with the most extreme phenotype resulting in the complete deletion of the basal region, including the roots and hypocotyls of the embryo. Deviations from wildtype development are first seen at the octant stage of embryo development (Laux and Jurgens, 1997). *Fackel* mutants reduce the hypocotyl size giving the embryo the appearance of having cotyledons that are attached directly to the embryo root. They fail to produce the characteristic elongated vascular precursor cells associated with the hypocotyl (Laux and Jurgens, 1997).

### 1.1.2 Radial patterning

Radial mutants, while possessing apical and basal regions, have missing or altered radial cell layer patterning. Examples include *knolle, keule, shortroot, scarecrow, pinocchio, gollum* and *woodenleg* (Mayer et al., 1991, Benfey et al., 1993, Scheres et al., 1995). Radial patterning appears to be more plastic than apical–basal patterning with partial layer replacement occurring in some mutants (Mayer et al., 1991).
The mutant *knolle* does not form a proper epidermis, subsequently, the phenotype comprises vascular tissue surrounded by large cells. The phenotypic changes can be traced back to the early 8-celled embryo where in the wildtype two layers of cells can be distinguished. In the *knolle* mutant the cell layers are not visibly different and are more loosely packed than in the wild type, additionally the upper end of the suspensor is enlarged. KNOLLE protein is membrane associated, and is specifically expressed during mitosis (Lauber *et al.*, 1997).

*Keule* affects the epidermis with cells being more elongated and irregularly arranged than in the wildtype, whereas the ground and vascular tissue look normal. In this mutant the epidermal cell primordia are abnormal and the precursor of the root primordia are larger than normal. The mutants, *shortroot, scarecrow* and *pinocchio* show pattern defects at the early heart stage (Scheres *et al.*, 1995). In wildtype plants the first periclinal division in the developing meristem, cortex / endodermis initials, doubles the ground tissue layer number, however in these mutants, this division fails to occur. Thus, at heart stage only one layer of ground tissue is present. *Scarecrow* is required for all the asymmetric divisions in the root. One such asymmetric division forms the endodermis and the cortex cell lineages. In mutant seedlings the cells have features of both cell types, having failed to differentiate fully into either endodermis or cortex (Laurenzio *et al.*, 1996). This may be the result of the failed asymmetric division producing a single cell type instead of two, which responds to both endodermal and
cortical positional signals. Alternatively *scarecrow* may be required to stabilise the cell in one or the other cell fate (Dolan, 1997).

*Gollum* and *woodenleg* show defects in their stele cells, with *woodenleg* producing a reduced number of pericycle like cells (Scheres *et al.*, 1995).

### 1.1.3 Shape mutants

Shape mutants are able to produce apical-basal and radial patterning in the early embryo but are unable to undergo the required number of appropriate divisions, or they produce incorrect cell shapes resulting in an embryo with all the tissue types but with a deformed shape. Mutants that change the embryo shape include *fass*, *knopf* and *mickey* (Mayer *et al.*, 1991, Fisher *et al.*, 1996).

*Fass* mutants are shorter and wider in circumference than wildtype *Arabidopsis*. In some individuals 3 cotyledons may be present. Sectioning of the seedlings at various stages of development show that although *fass* interferes with the pattern of cell division, cell elongation and the orientation of cell walls, it does not interfere with the type of cells that develop or indeed the cell polarity (Torres-Ruiz and Jurgens, 1994).

*Fass* mutants have elevated levels of both auxin and ethylene, suggesting that in *fass* the high level of ethylene may be a result of the high level of auxin, with the combination of the effects of the two hormones resulting in the phenotype (Fisher *et al.*, 1996).

*Knopf* and *mickey* mutants also change the shape of the embryo with the *knopf* embryo being smaller and more rounded than wildtype embryos. The vascular tissue
appears to be replaced with ground tissue in the hypocotyl, although vascular
primordia are present in heart stage embryo. *Mickey* mutations have thicker
cotyledons with short hypocotyls and roots. The epidermal cells are bloated and
vascular regions have reduced organisation (Mayer et al., 1991).

1.1.4 Abnormal suspensor mutants

Mutants that affect the development of the suspensor known as *sus* mutants have also
been isolated (Schwartz et al., 1994). These mutants consist of an enlarged
suspensor with a distorted embryo proper (Schwartz et al., 1994). Morphological
defects in these mutants are first visible in the globular stage embryo proper, with
abnormal divisions in the suspensor appearing at heart stage of development. Cells at
the core of the embryo undergo cell death with cells occasionally showing signs of
differentiation into xylem cells.

In *sus1* mutants starch grains appear in both the embryo proper and the suspensor
cells. The production of starch grains does not occur until after the heart stage of
development in the wild type and then only in the embryo proper. Thus, cellular
differentiation in the mutant embryo proceeds at an earlier stage in development than
in the wildtype. In the wild type, the suspensor normally produces a column
consisting of 6-8 cells. These cells undergo cell death prior to seed maturation. In the
*sus* mutants the suspensor fails to die. The default developmental programme of the
suspensor is embryogenesis, however, during normal development the suspensor cells
are inhibited from undergoing embryogenesis. In *sus* mutants, however, the
suspensor does not appear to be inhibited but starts to expand and develop into an
embryo. As the first developmental abnormalities appear in the embryo proper, the
suggestion is that the embryo proper inhibits the embryogenic development of the suspensor cells, possibly by the transmission of positional signals (Schwartz et al., 1994).

1.1.5 Meristem associated mutations

Shoot and root meristems are first visible at the heart stage of embryo development (Lindsey and Topping, 1993, Scheres et al., 1994). Meristems function to produce new cells by the division of totipotent or pluripotent cells. At least two groups of genes, shoot meristemless and root meristemless are involved in regulating meristem development. (Cheng et al., 1995, Endrizzi et al., 1996). Mutant plants of these two genes groups have much reduced or absent meristems of the respective type. In both cases the genes appear to regulate the incorporation of cells into the meristem during embryogenesis and lateral meristem development. They also regulate the size of the meristem. The gene products are required to activate cell division and maintain the relatively undifferentiated state of the cells within the meristem.

1.2 Development and growth of the Arabidopsis root

The basal cell, which results from the first asymmetric division of the zygote, subsequently develops into the hypophyseal cell and the suspensor cells. The hypophyseal cell contributes to the development of the root end of the embryo. The first division of the upper cell divides the embryo proper into an upper layer and lower layer, with the lower layer comprising of procambium, protoderm and ground tissue. Further divisions occur and at late heart stage the lowest of the protoderm cells start to undergo the periclinal divisions characteristic of the root meristem, producing
the root cap. The neighbouring cells are defined as cortex cells, pericycle cells and vascular initials. The main radial patterning of the root is completed between the late heart and torpedo stages of development. However, further pattern development occurs specifically in the root epidermis, with the development of root hair (trichoblast) cells and non-root hair (non-trichoblast) cells.

Several mutants, including those described above, influence root development. The following lack specific cell layers in the root. The mutants, shortroot, scarecrow pinocchio, knolle and keule (Benfey et al., 1993, Scheres et al., 1995, Mayer et al., 1991) lack one of the ground tissue layers. Shortroot lacks the endodermal layer, whereas the single ground tissue layer in scarecrow and pinocchio appears to have characteristics of the endodermal cell layer (Scheres et al., 1995). Knolle and keule appear to lack epidermal cell layers (Mayer et al., 1991). These mutants all appear to arise from a failure in an asymmetric division in the embryo, which may result in cells which receive conflicting positional information or that are unable to respond, or respond abnormally, to the information supplied to them.

1.3 The role of positional information and cell to cell signals involved in plant development

The differential state of cells can be determined by either cell lineage or by positional information (Dolan et al., 1994, Lindsey and Topping, 1993, Sachs, 1994). However, although lineage is important (Sachs, 1994), evidence is accumulating that positional information plays a pivotal role in the development and maintenance of cell layers and pattern.
Several studies have been undertaken using periclinal chimeras to study cell to cell signalling (Szymkowiak and Sussex, 1992, Irish and Bouhidel, 1993, Tian and Marcotrigian, 1993). Plant tissue can be divided into L1, L2 and L3 layers, with the L1 layer being the most external epidermal layer. Periclinal chimeras have a different genotype in at least one of these layers.

The inflorescence meristem of Arabidopsis consists of approximately 100 cells, and consists of 4 whorls; the 4 four sepals, 4 petals, 6 stamens and 2 fused carpels. The L2 cell layer gives rise the green parts of the flower. The recruitment of cells into the flower is predictable but not linked to lineage. Cells become incorporated on a probability basis (Furner, 1996). This suggests that signalling may be involved, since cells must determine their position in order to differentiate.

Szymkowiak and Sussex (1992), studied flower development using periclinal chimeras. Using plants that were expressing the mutant fasciated, they were able to show that both the floral meristem size and carpel number were determined by the genotype of the cells in the L3 internal layer. Since carpel initiation primarily involves cells of the L1 and L2 layers, these cells must be receiving positional information from the L3 layer in order to determine the carpel phenotype.

Irish and Bouhidel (1993) also used chimeras, but with the pistillata mutant genotype in sectors of the internal layers L2 and L3. Pistillata causes conversion of petals into sepal-like organs. L1 and L2 derived tissue contribute to the flower. Examination of flower buds suggested that the L1 layer did not function autonomously, and that it required a functional Pistillata gene in order for normal floral organ development.
Tian and Marcotrigiano (1993) used periclinal chimeras of *Nicotiana* to demonstrate the influence of one cell layer on the phenotype of another. *N. glauca* and *N. tabacum* have different body patterns with *N. glauca* producing lateral meristems on vegetative plants, whereas *N. tabacum* does not produce lateral meristems on vegetative plants. Chimeras were produced that comprised of cell layers of a mixture of *N. glauca* and *N. tabacum*. Detailed analysis of chimeras that produced multiple lateral meristems on vegetative tissue i.e. a *N. glauca* phenotype, showed that the genotype of the L3 layer strongly influenced the phenotype in respect to the number of lateral meristems produced. Chimera plants with the *N. glauca* as the L3 layer produced lateral meristems on vegetative plants, whereas those with *N. tabacum* as the L3 did not produce lateral meristems in the vegetative stage. The study further showed that other layers influenced the number of lateral meristems but none were as strongly influencing as the L3 layer.

Studies of genes such as *knotted* and *shoot meristemless*, have also shown that positional signals control shoot meristem development. *KNI* is a gain of function mutation. In the wild type the gene is expressed in the meristem cells and is thought to be involved in the maintenance of the non determination of the meristematic cells (Smith and Hake, 1993). *Knotted* was originally studied in maize, but two similar genes have been cloned in *Arabidopsis* (Lincoln *et al*., 1994). Over expression of one of these genes results in highly lobed leaves, this involves cellular proliferation in the epidermis or L1 layer. Studies of the expression pattern of *KNI* have shown that the mRNA is expressed in the L2 layer of cells, but that the protein is found in both L1 and L2 layers (Langdale, 1994). Fluorescence labelled KN1 protein has been shown
to move readily between cells (Lucas et al., 1995). The KN1 protein also facilitates the transport of KN1 sense mRNA and of dextran molecules up to 20kDa between cell layers (Dangl et al., 1995).

Positional information is also important during the development of roots. Van den Berg et al. (1995) have used laser ablation to study the relationship between different cells in the Arabidopsis root. Roots consist of a vascular region surrounded by pericycle, endodermis, cortex and epidermis cells. Cortical initial cells in the root meristem divide asymmetrically to produce cortex and endodermis cells. Pericycle cells will replace the cortical initial cell if it is destroyed by laser ablation. The invading pericycle cells start to divide asymmetrically, generating the cortex and endodermis cell files that the cortex initial cells would have given rise to. Thus the pericycle cells start to behave like the ablated cortical initial cells, with their new position rather than their original cell lineage determining their cell fate.

Ablation of epidermal initials leads to their replacement by cortical cells, which respond to their change of position by dividing periclinally and developing into epidermal and root cap cells thereby replacing the epidermal initials. Further ablation experiments demonstrated that the meristem produces and specifies the initials, but it is the more mature cells that act as a template determining the developmental pathway which leads to the cell types produced (van den Berg et al., 1995). This suggests a signal movement towards the meristem. Since cells develop according to position, positional information must be dissipated thus requiring some kind of cell to cell communication. Several mechanisms of cell to cell communication have been
proposed including signalling occurring via plasmodesmata, or by the use of diffusable signal molecules.

1.4 Methods of cell-cell signalling

The known functions of the plant hormones auxin, ethylene, cytokinin, gibberellin and abscisic acid have recently been reviewed (Kende and Zeevaart, 1997). These hormones and a growing number of other compounds are involved in plant differentiation and development. Some factors such as arabinogalactan-proteins appear to act via the plasma membrane and the cell wall (Kreuger and van Holst, 1996).

1.4.1 Signalling via the plasmodesmata

Plasmodesmata are complex channels that connect the cytoplasm of neighbouring cells, providing a possible intercellular route for signal molecules involved in patterning and differentiation (Mezitt and Lucas, 1996).

Cells of the embryo are initially all connected by plasmodesmata. As the plant develops, groups of cells become isolated into separate symplastic groups. An example of an isolated symplastic group is the epidermis of Arabidopsis roots. Injection of dye into cells of the root epidermis showed that undifferentiated cells in the root epidermis are symplastic connected. As cells become differentiated they become more symplasticly isolated from their neighbours with a reduction in the transfer of dye between neighbouring cells (Duckett et al., 1994). Cells in the epidermis of the hypocotyl remain linked symplastically to their neighbours, but the
group as a whole becomes isolated symplastically from the other plant organs. The knolle mutant mentioned earlier is unable to establish symplastic domains, due to an inability to produce complete cell walls. These embryos are severely defective in development. Further symplastic isolation is necessary in order for the development of guard cells in leaves (McLean et al., 1997).

In general, molecules up to 1000Da can pass through the plasmodesmata without active transport. However, evidence is accumulating that larger molecules, including proteins that control development, can pass through the plasmodesmata (Mezitt and Lucas, 1996). For example, the transport of the knotted protein (42kDa) described earlier is via the plasmodesmata (Langdale, 1994, McLean et al., 1997).

1.4.2 The role of the cell wall in cell competence and cell signalling

The cell wall plays an important role in cell development. The cell wall physically constricts the cell into a particular shape (Cosgrove, 1997), but there is also evidence that the cell wall is an important part of the cell-cell signalling/differentiation controlling mechanism (Brownlee and Berger, 1995, Dangl et al., 1995, Quatrano and Shaw, 1997).

The initial divisions of the brown alga Fucus embryo are similar to those of the Arabidopsis embryo, with the zygote undergoing an asymmetric division followed by further specific divisions (Quatrano and Shaw, 1997). The differences between the apical and basal cells are maintained as the cells divide and differentiate to become the rhizoid and thallus. Laser ablation of the rhizoid cell after this initial division leaves the thallus cell in contact with the cell wall. This results in a division pattern of
the thallus tissue similar to that of the intact embryo. If either cell is isolated as a
protoplast, they dedifferentiated, thus the cell wall appears to help maintain the
differentiated state of the cell. After further cell divisions, a cell will come into
contact with the portion of the cell wall that was previously in contact with the rhizoid
cell, and this cell will differentiate into a rhizoid cell (Berger et al., 1994). This
suggests, there must be a fate-determining factor present in the rhizoid cell wall. It
must be stable enough to be maintained by the rhizoid cell wall, but it must also be
able to diffuse across the cell wall of the thallus cell. Oligosaccharides in the cell wall
were considered to be a possible candidate for this role (Brownlee and Berger, 1997).

The polarity of the first asymmetric division and division angle is determined by
external light, with the division being perpendicular to the light source. The
unfertilised Fucus egg does not possess a cell wall but within minutes of fertilisation a
cell wall is formed. The cell wall does not at this stage appear to have any
polarisation. The cell wall contains two sulphated fucoidans. A third fucoidan is
sulphated in the golgi vesicles, these are called F-granules. After sulphation F-
granules are transported to the position of polar growth or target site i.e. the point of
rhizoid growth. Polar disposition of cell wall material from F-granules occurs while
the zygote is still symmetrical. The fixation of the polar axis relies on the cell wall.
Zygotes form a polar axis in response to polar light and will fix this axis even if the
direction of the light is changed. Protoplasts of these zygotes are also able to form a
polar axis in response to polar light, but they are unable to fix the polar axis if the
light direction is constantly changed.
At the two-cell stage, rhizoid cells that have their cell walls removed act as zygotes, regenerating a cell wall, and becoming polarised in a different direction to the previous polarisation. An asymmetric division follows the polarisation. Cell wall asymmetry is required for the correct orientation of the initial division, thus the cell wall is important in determining the patterning of the early embryo and further differentiation. Positional information is passed from the cell wall back to the zygote, to fix the axis and determine the angle of the first division. (Quatrano and Shaw, 1997).

Changes in the cell wall or polarisation of the cell wall during asymmetric division are also found during the early stages of somatic embryo development in carrot (Pennell et al., 1992, McCabe et al., 1997). As well as light, classical plant hormones and other signal molecules influence the direction of polarity, asymmetric divisions and differentiation patterns in plants.

1.5 Hormonal signals - Auxin, ethylene, cytokinin involvement in the development of lateral roots, and root hairs

1.5.1 Effects of auxin on roots and the production of lateral roots

Auxin has a role in determining the growth rate of roots, the number of lateral roots produced and in enabling the roots to perceive gravity. Mutations that have an altered response to auxin include aux1, axr1, axr2, axr3 and axr4 (Estelle and Somerville, 1987, Leyser et al., 1996, Wilson et al., 1990, Pickett et al., 1990, Hobbie and Estelle, 1995).
The *aux1* mutant is resistant to both auxin and ethylene, displaying reduction in both growth and gravitropic responses of the root, while the aerial parts of the plants have wild type appearance. *aux1* plants have a reduced size of rosette with crinkly leaves and short petioles. The flowering stems are much reduced and highly branched with reduced fertility or self-sterility. *aux2-1* mutant plants display dwarfism along with reduced number of root hairs and resistance to ethylene and abscisic acid. *aux3* mutants show reduced apical dominance, reduced root elongation and increased adventitious rooting.

1.5.2 Root hairs

The epidermis of the primary root of *Arabidopsis* has two cell types, hair (trichoblast) cells and non-hair (atrichoblast) cells. The cells are arranged in cell files with hair and non-hair cell files. Under control conditions hair cells develop over the join between two underlying cortex cells. Non-hair cells develop in between, in complete contact with a single cortex cell, Figure 1.2 (Dolan and Roberts, 1995, Dolan et al., 1994).

Ethylene promotes hair growth in *Arabidopsis* promoting the emergence of ectopic hair cells i.e. hair cells that are produced in cells in complete contact with a single cortex cell (Tanimoto et al., 1996). Ethylene is produced from methionine via s-adenosylmethionine (SAM), 1-aminocyclopropane-1-carboxylic acid (ACC) using the enzymes, methionine adenosyl transferase, ACC synthase and ACC oxidase. ACC synthase and ethylene perception is inhibited by aminovinylglycine (AVG) and silver ions (Ag⁺) respectively (Tanimoto et al., 1996). Using ACC to increase the
Figure 1.2 Cell type patterning in the *Arabidopsis* root


level of ethylene in *Arabidopsis* root, Tanimoto *et al.* (1996) showed that an increase in ethylene produced hair cells in positions normally occupied by non-hair cells. This increase in ethylene could be blocked by inhibiting the biosynthesis of ethylene using AVG, or by blocking the perception of ethylene using silver ions (Ag⁺). This decreased the number of ectopic hair cell produced. Thus, ethylene can promote hair growth in all files. Auxin also promotes the formation of ethylene, but auxin also has a separate role in the promotion of root hairs. Treatment of wildtype seedlings with AVG, blocks the production of ethylene, and thus reduces the number of root hair cells produced. However, this phenotype can be rescued by the addition of the auxin indole acetic acid (IAA). Thus, auxin can act indirectly to promote root hair growth by inducing the production of ethylene as well as operating directly to induce root hair production (Massucci and Schiefelbein, 1996).
Several mutants have been isolated that are altered in their response to auxin, ethylene, cytokinin and/or root hair production (summarised in Appendix 1). The auxin resistant mutant axr2-1 has a reduced number of hair cells although all are in the normal positions. This mutation also confers resistance to ethylene and cytokinin. The gene TTG is a negative regulator of hair development with the mutant line ttg-1 having almost double the number of hair cells compared to wildtype plants (Masucci and Schiefelbein, 1996).

The characteristics of these and other mutants allowed Masucci and Schiefelbein (1996) to propose a model for hair production in *Arabidopsis* (Figure 1.3). In the wildtype, the level of auxin/ethylene present is enough to promote hair growth. In some cells the genes TTG and GL2 block the auxin/ethylene promotion of hair cell growth resulting in an atrichoblast. However, if the auxin or ethylene concentrations are increased, the blocking effect of the TTG/GL2 genetic pathway is not enough to counter the inductive effect of the auxin/ethylene, and hair cells develop in positions where normally non-hair epidermal cells are found. However, certain characteristics of the non-hair cells are retained in these hair files. Cells which will become hair cells are very dark staining with the histological stain toluidine blue, this indicates a dense cytoplasm. Non-hair cell files stain more weakly.
The CTR-1 (*constitutive triple response*) gene is a negative regulator of ethylene response
(Keiber *et al.*, 1993) with the mutant *ctr1* genotype resulting in a phenotype which
phenocopies the effects of high ethylene treatment. Both the *ctr-1* mutant and
seedlings treated with ethylene precursor ACC have differential staining in the normal
untreated seedling position, with only darkly staining cells across the cortex cell
borders, despite the fact that ectopic hair cells develop later in development. The cells
in the epidermis of wildtype TTG all stain darkly at this stage of development. Thus,
in roots where the ethylene or auxin pathway is up regulated, either by addition of the
hormone or by genetic means, the TTG/GL2 cell patterning is still present but is being
influenced later in epidermal cell development.

1.6 Arabinogalactan-proteins: a marker of cell position and
pattern formation or an integrated part of cell to cell
signalling?

Arabinogalactan-proteins (AGPs) and related glycoproteins are complex
glycoproteins consisting of a protein backbone with carbohydrate side-chains.
Several AGPs have been isolated and/or their distribution characterised (Appendix 2).
The function of AGPs has been under discussion for several years (Fincher et al.,
been linked with plant development, initially due to their cellular expression patterns.
For example, studies using the monoclonal antibodies MAC207, JIM4, JIM13 and
JIM15 demonstrated that AGPs are expressed on the cell surface of developing and
mature cells in a pattern related manner (Knox et al., 1989, Knox et al., 1991). A
summary of AGPs isolated and the antibodies used in detecting AGPs can be found in
Appendix 2.

In carrots, the JIM4 epitope is associated with vascular tissue and particularly with
two sections of the pericycle (Knox et al., 1989). The pattern of expression reflects
cell position rather than any particular cell type. The JIM4 epitope appears early in
the development of the root prior to any visible differentiation in the cells (Stacey et
al., 1990). JIM13 and JIM15 epitopes can also be visualised in the carrot root with
almost opposite expression patterns. JIM13 reacts with future xylem cells,
metaxylem, root cap and the epidermis. JIM15 reacts with all cells including root cap cells, except the epidermis and future xylem cells (Knox et al., 1991). A further antibody JIM8 labels pre-xylem, fully differentiated xylem and root cap cells. Carrot cells grown in suspension culture also express these epitopes. Figure 1.4(i) shows examples of suspension culture cells labelled with the monoclonal antibodies JIM15 and JIM8. A particular cell may express several different epitopes simultaneously (McCabe et al., 1997). The epitopes are found on glycoproteins associated with the plasma membrane and cell wall, plus proteoglycans released into culture media of suspension culture cells.

