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**The Role of Auxin in Host Responses During Cauliflower
Mosaic Virus Infection**

By

Lorna Ann Smith

A thesis presented for the degree of Doctor of Philosophy

In

The Institute of Biomedical and Life Science
Division of Biochemistry and Molecular Biology
At the University of Glasgow

October 2006

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Declaration

I hereby declare that the work submitted in this thesis is the result of my own investigations except where references are mentioned and assistance is acknowledged. Therefore no part of this thesis has been previously presented for any degree.

Lorna A. Smith

October 2006

Dedication

To all who have supported me.

Acknowledgements

I would like to thank all who have supported and guided me throughout my studies. In particular I would like to thank my supervisor Dr Joel Milner who has offered invaluable advice and shared his knowledge and expertise with me. I would also like to acknowledge the help and support of all of the members of the Wolfson lab, especially Dr Love for his encouragement and guidance. This work would also not have been possible if it was not for the technical support offered by our technician Janet Laird.

Publications

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Abbreviations

AAAP	: Auxin amino acid permease
ABA	: Abscisic acid
ACMV	: African cassava mosaic virus
ATF	: Aphid transmission factor
AOX	: Alternative oxidase
ATP	: Adenosine triphosphate
BCTV	: Beet curly top geminivirus
BYDV	: Barley yellow dwarf virus
CaMV	: Cauliflower mosaic virus
CMV	: Cucumber mosaic virus
CP	: Coat protein
dCTP	: Deoxycytosine 5'-triphosphate
dNTP	: deoxyribonucleotide triphosphate
dpi	: Days post inoculation
EACMV	: East African cassava mosaic virus
GA	: Gibberellic acid
GFP	: Green Fluorescent Protein
GLM	: General Linear Model
GUS	: β -glucuronidase
HR	: Hypersensitive Response
IAA	: Indol-3-acetic acid
JA	: Jasmonic acid

LB	: Luria-Bertani media
MeJA	: Methyl jasmonate
MP	: Movement protein
MS	: Mass spectrometry
NLS	: Nuclear localization signal
NPA	: 1-naphthaleneacetic acid
ORF	: Open reading frame
PCR	: Polymcrase Chain Reaction
PD	: Plasmodesmata
PAT	: Polar auxin transport
PAV	: Barley yellow dwarf virus strain PAV
PPV	: Plum pox virus
PR	: Pathogenesis-related
PVM	: Potato virus M
QPCR	: Quantitative poymerase chain reaction
RdRp	: RNA dependent RNA polymerase
ROS	: Reactive oxygen species
RT-QPCR	: Reverse transcriptase quantitative polymerase chain reaction
SA	: Salicylic acid
SAR	: Systemic Acquired Resistance
SEL	: Size Exclusion Limit
TAV	: Transactivator domain
TBSV	: Tobacco bushy stunt virus

TCV	: Turnip crinkle virus
TGB	: Triple gene block
TIBA	: Triiodobenzoic acid
TMV	: Tobacco mosaic virus
ToMV	: Tomato mosaic virus
TRSV	: Tobacco ringspot nepovirus
TSV	: Tobacco streak virus
TSWV	: Tomato spotted wilt virus
UBQ	: Ubiquitin

Abstract

The role of auxin in the host response during compatible virus infection was examined in *Arabidopsis* with Cauliflower mosaic virus (CaMV). A genetic approach was adopted and a number of auxin signalling and transport mutants were used.

The host responses including, symptom type and severity, virus movement and virus titre were not affected by disruptions to auxin signalling conferred by mutations of the *AUX1* influx carrier or by mutations in *TIR1* or *TIR2*. The *tir3-1* mutation which contains a mutation in *TIR3* locus which is involved in the synthesis, localisation and functioning of the NPA binding site within the efflux carrier as well as protein trafficking did show an altered response to infection. *tir3-1* plants showed a consistent delay in the development of symptoms as well as delayed and reduced virus titre and viral spread.

Virus infection up-regulated the expression of a number of auxin responsive genes, *SAG21*, *SAUR_e* and *GH3*. This upregulation was significantly reduced in *aux1-100* and *tir3-1* mutants. The expression of another auxin responsive gene *LAX2* was found to be reduced in response to infection. From these findings it would appear that auxin signalling and transport is involved in some way during the infection process.

Auxin levels were quantified in the *AUX1* mutant allele *aux1-100* and the wild type WS. No differences in levels of free IAA were observed. A synthetic auxin response element was also examined using both GUS and GFP reporters

but again no alterations in auxin could be found. This could be due to the changes in auxin concentration being very subtle and therefore the techniques used failed to identify these changes, or it could be that the virus is interacting with specific components of the auxin signalling and transport pathways and the auxin responses are as a consequence of this rather than direct changes in auxin levels.

One CaMV gene product which may be interacting with the auxin pathway is P6. Previous studies have shown that P6 is responsible for symptom determination and host response and that it interacts with other signalling pathways including the ethylene and SA pathways. It has been found here that P6 confers resistance to the auxin efflux inhibitor TIBA and that it increases the expression of the auxin responsive gene *SAUR_e*. This resistance is independent of the chlorosis and stunting associated with P6. Preliminary genetic analysis of this interaction has identified an interaction between *tir3-1* and P6. Progeny of crosses between *tir3-1* mutants and the P6 transgenic Arabidopsis line A7 show an aberrant segregation ratio which suggests that *tir3-1* is able to suppress the P6 phenotype.