Certain carrot cells can be induced to undergo somatic embryogenesis in suspension culture. Suspension cultures of cells of hypocotyl origin, in auxin supplemented media, contain a mixture of cells of varying embryogenic potential. On removal of auxin from the media, embryos can develop from single cells and small cell clusters called proembryogenic mass. The level of embryogenic potential in the culture has been linked to the number of single cells in the culture that express an epitope in the cell wall, recognised by the monoclonal antibody JIM8 (Pennell et al., 1992). Epitopes of this antibody are expressed on small spherical cells, some elongated cells, at the edge of some cell clusters in carrot embryogenic cultures (Pennell et al., 1992), and transiently in the ovule of oilseed rape flowers (Pennell et al., 1991). Epitopes are also found in xylem and developing xylem cells. The antibody also recognises plasma membrane associated arabinogalactan-protein (AGPs) and also an epitope associated with golgi-derived vesicles in synergid cells and some embryo cells (Pennell et al., 1991). JIM8 antigens are also found on molecules released into the culture medium (Pennell et al., 1992).
Observation of cell cultures showed that one way embryos develop was through a series of cell changes from a JIM8 positive cell. The small spherical cell undergoes an asymmetric division during which a change in the distribution of the JIM8 antigens occurs. This results in a dividing cell with JIM8 antigens at one end of the cell. The division results in two daughter cells, one expressing the JIM8 antigen and one not (Figure 1.4(ii) and (iii), McCabe et al., 1997). The daughter cell which expresses the JIM8 antigen may undergo programmed cell death. The other daughter cell has a very dense cytoplasm, and this cell divides to become a pro-embryogenic mass. The cells produced by the asymmetric division usually become separated after the division.

Using the cell cultures, McCabe et al., (1997) separated cells expressing the JIM8 antigen using the JIM8 monoclonal antibody and a secondary antibody conjugated to paramagnetic beads. This enabled them to separate cells into populations containing JIM8+ cells, i.e. the cells expressing the JIM8 antigen, and JIM8 – population. The cell type or developmental stage can be described as a cell state. The JIM8 + cell population contains the pre-asymmetric division cells (state B), plus the asymmetric division daughter cell that also expresses JIM8 (state F, after death state G). The JIM8 – population contains the second daughter cells (state C), which normally develop into the pro-embryogenic mass. Thus both cell populations contain cells that are capable of developing into embryos. These populations were cultured separately. Embryos developed in the JIM8 + population but not in the JIM8 – population. Light microscopy of the JIM8 – population revealed the presence of state C cells, but that
Figure 1.4 Arabinogalactan-protein labelling of cell walls of cells from embryogenic cultures, and the relationship to embryogenesis

Reproduced from McCabe et al. (1997)
Labelling of cells in embryogenic suspension culture with monoclonal antibodies and secondary antibody conjugated to a fluorescent molecule.

(i)  a  Single cells labelled with JIM15  
b  Single cells labelled with JIM8  
c  Cell cluster labelled with JIM8  
Bar = 40μm (a, b), 10μm (c)

(ii) Changes in the cell wall labelling pattern during the asymmetric division of a state B cell. a-d labelled with JIM8. Bar=10μm

(iii) Schematic representation of the asymmetric division of a state B cell. Cells are depicted as circles. Circles with a green border indicate cells that would label with the monoclonal antibody JIM8. The asymmetric division of the state B cell produces a daughter cell which also labels with the JIM8 antibody (state F) which may go on to die (State G). The other daughter cell (state C), which does not label with the JIM8 antibody, may go on to produce an embryo.
these cells did not develop into embryos. The embryogenesis in the JIM8 – culture could however be rescued by transferring these cells into culture media that had been pre-conditioned by JIM8 + cells. Pre-conditioning of media involved the collection of media in which JIM8 + cells had been cultured. This demonstrated that cells in the JIM8 + population produce a signal that is secreted into the media. This signal is required by cells in the JIM8 – populations in order for them to progress into the somatic embryogenesis developmental pathway.

1.7 Other soluble signals involved in plant development

Other small signals have been isolated that influence plant development. These are summarised in Table 1.1. These will be discussed in the context of their relation to the cell wall and to nodulation.

1.7.1 Small carbohydrate signal molecules

Pectin is a major polysaccharide found in the primary cell walls of plants. Homogalacturonans are mainly composed of α-1,4-galacturonic acid linked subunits, whereas other pectins contain galactose, arabinose and rhamnose. Several studies have suggested that semi-purified pectins can antagonise auxin at low concentration. In one particular study, tobacco thin cell layers which comprise epidermis, chlorenchyma, collenchyma and parenchyma layers were induced to develop roots, flowers or vegetative shoots, depending on the auxin/cytokinin ratio (Creelman and Mullet, 1997). Flower formation or root inhibition can also be produced by exposure to oligogalacturinides. The mechanism of action of these oligogalacturinides is not known. Xyloglucan oligosaccharides have also been implicated in the growth of
<table>
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<tr>
<th>Small signal molecule</th>
<th>Common functions</th>
<th>Isolated mutants</th>
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<td>Small signal molecule</td>
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<tr>
<td>Polyamines</td>
<td>Changes in levels of polyamines can affect leaf morphology, root growth and flower formation.</td>
<td></td>
<td>Walden <em>et al.</em> (1997).</td>
</tr>
<tr>
<td>Jasmonic acid (JA)</td>
<td>Exogenous JA promotes senescence. Inhibits photosynthetic genes and possibly involved in UV protection. Inhibits germination of non-dormant seed and stimulates the germination of dormant seeds. Inhibits roots growth. IAA antagonist in coleoptiles. Possible roles in flower, fruit and seed formation. Pollen production. May be involved in protein storage. Involved in insect and disease resistance.</td>
<td><em>Coil</em> coronatine insensitive (also JA insensitive), <em>fad3-2</em>, <em>fad7-2</em> and <em>fad8</em> (fatty acid desaturase, JA deficient).</td>
<td>Creelman and Mullet (1997).</td>
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plants, with the mutant murl (deficient in fucose) unable to produce the xyloglucan XXFG (Figure 1.5) (Reiter et al., 1997). A similar mutant mur4 is deficient in arabinose. The double mutant of murl, mur4 has a severely dwarfed phenotype (Reiter et al., 1997).

Further evidence that carbohydrate molecules can influence development comes from several studies which have isolated tri- or tetra-saccharides with biological activity. When cells are cultured at low-density, cell death occurs. However these cells can be rescued by growing them in media supplemented with media conditioned by cells grown at high density (Steinbrenner et al., 1989). The active compound in the conditioned media was found to have a molecular mass of about 1000Da with a proposed structure as shown in Figure 1.5 (Schroder and Knoop, 1995). Other similar small carbohydrate molecules have been isolated, from flax fibres and from rice anthers. Extraction of semi-retted flax with water produced a mixture of pectic oligosaccharides and two acidic rhamnogalacturonide tetrasaccharides. When added to protoplasts one of the acidic rhamnogalacturonide saccharides was shown to cause increased activity of enzymes or proteins related to defence mechanisms (Dinand et al., 1997). The tetra-saccharide molecule isolated from rice anthers has the following structure; O-β-L-arabinofuranosyl-(1→3)-O-α-L-arabinofuranosyl-(1→3)-O-β-D-galactopyranosyl-(1→6)-D-galactopyranose. This structure is closely linked to part of a glycan chain of an arabinogalactan-protein found on the surface of cells in rice anthers and therefore could be derived from an AGP (Kawaguchi et al., 1996).
Figure 1.5 Small signal molecules isolated from plants and plant / *Rhizobium* symbiotic systems

Reproduced from Creelman and Mullet (1997), Schroder and Knoop (1995), Long (1996), (a) Xyloglucan signal molecules, Carbohydrate residues are shown as Fuc-fucose, Gal-galactose, Xyl-xylose, Glc-glucose. (b) Carbohydrate factor which enables cells to survive when grown at low density, Fuc-fucose, GalA-Galacturonic acid, Glc-Glucose. The enzymes α-Fucosidase, β-glucosidase, Polygalacturonase and lectins UEA, Con A, LCA were used in the calculation of the signal structure. (c) NOD factor involved in the signalling in *Rhizobium* / Plant symbiosis.

(a) Xyloglucan

```
                Fuc
               |
               Gal     Gal     Gal
               |
             Xyl     Xyl     Xyl     Xyl     Xyl
               |
               Glc — Glc — Glc — Glc     Glc — Glc — Glc — Glc
               |
XXFG          XLLG
```
Figure 1.5 continued

(b) Viability factor

\[ \text{ConA, LCA} \]
\[ \beta - \text{Glucosidase} \]

\[ \text{UEA I} \]
\[ \alpha - \text{Fucosidase} \]

\[ \text{Polygalacturonase} \]
\[ \alpha - 1,2/1,3 \text{ or } 1,4\text{-linked} \]
\[ \beta - \text{linked} \]
\[ \alpha - 1,4\text{-linked} \]

(c) Lipo-oligosaccharide (NOD factor)
1.7.2 Nodulation and plant development

Nodulation is the process by which the symbiosis of legumes and *Rhizobium* produces nitrogen-fixing nodules on the roots of leguminous plant species. Nodules on leguminous plants are initiated by root hair deformation and the dedifferentiation of cortical cells in response to lipo-oligosaccharides (Nod factors) produced by rhizobial bacteria. Each species of *Rhizobium* appears to produce a variety of Nod factors consisting of an oligosaccharide backbone and specificity producing side groups (Figure 1.5). As yet no Nod receptors have been isolated, but there is the suggestion that more than one receptor may be involved in the plant response, as experiments have shown that both ends of the Nod factor are important in triggering the full response (Long, 1996, Fisher and Long, 1992). Nod-like factors have not been found in plants that are not infected by *Rhizobium*, so there is no evidence that Nod-like factors act *in planta* as normal hormonal signals. Nod factors could be mimicking natural plant signals or could be taking advantage of plant defence responses that often respond to chitinaceous elicitors (Long, 1996).

Application of auxin transport inhibitors or cytokinin can produce developmental changes similar to nodule morphogenesis, and activate early nodulin induced genes (ENOD). Nod factors also change the local auxin / cytokinin ratio thereby inducing cell division suggesting that the Nod factors may be mimicking an as yet unknown plant signal.

Other bacteria such as *Agrobacterium tumifaciens* and *A. rhizogenes* cause changes in the root growth by altering the production of auxin and cytokinin or changing the
plant's response to these hormones (Long, 1996). The link is further demonstrated in plants with the \textit{axr1} mutation. This reduces both the frequency and size of crown galls and hairy root tumours induced by \textit{A. tumefaciens} and \textit{A. rhizogenes} (Lincoln \textit{et al.}, 1992). Crown gall tumours had a different morphology compared with wildtype tumours and did not survive if removed from the root. Hairy root tumours on \textit{axr2} plants did survive after removal from the main plants. The roots have reduced hair number, as do \textit{axr2} plants themselves. Synthetic lipo-chitoooligosaccharides have been shown to alleviate the requirement for auxin/cytokin in to sustain tobacco protoplast growth and stimulate the \textit{axil} promoter (Rohrig \textit{et al.}, 1995).

Ethylene has also been shown to be involved in the nodulation process (Spaink, 1997). \textit{ACC}, the enzyme involved in the biosynthesis of ethylene, accumulates in the pericycle opposite to the nodule primordia. The mutant \textit{sickle} of \textit{Medicago} shows a decreased sensitivity to ethylene. These mutants show an increased infectivity to their \textit{Rhizobium} symbiont, with infection threads passing into the cortex cells, rather than just the epidermis, as in wildtype plants.

One of the early expressed genes in response to Nod factors is the ENOD 40 gene. This particular gene has raised the possibility that small peptides influence the differential state of cells and thus the development of plant roots (Brewin and Legocki, 1996, van de Sande \textit{et al.}, 1996). ENOD40 is first expressed in the pericycle opposite to the nodule primordia, prior to cortical cell divisions. Clones of this gene contain two conserved regions, a small open reading frame at the 5' end encodes a peptide of 10-13 amino acids (tobacco 10, soybean 12 and pea, alfalfa, vetch 13) and a region in the middle of ENOD40 that does not contain an open reading frame. The
open reading frame was translated in tobacco protoplasts and the expression correlated with an increase in the tolerance to auxin. Further evidence suggested that the effect of auxin is not the primary factor in inducing protoplast division, but that another factor, possibly a small peptide, acts firsts to specify auxin action (Miklashevichs et al., 1996). Whether the ENOD40 protein is involved in cell-cell signalling between the pericycle and the dividing cortex cells has yet to be established.

In summary, Nod factors from the bacterial symbiont induce a variety of changes in the plant. Some of these can be explained by changes in the ratios of known hormones, however there is evidence that other molecules are involved in this process and it is important to clarify whether Nod factors themselves are acting as a mimic for some previously unknown plant morphogen.
1.8 Aims of project

For direct studies of cell-cell signalling in plants, it is necessary to find a source of signals. Whole plant extracts involve many stages for purification and require large starting quantities, with the likely result of very low yields. Many of the AGPs that are expressed in cell cultures are also expressed in plant roots. Cell suspension cultures are composed of single cells and small cell clusters. This makes the cell wall accessible, and also opens up the possibility of easy extraction of cell signals. Experiments studying the early stages of embryogenesis have shown that cells in carrot embryogenic cultures produce soluble signals that effect the development of other cells. Using these cultures, this project aimed:

1. To see if soluble signals extractable from the cell walls of embryogenic cultures caused developmental changes in seedling roots.
2. To characterise the effects of the signals on the seedling roots.
3. To purify cell signals
4. To isolate mutants of Arabidopsis with reduced susceptibility to the cell signals.
5. To begin characterisation of a sample of these mutants.
Chapter 2 – Materials and Methods

2.1 Plant material, cell and seedling culture

2.1.1 Plant material source

Carrot seeds, (Daucus carota L. Early Nantes) were purchased commercially from Covent Garden. Arabidopsis seed lines were obtained from the Nottingham Arabidopsis Stock Centre, the Arabidopsis Biological Resource Centre-Ohio, USA or Lelhe Seeds, USA. Seed lines were bulked, by sowing directly onto New Horizon compost and grown either in a greenhouse with supplementary lighting to produce a long day photoperiod or in temperature controlled growth rooms at 25°C, with a 16 hour photoperiod.

2.1.2 Base culture media

Cells and seedlings were grown using B5 culture media (Table 2.1) made as per manufacturer instructions, with 2% sucrose. This is referred to as B5.0 medium. Cell proliferation cultures were grown in B5.44 medium; B5.0 supplemented with 0.442mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Seedlings were also grown in medium supplemented with cell wall signal extract (CWSE); this medium is based on B5.0 and is described in section 2.3.1. Media were sterilised by autoclaving at 121°C for 20 mins.

2.1.3 Seed sterilisation

For sterile tissue culture experiments, using carrot or Arabidopsis seeds, seeds were first surface sterilised using one of two methods. The more pitted nature of the carrot seed coat necessitates a much harsher and longer sterilisation treatment than is needed
<table>
<thead>
<tr>
<th>Table 2.1 Composition of Gamborg’s B5 Medium</th>
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<tr>
<td>Reproduced from Gamborg (1970)</td>
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<td></td>
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<tr>
<td>Macroelements</td>
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<tr>
<td>KNO₃</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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<tr>
<td>NaSO₄.7H₂O</td>
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<tr>
<td>MgSO₄.7H₂O</td>
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<td>CaCl₂.2H₂O</td>
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<td>Microelements</td>
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<td>MnSO₄.4H₂O</td>
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<td>H₃BO₃</td>
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<td>CuSO₄</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
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<tr>
<td>KCl</td>
</tr>
<tr>
<td>Vitamins</td>
</tr>
<tr>
<td>Nicotinic acid</td>
</tr>
<tr>
<td>Thiamine-HCl</td>
</tr>
<tr>
<td>Pyridoxine-HCl</td>
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<tr>
<td>myo-inositol</td>
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<tr>
<td>Carbon</td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>pH 6.0</td>
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</table>
for *Arabidopsis* seeds. The methods described, produce between 70-95% sterile carrot seedlings and almost 100% sterile *Arabidopsis* seedlings.

Carrot seeds were surface sterilised by immersion for 10 mins in 70% ethanol followed by 10% Sodium hypochlorite for 20 min. After three washes in dH₂O the seeds were sown onto solidified media (see below). Sterile germinating seedlings were transferred to fresh medium after 2-3 days incubation at 25°C.

*Arabidopsis* seeds were surface sterilised, by a similar method to carrot seeds but with reduced exposure to Sodium hypochlorite i.e.10 min, or by immersing the seeds in 70% ethanol to wet the seeds followed by two applications of 90% ethanol. The seeds were air-dried before sowing onto solidified media.

### 2.2 Production of cell wall signal extract (CWSE) and culture media auxin control (*B₅₄₋₉*)

#### 2.2.1 Initiation of carrot cell suspension cultures

The carrot embryogenic suspension cultures were initiated by surface sterilising carrot seeds as described (Section 2.1.3) and sowing them onto *B₅₋₀* media solidified using 0.7% bactoagar (Difco). After incubating at 25°C in the dark for 2-3 days, seeds were transferred to fresh sterile *B₅₋₀*. Seedlings were then cultured for a further 4-5 days, after which seedling hypocotyls were cut into 10mm long sections. 10 sections were incubated in a 50ml conical flask containing 10ml *B₅₋₄₋₄* media at 25°C on a shaker rotating at 100rpm for 21 days. The hypocotyls were removed by sieving the resulting cell and hypocotyl suspension through a 250μm nylon mesh. 10ml of fresh *B₅₋₀* was added and the culture incubated for a further 14 days. The cell suspension
was added to 50ml fresh B₅₋₄₄ in a 250ml conical flask and incubated as before. Cultures were subcultured every 7-12 days; 10ml culture was added to 50ml sterile B₅₋₄₄ in a 250ml conical flask. Cultures become embryogenic after approximately 1 month and were used until they were 9 months old. After this time the embryogenic potential of the culture begins to decrease.

Embryogenic suspension cultures that are aged between 1 and 9 months contain a large number of single cells that express an epitope to the monoclonal antibody JIM8 (Pennell et al., 1992). Some of these cells are capable of dividing asymmetrically and producing a daughter cell that has the potential to develop into an embryo. This daughter cell requires signals in order to develop, via a small cell cluster group called a proembryogenic mass, into a full embryo (McCabe et al., 1997). Within the embryogenic suspension culture the daughter cell can develop up to the proembryogenic mass stage. Thus, the signals that support this early growth stage must be produced by cells in embryogenic suspension cultures aged 1-9 months. These cultures were therefore used as a source of developmental signals.

As a contrast the L1 culture was used as source of signals from a culture with extremely low embryogenic potential. The L1 line was originally set up using a similar methodology as described above at least 16 years ago. The culture has been subcultured weekly since being set up, however it now has an extremely low embryogenic potential (Lloyd et al., 1979).
2.2.2 Production of cell wall signal extract

CWSE production was based on extraction methods described by Pennell et al. (1991). The aqueous fraction collected contains a high concentration of carbohydrates, which include antigens of the monoclonal antibodies JIM7, JIM8, JIM13 and JIM15. These correspond to the range of antibody epitopes found on the cell walls of the suspension culture cells.

Pennell et al. (1991) extracted cell walls with dH₂O for 2 hours. Since experiments showed that the carbohydrates were released from the cell walls almost immediately (Roger Pennell personal communication), the time of the extraction was reduced. Suspension cultures, 8 days after subculture, were centrifuged at 1500rpm for 3 mins to collect the cells. The supernatant or conditioned media was decanted off. The packed cell volume and remaining conditioned media, usually had a volume of approximately 2ml, to this 16ml of dH₂O was added. The cell suspension was agitated for 5 mins before centrifuging at 1500rpm for 3 min. The supernatant or cell wall signal extract (CWSE) was removed and the cells discarded. The conditioned media and CWSE were filtered through Whatman's number 1 filter paper, to remove any cells or cell debris not removed by centrifugation, and frozen (-20°C) until use. Prior to assaying or purification CWSE or conditioned media were re-filtered through filter paper followed by a 0.45µm filter and 0.22µm filter (Micropore). The 0.22µm filter served to remove any small cell wall debris and to sterilise the solution. Filter sterilisation was used in preference to autoclaving as it would produce the least level of damage to possible signal molecules.
2.2.3 Production of culture media auxin control (B\textsubscript{5.4.9})

The culture media in which the cells are grown contains the auxin 2,4-D (B\textsubscript{5.44}). Auxin can affect the development of seedling roots at concentration in the region of $10^{-7}$ M, however 2,4-D has an effect on root growth at $10^{-8}$ M (Estelle and Somerville, 1987). I therefore decided to use a diluted form of the culture media in which the suspension culture cells were grown as a control. This enabled me to compare the chemical characteristics and partition characteristics of the auxin and other media components to any potential signal molecules in the CWSE.

During the production of cell wall extract, following the first centrifugation used to separate the carrot cells from the culture media, a small amount of the culture medium remains along with the cells as the pellet. The amount of medium in this pellet cannot be estimated, however the volume of the total pellet could be estimated as approximately 2ml. The pellet was then washed with 16ml H\textsubscript{2}O. To prepare an auxin control for the CWSE, fresh B\textsubscript{5.44} medium was diluted 1:8 with dH\textsubscript{2}O. This was named B\textsubscript{5.4.9}. Thus this control contains the maximal level of auxin contamination that could be present in the CWSE as a result of contamination from the original culture medium.

2.3 Effect of CWSE on the root development of carrot and Arabidopsis seedlings

2.3.1 Effect of samples on seedling root development

Cell wall signal extracts, B\textsubscript{5.4.9}, or fractions from purification experiments were tested for their effect on the growth of Arabidopsis and carrot seedlings. Carrot or
Arabidopsis seeds were surface sterilised and sown onto solidified media supplemented with CWSE, B₅₋₀, or the purification fractions.

Initially B₅₋₀ medium was prepared using Gamborg’s B5 media (Table 2.1) and low temperature melting agarose No VII (Sigma) (1 %w/v). At this stage the media were made to 10, 75 or 90% of the final volume using dH₂O. The media were sterilised by autoclaving at 121°C for 20 mins. Media were either maintained at 55°C to prevent solidification and used immediately, or allowed to cool, stored sterile at room temperature for up to 1 month prior to heating to 70°C to melt the agarose, then maintaining it at 55°C prior to use.

The CWSE, purification fraction or control solution (B₅₋₀ or dH₂O) to be tested was filter sterilised through a 0.22μm filter, and diluted to the required concentration using filter sterilised dH₂O. Samples were mixed with the pre-prepared B₅₋₀ to give the required media and sample concentrations. The supplemented media were poured into 150mm diameter petri dishes for all carrot assays, 60mm diameter petri dishes for Arabidopsis assay and 100mm petri-dishes for Arabidopsis mutant selection (Section 2.13).

Surface sterilised Arabidopsis or carrot seeds were sown onto the media and stored for 3-7 days in the dark at 4°C. Petri dishes were placed semi-vertical at 25°C, in long-day conditions (16h day, 8h night), unless otherwise stated. Where possible seeds were sown 10 per petri dish. A minimum of two replicates were used, although the usual number was three. The exception to this was during the retesting of some mutant phenotypes, where the low numbers of seeds available restricted the replicate
number to one. Seedlings were scored for root length and the number of lateral roots
produced per seedling. Graphs showing data from these root biassays include error
bars, which show the standard error of the data.

2.3.2 Sectioning of carrot seedlings

Short segments of carrot roots grown on control BS0 media, and CWSE supplemented
media were fixed in 2.5% glutaraldehyde in PEM buffer for 1 hour (Sigma). After
washing thoroughly with dH2O the segments were exposed to 2% osmium tetroxide
(Sigma) for 30mins and washed again in dH2O. Segments were dehydrated using a
sequential series of 20%, 40%, 60%, 80%, and two stages of 100% acetone. The
segments were incubated at room temperature in each dilution of acetone for 20mins
each except for the last 100% acetone incubation, which was incubated at 4°C
overnight.

Epoxy resin (Sigma) was prepared by mixing equal quantities of solution (A) 38%
SPI-PON 812 Resin, 62% 2 dodeden-1-y1 succinic anhydride and (B) 53% SPI-PON
812 Resin, 47% Methyl-5-norbornere-2,3-dicarboxylic anhydride. 12.5ml of this
resin solution was mixed with 375µl 2,4,6-tris (dimethyl aminomethyl) phenol to
produce the infiltration resin. The segments were infiltrated with this resin using a
sequential series of 30% or 60% resin diluted with acetone and two stages of 100%
resin. Segments were incubated in each dilution for at least 3 hours. The last 100%
infiltration was incubated overnight. Seedling segments were arranged in mould
blocks and covered with 100% infiltration resin. The resin was solidified by heating
the blocks to 60°C overnight.
Sections of approximately 10μm thickness were cut and stained using 0.5% toluidine blue.

2.3.3 Sectioning of *Arabidopsis* seedlings

*Arabidopsis* seedlings were sectioned in blocks of Historesin (Leica). The seedlings grown for either 5, 10 or 14 days on B5-0, B5.4.9 or carrot CWSE supplemented media were fixed by flooding the petri-dishes in 1% glutaraldehyde (Sigma), 50mM sodium cacodylate (Sigma) for 10 mins and washed with dH2O. Seedlings were removed from the agarose medium and washed further with dH2O. Seedlings were dehydrated using a sequential series of 33%, 66% and two changes of 100% ethanol in dH2O for 30 mins each. Seedlings were then infiltrated using a sequentially diluted series of infiltration resin (100ml Historesin + 1 packet of activator (Leica)) diluted to concentrations of 33%, 66%, and 100% with ethanol. The sections were incubated in each dilution for 30 min. Samples were finally incubated in 100% infiltration resin overnight. Seedlings or small sections of seedlings were arranged in moulds and embedded by covering the samples with embedding resin (100% infiltration resin mixed at a ratio of 15ml resin to 1ml hardener dimethyl sulphoxide (Leica)). The blocks were covered with plastic covers. This resin sets in 1-2 hours at room temperature. Blocks were cut into 8μm-10μm sections and stained by dipping slides into 0.5% toluidine blue solution for 30 secs prior to rinsing with dH2O.
2.4 Determination of fraction chemical during fractionations and purification

2.4.1 Protein

The presence of protein and aromatic compounds was assayed by recording the absorbance of UV light at 280nm by the sample.

2.4.2 Carbohydrate

Total carbohydrate concentration was assayed using a method based on the carbohydrate assay published by Dubois et al. (1956). Briefly 0.4 ml of fraction was mixed with 10μl of 80% phenol in a 1ml eppendorf tube. 1ml concentrated sulphuric acid was added quickly using a Gilson pipette. This ensured rapid mixing. The chemical reaction between the phenol and sulphuric acid is exothermic thus, fractions were allowed to cool before being transferred to plastic cuvettes and the absorbance at 485 nm was recorded. Increasing absorbance was proportional to increasing carbohydrate concentration. The absorbance produced by individual carbohydrates varies. As sucrose is the major component of B5,0 media, it was used as a standard.

2.4.3 Immunochemistry – ELISA

20μl of column eluate was pipetted into wells of Immunon 1 ELISA 96 well plates, and diluted with 60μl of TBS (1mM Tris, 150mM NaCl). Standards for monoclonal antibodies (MAbs) were JIM8 – gum arabic, JIM5 pectin. Plates were sealed and placed at 4°C overnight. Plates were blocked using 0.2% dried milk (Marvel) in PBS (dmPBS) for at least one hour. After washing wells five times with PBS pH 7.2 (16mM Na2HPO4, 8.5mM NaH2PO4, 150mM NaCl), 80μl of a 1:9 dilution of primary antibody diluted with dmPBS, was added. The plates were incubated, sealed with
Parafilm at 4°C for 2 hours. The primary antibody solution was removed by washing five times in PBS solution. 80μl of a 1 in 2000 dilution of the secondary antibody antiRat IgG conjugated to hydrogen peroxidase (Sigma) in dmPBS was added to each well, followed by incubation at 4°C for one hour. After washing each well five times with PBS solution plates were developed using 80μl TMB microwell peroxidase substrate. The reaction was stopped after 30 mins by added 20μl of 2N H₂SO₄. Absorbance at 450nm were recorded.

2.5 Stability of CWSE to heat treatment and stability over time

The stability of the activity in the CWSE was tested by heating CWSE to a range of temperatures between 20°C (room temperature) and 121°C prior to cooling the samples and testing them for root altering activity. The bioassay of the samples using Arabidopsis included a cold treatment for up to 5 days followed by incubation for up to 14 days at 25°C. To test the length of time the CWSE was active, samples were stored at either 4°C or 25°C for up to 14 days, and frozen at -20°C after this incubation. Each sample was then assayed at the same time for root altering activity.

2.6 Fractionation of CWSE using size exclusion chromatography

2.6.1 Dialysis of CWSE taken from cultures of different ages

The embryogenic potential of a particular culture changes over time with the culture having it’s greatest embryogenic potential between 3 and 9 months (90-270 days) (Pennell et al., 1992). To see if the activity level obtained from the CWSE was linked to the age of the culture from which the CWSE was obtained, CWSE was collected from cultures over a period of 320 days. Samples were stored at –20°C. CWSE was
also obtained from an L1 culture. Half of the sample was dialysed using a 25,000 cut-off dialysis membrane against 2 changes of 10L dH2O. Samples were tested for their root altering activity as described in section 2.3.

2.6.2 Fractionation of CWSE using Sephadex test columns

Dialysis of the CWSE showed that the CWSE appeared to contain at least two active molecules of differing molecular mass. To separate these more efficiently, Sephadex columns were used. CWSE was freeze-dried or concentrated using rotary evaporation under vacuum at 40°C. 1 ml of 10x CWSE was loaded onto 20ml columns of Sephadex G-10, G-15 or G-25 (Sigma). 2ml fractions were eluted using dH2O. The activity found in the CWSE sample was used as a positive control. This was prepared by diluting a sample of the 10x CWSE, 1 in 5 using dH2O. Culture media were supplemented to a concentration of 5% with column fractions or CWSE control and were tested for root altering activity using the method described in section 2.3.1.

2.6.3 Comparison of the elution of CWSE and B5.4.9 from 20ml Sephadex G-10 column

1ml of 5x CWSE produced by rotary evaporating CWSE, or 1 ml of 5x B5.4.9 was loaded onto 20ml Sephadex G-10 column equilibrated with dH2O. 1ml fractions were eluted using dH2O, and tested for root altering activity at a concentration of 5% using the methods described in section 2.3.1.
2.7 Fractionation of CSWE and B_{5.4.9} using size exclusion membranes

CWSE, B_{5.4.9} (or fraction F collected from Sephadex G-10 column (Section 2.10)) were separated by passing samples through molecular weight cut-off membranes with pore sizes 500, 1000, and 3000 Da using a 10ml Amicon pressurised unit. Compressed air was used to supply pressure. The membranes were either used serially starting with the larger pore size or individually. Fractions not passing through each membrane were also collected. This was achieved by adding 10ml dH_{2}O to the pre-filter chamber in the Amicon unit after the sample separation prior to membrane removal. The solution was stirred under atmospheric pressure for 30 mins, collected, and treated as the solution that had passed through the membrane.

Samples for the initial fractionation shown in section 4.3.4 were used to supplement B_{5.0} at a concentration of 25%.

2.8 Partitioning of CWSE and B_{5.4.9} with ethyl acetate

30ml of CWSE or B_{5.4.9} was shaken with 10ml of ethyl acetate. The solvents were separated using a separating funnel, and the process repeated twice. The two sets of ethyl acetate fractions were pooled. Excess anhydrous sodium sulphate was added to each set to remove any contaminating H_{2}O. The ethyl acetate was decanted away from the sodium sulphate using a glass pipette. The ethyl acetate was removed from the ethyl acetate fractions and the aqueous fractions by rotary evaporation under vacuum at 40°C. Both samples were re-dissolved/made up to 30ml with dH_{2}O, before assaying for root inhibiting activity.
2.9 Isolation of a JIM8 antigen using FPLC ion exchange chromatography in preparation for carbohydrate residue analysis

50ml of CWSE was loaded onto a mono Q 5/5 column and washed with 30ml solution A at a flow rate of 1ml/min. Fractions were eluted using a gradient to 100% solution B at 1 ml/min over 60 min. The composition of solution A and B are described below, sections 2.9.1 and 2.9.2. Fractions were collected during the loading and washing of the column; these were pooled as “load” or “wash”. Two different sets of buffers were used with different desalting methods (Sections 2.9.1 and 2.9.2).

2.9.1 NaCl gradient

Buffers
A 10mM Tris pH7.2
B 10mM Tris pH7.2, 1.2M NaCl.

Desalting of fractions. Fractions from 3 separate fractionations were pooled, freeze-dried and re-suspended in 3ml of dH2O. 2.5ml of these fractions were loaded onto a PD-10 desalting column (Pharmacia). 4 fractions were collected during and after sample loading, while eluting the column with dH2O. These represent fraction 1 (load 0-2.5ml), 2 (void volume, 3-6ml) containing molecules with molecular mass >2500Da, 3 (column separation zone 6.5-9.5ml) containing the majority of the NaCl and 4 (Late separation zone, 10-12.5ml) the fractions where the majority of the CSWE activity was shown to elute. Fractions were assayed for root altering activity using the Arabidopsis bioassay at 10% of the fraction concentrations.
2.9.2 Ammonium bicarbonate gradient
Buffers

A 10mM Ammonium bicarbonate
B 2M Ammonium bicarbonate

Desalting of fractions. Fractions were rotary evaporated at 40°C and were re-dissolved in 5ml dH₂O. 1ml of this was made up to 5ml with dH₂O. The 5ml were evaporated as above, before re-suspending the samples in 5ml dH₂O. The evaporation and re-suspension was repeated. Untreated CWSE was mixed with ammonium bicarbonate to a concentration of 2M, evaporated and re-suspended three times. Fractions were added to media at a concentration of 25% to assay the fractions for root altering activity (Section 2.3).

2.10 Isolation of compounds exhibiting root altering activity in preparation for carbohydrate residue analysis

2.10.1 Fractionation of CWSE using 250ml Sephadex G-10 column
1L of CWSE was freeze dried and re-dissolved in 30ml dH₂O. 10ml of concentrated CWSE was loaded onto a 250ml Sephadex G-10 and eluted using dH₂O. 5ml fractions were collected and pooled from each of the 3 runs. Fractions were further pooled as follows and tested for activity.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>8ml volume eluted by 0-50ml dH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.5ml each elution volume fractions 55-80ml dH₂O</td>
</tr>
<tr>
<td>B</td>
<td>0.5ml each elution volume fractions 85-105ml dH₂O</td>
</tr>
<tr>
<td>C</td>
<td>0.5ml each elution volume fractions 110-135ml dH₂O</td>
</tr>
<tr>
<td>D</td>
<td>0.5ml each elution volume fractions 140-165ml dH₂O</td>
</tr>
<tr>
<td>E</td>
<td>0.5ml each elution volume fractions 170-190ml dH₂O</td>
</tr>
</tbody>
</table>
G

0.5ml fraction eluted with 195ml dH₂O made up to 9ml
with fraction eluted with 200-250ml dH₂O.

Each pooled fraction was made up to 9ml with dH₂O and assayed for root inhibiting
activity.

2.10.2 Preparation of fraction F from Sephadex G-10 column for thin
layer chromatography (TLC) or Flash chromatography

CWSE was prepared for TLC or Flash chromatography by initially separating the
CWSE on a 250ml Sephadex G-10 column as detailed above (Section 2.10.1). 50ml
of fraction F collected from this column (eluting at 170-190ml) was passed through a
500Da molecular cut-off filter as described in section 2.7. Both >500Da and <500Da
fractions were collected. 50ml of <500Da fraction was concentrated to a volume of
2ml on a rotary evaporator at 40°C. This will be referred to as fraction F <500.

2.10.3 Thin layer chromatography (TLC) of fraction F <500

Determination of mobile phase

Fraction F <500 was separated using TLC. 3 x 10μl of the fraction was dotted onto a
2 x 10cm TLC silica plate impregnated with a fluorescent dye allowing each sample
to air dry in between each application. After air drying the sample was visible as a
dark spot in a fluorescent background under short-wave UV and as a fluorescing spot
under long-wave UV. Plates were developed using mobile phases including,
methanol, ethanol, acetone, and chloroform and mixture of these solvents including
dH₂O until a separation of the visible components under the two types of UV had
taken place (data not shown). Appendix 3 shows the polarity of solvent used during the TLC experiments.

*Viewing of possible carbohydrate components found on TLC plates after fractionation of fraction F <500.*

Carbohydrate was viewed on TLC plates by spraying the plates with a fine mist of phenol, $\text{H}_2\text{SO}_4$ solution (3g phenol dissolved in 5ml $\text{H}_2\text{SO}_4$ followed by 95ml in ethanol) followed by heating the plate to 130°C for 15min. Carbohydrate appears as brown dots on the TLC plate.

*Recovery of activity from TLC plates.*

To test if the active compounds could be recovered from a silica plate, a plate was developed using a selected mobile phase of 4: 4: 2 methanol: acetone: $\text{dH}_2\text{O}$. The developed TLC plate was viewed under UV light and cut into 6 sections corresponding to dots visible on the plate. Separated components were retrieved from the TLC plate, by scraping the silica from the background plate into eppendorf tubes. The silica was covered in 2ml of the mobile phase and agitated for about 5 mins before centrifuging the samples at 10,000rpm. The liquid supernatant was removed and the process repeated with the pellet of silica. The two supernatants were pooled and the solvents removed by evaporation at 40°C. Samples were dissolved in 2ml $\text{dH}_2\text{O}$. The $\text{H}_2\text{O}$ was removed at 40°C and samples were dissolved in 2ml $\text{dH}_2\text{O}$. Half the samples was made up to 6ml with $\text{dH}_2\text{O}$, filter sterilised and bioassayed. The other half was pooled and filter sterilised to form the “Total” fraction. 30µl of the sample used for the TLC was diluted to 6ml using $\text{dH}_2\text{O}$ and filter sterilised to form the “pre”
sample. The fractions were added to media at a final concentration of fraction of 25% and assayed for root altering activity (Section 2.3.1).

2.10.4 Fractionation of fraction F <500 using Flash Chromatography

1 ml of fraction F <500 was loaded onto a 250ml silica column attached to Flash 40 unit (Biotage UK LTD) and fraction eluted using the following series of solvents:

9: 1 Chloroform: Acetone
followed by 1: 1 Chloroform : Acetone
followed by 1: 8 : 1 Chloroform : Acetone : H₂O

For the polarity of these solvent see Appendix 3.

Fractions of approximately 25ml were collected. The solvents were removed at 30°C under a vacuum. Residues were dissolved in 1ml H₂O. 80µl of each fraction were pooled into groups of fractions as follows and made up to 8 ml with dH₂O prior to assaying the pooled fractions for root inhibiting activity at a concentration of 25% pooled fraction (Section 2.3).

Group A Fractions eluted by 1L of 9: 1 chloroform: acetone.
Group B Fractions eluted by first 500ml of 1: 1 chloroform: acetone.
Group C Fractions eluted by second 500ml of 1: 1 chloroform: acetone.
Group D Fractions eluted by 250ml 1: 8: 1 chloroform: acetone: H₂O

The solvents generated 35, 40 and 10 fractions respectively. 1ml of each fraction was pooled into the following groups, 1-35, 36-55, 56-75, and 76-85. The solvents were
removed under a vacuum at 40°C and the samples were suspended in 8ml dH₂O. Fractions were tested for root altering activity at a concentration of 25%.

The individual fractions, numbered 1-55 and 76-85 were also tested for root altering activity by removing the solvents under a vacuum at 40°C and resuspending each in 1ml dH₂O. The absorbance at 280nm was recorded. 80ul of the sample was diluted to 8ml with dH₂O, filter sterilised and assayed for activity using the Arabidopsis root bioassay at a concentration of 25%.

2.11 Glucosyl chemical analysis of fractions

Fraction 16-18ml from an ion exchange separation (Section 2.9.1), fraction C from the large Sephadex fractionation (Section 2.10.1), fraction F >500 Da (section 2.10.2), fraction F<500 (Section 2.10.2), and Flash Biotage individual fractions 27, 38 and 79 (Section 2.10.4) were analysed for glucosyl composition by Dr L. Scott Forsberg at the Complex Carbohydrate Research Center at the University of Georgia, USA. The method used is as described in McCabe et al. (1997). Fractions were freeze dried and hydrolysed in 1M methanoic HCl at 80°C for 18 hours and N-acetylated and trimethylsilylated (York et al., 1985). The trimethylsilyl glycosides were subjected to gas chromatography-mass spectrometry (electron impact) (GCMS) analysis, using a 30-m DB-5 fused silica capillary column.
2.12 Isolation of Arabidopsis mutants resistant to the effects of the carrot CWSE

2.12.1 Isolation of Arabidopsis mutants resistant to carrot CWSE from EMS mutagenised M2 populations

CWSE was filter sterilised and mixed in a ratio of 1:9 with B\textsubscript{5.0} medium such that the final concentration of components were, B\textsubscript{5} salts (Table 2.1), 2% sucrose, 1% agarose and 10% CWSE. M2 ethyl methane sulphanate (EMS) mutagenised seeds obtained from Lelhe seeds, USA, were surface sterilised (Section 2.1.3) and sown at a density of 100 / 100mm diameter petri dish. Petri dishes were stored at 4°C for 4 days before transferring to continuous light for 3 days and short-days (8h day, 16h night) for a further 4 days. Petri dishes were incubated horizontally. Putative mutants were selected on the basis of their resistance to root growth inhibition by the CWSE or a change in the response of the lateral roots to the CWSE. Seedlings were transferred to compost after 10 days and grown under short-day conditions until seeds were harvested.

2.13.2 Mutant re-screen

M3 seeds were surface sterilised and sown 10 seeds per petri dish on 10% CWSE and B\textsubscript{5.0} medium. At least 2 replicates of each treatment were produced although 3 were used where possible. Petri dishes were incubated at 4°C for 3-5 days before sealing with Nescofilm and transferred to 25°C and long day conditions. Petri dishes were placed semi-vertical. The root length and the number of lateral roots produced were scored after 10 days growth. 3 seedlines that were representative of the range of phenotypes found, were grown on dilution series of CWSE.
2.14 Initial characterisation of individual mutants

To investigate the genetic dominance of the mutants isolated, chosen mutants were crossed to the wildtype seedline *coll, gll*. This seedline was the wildtype used in the mutagenesis. Plant flowers were prepared for crossing by emasculating closed mutant flowers using forceps. All other flowers, including those setting seed, open flowers and very immature flowers were removed. This left only carpels containing unfertilised eggs in ovaries. Pollen from the *coll, gll* plants was transferred, by brushing mature stamens across the stigmas of the mutants. The resulting F1 seeds were collected and screened for resistance by sowing onto media containing 25% CWSE. Both resistant and susceptible plants were transferred to soil, and grown at 25°C, under long-day conditions. The plants were allowed to self-fertilise and F2 seed was collected. The F2 populations were screened for the resistance phenotype on 10% CWSE.
Chapter 3: Investigation of the effects of carrot cell wall signal extract (CWSE) on the growth and development of carrot and Arabidopsis seedlings

3.1 Introduction

Plant cell walls are made up of a complex arrangement of cellulose, pectins, proteins and glycoproteins (York et al., 1985). Many of the different molecules are bound by chemical bonds. Therefore, cell walls can be extracted using a range of solvents, producing solutions with different proportions of cell wall components (Zablackis et al., 1995). AGPs are expressed in cell walls and cell membranes of carrot embryogenic cultures. Similar AGPs are present in seedling roots (Stacey et al., 1990, Knox et al., 1991, Rae et al., 1991, Li et al., 1992, Pennell et al., 1992, Schindler et al., 1995). In both systems the expression patterns of the AGPs are developmentally related.

McCabe et al. (1997) showed that cells of embryogenic cultures produce soluble signals that controlled cell fate. A population of cells, designated by the AGP epitopes expressed in the cell walls, produced a set of signals that enabled other cells to undergo differentiation. Using distilled water, or NaHCO₃, extracts can be produced which contain antigens that label with anti-AGP MAbs (Pennell et al., 1992). AGPs are considered to be a candidate for cell-cell signals (Chasen, 1994), for example, an AGP which binds to the ZUM 18 antibody has been shown to increase the number of embryos produced by an embryogenic culture (Kreuger and van Holst, 1995).
Chapter 3 - Results

This study aimed to study signalling in plants, using the fact that AGP epitopes expressed in cell cultures are also expressed in the cell wall of root cells. Since, the cell wall of suspension cultured cells is more accessible to extraction than root cell walls, embryogenic cultures were used as the source of cell wall signals.

In this chapter, I will describe the effects of the cell wall signal extracts (CWSE) on seedling roots of carrots and *Arabidopsis*. Seedlings were grown on culture media which had been supplemented with the CWSE produced by extraction of the suspension-cultured cells. This was to assess any biological activity of these signals on cells in normal plant tissue, as opposed to the suspension culture cells, which are exposed to the more artificial cell culture conditions.

The suspension culture cells are grown in a medium containing 0.44mg/L of the auxin 2,4-D. Since this hormone is known to causes changes in root development (Estelle and Somerville, 1987), seedlings were also grown on a diluted form of this medium. The aim was to compare the possible level of auxin contamination in the CWSE and the quantitative and qualitative effects of the diluted culture medium (B$_{5,4,9}$).

The signal transduction pathways of several hormones eg, auxin, ethylene and cytokinins are thought to interact (Kende and Zeewaart, 1997). Therefore, seedlings of plants that have reduced sensitivity to auxin, ethylene and cytokinins were also grown on the CWSE and control B$_{5,4,9}$ media, to test their resistance to the effects of the CWSE in relation to their response to the auxin 2,4-D.
3.2 Effect of CWSE on the growth and development of carrot seedling roots

Carrot seeds were surface sterilised and sown onto media supplemented with CWSE. The seeds were germinated under long day conditions, 16 hour day, 8 hour night and scored for root length and the number of lateral roots produced over a 29 day period (Figure 3.1a). The effect of the CWSE was concentration dependent, with increasing concentration producing shorter roots and an increase in the ratio of lateral roots to primary root length (Figure 3.1b). Lateral roots produced were also much shorter than control roots (Figure 3.2a, d, and e). At high concentrations there is a breakdown in the normal cellular arrangements of the root, with an increase in overall diameter of both primary and secondary roots (Figure 3.2 b-e). To investigate the nature of the cellular breakdown, seedlings were embedded in epoxy resin and sectioned transversely (Figure 3.2 f, g). Sections showed an increase in the number of cells in the epidermal layer. The number of cells in the cortex also increased but in this layer there was a change in the shape and organisation of the cell layer. Cells often became detached from their neighbours and were not arranged in longitudinal columns as in the control grown seedlings.
Figure 3.2 Effect of CWSE on the structure of carrot seedling roots

Carrot cell wall signal extract causes inhibition of root growth and expansion in root diameter. Root cellular organisation is also disrupted.

(a) Carrot seedlings grown on B5 control media.
(b, c) Carrot seedlings grown in CWSE supplemented media. All are 24 day old seedlings.
(c) Carrot seedling root. (b, c) Primary root tips, (d, e) Lateral roots
(f, g) Transverse sections of primary roots.

E = Epidermis,
C = Cortex,
En = Endodermis,
P = Pericycle,
X = Xylem,
Ph = Phloem,
Bar = 50µm.
3.3 Effect of CWSE on the growth and development of Arabidopsis seedling roots

*Arabidopsis thaliana* is a small plant with a rapid life cycle (6-12 weeks), with >7000 mutant seedlines available. To test if the signals present in the CWSE could also influence the development of *Arabidopsis* seedling roots, seeds were sown onto media supplemented with CWSE. The seeds were kept at 4°C for 4 days before being transferred to 25°C, long-day conditions.

The effects of the CWSE on the *Arabidopsis* roots were similar to the effects of the CWSE on the carrot seedlings (Figure 3.3 to 3.7). The main difference was a reduction in the level of cellular detachment of cortex cells. In carrot seedling roots exposed to CWSE, the cortex became highly disorganised with cells becoming detached from their neighbours. In *Arabidopsis* cell detachment only occurred at very high concentrations/activity with greater than 95% root inhibition.

The effects were concentration dependent with some effects visible at concentrations of 1% CWSE. The roots were reduced in length and the number of lateral roots produced changed (Figure 3.3a). Roots were increased in diameter and there was also occasional flattening of both primary and secondary roots, so that transverse sections showed an asymmetric root pattern (Figure 3.4 and 3.5). Flattening was more frequent in lateral roots, and was often the result of root fasciation (Figure 3.4f).

There were no visible effects on the seedling shoot, which was not in contact with the agarose, except on very high concentrations of CWSE, where leaf expansion was

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Figure 3.3 Effect of CWSE on the growth of *Arabidopsis* seedlings

*Arabidopsis* seeds were sown onto media supplemented with CWSE made from carrot embryogenic culture cells. The graph shows the effect on the growth and development of *Arabidopsis* seedling roots over time compared with B₅₀ grown seedlings.
(a) Root length (mm).
(b) Number of lateral roots.
(c) Ratio of the number of lateral roots produced per mm primary root growth.
(d) Effect of varying concentration of CWSE on the growth of *Arabidopsis* seedlings. Root length, laterals roots, and ratio of lateral roots / mm primary root growth expressed as % inhibition of control grown seedlings (14 days).

- ▢ CWSE - root length
- □ Control - root length
- ▢ CWSE - lateral roots
- □ Control - lateral roots
- ▢ CWSE - lateral roots/mm root
- □ Control - lateral roots/mm root
Figure 3.4 Effects of CWSE on *Arabidopsis* seedling roots

*Arabidopsis* seeds were sown on CWSE and B₃₆₀ media. The CWSE causes inhibition of elongation and changes the shape of roots produced.
a, c 5 days old control
b, d 5 days old CWSE
e, g 15 days old control
f, h, i 15 days old CWSE.
Figure 3.5 Sections of *Arabidopsis* seedling roots grown on CWSE supplemented medium

Transverse sections of 28 day old *Arabidopsis* seedling roots grown on; B<sub>5</sub> medium (a) or CWSE supplemented medium (b).

- at = atrichblast.
- t = trichoblast.
- et = ectopic trichoblast.
- c = cortex cell layer.
- c? = multiple cell layers, possibly of cortex cell type.
- x = xylem.

Bar = 50 μm
reduced. This is likely to be the result of the reduced root growth and a possible concurrent reduction in the seedling’s ability to absorb media nutrients. Alternatively it may be that the leaves of seedlings receive a reduced dose of the signal or they may only be sensitive to higher concentrations of the CWSE signal.

*Arabidopsis* seedlings grown for 5 or 14 days on CWSE supplemented media were embedded using Historesin and sectioned. Sections showed the increase in diameter of the roots was due to an increase in the number of cells layers with a concurrent increase in the number of cells/layer (Figure 3.5, 3.6, 3.7). In roots, which have become flattened, a plate or sheet of xylem tissue was occasionally present (Figure 3.5).

Transverse sections of the seedling roots also showed the presence of ectopic trichoblasts i.e. hair cells in positions that are not over the join of two cortex cells (Figure 3.5 and 3.7).

In *Arabidopsis*, growth on CWSE changed the overall shape of the roots. The diameter of the root increases and in some cases, roots flatten, such that they became asymmetrical instead of radial in transverse section (Figure 3.5). Two processes control the rate of root growth, the increase in the number of cells and the elongation of those cells (Doerner et al., 1996). The changes in structure of the root appeared to be the result of a change in the plane of division within the meristem such that extra layers of cells were produced, combined with a decrease in cell expansion (Figure 3.6 and 3.7). The cells also do not appear to differentiate as quickly as cells in the meristems of control grown plants (Figure 3.6). This may suggest an effect on the
Figure 3.6 Serial sections of the apical region of *Arabidopsis* primary root grown on CWSE supplemented media and B$_{5.0}$ media

Approximately 10µm transverse sections for the root apical region of 14 day old *Arabidopsis* seedling. Bar = 50 µm. All sections at same magnification.

From left to right:

Column a-c = serial sections of CWSE grown root. Sections run from top to bottom and left to right, (i.e. top section of column b is section 6)

Column d = section of control root grown on B$_{5.0}$ medium. Sections run from top to bottom and are the growth stages equivalent to approximately every third section of the CWSE sequence.
Figure 3.7 Sections of *Arabidopsis* seedling roots grown on CWSE supplemented medium

*Arabidopsis* seedlings were grown for 14 days on 10% CWSE. 10um transverse section were cut. This figure shows example of sections taken approximately every 50um.

l – lateral roots emerging

t – trichoblast, et – ectopic trichoblast

Bar = 50 μm All sections at same magnification.
quiescent centre of the meristem, which functions to suppress differentiation, and maintain the initial cells which surround it (Doerner, 1997).

On germination the primary meristem initially appeared to produce the correct number of cell layers as an almost normal radial pattern was visible in the older root tissue (Figure 3.7j-l). However, younger root tissue found nearer the meristem, had an increased number of cell layers present in transverse section (Figure 3.6, Figure 3.7b-d). The number of cells present in the meristem increases as the root grows, producing a root with an increased number of cell layers and consequently an increased diameter. In some seedlings this meristematic expansion occurs in an asymmetric manner, creating a root with an asymmetric transverse section (Figure 3.5).

Patterning in roots is established in the embryo for primary roots and prior to emergence for secondary roots. The number of periclinal divisions in the meristem determines the number of cell layers that will be present in the root (Dolan et al., 1994). After embryogenesis, the differentiation of the cell in the root is influenced by signals that pass from the older more mature differentiated cells down to the differentiating cells (van den Berg, 1995). Under these circumstances the CWSE must be interfering with this signalling process, either by changing the signalling transport or overwhelming the signalling system by increasing the amount of signal present. If the signals are the limiting factor which determines the diameter and number of cell layers within the root, then the latter explanation may explain the increase in cell layers supported within the CWSE grown roots. High concentrations of ethylene or auxin also have similar effects on *Arabidopsis* roots.
3.4 Growth on CWSE causes irreversible changes to the pattern of the primary root in Arabidopsis

The changes in root caused by the CWSE could result in irreversible changes to the root structure and growth rate, or alternatively changes to the patterning could be reversible. If the changes are reversible then on removal from the CWSE, the root would change its growth rate and growth structure such that any new growth should be similar to roots of seedlings grown on control medium. It was also possible new growth may be a mixture of reversible and irreversible patterns.

To investigate these possibilities, seeds were sown onto CWSE or control B5.0 media. After 4 days at 4°C the seeds were transferred to 25°C. Seedlings were transferred from CWSE supplemented media or B5.0 media to B5.0 medium, after 4 days at 4°C or 1, 2, 4 or 8 days at 25°C. The seedling growth for those seedlings transferred on 1, 2 or 4 days were recorded on days 1, 2, 4, 8 days (Figure 3.8). The growth of the seedlings incubated at 4°C was also recorded on these days. For these seedlings, day 0 is the 4th day of incubation when seedlings were transferred to fresh B5-0 medium and 25°C.

During growth on CWSE the growth rate of the root is reduced. However, after seedlings were transferred to control medium the growth rate of the primary root increases to match the growth rate of seedling roots transferred from control medium to control medium (Figure 3.8). This implies the reduction in longitudinal growth rate caused by the CWSE is a reversible effect.
Figure 3.8 Changes in the structure and development of *Arabidopsis* roots treated with CWSE

*Arabidopsis* seeds sown on CWSE supplemented and B₅₀ media were incubated at 4°C for 4 days after which they were transferred to 25°C (day 0). Seedlings were transferred to fresh B₅₀ on days 0, 1, 2 or 4 days after transfer from to 25°C. The graphs show the effect of this treatment on the growth of the primary root (a) and on the number of lateral roots/mm primary root (b). Transferring seedlings reduced the No. of lateral roots produced per mm root length in both control and extract seedlings. Seedlings grown on CWSE exhibit inhibition of primary root growth. After transfer to B₅₀ the rate of this root growth increases to almost match the rate of growth of the control roots.
After about 8 days on CSWE, some roots started to exhibit flattening of the primary root. When these seedlings were transferred to B5.0, the longitudinal growth rate of these roots increased to match the control root growth. However the new growth produced by these meristems also have the transverse flattened shape (Figure 3.9). Thus the patterning changes produced by the CWSE were irreversible. This indicates that the CWSE effects on the processes of pattern development and the root elongation are separate processes.

Signal molecules that influence the type of cell an undifferentiated cell develops into, are thought to pass from the more mature cells down to the undifferentiated cell (van den Berg et al., 1995, Wysockadiller and Benfey, 1997). The maintenance of the patterning after removal of roots from the CWSE is consistent with this theory since the new portions of the root produced are following the altered template, rather than reverting to the normal root patterning.

The maintenance of this alternative root shape also requires the maintenance of a larger root meristem. The fact that the larger meristem is maintained suggests that either permanent changes have occurred to the meristem, or alternatively an increased level of signal being passed down towards the meristem from the increased root size maintains the larger meristem.

The transfer between media also affects lateral root production. The number of lateral roots produced is reduced, the later the transfer occurs on both control and CWSE transferred seedlings. Lateral roots are mostly produced after 4 days incubation. Since there is no statistical difference between the number of lateral roots produced
Figure 3.9 Growth of seedlings on CWSE causes irreversible changes to the root structure

8 day old CWSE grown seedlings, which have flattened root structure, were transferred to B5-0. 
(a) The rate of length growth increases to match the growth rate of control grown roots however, the flattened asymmetric structure is maintained. Arrows show extended flattened root 
(b) Lateral roots produced post transfer appear to have normal morphology. An extended fasciated coiled root shown by blue arrow. Black arrow shows lateral root produced after transfer with normal morphology. 
Bar = 100 μm
on the seedlings originally grown on CWSE or those original grown on B5.0 their appears to be no permanent effect of CWSE on the number of lateral roots produced.

Longitudinal expansion of lateral roots initiated prior to transfer, returns to control level when seedlings are transferred to B5.0. New lateral roots that are generated after the transfer to control medium develop as normal control grown lateral roots as regard to diameter and shape (Figure 3.9b). Thus, new lateral root meristems are able to develop normally.

3.5 Effect of carrot CWSE and B5.4.9 on Arabidopsis seedlings

When Arabidopsis seedlings are grown on CWSE, the roots are stunted, the number of lateral roots is changed and hair cells appear in ectopic positions. The CWSE is produced from cells which have been cultured in the auxin 2,4-D. Auxin and ethylene also produce similar effects on root development when seedlings are grown on media supplemented with them.

The cell cultures are grown in B5.44 medium that contains 0.44mg/L of the auxin 2,4-D. During CWSE production, the cells of the suspension culture are centrifuged to pellet them and washed with distilled water. This suspension is centrifuged and the supernatant is collected, this is the CWSE. It is possible that a small amount of 2,4-D from the culture medium could contaminate this CWSE.

To test if the maximum 2,4-D contamination could account for the root altering activity in the CWSE, carrot cell culture was centrifuged and the supernatant


Figure 3.10 Effect of CWSE and auxin on the root growth of *Arabidopsis* seedlings

(a) During production of cell wall extract from 50ml of culture, 2ml of cells and culture medium remains after the first centrifugation. 16ml distilled H₂O is added to these cells. The maximum contamination from B₅₄.₄ in CWSE is thus 11%. B₅₄.₄ diluted to this ratio will be called B₅₄.₉. This represents the 0.049mg/L of 2'4D present in this solution. The effects of the CWSE or B₅₄.₉ on the growth of *Arabidopsis* seedling roots was tested. The CWSE contained 3-5 times the root altering activity found in the B₅₄.₉.

(b) Extract

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(c) Extract
discarded. The total volume of the cells plus small amount of remaining medium was estimated to be approximately 2mls. Since 16mls distilled H₂O is added at this stage in CWSE production, a 2,4-D control was prepared by diluting 2 ml B₅₄₄ with 16 ml H₂O. This is referred to as B₅₄₉.

The CWSE and B₅₄₉ were serial diluted and assayed for activity using the Arabidopsis root bioassay. Seedling root length and number of lateral root were scored after 14 days (Figure 3.10). The CWSE showed approximately 3-5x the level of root inhibiting activity found in the B₅₄₉. This was estimated by recording the amount of CWSE that was required to produce 40% root growth inhibition. 40% inhibition was chosen since this is half of the maximum root inhibition produced by the two treatments under the experimental conditions used. To reach this level requires 0.33% CWSE whereas, 3 times the level or 1% B₅₄₉ was required to produce the same level of inhibition. 2% B₅₄₉ was required to produce the maximum level of increase in the ratio of lateral roots produced per mm root length compared with only 0.2% of CWSE. It is also important to restate that the volume of B₅₄₄ used to produce the B₅₄₉ is equivalent to the maximum possible contamination level.

Kreuger et al., (1996) showed that in fresh embryogenic cucumber culture the 2,4-D concentration falls from 9μM to 0.5μM after about 5 days. The fact that the concentration of 2,4-D medium used produces less activity than the CWSE, even though the auxin contamination level in the CWSE must in fact be much less than this, suggests that auxin contamination from medium is not the source of the majority of the activity in the CWSE. This does not however discount the possibility that cells are sequestering auxin in the cell walls, which is then released during water extraction.
3.6 Effect of carrot CWSE on mutants of Arabidopsis known to effect the growth and development of the root

Auxin and ethylene influence the development of the root. Auxin acts both directly on cells and indirectly by increasing the levels of ethylene present in roots (Romano et al., 1993, Roman et al., 1995). The previous section demonstrated that the level of root altering activity found in the CWSE was higher than would be expected if the activity was the result of auxin contamination from the culture medium alone. However, the molecules found in the CWSE may be using the same signal transduction pathways as auxin or ethylene, in order for the phenotype to develop. Alternatively, the CWSE molecules may be altering the levels of auxin or ethylene within the root or changing the cell's response to the hormones.

ENOD 40 is a gene found in legumes which encodes an oligopeptide of about 10 amino acids. The gene alters the legumes response to auxins (van de Sande, 1996). Changes in the sensitivity to auxin, in auxin resistant mutants, can also alter the sensitivity to infection by Agrobacterium rhizogenes and Agrobacterium tumefaciens (Lincoln et al., 1992). The sensitivity to auxin influences the response of plants to infection, and to the external signals involved with infection. However, the sensitivity to auxin can be also be influenced by signals from the infecting bacteria via NOD factors, and the resulting peptide produced by the ENOD40 gene.

A homologue of the soybean ENOD40 gene has been isolated from the non-legume, tobacco. When constituitively expressed in protoplasts this gene alters the sensitivity of these protoplasts to auxin, demonstrating that both non-legumes and legumes sensitivity to auxin can be influenced by this gene (van de Sande, 1996).
Some of the genes involved in root development were discussed in Section 1.5 and they are also listed in Appendix 1. The interaction of some of the wildtype genes are shown in Figure 1.3. Auxin and ethylene are known to influence both the growth of *Arabidopsis* root and the development/differentiation of individual cell types within the root. To test if any of the genes involved in the transduction of the hormones signals were involved in the seedling response to the CWSE, seedlings of several hormone transduction pathway mutants were grown on CWSE and B$_{5,4,9}$ supplemented media. These experiments aimed to see if the seedlings were resistant to the effect of the CWSE and B$_{5,4,9}$. If the seedlings were resistant to both treatments, the experiments would further ascertain if they were resistant to the same degree. The wildtype seedline for all the mutants tested was *coll*.

*AUXI* and *AXRI* act as two branches of the ethylene/auxin transduction pathway that converge at *AXR2*. The pathways are stimulated by ethylene. Ethylene production is induced by auxin. Auxin is also thought to act directly on the *AXRI* pathway. *AXR4* is thought to be epistatic to *AUXI* with respect to root elongation but act additively with respect to the production of lateral roots. *axr1, axr4* double mutant is more resistant to auxin than either of the parental mutants, producing less lateral roots than either parent. *AXRI* and *AXR4* thus appear to act additively. If both *AXRI, AUXI* genes contain mutations, the double mutant is more resistant to auxin than either parental mutant. The mutants *axr1-3* and *aux1-7* have approximately the same level of resistance to the auxin 2,4-D (Tempte *et al.*, 1995).
The genes *ETR1, EIN4, CTR1, EIN2, EIN3* and *EIN5* are also all involved in a third pathway between ethylene and induction of root hair formation, and root growth (Guzman and Ecker, 1990, Keiber *et al.*, 1993, Roman *et al.*, 1995). Mutants of these genes are available and have the following phenotypes: *etr1-3* has longer roots than wildtype; *ein4-1* has approximately the same length roots as wild type; whereas *ctr1-1* mutants have much reduced root elongation. *ctr1-1* plants appear very similar to those supplied with ethylene, with reduced root length caused by a reduction in cell elongation (Kieber *et al.*, 1993). The mutant *eto1-1* produces 2-5 times the normal levels of ethylene compared to light grown wildtype seedlings (Guzman and Ecker, 1990). The mutation is believed to cause increased conversion of SAM to ACC, the rate limiting step, in ethylene production. *ein2-1, ein3-1* and *ein5-1* act epistatically to *etr1-3, ctr1-3* and *ein4-1* (Roman *et al.*, 1995).

The reaction of the roots of the auxin and ethylene single mutants, to both CWSE and B$_{5.49}$ was tested. Figure 3.11(a) and Figure 3.11(b) show that some of the mutants were resistant to the effects of the CWSE and to B$_{5.49}$. The level of resistance shown by each mutant to B$_{5.49}$ and CWSE are summarised in Table 3.1.

With respect to root length growth, the single mutants *axr1-3, axr4-2, axr2-1, ein2-1, ein3-1* and *ein5-1* were equally resistant to the CWSE as they were to B$_{5.49}$. However responses with respect to lateral root production varied. There was no significant difference between the effect of CWSE and B$_{5.49}$ on the mutants, *axr1-3, axr4-2* and *ein3-1*. Both *axr1-3* and *axr4-2* have significantly less lateral roots when grown on control medium than *coll* grown on control medium. In this particular experiment (test
Figure 3.11 (a) Effect of CWSE and B₅-4.9 on mutants involved in root development

Mutants of *Arabidopsis*, which were resistant to ethylene and/or auxin, were grown on CWSE or B₅-4.9 supplemented media. The percentage inhibition of root growth and of lateral root production was recorded for mutants grown on CWSE and B₅-4.9 treatments compared with mutants grown on control medium.
Figure 3.11 (b) Effect of CWSE and B$_{5-4.9}$ on mutants involved in root development

Mutants of Arabidopsis, which were resistant to ethylene and/or auxin, were grown on CWSE or B$_{5-4.9}$ supplemented media. The percentage inhibition of root growth and of lateral root production was recorded for mutants grown on CWSE and B$_{5-4.9}$ treatments compared with mutants grown on control medium.
### Table 3.1 Summary of effect of B5-4.9 and CWSE on mutants of Arabidopsis resistant to auxin and/or ethylene

<table>
<thead>
<tr>
<th>Mutant genotype</th>
<th>Root length growth</th>
<th>Number of lateral roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistance to B$_{5,4.9}$</td>
<td>Resistance to CWSE</td>
</tr>
<tr>
<td>axr1-3</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>axr1-3, axr4-2</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>axr4-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>aux1-7, axr4-2</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>aux1-7</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>axr2-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>etr1-3</td>
<td>0/+</td>
<td>0/-</td>
</tr>
<tr>
<td>ctr1-1</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>eto1-1</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ein2-1</td>
<td>0/-</td>
<td>0</td>
</tr>
<tr>
<td>ein3-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ein4-1</td>
<td>0</td>
<td>0/-</td>
</tr>
<tr>
<td>ein5-1</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Table 3.1** B$_{5,4.9}$, ethylene and CWSE cause root inhibition. Mutants that are resistant to auxin or ethylene were grown on B$_{5,4.9}$ and CWSE to test their response to these treatments. The Table summarises the level of resistance to the treatments found for each mutants.

**Primary root growth**
- root inhibition is more than the root inhibition caused in wildtype.
- no difference between mutant inhibition and wildtype inhibition.
+ to ++++ root inhibition is less than the root inhibition caused in wildtype.

**Lateral root production**
- increased level of inhibition in mutant compared with wildtype.
- no difference between mutant inhibition and wildtype inhibition.
+ to ++++ reduced level of lateral root inhibition.

S : No difference in response between response to CWSE and B$_{5,4.9}$ was found,
D : Difference in response to CWSE and B$_{5,4.9}$ was found.
d : small but not conclusive difference found.
of axr1-3 and axr4-2 responses), there was no significant difference between the response of coll to the CWSE and its response to the B5.4.9. This was due to the high concentration of CWSE and B5.4.9 used, which resulted in near maximal inhibition levels. There was also no difference in response to the CWSE and B5.4.9 by axr1-3 and axr4-2. axr1-3 did not change the number of lateral roots produced compared with its control grown seedlings, in contrast to the coll plants which decreased the number of lateral roots produced. The axr4-2 mutant actually increases the number of lateral roots produced in response to both CWSE and B5.4.9. The responses shown represent approximately the same level of resistance to both auxin and CWSE.

The other mutants axr2-1 and ein5-1 react differently to the CWSE and B5.4.9 with respect to their lateral root production. Although axr2-1 appeared to be resistant to both CWSE and B5.4.9 with regard to lateral root inhibition, the mutant appeared to be more resistant to the effect of CWSE than B5.4.9. ein 5-1 appeared to be more resistant to B5.4.9 than CWSE with respect to lateral root production, with B5.4.9 inducing an increase in the number of lateral roots produced whereas the CWSE grown seedlings had a relatively unchanged number of lateral roots compared with control grown mutant. Control grown ein5-1 did not produce significantly different number of lateral roots compared with coll. The reaction to CWSE of ein5-1 is also not significantly different to coll, with the number of lateral roots produced by the CWSE treated ein5-1 and coll not being significantly different. However, auxin produced a significant increase in the number of lateral roots produced by the mutant ein5-1 plants.
Several single mutants tested were more resistant to CWSE than to B5.4.9 with respect to the inhibition of root length. For example, B5.4.9 produces 65% inhibition of *coll* root growth, and only 30% inhibition of *auxl-7* root growth. However, the reaction to CWSE was significantly different. CWSE inhibits *coll* seedling root growth by 80% whereas there was no significant inhibition of root growth in *auxl-7* seedlings on the same CWSE concentration.

At the concentration used both B5.4.9 and CWSE inhibit the number of lateral roots produced by the *coll*. Both these treatments increased the number of lateral roots produced by *auxl-7*. However, the change in the number of lateral roots produced by the B5.4.9 was much less than the difference produced by the CWSE. The B5.4.9 produced a reduction in lateral roots of 35% in the wildtype, whereas in the *auxl-7* mutant the same level did not produce a significant change in the number of lateral roots produced compared with control grown *auxl-7* seedlings. The CWSE inhibited root production to the higher level of 82% in the wildtype, which is converted to an increase in lateral root production of 95% in the *auxl-7* mutant.

Both the lateral root results and the root elongation data suggests that *auxl-7* is more resistant to CWSE than it is to the auxin in B5.4.9.

Four other single mutants, *etr1-3, ein4-1, ctr1-1, ein2-1* and *eto1-1* were slightly more resistant to CWSE that B5.4.9, with respect to root length with the amount of inhibition of root growth being equal with both CWSE and B5.4.9, whereas the two treatments has a significantly different effects on *coll*. *etr1-3* and *ctr1-1* were also more resistant to CWSE than B5.4.9 with respect to inhibition of lateral roots. *ein2-1* and *ein4-1*
responded to B$_{5.4.9}$ by increasing the inhibition of lateral roots produced. This was in contrast to the reaction of these mutants to CWSE. The level of inhibition of lateral root production in response to CWSE was the same for *ein4-1* and for *coll1*. *ein2-1* reacted to CWSE by inhibiting lateral root production to less than, or the same as, the inhibition of lateral root production by *coll1* plants grown on CWSE. *eto1-1* was equally resistant to CWSE and B$_{5.4.9}$ with respect to lateral root inhibition.

Two double mutants were also tested. Hobbie and Estelle (1995) showed that with respect to root length inhibition, the double mutant *axr1-3, axr4-2* is more resistant to auxin than either of its two parental lines. This was consistent with the results in Figure 3.11a although the difference between *axr1-3* and *axr1-3, axr4-2* was not significantly different in this particular set of data due to the large standard errors. Although, the difference between the reaction of *axr1-3, axr4-2* to CWSE and B$_{5.4.9}$ was just outside the normal levels of significant difference (p=<0.1), the level of resistance shown by the mutants to B$_{5.4.9}$ is consistent with published data. Thus the mean resistance for this mutant is likely to be correct. However, this would result in it being unlikely that the mutant is as resistant to CWSE as it was to B$_{5.4.9}$. The double mutant *axr1-3, axr4-2* reacted to the B$_{5.4.9}$ and CWSE equally with respect to the number of lateral roots produced.

A second double mutant *aux1-7, axr4-2* produced a different resistance pattern to CWSE compared with the B$_{5.4.9}$ supplemented medium. As stated above the *axr4-2* mutant was equally resistant to both B$_{5.4.9}$ and CWSE with respect to both root elongation and the number of lateral roots produced. Figure 3.11a shows that the double mutant of *axr4-2* and *aux1-7* reacted differently to CWSE compared with
B₅₄.₉. The double mutant had a resistance to B₅₄.₉ comparable to the auxl-7 parental line, consistent with Hobbie and Estelle (1995). However, although the level of inhibition produced by the CWSE is the same as the inhibition by B₅₄.₉ in the double mutant, in col1 the CWSE produced a higher level of inhibition. This suggested a higher level of resistance to CWSE. This was probably the result of the effect of auxl-7, which was more resistant to CWSE than B₅₄.₉ in the single mutant. Hobbie and Estelle (1995) suggested auxl-7 acted epistatically to auxr4-2 with respect to its response to auxin induced root inhibition. However, the high resistance to CWSE exhibited by auxl-7 was attenuated in the double mutant of auxr4-2, auxl-7 such that the resistance recorded was approximately half way between the resistance of the single parental mutant lines. This suggests a less directly epistatic relationship with respect to CWSE activity than suggested in Hobbie and Estelle (1995) for auxin activity.
3.7 Discussion

Cell walls are thought to play an important role in cell development and
differentiation and possibly in cell-cell signalling (Brownlee and Berger, 1995,
McCabe et al., 1997). Arabinogalactan-proteins are developmentally regulated and
changes in the AGPs on a cell can predict it’s developmental pathway (Pennell and
process of carrot somatic embryogenesis in vitro is also linked with changes in the
AGPs present in the cell walls. AGPs are found on the plasma membrane, cell wall
and are excreted into the culture media of these cultures (Pennell et al., 1991, Kreuger
and van Holst, 1993, Serpe and Nothnagel, 1994). Aqueous extracts of the cell walls
from embryogenic cultures (CWSE) contain AGP related molecules (Pennell et al.,
1991, McCabe et al., 1997).

This chapter demonstrates that this CWSE influences the development of seedling
roots in culture, causing inhibition of longitudinal root growth and changes in the ratio
of lateral roots to the root length, in carrot and Arabidopsis. In carrot, roots increase
in diameter and cortex cells become disorganised. Arabidopsis roots increase in
diameter with some becoming flattened. There is an increase in the number of cell
layers and number of cells within each cell layer, with a concurrent decrease in cell
size. Investigations comparing the level of root inhibiting activity found in CWSE
and an auxin containing medium suggests that the root inhibiting activity found in the
CWSE could not be due to contamination of the CWSE by culture medium. This was
supported by the reaction of auxin/ethylene mutants, which are involved in root
development. Some of the mutants tested reacted differently to CWSE and the auxin
containing control, confirming that the root inhibiting activity in the CWSE is not the result of contaminating 2,4-D from the cell culture medium.

It may be that the CWSE is changing the sensitivity the root to auxin or that it alters the bias in which the signal is transferred down each of the branch pathways. The data suggests that the cell walls of carrot somatic embryogenic cells contain active compounds that are either influencing the known auxin/ethylene signal transduction pathway directly, or that are able to change the sensitivity of the root cells to auxin or ethylene.

In the next chapters I will discuss studies of the stability and chemical composition of the CWSE including further comparisons with B_{5,4,9} (Chapter 4,) prior to purification of the activity in the CWSE (Chapter 5).
Chapter 4: Initial analysis of Carrot cell wall signal extract properties and composition

4.1 Introduction

The results discussed in Chapter 3 showed that aqueous carrot wall signal extract (CWSE), isolated from the carrot somatic embryogenic cultures affected the root development of carrot seedlings and Arabidopsis seedlings. The effects of the CWSE on Arabidopsis seedlings can be summarised as an increase in root diameter, with a decrease in length of both the primary and secondary roots. The ratio of the number of lateral roots produced per mm of primary root length is usually increased.

Studies of the effects of CWSE and B_{5,4,9} on several mutants of Arabidopsis, that have altered sensitivity to several plant hormones, showed that although some of these mutants reacted to both treatments in a similar way, some of the mutants reacted to CWSE differently to the auxin containing B_{5,4,9}. This suggests that although the CWSE may possibly contain some contaminating auxin from the cell culture medium, it is likely that the CWSE also contains another compound that causes the root growth changes described above. This is supported by the fact that more root inhibiting activity was found in the CWSE than could be accounted for by simple contamination by culture medium.

The carrot cell suspension cultures used to produce the CWSE will produce somatic embryos on the removal of auxin from the culture medium. The number of embryos produced is proportional to the number of cells expressing an epitope recognised by the monoclonal antibody JIM8 (Pennell et.al., 1992). Separation of the cells into populations that express or did not express the JIM8 antigen showed that this antigen
defines a particular population. The JIM8 + cells produce a signal that is required by some of the JIM8- cells in order for them to develop into embryos. The CWSE contains molecules that react to the JIM8 antigen (McCabe et al., 1997). Cell cultures remain embryogenic for up to 9 months after being established. The level of embryogenic potential varies over this time. Thus, using CWSE taken from cultures of different ages I tested whether the level of root altering activity found in the CWSE is linked to the age of the culture.

This chapter also discusses experiments investigating the stability of the activity when exposed to heat treatments. Experiments which initially fractionate the CWSE are discussed. These include size exclusion and solvent partitioning. The culture medium, auxin control, B$_{5,4,9}$ was also separated using some of these methods to investigate any further differences between the root inhibiting activity in the CWSE and the B$_{5,4,9}$, which would allow complete removal of any contaminating auxin.

**4.2 Heat and time stability of CWSE activity**

Molecules such as proteins lose their activity when heated. This disrupts hydrogen bonding within the molecular structure. Activity of signal molecules can also be reduced by degradation over time when a solution is stored. Carbohydrates are more stable and 2,4-D, the major possible hormone contaminant in the CWSE is known to be stable up to 20mins at 121°C and over several weeks at room temperature.
Figure 4.1 Effect of heat treatment and incubation time on the stability of carrot wall signal extract

CWSE was treated by (a, b) storing at 4°C for 4 days or 25°C for varying lengths of time prior to freezing at -20°C or (c, d) heating to temperatures between 20°C and 121°C for 20 minutes. Each fraction was tested for root inhibiting activity using Arabidopsis seedlings. (a, c) % Inhibition of primary root growth induced by cell wall extract after treatment, (b, d) Inhibition of the number of lateral roots produced / mm primary root length induced by cell wall extract after treatment. The concentrations of cell wall extract used for the bioassay are shown below each graph.
Chapter 4 - Results

In the previous Chapter, *Arabidopsis* seeds were grown on CWSE media. The plates were incubated for up to 5 days at 4°C and were then transfer to 25°C for a further 14 days. To test whether the CWSE was active over the full assay period, CWSE was stored at 4°C or 25°C for varying lengths of time then tested using the full bioassay procedure. Figure 4.1a, b shows that both the root inhibiting activity and the lateral root production altering activity levels in the CWSE were very stable over time at 4°C and 25°C. CWSE was also heated to temperatures between 20-121°C for 20mins prior to assaying for root altering activity (Figure 4.1 c, d). The activity in the CWSE was also stable over this range of treatments.

4.3 Size exclusion using Sephadex test columns and molecular weight cut-off membranes

4.3.1 The size of compounds contributing to the root inhibiting activity in the CWSE varies over the life of the somatic embryogenic culture

Embryogenic potential is a measure of the number of somatic embryos that could be produced by the embryogenic culture under inductive conditions. The embryogenic potential reaches a maximum after about 40 days and starts to decline after around 150 days (Pennell *et al.*, 1992). Figure 4.2 shows the level of activity obtained in CWSE taken from cultures of different age. The activity was assayed either with no treatment, or after dialysis using a 25kDa cut-off membrane. Figure 4.2 shows that the root altering activity in the CWSE is the result of more than one molecule. The 25kDa dialysis tubing retained some activity.
Figure 4.2 Activity units found in CWSE with no treatment or after dialysis

Cell wall signal extract was tested for units of activity using the Arabidopsis root bioassay. The extract was either used without treatment or after dialysis using a 25kDa cut-off membrane. The activity is quoted in units/ml calculated to be present in the extract post treatment. 1 unit/ml in the CWSE supplemented medium produces a 50% inhibition in root length growth.
However, this does not account for all the activity found in the CWSE, thus some of the activity must be of molecular mass < 25kDa.

The total level of estimated activity reduces slightly at just after 200 culture days, with an increase in the amount of activity being accounted for by molecules < 25KDa. The activity of these molecules reduces as the age of the culture increases.

Thus, this suggests that the root altering activity found in the CWSE, is the result of at least two molecules of different sizes, the ratio of which alters in relation to the age of the culture.

4.3.2 Fractionation of CWSE using Sephadex size exclusion gel

CWSE was separated using size exclusion chromatography test columns of 20ml volumes of Sephadex G-10, G-15 or G25 gel. The void volume of each column was estimated as 9, 10 and 11 ml respectively, as determined with dextran blue in which molecules of 1000, 1500 or 2500 Da were present. Smaller molecules were partitioned between the solute mobile phase and the solute stationary phase within the gel particle pores. 1ml of 10x-concentrated CWSE was loaded onto the test columns and 2ml fractions were eluted using distilled water. Fractions were collected and tested for activity using the *Arabidopsis* seedling bioassay.
Figure 4.3 Separation of CWSE using Sephadex test columns

(a) G-25

10 x concentrated CWSE was loaded onto 3 columns containing three different types of Sephadex. Fractions were eluted with H2O. Graphs (a), (b) and (c) show the effect of the collected fractions on the root growth of *Arabidopsis* seedlings. Graph (d) shows the elution profile of a mixture of Cobalt chloride and Dextran Blue.

- **Root length**
- **Number of lateral roots**
- **Laterals/mm root length**

(b) G-15

(d) G-10

absorbance at 510 nm follows Cobalt chloride

absorbance at 625 nm follows Dextran Blue
Figure 4.3 shows the root inhibiting activity found in each fraction eluted from the three different columns. The separations confirmed the presence of more than one active molecule. The majority of the root inhibiting activity was late eluting requiring elution volumes of 15-18 (G-10), 17-20 (G-15) and 17-20 ml (G-25). This suggests a molecule of small molecular mass, although as the activity on the Sephadex G-10 elutes after the cobalt chloride, it is not possible to estimate an exact size. There is also the possibility that the very late elution is assisted by an interaction between the active compound and the solid matrix of the gel. This would delay the elution of the compound giving the impression of a very small molecule.

All columns also showed a second low activity pool, which eluted prior to the main activity indicating a larger molecule. This second pool elutes at, 9/10 ml from the G-25 column, 11/12ml from the G-15 column and 11/12ml from the G-10 column. The estimates of molecular mass for these different pools would be different for each column. For example the molecular mass of the pool eluting from the G-10 column would be estimated at <1000Da, whereas the pool eluting from the G-25 column elutes in the void and thus would be estimated as having a molecular mass >2500Da. Thus, these extra activity pools need to be confirmed, either using a higher concentration of CWSE for the separation or by using a larger Sephadex G-10 column, such that more activity can be isolated.

4.3.3 Fractionation of CWSE and B₅₋₄₄ media using Sephadex G-10

Section 3.5 discussed the maximum level of 2,4-D contamination possibly present in the CWSE. A diluted medium control was prepared to account for this maximum level and is referred to as B₅₋₄₉. CWSE and B₅₋₄₉ were separated using the 20ml Sephadex
G-10 column. 1 ml of 5x CWSE or B5.4.9 was loaded onto the column and 1 ml fraction eluted using dH2O. Fractions 14-19 were tested for root inhibiting activity on Arabidopsis seedlings. This purpose of this experiment was to see if the elution characteristics of the B5.4.9 activity were the same as the CWSE and if the elution characteristics were shown different, an estimate the level of 2,4-D contamination in unseparated CWSE could be calculated.

Figure 4.4 shows that the root inhibiting activity from the CWSE elutes during the 15th and 16th ml of dH2O elution, whereas the elution of root inhibiting activity from B5.4.9 elutes during the 16th and 17th ml of dH2O elution. Roots were also scored for the number of lateral roots produced per mm of primary root length. The expected increase in this measurement was also separated by one fraction. Thus the root inhibiting activity found in the B5.4.9 is not the same molecule as that in the CWSE. It is possible that a difference in pH can have an effect on the elution characteristics of different molecules, however, there was no difference in the pH of the CWSE and the B5.4.9. This adds to the evidence that the activity found in the CWSE was not caused by direct contamination of the CWSE by 2,4-D, but by a molecule that is possibly larger than the auxin.

Since the root inhibiting activity found in the CWSE and the B5.4.9 overlap it is not possible to completely confirm whether there is 2,4-D contamination in CWSE. However, if there is contamination it is likely to be very low, based on the levels of activity eluted by 17ml of dH20. Half of the total activity eluted from the B5.4.9 separation was detectable in the 17ml fraction. However, only 10% root inhibiting activity was detectable in this fraction in the CWSE elution curve.
Figure 4.4 Separation of CWSE and B$_{5.4.9}$ using Sephadex G-10

1 ml 5x CWSE extract or 1 ml 5x B$_{5.4.9}$ was loaded onto a 20 ml Sephadex G-10 column. Fractions were eluted using distilled H$_2$O, and tested for root growth inhibiting activity. The graphs show the root length, number of lateral roots, and number of lateral roots produced in relation to 1 mm primary root length produced by 10 day old Arabidopsis seedlings grown on extract supplemented with 5% of the individual fractions.

(a) CWSE,
(b) B$_{5.4.9}$.

x = loaded sample diluted 1 in 5 prior to assaying for activity.
4.3.4 Fractionation of CWSE using molecular sieve membranes

The elution position of the majority of the root inhibiting activity of the CWSE from the Sephadex column suggested that at least one of the active compounds was relatively small. The molecular mass could not be estimated more closely since the activity elutes after smallest coloured known molecular mass marker cobalt chloride (Figure 4.3 (c,d)). As discussed above this suggests that there is some interaction between the column and the active compound. The % inhibition of root growth produced by the untreated CWSE and B_{5,4,9} in this experiment is similar. This is due to the high level of activity in both samples which produces the maximum level of inhibition.

To further investigate the size range of the active molecules present in the CWSE, CWSE was passed serially through molecular sieve membranes with molecular cut-off values of 3000, 1000 and 500Da. The molecules retained by each membrane were recovered from the membrane surface using distilled water. This produced fractions with molecular mass of, >3000, <3000 >1000, <1000 >500 and <500Da.

Each fraction was tested for root inhibiting activity (Figure 4.5). This serial fractionation separated three pools of root inhibiting activity. The first with a molecular weight >3000 exhibits low inhibition of root growth and inhibition of lateral root production. The second possible pool of activity is retrieved between the 1000 and 500Da cut-off membranes, this has similar levels of activity to the >3000Da activity pool. These first two do not exhibit the increase in ratio of the number of lateral roots/mm primary root normally associated with untreated CWSE.
Figure 4.5 CWSE fractionated using molecular weight cut-off membranes

CWSE or B_{s4.9} was separated using a series of molecular cut off membranes. Fractions were collected and tested for their ability to effect the root growth.

(a) CWSE
(b) B_{s4.9}
(c) total carbohydrate concentration.
(d) Absorbance of each fraction at 280nm. 
U= Untreated, >3 =>3000Da,
<3 >1 =>3000 <1000Da,
<1 >0.5 <=1000 >500 Da, <0.5 = 500Da
The third and most active pool is found to have a molecular weight <500Da, with a high level of both root inhibition and inhibition of lateral root production with an overall increase in the number of laterals / mm primary root length. This is consistent with the elution data from the Sephadex column (Sections 4.3.2).

The B_{5.4.9} exhibits two pools of activity in the <500 and >500, <1000Da fractions. Both of these are likely to be the result of the 2,4-D in the diluted medium since the 500Da membrane may have retained some of the hormone.

It is known that the separation of solutes can be affected if the solutions have a high osmotic potential (Manufacturers information). Further experiments using the 500Da cut-off membrane alone to separate B_{5.4.9}, followed by washing the membrane with dH_2O showed that all this activity passed through the membrane (data not shown).

Figure 4.5 also shows the distribution of the carbohydrate found in each fraction and the absorbance of each fraction at 280nm. Although the CWSE and the B_{5.4.9} appear to contain nearly the same level of overall carbohydrate, the separation distributed the B_{5.4.9} carbohydrate between the fractions >500 <1000 and <500, whereas all the detectable carbohydrate transferred through to the <500 fraction during the CWSE separation. The distribution of molecules that absorbed at 280nm was similar to the carbohydrate distribution for both separated samples, although detectable absorbance was found in the >3000 and <3000 >1000 fractions of both samples.
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The total recovery of carbohydrate appeared lower than expected. This is likely to be a consequence of the methodology, since molecules not passing through the membranes are recovered by washing the membrane surface. This process is unlikely to be 100% efficient.

4.4 Phase partitioning of CWSE and B_{5.4.9}

The root inhibiting activity in the CWSE has been shown to separate slightly from any possible contaminating 2,4-D activity when fractionated using Sephadex G-10. However, in order to purify the activity further, a different method of separation would be necessary. To test further the chemical properties of the activity in the CWSE and B_{5.4.9}, samples were partitioned with the polar solvent, ethyl acetate. Ethyl acetate and water are immiscible.

After separation, fractions were assayed for root altering activity using the *Arabidopsis* bioassay, with the root lengths and the number of lateral roots being scored after 10 days (Figure 4.6). This method produced a partitioning of the activity in the B_{5.4.9} between the ethyl acetate layer and the aqueous layer with a ratio of 1.137 (ethyl acetate concentration/ aqueous concentration). The activity in the CWSE had very different partitioning characteristics, with a ratio of <0.01 between the two solvents. This suggests that the root inhibiting factors in the CWSE are more hydrophilic than the auxin 2,4-D. The resulting partitioning of the 2,4-D is expected, considering the chemical properties of the 2,4-D molecule. The chemical structure of auxin and auxin related molecules allow molecules to enter a cell by passing directly through the plasma membrane, by diffusion (MacDonald, 1997). Since the activity
Figure 4.6 Partitioning of CWSE and B_{5.4.9} using ethyl acetate

(a) Root length after 10 days.
(b) Ratio of lateral roots produced per mm root length after 10 days.

Cell wall signal extract and B_{5.4.9} was partitioned against the polar solvent ethyl acetate. Fractions were tested for the root inhibiting activity using the *Arabidopsis* root bioassay.
from the CWSE remains almost entirely in the aqueous phase during partitioning, it is unlikely to pass through the plasma membrane by this method, but would require either a receptor on the cell surface or would have to use a hydrophilic pore.
4.5 Summary

In this chapter I have further explored the possible relationship between the root inhibition activity found in the CWSE and the activity resulting from the auxin 2,4-D in the control B_{5.4.9}. In addition to the quantitative analysis of activity obtained in chapter 3, which revealed more activity than could be accounted for by possible 2,4-D contamination, and differences in the reaction of some hormone resistant mutants to the two treatments, the experiments have revealed further differences.

Size partitioning of CWSE revealed that the activity in the CWSE was the result of 3 pools of activity, with molecular weights of >3000Da, 1000-500Da and <500Da. Further comparisons with the elution of B_{5.4.9} from the same columns, indicated that less than 10% of the activity and thus less than 2% of the concentration (%) of the CWSE could possibly be the result of contaminating 2,4-D. This was further confirmed by partitioning the CWSE and B_{5.4.9} with polar solvents. The activities present in the two solutions were found to have very different partitioning coefficients, thus suggesting that the activity is unlikely to be of the same chemical composition.

In summary, CWSE caused root inhibition and changes the number of lateral roots produced in *Arabidopsis* seedlings. There was a variation in the type/size of molecules producing the activity over the life of the embryogenic culture. Less than 10% of the root inhibiting activity in the CWSE was the result of possible contaminating 2,4-D. The CWSE however, contained at least 3 pools of active molecules, which could be separated using size exclusion. All the active compounds are hydrophilic.
Chapter 5: Purification of active signal compounds from carrot CWSE

5.1 Introduction

In chapter 3, I showed that the carrot CWSE causes changes in the development of Arabidopsis roots, with a reduction in root length and change (usually a large increase) in the number of lateral roots produced. Experiments described in chapter 4 showed that the activity in the CWSE is both heat stable up to 121°C for 20 mins and stable at 25°C for up to two weeks. Studies also showed the activity in the CWSE is unlikely to be caused by 2,4-D contamination of the CWSE from the culture medium since the active compound/s are more hydrophilic than activity caused by 2,4-D. Separation of CWSE or B5,4,9 using Sephadex columns showed the root inhibiting activity of each had different elution profiles.

This chapter describes initial purification of the active compounds in the carrot CWSE. A combination of recording the absorbance at 280nm and ELISA, to follow the presence of the JIM8 antigen, are used. Fractions were assayed using the Arabidopsis root bioassay to assess their root altering activity.

Several pilot purification studies have been described in the previous chapter. These included size exclusion, using Sephadex gel and molecular weight cut-off membranes (Section 4.3). The possibility of using solvent partitioning was also investigated and showed the active compounds in the CWSE were hydrophilic. Using the information gained about the stability and partition characteristics of the CWSE active compounds, purification of the compounds was undertaken.
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5.2 Purification of an AGP-like carbohydrate from the CWSE using ion exchange chromatography

The CWSE has been shown to contain an antigen recognised by the monoclonal antibody JIM8 (Pennell et al., 1992). An antigen recognised by this Mab, hereby referred to as the JIM8 antigen, was isolated using ion exchange chromatography. Two different ion gradients were used 0-1.2M NaCl, or 0-2M NH₄HCO₃.

Figure 5.2 shows the elution characteristics of CWSE separated using Cl⁻ as the counter ion. Two main peaks of absorbance at 280nm are found eluting at 0.10-0.14M NaCl and 0.22-0.24 NaCl and three main peaks of JIM8 antigens eluting at 0.018-0.2M, 0.3-0.34M and 0.76-0.78M NaCl. Although there are three main peaks of JIM8 antigens, these are surrounded by smaller peaks and shoulders suggesting several different molecules may be combining to produce the JIM8 reactive peaks. The main JIM8 antigen peak eluting between 0.18-0.20M NaCl was analysed for carbohydrate composition. The results are discussed in Section 5.7. Elution using NH₄HCO₃ as the eluting buffer, produced a similar profile.

Fractions were desalted and tested for root altering activity. No obviously active fraction was obtained from either column. This may result from the activity being the product of two compounds, as the column may separate the two compounds. This is unlikely, as a sample made up of all fractions isolated by NH₄HCO₃ elution was also found to be non-active.
Figure 5.1 Fractionation of CWSE using NaCl ion exchange chromatography
The graph shows the absorbance at 280nm, or the concentration of JIM8 antigens found in fractions eluted from a Mono Q 5/5 ion exchange column, using a gradient to 1.2M NaCl. The concentration of epitope is shown as the % gum arabic that give the same ELISA reading. Fractions eluting between 0.18 and 0.20 NaCl were desalted and analysed for carbohydrate residue composition.
Another possibility is that the exposure to the high NaCl level may deactivate the compounds in the CWSE. Exposure of CWSE to higher NaCl followed by removal of the NaCl, showed that exposure to NaCl reduced the activity level in the CWSE by approximately 25% (Appendix 4). However, this could not explain the lack of activity in the NH$_4$HCO$_3$ eluted fraction, since if CWSE is exposed to 2M NH$_4$HCO$_3$ the resulting activity is the same as non-treated CWSE. A further possibility is that the active compounds bind to the column in a form that cannot be removed by the 1.2M NaCl or 2M NH$_4$HCO$_3$. The full data for the bioassays of these fractionations are presented in Appendix 4.

Since no activity was obtained from either of the ion exchange experiments, a different method of purification was pursued.

5.3 Size exclusion chromatography of CWSE and preparation of active fractions for thin layer chromatography and partition column chromatography

Fractionation of CWSE using Sephadex size exclusion revealed one low activity fraction with a large molecular mass > 1500Da and one low molecular mass fraction circa 500Da. Fractionation of the CWSE using molecular weight cut-off membranes revealed three pools of root inhibiting activity with molecular mass in the regions of >3000Da, >500 and <1000Da and >500Da. When separating using Sephadex G-10 column the higher molecular mass fraction is likely to contain the >3000Da molecules
Figure 5.2 Chemical composition of fractions from the initial fractionation of CWSE using a 250ml Sephadex column

Cell wall extract was concentrated approximately 30X and loaded onto a 250ml Sephadex G-10 column in 10ml samples. 5ml fractions were eluted using distilled H$_2$O. The absorbance of each fraction at 280nm, the level of JIM8 antigens and the background level of carbohydrate were recorded. Fractions were pooled into groups A-G.
and some of the active molecules isolated between 1000 and 500Da cut-off membranes. The small molecular mass fraction isolated from the Sephadex column is likely to contain the active molecules isolated with molecular mass <1000Da.

Thus, to fractionate the three activity pools, a combination of the Sephadex G-10 column and molecular weight cut-off membranes was used. Initially a 250ml Sephadex G-10 column was used to fractionate concentrated CWSE into several fractions including 2 active fractions. This allowed large-scale separation of the larger active molecules from the active fraction of lower molecular mass.

Figure 5.2 shows the absorbance at 280nm, the concentration of the JIM8 antigens as scored by ELISA and the total carbohydrate concentration for each of the fractions collected. There were several peaks in the 280nm recordings, suggesting possible protein, some were associated with either carbohydrate peaks, or peaks in JIM8 antigens.

Three main peaks of carbohydrate eluted at 130, 140 and 180 ml. It is important to note that the absorbance resulting from this particular assay varies according to the type of carbohydrate present, with differences in absorbance of 5 times produced between the same concentration of D-mannose and 2,3,4,6-tetra-O-methyl-D-glucose (Dubois et al., 1956). The sugars must also have a free, or potentially free, reducing group. The major carbohydrate used in the B5-44 medium is sucrose. 0.1 mg/ml sucrose produces an absorbance of approximately 2.5 at 585nm. This is relatively high for this assay. Against a standard curve of sucrose the three peaks approximate to
Figure 5.3 Effect of fractions of CWSE separated using Sephadex G-10 on the growth of *Arabidopsis* seedling roots

CWSE was concentrated approximately 30X. 10ml was loaded onto a 250ml sephadex column and eluted using distilled H2O. The 5ml fractions collected were pooled into fractions:

- A  0-50ml fractions
- B  55-80ml
- C  85-105ml
- D  110-135ml
- E  140-165ml
- F  170-190 ml
- G  195-250ml

The graph shows the effect of the pooled fractions on the growth of 10 day old *Arabidopsis* seedlings.
0.05, 0.2 and 0.01 mg/ml carbohydrate. The minimum level of sucrose detectable in this assay is 10 ug/ml. The first peak of JIM8 antigens eluted is associated with a peak in possible protein, but the level of carbohydrate recorded is only just in the sensitivity range of the carbohydrate assay. Further peaks of JIM8 antigens are associated with carbohydrate peaks; this along with the elution pattern suggests smaller molecules of carbohydrate that bind to the JIM8 monoclonal antibody. Although the full epitope may be relatively large, antibodies can react with smaller molecules than is necessary to induce the antibody production. Using a combination of these peaks the fractions were pooled into 7 groups as represented in Figure 5.2. The pooled fractions A-G were assayed for root inhibition activity using the Arabidopsis root bioassay (Figure 5.3). Root altering activity was found in fractions A, C, F and G.

Fractionation of CWSE using molecular sieves showed the CWSE contained root altering activity with molecular weights in the ranges, >3000, >500 <1000, and <500. The Sephadex G-10 will restrict all molecules larger than 1000Da to the void volume. It is therefore likely that the activity retained by the 3000Da membrane are the same compounds eluted from the Sephadex column in the pooled fraction C, confirming the presence of a low activity pool in fraction C. This fraction contained at least one JIM8 antigen and was therefore concentrated and analysed for carbohydrate residue composition (Section 5.7).

The majority of the activity was found in fraction F and G. Fraction F also contains a JIM8 antigen associated with peaks in carbohydrate and a 280nm absorbance peak. It was anticipated that this fraction would be a mixture of small ions and hormones from
the culture, medium, or hormones that had been produced by the carrot cells, thus the fraction was further fractionated using a 500Da cut-off membrane.

5.4 Fractionation of fraction F isolated using Sephadex G-10 column into two active fractions using a 500Da molecular cut-off membrane

Fraction F and G isolated from CWSE using Sephadex G-10 could account for both the >500<1000 and the <500 pools of activity fractionated using molecular weight cut-off membranes. Since fraction G appeared to be the tail end of the peak present in fraction F, it was not studied further at this stage. This decision was based on the elution curves from the early test columns (Section 4.3).

60ml of fraction F was therefore separated using the 500Da cut-off membrane. Figure 5.4 shows the segregation of activity produced by this membrane. The majority of the activity passed through the membrane, however a significant level was retained by the membrane. This result is consistent with the molecular weight cut-off membrane fractionation of whole CWSE and confirms the presence of an active compound with a molecular mass in the region of <1000 and >500 Da. This fraction, designated “fraction F >500” was freeze-dried and analysed for carbohydrate composition (Section 5.7).
Figure 5.4 Fractionation of Sephadex G-10 fraction F, using a 500Da cut-off membrane

Fraction F from the Sephadex G-10 separation (Figure 4.2, 4.3) was separated using a 500Da cut-off membrane. Extract = original extract separated, >500 = fraction retained by membrane, <500 = fraction passing through membrane, pre-bio = fraction <500 after it has been concentrated 50x using a vacuum at 40°C, then diluted 1 in 50 with dH2O.

(a) Effect of each fraction on *Arabidopsis* seedling roots.
(b) Absorbance at 280nm of each fraction, pre-bio sample absorbance was recorded prior to 1 in 50 dilution.

![Graph (a)](image1)

![Graph (b)](image2)
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The fraction which passed through the 500Da cut-off membrane was designated fraction F <500. This is likely to be a mixture of several compounds and any growth regulators from either the culture medium or produced by the carrot cells. This fraction was concentrated so that further separations could be undertaken.

At this stage of isolation the following fractions, assessed by root altering activity, had been isolated:

1 non-active fraction isolated using ion exchange chromatography

(1) associated with the main peak in JIM8 antigen elution, molecular mass approximately 2000 Da.

3 active fractions isolated using size exclusion

(2) fraction C >1000Da, associated with JIM8 peak,
(3) Fraction F >500, molecular mass <1000 >500Da, associated with JIM8 and 280nm absorbance peak during Sephadex separation.
(4) Fraction F <500, molecular mass <500Da, associated with JIM8 and 280nm absorbance peak during Sephadex separation.
5.5 Thin layer chromatography of fraction F <500

5.5.1 Separation of CWSE Sephadex fraction F <500 using thin layer chromatography (TLC)

The activity present in fraction F <500 is likely to be a combination of several plant hormones which have molecular mass less than 500Da. 50ml of fraction F <500Da was concentrated to 2ml. 60μl of this sample was separated by thin layer chromatography (TLC) using a silica TLC plate. The TLC plates were examined using short wave ultraviolet and long wave ultra violet light. The TLC plates used were impregnated with a dye that fluoresces under short-wave ultraviolet. Compounds appear on these TLC plates as dark patches in a fluorescing background. TLC plates were examined under long-wave UV since many compounds have fluorescence under this type of light.

Initially several pure solvents were used to separate the fraction F<500. Viewing of the separations aided the choice of solvents to be mixed, until a separation was achieved using a mobile phase of 4:4:2 methanol: acetone: distilled water. Figure 5.5 (a) shows the plate viewed under short and long wave ultraviolet light.

Both the position of the activity and whether it could be removed from the silica TLC plate were tested. The silica plate was therefore cut into fractions as indicated by Figure 5.5 (a). The sections cut were related to the visible fractions under short/long wave UV. The separated molecules were extracted from the silica sections.
Figure 5.5 Fractionation of fraction F<500Da using thin layer chromatography

(a) Fraction F <500 was separated by thin layer chromatography (TLC) on a silica plate impregnated with a dye which produces fluorescence under shortwave UV. The mobile phase used was 4:4:2 methanol:acetone :distilled H2O. Pre = concentrated Fraction F <500.

(a) Left: TLC plate under longwave UV. Middle: TLC plate under shortwave UV. Right: Diagram to show sections of TLC taken for activity analysis
(b) Effect of fractions on the growth of Arabidopsis seedlings.
(c) Effect of fractions on growth of the primary root and the number of lateral roots produced by 10 day old seedlings.

(b) Control Blank Pre Total 1 2 3 4 5 6

(c) % inhibition

-125 -75 -25 25 75

- Root length - Number of laterals - Laterals/mm root length
with 2 x 2ml 4:4:2 methanol: acetone: dH₂O. Any remaining silica was removed from the fraction by centrifugation. Fractions were concentrated to 2ml, split into two, and one half tested at a concentration of 4.2% fraction for activity using the Arabidopsis root bioassay. The other half was pooled and used as “total” compounds recovered sample and was tested for root altering activity at a concentration of 25%.

The more concentrated positive control or pre-sample was tested at a concentration at 0.000125%. This produces the same dilution factor as estimated for the “total” sample. The pre-sample produced an inhibition of root growth relative to the control grown plants of 52.9%. In comparison the “total” sample gave an inhibition of root growth of 47.7%. The recovery efficiency of the method was thus calculated to be approximately 82%.

Fractions 4 and 5 were shown to contain the root altering activity (Figure 5.5 b, c). The wide band of activity may be due to smearing of the compounds due to the solvent or an incomplete separation of a mixture of active compounds.

5.5.2 Reduction of RF values during thin layer chromatography in preparation for flash chromatography

The compounds in fraction F<500 can be fractionated using silica thin layer chromatography. Column chromatography is preferable to thin layer chromatography for obtaining large samples. Thus a Flash 40 chromatographic unit, using silica columns was chosen for large scale separation of the fraction F<500 sample. The Flash 40 unit is a high-pressure solvent column liquid chromatography system that can be used with a variety of solvents.
The mobility, or Rf value, for a compound in a particular solvent can be calculated as the distance moved by that compound divided by the distance moved by the solvent front. While giving an indication of the order in which compounds will elute from a silica column, the relationship between the Rf value and elution volume from a column also made of silica is not direct. The Rf value of a compound on a TLC plate is inversely proportional to the elution time of a component from the chromatographic column (manufacturer information).

The Rf value for the root altering active compounds found in fraction F<500 could be estimated at between 0.56 and 0.9. To prepare for flash chromatography it was necessary to decrease the polarity of the mobile phase, used to separate the fraction F<500 on the TLC plates, such that the Rf values were reduced to between 0.15 and 0.35 (manufacturers data sheets).

Previously a good separation of compounds was achieved using a mobile phase of 4:4:2 acetone: methanol: dH2O. The polarity of this mixture is estimated to be approximately 0.99. In order to reduce the Rf values it was necessary to replace methanol with chloroform. Figure 5.6 shows the results of separations of fraction F<500, using various ratios of chloroform: acetone: dH2O. The Rf values reduce with decreasing polarity of the mobile phase, with a ratio of 1:8:2 reducing the majority of the Rf values to below the required 0.35.
Figure 5.6 Reduction of Rf values of components detectable using UV and carbohydrate assays by mobile phase polarity reduction

Fraction F<500, isolated from CWSE using size exclusion was fractionated using thin layer chromatography (TLC). The fraction was resolved on silica coated thin layer chromatography plates impregnated with a dye that fluoresces under short wave UV light. The following solvent mixtures were used, with polarity reducing from left to right. Relative proportion of solvents: Chloroform, acetone, dH₂O = polarity.

1. 1:8:2 = 5.96
2. 1:8:1 = 5.66
3. 2:8:1 = 5.55
4. 1.25:8:0.75 = 5.55.

Top: Shortwave UV light.
Separated compounds are visible as dark patches on a fluorescent background.

Longwave UV light. Showing some of the compound exhibit fluorescence.

Bottom: Carbohydrate assay. Carbohydrate visible as brown patches on white background.
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This data suggested that the compounds in the fraction F <500 should be separable using column chromatography with a mobile phase of chloroform, acetone and dH₂O. The separation should start with a mobile phase with a high chloroform ratio, and ending with a solvent mixture of 1:8:2 chloroform: acetone: H₂O, as this moves all visible compounds in the fraction.

5.6 Separation of fraction F <500 using Liquid Chromatography (Flash Chromatography)

Fraction F <500 was prepared from CWSE by size exclusion as detailed in sections 5.3 and 5.4. After concentrating the sample, it was loaded onto a dry 250ml-silica Flash Biotage chromatography column and eluted using a series of solvents, 1L 9:1 chloroform: acetone, 1L 1:1 chloroform: acetone followed by 250ml 1:8:1 chloroform: acetone: d H₂O. The polarity of these solvent mixtures is 4.5, 4.9 and 5.6 respectively. The polarity of ethyl acetate, into which none of the CWSE activity passed is 4.3.

The solvents generated 35, 40 and 10 fractions respectively. 1 ml of each fraction was pooled to produce the following solvent groups including fractions 1-35 (A), 36-55 (B), 56-75 (C), and 76-85 (D). After solvents were removed, the samples were tested for root inhibiting activity (Figure 5.7). This revealed that three out of the four pooled fractions contained root altering activity. These pools contained fractions 1-35, 36-55 and 76-85. The only fraction not containing activity was the second half of the 1:1 chloroform: acetone separation, or fractions 56-75.
CWSE was separated using a Sephadex G-10 column, a peak of root inhibiting activity was then passed through a 500Da cut-off filter. The <500Da fraction was loaded onto a 250ml Flash Biotage silica column. Samples were eluted using 9:1 1L v/v chloroform:acetone (A), 1L 1:1 v/v chloroform:acetone (first 500ml. (B), second 500ml. (C)) followed by 250ml. 1:8:1 chloroform:acetone:H₂O. Fractions were tested for root altering activity.

(a) Effect of pooled fractions on the growth of seedling roots shown as inhibition of B₅₀ grown seedlings at 10 days.
(b) Examples of 14 day old seedlings grown on each pooled fraction. Activity is found in fractions A, B and D.
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The solvents were removed from the individual fractions of the groups exhibiting root altering activity and the samples were assayed for activity using the *Arabidopsis* root bioassay. Figures, 5.8, 5.9 and 5.10 show the effect of the fractions on root growth of each of the fractions from pooled groups A, B and D. The solvent mixture 9:1 chloroform : acetone eluted the fractions shown in Figure 5.8. The solvent appears to elute a complex mixture of molecules with smearing of the activity across many fractions. This may be a result of the solvent used, as some solvents do produce this effect, or it may suggest that the activity peak is made up from a group of very similar compounds. The majority of activity was found in fractions 26-29.

Figure 5.9 shows the effect of the fractions eluted by the first 500ml of the solvent mixture 1:1 chloroform: acetone. The activity is found to elute between fractions 36 and 39, with the majority of the activity being found in fraction 37. The activity in these fractions appears to lie specifically with an absorbance peak at 280nm.

The effect of fractions eluted by 1:8:1 chloroform: acetone: H₂O on the roots of *Arabidopsis* seedlings are shown in Figure 5.10. As with the active eluted fractions eluted with 1:1 chloroform: acetone the activity is limited to a small number of fractions, 79-81. The increase in activity occurs at a similar time to an overall increase in absorbance at 280nm. However after the root altering activity decreases, the high absorbance remains. This may be due to traces of solvent remaining in the samples which do not effect the root growth. Fraction 79 contained the majority of the activity.
1 ml of concentrated fraction F <500 was loaded onto a 250 ml silica Flash chromatography column and eluted using initially 1 L of 9:1 chloroform:acetone. The solvents were removed from the fractions by rotary evaporation and resuspended in 1 ml dH₂O. Each fraction was added to bioassay media to a concentration of 0.25% and tested for their effect on *Arabidopsis* root growth.
Figure 5.9 Fractionation of fraction F<500, fractions collected using 1:1:0 chloroform:acetone:dH₂O (pool B)

1ml of concentrated fraction F<500 was loaded onto a 250ml silica Flash chromatography column and eluted using initially 1L of 9:1 chloroform:acetone (Figure 5.8), followed by 1L 1:1 chloroform:acetone. The first 500mls was found to contain the active compounds (Figure 5.7). The solvents were removed from these fractions by rotatory evaporation and resuspended in 1ml dH₂O. Each fraction was added to bioassay media to a concentration of 0.25% and tested for their effect on Arabidopsis root growth.
Figure 5.10 Fractionation of fraction F<500, fractions collected using 1:8:1 chloroform:acetone:dH₂O (pool D)

1ml of concentrated fraction F <500 was loaded onto a 250ml silica Flash chromatography column and eluted using initially 1L of 9:1 chloroform:acetone (Figure 5.8), followed by 1L 1:1 chloroform:acetone.(Figure 5.9). Fractions were then eluted using 250ml 1:8:1 chloroform:acetone dH₂O. The solvents were removed from these fractions by rotary evaporation and resuspended in 1ml dH₂O. Each fraction was added to bioassay media to a concentration of 0.25% and tested for their effect on Arabidopsis root growth.
Fraction F<500 therefore contains three pools of active compounds, corresponding to fractions 27, 37 and 79 so these were analysed for carbohydrate composition.

5.7 Chemical composition of active fractions collected during separation of CWSE

The CWSE was shown to contain a complex mixture of compounds. From the CWSE a compound that binds to the JIM8 antibody was isolated using FPLC ion exchange chromatography. This compound was found to be non-active in the Arabidopsis root bioassay. 6 fractions of varying levels of root altering activity were also isolated from CWSE using size exclusion and partition chromatography. The isolation sequence of these 6 fractions is summarised in Figure 5.11. All fractions were analysed for their carbohydrate composition by Dr Scott Forsberg at the Complex Carbohydrate Research Centre, USA. The results of the composition analysis are shown in Table 5.1.

The carbohydrate composition of the fraction eluted from the FPLC column is characteristic of AGPs with approximately 7% arabinosyl, and 37% galactosyl residues (Fincher et al., 1983, Serpe and Nothnagel 1995, 1996). This fraction contains the majority of the JIM8 antigens eluted from the column by NaCl. This fraction along with all the other fractions eluted from the FPLC column did not exhibit a significant level of root inhibiting activity.
Figure 5.11 Summary of purification
This figure summarizes the purification of fractions with root inhibiting activity isolated from the carrot CWSE. The numbers in red relate to the fraction number listed in Table 5.1, and are the fractions analysed for their carbohydrate residue composition.

CWSE isolated from carrot embryogenic cultures and concentrated by freeze drying

↓

CWSE loaded onto Sephadex G-10 column
2 active fractions produced

↓

fraction C
>2500Da
(2)

Fraction F separated using 500Da molecular mass cut-off membrane
2 active fractions collected.

↓

Fraction F >500Da
(3)

Fraction F <500Da (4) concentrated and separated by Flash Chromatography. 3 active fractions isolated

↓  ↓  ↓

27  37  79
(5)  (6)  (7)
CWSE was also separated using a Sephadex G-10 column. This column fractionated two fractions with root altering activity, both of which contained JIM8 antigens. The first fraction to elute, fraction (2) shows similar carbohydrate composition to rhamnogalacturonan I and II (RGI and RGII) (Zablockis et al., 1995). RGI is a pectic polysaccharide and RGII is a substituted 4-linked homogalacturonan that contains some unusual residues, including 2-keto-3-deoxy-octulosonic acid (Kdo).

The analysis of the fraction found evidence of compounds with similar but not identical spectra to Kdo. The fraction is likely to be a combination of RGI and RGII or related compounds. The antibody JIM8 is known to bind to RGI and RGII (Pennell et al., 1991).

The second fraction eluted from the Sephadex G-10 column exhibited the majority of the root altering activity, and was further fractionated by passing it through a 500Da cut-off membrane. Root altering activity was found to be both retained by the membrane, fraction (3), and pass through the membrane, fraction (4) (Figure 5.11). Carbohydrate residue analysis showed that the fraction (3) contained almost equal levels of galactosyl and glucosyl residues at 34 and 40 mol%, the fraction also contained < 10 mol% of arabinosyl, rhamnosyl, xylosyl, mannosyl residues and inositol-O methyl ether. Fraction(4) contained the same residues except for the absence of the arabinosyl residue, which was replaced by the presence of glycerol and a reduction in the level of galactosyl residues.

Fraction (4) was further separated using partition chromatography. This revealed 3 pools of root altering activity contained in the fractions designated (5), (6) and (7).
### Table 5.1 Carbohydrate composition of isolated fractions

<table>
<thead>
<tr>
<th>Analysed fraction numbers</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg total carbohydrate per total sample</td>
<td>-</td>
<td>272.8</td>
<td>25.6</td>
<td>35.2</td>
<td>3.8</td>
<td>73.6</td>
<td>67.7</td>
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<td>Glycerol</td>
<td></td>
<td></td>
<td>0.4</td>
<td></td>
<td></td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>6.8</td>
<td>14.2</td>
<td>9.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhamnose</td>
<td>13.1</td>
<td>5.7</td>
<td>5.1</td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucose</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>8.8</td>
<td>8.5</td>
<td>3.5</td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>4.1</td>
<td>5.8</td>
<td>3.5</td>
<td>5.1</td>
<td>8.6</td>
<td>1.7</td>
<td>0.7</td>
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<tr>
<td>Galactose</td>
<td>36.2</td>
<td>34.2</td>
<td>34</td>
<td>13.4</td>
<td>0.6</td>
<td>0.6</td>
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<td>Glucose</td>
<td>11.5</td>
<td>18.8</td>
<td>39.8</td>
<td>63.3</td>
<td>57.3</td>
<td>96.5</td>
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<td>Glucuronic acid</td>
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<tr>
<td>Galacturonic acid</td>
<td>12.6</td>
<td></td>
<td>6.0</td>
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<td>Kdo-like</td>
<td>0.2</td>
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<td></td>
<td></td>
<td>6.4</td>
<td>0.8</td>
<td>0.9</td>
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<td>DHA-like</td>
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<tr>
<td>Inositol</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Inositol-O methyl ether</td>
<td>0.1</td>
<td>Trace</td>
<td>1.9</td>
<td>12.9</td>
<td>0.5</td>
<td>37.9</td>
<td></td>
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<tr>
<td>Unidentified</td>
<td>2.6</td>
<td>4.3</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td>11.4</td>
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</table>

Concentrations of different carbohydrate residues present in isolated fractions expressed as mol%. The method of composition analysis is described in section 2.3.9. 7 different fractions were analysed:

1. FPLC main JIM8 fraction, 0.32-0.38M NaCl (Figure 5.1).
2. Sephadex G-10, 85-105ml, fraction C, (Figure 5.2 / 5.3).
3. Fraction F >500Da, (Figure 5.4).
4. Fraction F <500Da, (Figure 5.4).
5. Fraction F, <500 Silica column fraction 27, (Figure 5.8).
6. Fraction F, <500 Silica column fraction 38, (Figure 5.9).
7. Fraction F, <500 Silica column fraction 79, (Figure 5.10).
All contained mannosyl, glucosyl, and a 2-keti-3-deoxy octulosonic acid like residue (Kdo-like) and the inositol-O methyl ether. Fraction (5) also contains a deoxyheptulosaric acid (DHA) like residue. Fractions (6) and (7) contain galactosyl residues and fraction (7) also contains inositol.

All active fractions tested contain a rare residue, inositol-O methyl ether. Myo-inositol is an important factor in the culture medium (present at 100mg/l) in which both the carrot cells and the Arabidopsis seedlings are cultured.
5.8 Discussion

CWSE of carrot embryogenic proliferating cultures has been separated using several separation techniques, including FPLC ion exchange, size exclusion and partition chromatography. Several types of root inhibiting activity were isolated and tested for their carbohydrate composition. All fractions were found to contain significant levels of carbohydrate, including some of the more unusual carbohydrate residues.

All fractions contain inositol-o methyl ethers and some fractions contain 2-keto-3-deoxyoctulosonic acid and deoxyheptulosaric acid. Inositols have previously been found in plants. Inositol phosphates are linked to internal signal transduction pathways of some glycoproteins and 1D-4-O-methyl-myo-inositol has been shown to accumulate during drought stress (Wanek and Richter, 1997). Further 1L-1-O-α-D-galactopyronosyl-myo-inositol is found in sugar beet sap and 1D-2-O-(α-D-galactopyranosyl)-4-O-methyl-chiro-inositol is found in seeds of several leguminous species (Loewus, 1990). Conjugates of indole-3-acetyl-2-O-myo-inositol glycosylated at carbon 5 of the inositol with either arabinose or galactose have also been found. The presence of the inositol residues in all the active fractions but not in the non active fraction from the FPLC column suggests that this residue may be required in activating the molecule. According to the literature inositols however have not been found as residues in AGP (Fincher et al., 1983).

Small oligosaccharides and O-methyl inositols have also been found in response to saline conditions. Gilbert et al. (1997) showed that in green tissues, increases in the o-methylated inositols, together with increased activity of galactan:galactan galactosyl
transf erase for synthesis of the oligosaccharides, and myo-inositol 6-O-methyl
transferase for O-methylation of myo-inositol were found. This was accompanied by
a change in the transport of glucose. In root, galactan:galactan galactosyl transferase
but not myo-inositol 6-O-methyltransferase was induced. If the methyl-O-inositol
are also involved in root growth signalling, the change in balance may effect the
growth of roots in saline conditions. Thus change in conditions can induce a change
in the level of these O-methylated inositol s.

The compounds isolated have similar effects on the roots of *Arabidopsis*. The
carbohydrate residue composition suggests that they may form a group of related
compounds. Further investigation of the effects of the individual compounds may
show subtle differences in the activity.
Chapter 6 – Results: Selection of *Arabidopsis* mutants with reduced sensitivity to carrot cell wall signal extract (CWSE)

**6.1 Introduction**

Carrot wall signal extract (CWSE) influences the development of *Arabidopsis* seedling roots. As stated previously, the main effects are inhibition of root growth, inhibition of lateral production, and a change in the ratio of lateral roots produced per unit length of primary root. Roots expand in diameter and frequently become asymmetric in cross-section, (Section 3.3). Some hormone resistant mutants are resistant to the CWSE; these include *axr1, aux1, axr4, ctrl, eto1* and *ein2-1*. Some of these mutants reacted differently to CWSE than to diluted media containing the auxin 2,4-D (Section 3.6). This suggested that although the CWSE was influencing the auxin ethylene pathway that is involved root development, it was not acting in the same way as the auxin 2,4-D.

Many of the auxin resistant mutants were isolated by screening for mutants that could elongate roots on auxin supplemented media (Lincoln *et al.*, 1990, Wilson *et al.*, 1990). Using the same principle, mutagenised M2 populations of *Arabidopsis* were screened for seedlings which were able to elongate roots on CWSE supplemented medium.
6.2 Screening for Arabidopsis seedlings that have a reduced sensitivity to CWSE

Approximately 45,000 M2 ethyl methane sulphanate (EMS) mutagenised Arabidopsis seeds were sown onto media supplemented with 10% CWSE. After 7 days of growth seedlings were scored for resistance to the effects of CWSE. The background growth rate was the reaction of the majority of the seedlings to the CWSE. Seedlings with roots that elongated to at least twice the length of the root growth of the control were chosen as putative mutants. These seedlings were transferred to compost (Figure 6.1). These plants were grown to maturity under short day conditions and seeds of the M3 generation were collected. Short day conditions were chosen as these give the greatest survival rates for transferred mutants seedlings.

A total of 188 seedlings were transferred to compost from the selection plates. This represents a selection rate of 0.42% (Table 6.1). Of these, seed was collected from 117 plants or 0.26% of the original screened seed. Compared with a screen for selection of mutants for resistance to the auxin IAA or the ethylene precursor ACC, this selection rate was high. The selection rate on IAA was between 0.005-0.01% (Lincoln et al., 1990, Wilson et al., 1990) and the selection rate on ACC was 0.09% (Pickett et al., 1990).

The high selection rate may be explained by the low level of increase in growth required in order that a plant was chosen as a putative mutant, i.e. only requiring double the background growth rate. 18 separate populations of EMS mutagenised seeds were screened. This is much higher than in the screens for IAA or ACC resistance, in which between 1 and 4 populations were screened (Picket et al., 1990,
Lincoln *et al.*, 1990). The increase in population number increases the chance of finding different genes in the signal transduction pathway, and thus increases the number of mutants that will be picked up in a screen.

The fertility of the plants selected varied significantly, from plants which produced several thousand seeds per plant, to those which appeared to be self-sterile. Some of these genotypes were rescued by fertilising them with the wildtype *coll, gl1* pollen.
Figure 6.1 Selection of resistant *Arabidopsis* seedlings from EMS M2 mutant populations

Populations of EMS mutagenised M2 seeds were sown onto 10% CWSE. Seeds were germinated and after 7 days were scored for their ability to grow roots longer than the control population. **(a)** shows a selection plate after 10 days growing time, arrow indicates a plant selected as a possible mutant. **(b)** Selected plants after transfer to soil and growth under short-day conditions at 8 weeks. **(c)** 14 weeks old plants grown under short-day conditions.
## Table 6.1 Selection, transfer and seed collection of seedlines from EMS mutagenised populations

<table>
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<tr>
<th>Population Number</th>
<th>Number seeds scored</th>
<th>Number of seedlings transferred</th>
<th>% plants selected as possible mutants</th>
<th>Number of seedlines from which M3 seed were collected</th>
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<tbody>
<tr>
<td>14</td>
<td>2519</td>
<td>10</td>
<td>0.40</td>
<td>7</td>
</tr>
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<td>19</td>
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<td>10</td>
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<td>3</td>
</tr>
<tr>
<td>28</td>
<td>2510</td>
<td>8</td>
<td>0.32</td>
<td>6</td>
</tr>
<tr>
<td>29</td>
<td>2472</td>
<td>7</td>
<td>0.28</td>
<td>3</td>
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<tr>
<td>30</td>
<td>2448</td>
<td>9</td>
<td>0.37</td>
<td>6</td>
</tr>
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<td>31</td>
<td>2500</td>
<td>10</td>
<td>0.40</td>
<td>6</td>
</tr>
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<td>32</td>
<td>2464</td>
<td>6</td>
<td>0.24</td>
<td>4</td>
</tr>
<tr>
<td>33</td>
<td>2501</td>
<td>9</td>
<td>0.36</td>
<td>8</td>
</tr>
<tr>
<td>35</td>
<td>2507</td>
<td>18</td>
<td>0.72</td>
<td>13</td>
</tr>
<tr>
<td>36</td>
<td>2495</td>
<td>19</td>
<td>0.77</td>
<td>12</td>
</tr>
<tr>
<td>37</td>
<td>2482</td>
<td>18</td>
<td>0.73</td>
<td>6</td>
</tr>
<tr>
<td>38</td>
<td>2461</td>
<td>11</td>
<td>0.45</td>
<td>8</td>
</tr>
<tr>
<td>409</td>
<td>2520</td>
<td>9</td>
<td>0.36</td>
<td>3</td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>44951</strong></td>
<td><strong>188</strong></td>
<td><strong>0.42</strong></td>
<td><strong>117</strong></td>
</tr>
</tbody>
</table>
6.3 Selected seedline re-tests

Natural variation in phenotype means that some plants selected as possible mutants during the initial selection process may not produce populations of resistant plants. Thus to test whether the apparent resistant phenotype found in the M2 plants were hereditary, and were the result of a genetic mutation rather than phenotypic variation, seeds from the M3 generation of the seedlines were sown onto 10% CWSE supplemented medium.

The seedlines to be tested were chosen on the basis of the number of seeds available and the level of apparent resistance in the M2 seedling screen. Seeds of each seedline were also sown onto B₅₀ medium so that a comparison could be made with the untreated phenotype of the mutant, as well as the col1, gl1 background phenotype. The complete results of this re-test are listed in Appendix 5.

Figure 6.2 shows the phenotype of several of these mutants on B₅₀ medium. Common phenotypes included a slight reduction in root growth compared to the wild type and also a reduction in lateral root production. The ratio of lateral root production to primary root length was reduced in all of these seedlines other than 409:01:01, where the ratio was unchanged. This is in contrast to the auxin resistant mutant test in Chapter 3, all of which grew longer roots on B₅₀ than wildtype plants (data not shown).

Each seedline tested was given two resistance scores. The average root length or number of lateral roots were usually calculated from at least 20 seedlings.
Figure 6.2 Phenotype of a selection of mutants in grown on control medium

M3 generation of a selection of the mutants were grown on B_{5.0} medium. The reduction in growth of the primary root, the number of lateral roots and the ratio of number of lateral roots produced per mm root length caused by the mutant is shown as inhibition of wildtype root growth on B_{5.0} medium.

![Bar chart showing % inhibition for different seedlines with error bars.](chart.png)
Table 6.2 Resistance scores of a selection of mutants re-tested

Resistance is scored as = ((X-Y)/X)*100
Where X = % inhibition of growth in wildtype caused by 10% CWSE
Y = % inhibition of growth caused by 10% CWSE in mutant.
The root length and the number of lateral roots produced were scored for at least 25
col1, gl1 seedlings grown on B5:0 and CWSE supplemented media.

<table>
<thead>
<tr>
<th>Seedline</th>
<th>% resistance in respect of primary root growth phenotype</th>
<th>% resistance in respect of the number of lateral roots produced</th>
<th>Number of mutant seedlings scored. B5:0 grown, CWSE grown.</th>
</tr>
</thead>
<tbody>
<tr>
<td>33:21:01</td>
<td>69.3</td>
<td>98.6</td>
<td>23,28</td>
</tr>
<tr>
<td>19:15:02</td>
<td>63.4</td>
<td>89.4</td>
<td>20,26</td>
</tr>
<tr>
<td>35:08:01</td>
<td>74.4</td>
<td>72.8</td>
<td>25,28</td>
</tr>
<tr>
<td>28:14:01</td>
<td>82.2</td>
<td>67.4</td>
<td>24,22</td>
</tr>
<tr>
<td>409:17:01</td>
<td>54.2</td>
<td>30.7</td>
<td>19,30</td>
</tr>
<tr>
<td>32:15:01</td>
<td>59.5</td>
<td>-0.4</td>
<td>31,26</td>
</tr>
<tr>
<td>409:01:01</td>
<td>87.0</td>
<td>-25.1</td>
<td>30,28</td>
</tr>
<tr>
<td>35:13:04</td>
<td>87.8</td>
<td>-153.5</td>
<td>30,21</td>
</tr>
</tbody>
</table>

The actual number of plants used are listed in the tables displaying the mutant scores.

These scores represented the resistance to changes caused by the CWSE on the growth of the primary root and the ratio of the number of lateral roots produced per mm root length. Table 6.2 shows the results of the phenotype re-test for a selection of mutants, which are discussed further in this chapter. The % mutation score shown in Table 6.2 are calculated as the as:

\[(X-Y) \times 100 \]

\[X\]

Where X is the % inhibition of root growth, or % increase of lateral root/length ratio of col1, gl1 grown plants on carrot CWSE supplemented medium compared with
Figure 6.3 Mutant M3 *Arabidopsis* grown on B<sub>5</sub>-0 and CWSE supplemented medium

Phenotype of the M3 generation of a selection of mutant lines. Plants are grown on B5-0 medium (left) or 10% CWSE supplemented medium (right).

(a) *coll*, (b) *gll*, (c) 28:14:01, (d) 409:01:01, (e) 35:13:04, (f) 33:21:01, (g) 19:15:02, (h) 35:08:01 (i) 409:17:01, (j) 32:15:01.
coll, gll seedlings grown on B_{5.0}. Y is the % inhibition of root growth, or % increase of the lateral root/length ratio, of the putative mutant growth on carrot CWSE supplemented medium compared with that seedline grown on B_{5.0}. Thus the higher the value the more resistant the putative mutant is to the effects of the CWSE. The % inhibition for each line was calculated as the inhibition of growth caused by the CWSE, compared with the mutant line grown on B_{5.0}. The full list of resistance scores are listed in Appendix 5. With respect to the effect on the primary root growth, 30 out of 78 seedlines tested were greater than 50% resistant to the CWSE.

Seedlines such as 28:14:01 (Figure 6.3 (b)), 33:21:01 (Figure 6.3 (e)) and 19:15:02 (Figure 6.3 (f)) developed roots that were very similar in phenotype on both the CWSE and the B_{5.0} medium. They were also similar in appearance to coll, gll B_{5.0} grown plants. Others such as 409:01:01 (Figure 6.3(c)) and 32:15:01 (Figure 6.3 (i)) have reduced root growth on the CWSE compared with their B_{5.0} grown phenotype, however they do not have the characteristic fattening in the diameter of roots found in wildtype plants grown on CWSE supplemented medium.

### 6.4 Effect of CWSE and B_{5.4,9} on a selection of the Arabidopsis mutants

Several of the auxin and ethylene resistant mutants tested in Chapter 3 were shown to be resistant to CWSE. Figure 6.3 shows the phenotype of each of the seedlines listed in Table 6.2 when grown on B_{5.0} medium. Three of these seedlines were further tested for their resistance against the effects of CWSE, and the auxin containing B_{5.4,9} medium (Figure 6.4).
Figure 6.4 Effect of CWSE and B<sub>5-4.9</sub> on the growth of mutants

Seeds from seedlines 19:15:02, 33:21:01, 409:01:01 and the wildtype *coll, gl1* were sown onto a dilution series of CWSE and B<sub>5-4.9</sub>. The figure shows the effect of these treatments on the root growth (left) and the number of lateral roots produced (right).

(a) 19:15:02

(b) 33:21:01
Figure 6.4 continued
Seeds from seedlines 19:15:02, 33:21:01, 409:01:01 and the wildtype coll, gl1 were sown onto a dilution series of CWSE and B5-4.9. The figure shows the effect of these treatments on the root growth (left) and the number of lateral roots produced (right).

(c) 409:01:01
All three of the seedlines tested were resistant to the effects of both the CWSE and the B_{5,4,9}. The level of the resistance appeared to be similar for mutants 19:15:01 and 33:21:01, but with 19:15:01 having a larger maximal increase in the number of lateral roots produced in response to CWSE than to B_{5,4,9} compared with inhibiting response by the *coll, gl1* plants.

The response of the mutant 409:01:01 to the CWSE and B_{5,4,9} appears to be different. *coll, gl1* plants responded to increasing concentration of B_{5,4,9} with a general increase in inhibition of root growth and a decline in the number of lateral roots produced. The mutant 409:01:01 responded with increasing inhibition of growth to both B_{5,4,9} and CWSE. The mutant was resistant to the CWSE extract, however it only appeared to be resistant to B_{5,4,9} at concentration above 10% B_{5,4,9}. Below this concentration the % inhibition is the same for both the mutant and the wildtype. This difference in resistance was also reflected in the effect of the treatments on lateral root production.

The difference in response of mutant 409:01:01 to CWSE and B_{5,4,9} is also exhibited in the production of lateral roots. Whereas, *coll, gl1*, plants show inhibition of root production in response to both treatments, the mutant shows no change in lateral root production in response to B_{5,4,9}. However, the mutant responds to CWSE with a significant increase in the number of lateral roots produced at CWSE concentrations of 6.25-12.5%, which turns to an inhibition of lateral root production equal to the control at 25% CWSE.
6.5 Segregation of resistant phenotype after cross fertilisation of mutant seedlines with col1, gl1 wildtype

The mutants tested for their resistance to both CWSE and B_{5.4.9} were chosen for segregation analysis. These were 19:15:02, 33:21:01 and 409:01:01. The flowers of mutant plants were emasculated prior to stamen maturity, to ensure that the individual flowers had not self-fertilised. The eggs in these flowers were fertilised using pollen from col1, gl1 plants by brushing the stamens from wildtype plants across the stigmas of the mutant plants. The seeds resulting from these crosses (F1) were collected and tested for their resistance to the root inhibiting effects of CWSE. Once resistance or susceptibility had been established, plants were transferred to soil and grown to maturity. Plants were allowed to self fertilise and the resulting F2 seeds were collected. These were also tested for their resistance to the effects of CWSE.

Table 6.3 shows the result of the segregation analysis. Two out of the three seedlines, 33:21:01 and 409:01:01, showed a segregation ratio that suggested the mutant phenotype maybe the result of a single recessive mutation, with the original mutant parent used for the cross being a homozygous mutant (Ayala and Kiger, 1984). The ratio of susceptible plants to resistant plants produced by 33:21:01, could also fit other segregation ratios, such as the ratios described in Figure 6.5 as a possible explanation of the segregation ratio of seedline 19:15:02.

19:15:02 showed a significant deviation from the expected segregation ratios of a single recessive mutant. Although all plants in the F1 generation were found to be susceptible, the ratio of resistant plants : susceptible plants deviated from the expected 1:3 in the F2 generation.
Table 6.3 Segregation analysis of examples of the 3 mutant types

Seedlines were crossed to *coll*, *gll*. The plants were grown to maturity and were allowed to self fertilise. The segregation data of resistant to non-resistant plants are shown.

**Seedlines 19:15:02**

<table>
<thead>
<tr>
<th>M2 phenotype</th>
<th>M3 phenotype</th>
<th>(Total 158) expected ratio</th>
<th>$X^2$ = 4.464</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Number of seedlings</td>
<td>Phenotype</td>
<td>Number of seedlings</td>
</tr>
<tr>
<td>Resistant</td>
<td>0</td>
<td>Resistant</td>
<td>51</td>
</tr>
<tr>
<td>Susceptible</td>
<td>4</td>
<td>Susceptible</td>
<td>107</td>
</tr>
</tbody>
</table>

Significantly different from expect ratio of 1:3 95% chance of not being 1:3

**Seedline 33:21:01**

<table>
<thead>
<tr>
<th>M2 phenotype</th>
<th>M3 phenotype</th>
<th>(Total 59) expected ratio</th>
<th>$X^2$ = 0.955</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Number of seedlings</td>
<td>Phenotype</td>
<td>Number of seedlings</td>
</tr>
<tr>
<td>Resistant</td>
<td>0</td>
<td>Resistant</td>
<td>18</td>
</tr>
<tr>
<td>Susceptible</td>
<td>22</td>
<td>Susceptible</td>
<td>41</td>
</tr>
</tbody>
</table>

Not significantly different from 1:3 ratio

**Seedline 409:01:01**

<table>
<thead>
<tr>
<th>F2 phenotype</th>
<th>F3 phenotype</th>
<th>(Total 176) expected ratio</th>
<th>$X^2$ = 0.273</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Number of seedlings</td>
<td>Phenotype</td>
<td>Number of seedlings</td>
</tr>
<tr>
<td>Resistant</td>
<td>0</td>
<td>Resistant</td>
<td>47</td>
</tr>
<tr>
<td>Susceptible</td>
<td>6</td>
<td>Susceptible</td>
<td>129</td>
</tr>
</tbody>
</table>

Not significantly different from 1:3 ratio
A possible explanation of this is that the mutant seedline 19:15:02 contains two recessive mutants with the presence of either in homozygous form producing a resistant phenotype. Figure 6.5 shows the possible genetic cross that would produce this 1:2 resistant to susceptible phenotype.

Another possibility, that would explain the lower than expected ratios of susceptible plants: resistant plants scored in the F2 generations, for all the seedlines, is that the mutant phenotype changes the seed phenotype in such a way that sowing bias occurs, rather than random sowing occurring which is a requirement for this type of study. An example maybe if the mutant phenotype produced a larger seed. It may also be possible that seed set or germination in the mutant lines was more successful than for wildtype or heterozygous plants.

The phenotypes of the segregating resistant and susceptible plants of the F2 generation from the crosses with parental mutants 19:15:02, 33:21:01 and 409:01:01 are shown in Figures 6.6, 6.7 and 6.8 respectively. There was no obvious phenotype that segregated with resistance to the CWSE. Since plants are grown on the CWSE in this experiment prior to transfer to soil, there was large variation in the phenotypes within the populations. Both resistant and susceptible plants can be severely affected by transfer from the culture medium to soil. Also the difference in root structure of the phenotypes on CWSE may influence their survival and growth capabilities after transfer. Further mutations other than those that give resistance to CWSE can produce segregating phenotypes, e.g. the multiple petal whorl flower phenotype which segregated from mutant 33:21:01 (Figure 6.7h).
Figure 6.5 Possible genotype segregation of mutant 19:15:02

Parental seedlines  
Mutant  Wildtype
Parental Phenotypes  Resistant  Susceptible
Parental Genotype  aaBb  AABB

F1 genotypes  
Wildtype pollen genotype

<table>
<thead>
<tr>
<th></th>
<th>AB</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>aB</td>
<td>AaBB</td>
<td>susceptible</td>
</tr>
<tr>
<td>ab</td>
<td>AaBb</td>
<td>susceptible</td>
</tr>
</tbody>
</table>

F2
Each genotype will produce the following offspring in the F2 generation.

Type 1 Genotype AaBB
AaBB self-fertilisation

<table>
<thead>
<tr>
<th></th>
<th>AB</th>
<th>AB</th>
<th>aB</th>
<th>aB</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB</td>
<td>AaBB</td>
<td>s</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>AB</td>
<td>AaBB</td>
<td>s</td>
<td>s</td>
<td>s</td>
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<tr>
<td>aB</td>
<td>AaBB</td>
<td>s</td>
<td>r</td>
<td>r</td>
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<tr>
<td>aB</td>
<td>AaBB</td>
<td>s</td>
<td>r</td>
<td>r</td>
</tr>
</tbody>
</table>

Type 1 genotype phenotypic ratio
Susceptible : resistant
12:4

Type 2 Genotype AaBb
AaBb self-fertilisation

<table>
<thead>
<tr>
<th></th>
<th>AB</th>
<th>Ab</th>
<th>aB</th>
<th>ab</th>
</tr>
</thead>
<tbody>
<tr>
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<td>AaBB</td>
<td>s</td>
<td>ss</td>
<td>s</td>
</tr>
<tr>
<td>Ab</td>
<td>AABb</td>
<td>s</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>aB</td>
<td>AaBb</td>
<td>s</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>ab</td>
<td>AaBb</td>
<td>s</td>
<td>r</td>
<td>r</td>
</tr>
</tbody>
</table>

Type 2 genotype phenotypic ratio
Susceptible : resistant
9:7

F2 Summary
F1 type 1 plant produces ratio of susceptible: resistant plant of 12:4
F1 type 2 plant produces ratio of susceptible: resistant plant of 9:7
If both types are in equal abundance in the F1 generation, as would be expected if the parental mutant genotype was aaBb, total F2 ratio would be predicted as 21:11, approximately a 2:1 ratio of susceptible to resistant plants. This is close to the F2 ratio for mutant 19:15:02.
Figure 6.6 Phenotype of F2 19:15:02 segregants

coll, gl1 pollen was used to fertilise emasculated mutant 19:15:02 plants. The F1 seeds produced were allowed to self-fertilise. This figure shows the phenotype of F2 plants.
(a), (c) non-resistant segregants
(b), (d), (e) resistant segregants
**Figure 6.7 Phenotype of F2 32:21:01 segregants**

*coll, gl1* pollen was used to fertilise emasculated mutant 32:21:01 plants. The F1 seeds produced were allowed to self-fertilise. This figure shows the phenotype of F2 plants.

(a), (c), (e) susceptible to CWSE (b), (d), (f), (g) resistant plants.

(h) Flower phenotype in both groups.
**Figure 6.8 Phenotype of F2 409:01:01 segregants**

*coli, gl1* pollen was used to fertilise emasculated mutant 409:01:01 plants. The F1 seeds produced were allowed to self-fertilise. This figure shows the phenotype of F2 plants.

(a), (c), (e) plants that were not resistant to CWSE.
(b), (d), (f) plants resistant to CWSE.
6.6 Summary

The CWSE extract has been shown to contain several carbohydrate molecules that can influence the development of the cells within the *Arabidopsis* root. This produces a root phenotype which is shorter and fatter that the roots of plants grown on control B₅₀ medium. Using this phenotype as a background, mutant *Arabidopsis* seedlings were screened for seedlings that could elongate roots on 10% CWSE. The M3 seed populations were retested, 30 seedlines were greater than 50% resistant to the effects of 10% CWSE on the growth of their primary root compared with the effect of this concentration on wildtype seedlings.

A common feature of many of the mutants isolated was a slightly reduced growth rate of the seedling root, and a reduction in the number of lateral roots produced.

One mutant 409:01:01, appeared to react differently to the auxin 2'4D and CWSE. Seedlines 19:15:02, 33:21:01 and 409:01:01 were backcrossed to the wildtype *coll*, *glI*. The latter two mutants, segregated the resistant phenotype in the F2 generation in ratios which suggested they are each the result of single recessive mutations. Seedline 19:15:02 resistance segregated in ratios that suggest the seedline may possibly contain 2 recessive mutations.

EMS generated mutant seedlines often contain several mutations in different genes. It would therefore be preferable to perform further back crosses to the wildtype *coll, glI*. prior to further phenotype investigations. These may involve investigations to see whether the changes in growth habit are due to any changes in the cellular structure of
the roots. Preliminary studies of mutant seedline 409:01:01 revealed a reduction in root hair formation (data not shown).

I would anticipate further work on these mutants to involve complementation tests to see how many genes, and alleles of the genes, have been isolated. It would also be useful to perform complementation tests with some of the auxin and ethylene genes, especially aux1, which appeared to be twice as resistant to CWSE as it was to the auxin containing B_{5,4,9}. 
References


References


References


References


# Appendix 1 - Auxin and ethylene mutants involved in root development and mutants affecting the development of root hairs

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>axr3-1</td>
<td>Enhanced apical dominance, reduced root elongation, increased adventitious rooting, no root gravitropism, ectopic expression of the auxin induced mRNA SAUR-AC1. Suggests increased auxin response.</td>
<td>Leyser et al. (1996)</td>
</tr>
<tr>
<td>axr4-1</td>
<td>Specifically resistant to auxin, and are defective in root gravitropism. Reduced number of lateral roots produced.</td>
<td>Hobbie and Estelle (1995)</td>
</tr>
<tr>
<td>Allele</td>
<td>Description</td>
<td>Reference</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>etr1-3</td>
<td>Ethylene resistant – lacks ethylene induced inhibition of cell elongation, promotion of seed germination, enhancement of peroxidase activity, acceleration of leaf senescence, and feedback suppression of ethylene production. Ethylene binding sites present in yeast cells expressing ETR1; mutant form no binding sites.</td>
<td>Bleeker et al (1988), Schaller and Bleeker (1995)</td>
</tr>
<tr>
<td>ein2-1</td>
<td>Produces increased amounts of ethylene, ethylene insensitive.</td>
<td>Chang et al (1993), Guzman and Ecker (1990)</td>
</tr>
<tr>
<td>ein3</td>
<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
<tr>
<td>ein4</td>
<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
<tr>
<td>ein5</td>
<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
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<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
<tr>
<td>ein7</td>
<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
<tr>
<td>eir1</td>
<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
<tr>
<td>eir2</td>
<td>Ethylene resistant</td>
<td>Roman et al. (1995)</td>
</tr>
<tr>
<td>eto 1-1</td>
<td>Overproduces ethylene</td>
<td>Guzman and Ecker (1990)</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>gl2-1</td>
<td>Ectopic hair cells, glabrous.</td>
<td>Masucci and Schiefelbein (1996)</td>
</tr>
<tr>
<td>rhd6</td>
<td>Reduction in the number of root hairs, change in the site of hair emergence, high frequency of cells with multiple hairs.</td>
<td>Masucci and Schiefelbein (1994)</td>
</tr>
</tbody>
</table>
## Appendix 2 - Distribution of glyco-proteins in plants

Glyco-proteins have been isolated from many plants. The distribution of some glyco-proteins has been studied using monoclonal antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Distribution/possible function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JIM4</td>
<td>Carrot cell cultures. Transient expression at the shoot root junction during transition from globular to heart shaped embryos. Transient expression in a single layer of cells on embryo cell surface. At torpedo stage expression in future stele and pro-vascular cells. Carrot roots – future pericycle.</td>
<td>Stacey <em>et al.</em> (1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Knox <em>et al.</em> (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Knox <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>JIM8</td>
<td>Carrot somatic embryo cultures, linked to early steps in embryogenesis. Expressed in the first cell of the embryo <em>in vivo</em>, with transient expression in other cell types linked with development of the ovary and stamens. Expressed in the suspensor. Xylem and pre-vacuolar tissue. Plasma membrane, cell wall, culture media. Periodic deposition in the cell wall of pollen tubes.</td>
<td>Pennell <em>et al.</em> (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Li <em>et al.</em> (1992)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pennell <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>JIM12</td>
<td>Carrot pericycle opposite the xylem poles. Future metaxylem cells.</td>
<td>Smallwood <em>et al.</em> (1994)</td>
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<tr>
<td>Antibody</td>
<td>Distribution / possible function</td>
<td>Reference</td>
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<tr>
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<tr>
<td>JIM15</td>
<td>Carrot roots – all cells except the epidermis and cells of the future xylem band. Root cap cells and cells of the apex.</td>
<td>Knox et al. (1991)</td>
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<tr>
<td>No antibody</td>
<td>Distribution / possible function</td>
<td>Reference</td>
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<tr>
<td>------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>No antibody</td>
<td>Rosa cultures, involved in cell proliferation.</td>
<td>Serpe and Nothnagel (1994)</td>
</tr>
<tr>
<td>No antibody</td>
<td>Plasma membrane and cells of tobacco cells. Lost in NaCl adapted cells. Implicated in cell expansion.</td>
<td>Zhu et al. (1993)</td>
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<tr>
<td>No antibody</td>
<td>Cell surface and intravacuolar multi-vesicular bodies.</td>
<td>Herman and Lamb (1992)</td>
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<tr>
<td>No antibody</td>
<td>AGP backbone cloned from pear. 144 amino acids long.</td>
<td>Chen et al. (1994)</td>
</tr>
<tr>
<td>No antibody</td>
<td>Hydroxyproline-rich glycoprotein cloned from soybean.</td>
<td>Ahn et al. (1996)</td>
</tr>
<tr>
<td>No antibody</td>
<td>From tobacco pistal stylar tissue. Stimulates pollen growth between stigma and ovary.</td>
<td>Cheung et al. (1995)</td>
</tr>
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<td>No antibody</td>
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<td>Qiu et al. (1997)</td>
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<tr>
<td>No antibody</td>
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<td>MAC265</td>
<td>Infection threads of in pea in nodules in symbiosis with Rhizobium leguminosarum. Also found in parenchyma of non infected nodules.</td>
<td>Baldwin et al. (1993) Rae et al. (1991)</td>
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<td>CCRC-M1</td>
<td>Root cap, epidermis, cortex, cortex (not radial cross-walls, pericycle, phloem.</td>
<td>Freshour et al. (1996)</td>
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<td>CCRC-M2</td>
<td>Atricoblasts, trichoblasts further than 2mm from apex.</td>
<td>Freshour et al. (1996)</td>
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<tr>
<td>CCRC-M7</td>
<td>All cells except lateral root cap.</td>
<td>Freshour et al. (1996)</td>
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<td>LM2</td>
<td>Media of proliferating rice suspension cultures.</td>
<td>Smallwood et al. (1996)</td>
</tr>
<tr>
<td>ZUM18</td>
<td>Carrot cell cultures, increases the percentage of embryogenic cells in the culture.</td>
<td>Kreuger and van Holst (1995)</td>
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### Appendix 3 Polarity and selectivity of solvents used in Thin layer chromatography and Flash Chromatography

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<td>H₂O</td>
<td>9.0</td>
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<td>1 : 1 chloroform : acetone</td>
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<td>1 : 8 : 1 chloroform : acetone: H₂O</td>
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Appendix 4 – Isolation and bioassay of major JIM8 antigen from CWSE using ion exchange chromatography

CWSE was fractioned using FPLC ion exchange chromatography to isolate the main antigen of the monoclonal antibody JIM8. This is discussed in Section 5.2. This appendix shows the results of assays of the ion exchange fractions for root altering activity.

Figure A4 shows the elution characteristics and the results of the bioassays when CWSE was eluted from the monoQ 5/5 column using a gradient to either 1.2M NaCl or 2M NH₄HCO₃.

Using NaCl and thus Cl⁻ as the counter ion, two main peaks of 280nm absorbance were found to elute at 0.10-0.14M NaCl and 0.22-0.024M NaCl. Further three main peaks of JIM8 antigens eluted at 0.018-0.2M, 0.3-0.34M and 0.76-0.78M NaCl. Although there are three main peaks of JIM8 antigens these are surrounded by smaller peaks and shoulders suggesting several different molecules may be combining to produce the JIM8 reactive peaks.

Fractions collected during the loading and sample washing were pooled into two separate groups, freeze dried and redissolved in 5ml each. 5ml fractions were collected from column during the elution gradient.
Figure A4 Effect of fractions isolated from CWSE using ion exchange on *Arabidopsis* seedling roots

(a) Absorbance (280nm)

(b) Absorbance (280nm)

(c) Absorbance (280nm)

Legend:
- Red: Absorbance 280nm
- Black: Root length
- Blue: Lateral roots
- Pink: Number of lats/length

Appendix 4 – Isolation of major JIM8 antigen
CWSE was separated using ion exchange chromatography on a mono Q 5/5 column, using either 1.2M Sodium chloride or 2M ammonium bicarbonate as counter ion. The graphs show the absorbance at 280nm and the root altering activity of each fraction as shown by the % inhibition of root growth, lateral root production and the ratio of lateral roots produced per mm root length. NaCl eluted fractions were desalted using a PD-10 column. Three fractions were isolated from each ion exchange fraction, representing the PD-10 column, void fraction (a), normal separation zone (containing the NaCl(b) and the late separation zone (c). Ammonium bicarbonate eluted fractions were desalted by subliming the solute from the solutions. The biassay results for these fractions are shown in graph (d).
The fractions could not be assayed for root inhibiting activity immediately due to the high NaCl concentration contained in some fractions. NaCl was removed using a PD-10 desalting column, which contains Sephadex G-25. When 2.5ml CWSE was loaded the vast majority of the JIM8 antigen detectable was found to elute in fractions 4-11ml. NaCl elutes mainly in fractions 7-10ml (manufacturers literature) and there was a range of absorbance at 280nm across the separation zone. Root altering activity was found in fractions 10-12ml. Stimulation of root growth and increase in lateral root production appeared to be associated with the JIM8 antigen peak between fractions 4-7ml. A similar growth stimulation was associated with the fraction eluted during loading of the sample, suggesting the stimulation in growth produced by fraction 4-7 may be not be due to the JIM8 activity but rather an increased background growth stimulation by the eluted fraction compared with the control grown seedlings. Compared with this background fractions 3-4 show inhibition of root growth.

2.5mls of FPLC ion exchange NaCl separated eluate was loaded onto a PD-10 column and eluted using dH2O. Fractions were collected as 1: load (0-2.5mls) 2: void volume (3-6mls) 3: separation zone containing majority of NaCl (6.5-9.5), and 4 post NaCl fraction (10-12.5) likely to contain majority of cell wall activity. NaCl was also added to CWSE and B5-4.9 to a concentration of 1.2M prior to removal of the NaCl using a PD-10 column. This allowed the effect of exposure to high NaCl levels on the root inhibiting activities to be investigated. The absorbance at 280nm of each fraction was recorded. All fractions were assayed for activity using the Arabidopsis seedling root bioassay the results of which are shown in Figure A4. The absorbance at 280nm
of the fractions, and the effect of the fractions on *Arabidopsis* seedlings are shown for fractions 2 (Figure A4 (a)-(c)).

The effect of high NaCl concentration on root growth can be seen in Figure A4 (b). Some of the later fractions in Figure A4 (c) also have a significant level of NaCl.

The graphs show that no obviously active fraction was obtained. This may result from the activity being the product of two compounds, as the column may separate the two compounds activity. Another possibility is that the exposure to the high NaCl level may deactivate the compounds in the CWSE. NaCl was added to CWSE to a concentration of 1.2M, and the NaCl removed using the PD-10 column. This showed that exposure to NaCl reduced the activity level in the CWSE by approximately 25%, (Figure A4 (b)). A further possibility is that the active compounds bind to the column in a form that cannot be removed by the 1.2M NaCl.

Since no activity was obtained from the ion exchange column using NaCl, it was decided to use a different counter ion that would involve a different desalting method. The chosen counter ion was bicarbonate in the form of Ammonium bicarbonate. The fractions were eluted using a gradient to 2M Ammonium bicarbonate and desalted by freeze drying which allowed the Ammonium bicarbonate to sublime from the samples as ammonia and carbon dioxide. Figure A4 (d) shows the absorbance of fractions at 280nm, and the results of assaying the fractions using the *Arabidopsis* root bioassay. No activity was found in any of the fractions. To check whether several of the fractions were needed to produce the activity, fractions were pooled to form one fraction, which was tested for root inhibiting activity. This was also non-active. The
lack of activity was not due to exposure to the ammonium bicarbonate, since if CWSE is exposed to 2M ammonium bicarbonate the resulting activity is the same as non-treated CWSE. This leaves the possibility that the activity is binding to the column in such a way that it cannot be removed by the counter ion.
Appendix 5. Re-test of putative mutants resistant to CWSE

The selection of putative mutants from EMS M2 populations that were capable of elongating roots on 10% CWSE produced over 100 possible mutants (Section 6.2). The M3 generation of some of these mutants were re-tested for this resistance characteristic by germinating seeds on 10% CWSE supplemented medium, and B$_{5.0}$ control medium. This allowed comparison of the control grown phenotypes of the mutant and the coll, gll background ecotype and the phenotype of the seedlines grown on cell wall supplemented medium. Seedlines were chosen on the basis of the number of seeds available and the level of apparent resistance as shown during the original M2 screen. Some examples are discussed in Section 6.3.

Tables A5.1 and A5.2 shows the results of the re-test. The phenotype of the mutant seedlines grown on B$_{5.0}$ are shown as % reduction of the average coll, gll plants grown on B$_{5.0}$. Mutants were also given a score of % mutant for the primary root growth. This percentage is calculated as:

\[
\frac{(X-Y)}{X} \times 100
\]

Where X is the %inhibition of root growth, or % increase of lateral root/ length ratio of coll, gll grown plants on carrot CWSE supplemented medium compared with coll, gll seedlings grown on B$_{5.0}$. Y is the % inhibition of root growth, or % increase of the lateral root/ length ratio, of the putative mutant growth on carrot CWSE supplemented medium compared with that seedline grown on B$_{5.0}$. Thus the higher the
value the more resistant the putative mutant is to the effects of the CWSE. This test thus separates out the mutant plants which simply grow quicker than *col1*, *gl1* on both CWSE and B₅.₀, thus will always have longer roots, but that are equally effected by the CWSE from those that may be shorter than the *col1*, *gl1* plants when grown on B₅.₀ medium but in percentage terms are not effected by the CWSE at all, and thus are truly resistant to the CWSE.
### Table A5.1 M3 Root phenotype of selected seed lines

Root phenotype of mutant seedlines when grown on B<sub>5.0</sub> medium expressed as % inhibition of coll, gll phenotype grown on B<sub>5.0</sub> medium. >25 coll, gll plants were scored for root phenotype. Seedlines highlighted in blue are discussed in chapter 6. Those in red are other mutants of possible interest.

<table>
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<tr>
<th>Seedline</th>
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<th>% increase in laterals/length ratio</th>
<th>No. of mutant plants scored</th>
<th>Seedline</th>
<th>% inhibition root length</th>
<th>% increase in laterals/length ratio</th>
<th>No. of mutant plants scored</th>
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<td>83.1</td>
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Table A5.2 Phenotype of M3 generation of selected seedlines

% mutant as calculated as described in the text, the higher the number to more resistant the seed line. X seedlines were mutant % could not be calculated as no lateral roots were produced by the control grown seedlings. * seedlines shown in Figure 6.2 Mutant category, character gradation of mutant. Number scored = Number of control grown mutant seedlings, CWSE grown mutant seedlings scored for root phenotype. Seedlines highlighted in blue are discussed in chapter 6. Those in red are other mutants of possible interest.

<table>
<thead>
<tr>
<th>Seedline</th>
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<th>% increase in the ratio of lateral roots produced / mm primary root growth</th>
<th>% mutant in respect of primary root growth</th>
<th>% mutant in respect of the ratio of lateral roots produced / mm primary root growth</th>
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<th>No. scored</th>
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