These results suggest that a specific component of the auxin signalling pathway is involved in the host response to compatible infection. It is possible that P6 interacts directly with *TIR3* rather than altering IAA levels. *TIR3* is involved in protein trafficking and therefore could be a requirement for successful infection. Alterations in this gene could affect the trafficking of auxin signalling

components such as PIN1 and lead indirectly to the auxin responses seen during infection.

Chapter One

Introduction (1.1)

Plants are vulnerable to attack by a variety of different pathogens, including bacteria, fungi and viruses, and so must therefore continually defend themselves against infection. The effects of bacterial and fungal pathogens on plant growth and the pathogenic effects of plant viruses on plant growth, development and symptom production are well documented in plant literature but the mechanisms by which plant viruses cause these effects are less well understood at a molecular level.

The speed and pattern at which a virus moves in nature depends on the relationship between the virus and the vector, on the numbers and activity of the vectors and on many other factors (Hull, 2001). Some viruses under specific conditions may infect a plant without producing any obvious signs of disease whilst others may lead to the rapid death of the whole plant. One of the main reasons for studying plant viruses is the impact that the diseases they cause have on crop productivity worldwide. It has been suggested that at least 10% of global food production is lost due to plant disease (Strange and Scott, 2005). Viruses are of economic importance when they cause some significant difference in the growth of the host plant compared to a normal uninfected plant, or when they reduce the quality of the crop. It is difficult to obtain accurate data on the scale of the losses caused each year by viruses, as these losses are commonly less obvious than those caused by other plant pathogens and tend to last for much longer. Barley yellow dwarf virus (BYDV), for example, is distributed worldwide and infects over 150 species of the Poaceae, including the majority of

the staple cereals, such as wheat, rice, barley, oats and maize (Strange and Scott, 2005). A number of studies have demonstrated the debilitating effect these viruses have on cereals, for example, grain yield of maize infected with the PAV serotype of the virus was 15-20% less than in uninfected controls because the infected plants produce fewer kernels per ear (Beuve *et al.*, 1998). Whereas barley infected with the same serotype showed reductions in yield up to 38% (Edwards *et al.*, 2001).

Cassava is a very important food crop for farmers particularly in low-income, food deficit countries. However, it is prone to diseases caused by mosaic viruses. The sensitivity of the crop to such viruses as African cassava mosaic virus (ACMV), East African mosaic virus (EACMV) and a more severe recombinant virus called UgV, and the extent of the losses caused by these isolates, has led to many farmers abandoning cassava cultivation (Zhou *et al.*, 1997). This has serious consequences for food production and security in the African countries affected.

It is the physical symptoms of disease e.g. stunting, vein clearing and chlorosis that cause the loss of viable crop plants and hence lead to economic losses (Gibbs and Harrison, 2002). Therefore it is necessary to investigate the causes of these symptoms of disease because it is only once we understand the causes that we will be able to start addressing the issue of how we can control them. The ability to control the development of symptoms during viral infection would allow a better understanding of how to reduce the economic losses that are

caused by viral diseases throughout the world. A detailed study of plant-microbe interactions will also allow us to elucidate the signalling mechanisms by which plant cells deal with a stress situation. In order to study the effects of plant viruses on host species model systems have been exploited using viruses that are well characterised and host plants that are equally well understood. One such system is the Caulimovirus, Cauliflower mosaic virus (CaMV) and *Arabidopsis thaliana* (*Arabidopsis*).

Symptoms (1.2)

When a host plant is infected with a virus either a compatible or incompatible interaction may occur. Recognition of a genetically incompatible pathogen results in the activation of a complex series of defence responses (DeWit, 1997; Dangl and Jones, 2001). The process is co-ordinated both temporally and spatially to ensure that only the necessary numbers of plant cells are recruited from primary metabolism into a defensive role (Hull, 2001; Hammond-Kosack and Jones, 2000). This rapid and highly localised induction of plant defence response results in the creation of unfavourable conditions for pathogen growth and reproduction, at the same time, the responding cells detoxify and impair the spread of harmful enzymes and toxins produced by the pathogen. Full activation of this intense response to the pathogen generally occurs within 24 hours and usually leads either directly or indirectly to localised cell and tissue death known as the Hypersensitive Response (HR; Heath, 2000; Shirasu and Schulze-Lefert, 2003) and eventually localised and systemic resistance (Hull, 2001; Dangl *et al.*,

1996). A compatible reaction however, is one in which the pathogen can spread throughout the plant and cause a systemic infection. During compatible interactions the expression of specific host and viral genes leads to the development of viral disease and the symptoms associated with such disease.

The fact that both viruses and viroids induce disease symptoms demonstrates that nucleic acids themselves, as well as proteins, are sufficient to cause symptoms. However, the severity of symptoms is not necessarily related to pathogen titre, indicating that disease is often the result of specific interactions and not general distress (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002; Lee *et al.*, 1996). For example, a spontaneous mutant of Tobacco streak virus (TSV) that has a single nucleotide change in the intergenic region of RNA3 induces more severe and prolonged symptoms in tobacco than the parental virus, despite a lower titre (Xin and Ding, 2003).

The symptoms associated with disease differ greatly depending on the virus, host plant and environmental conditions (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002). Leisner *et al.*, (1992) demonstrated in CaMV infection of *Arabidopsis* that if the development of early flowering plants is retarded by growth under sub-optimal conditions inoculated plants become more susceptible to the virus and systemic infections more widespread.

Local symptoms tend to include localised lesions that develop near the site of entry, a loss of chlorophyll, and the development of necrotic lesions where

infected cells have died (reviewed by Hull, 2001). Often these localised lesions are not of any great economic significance. Systemic infection however, is of much more importance economically and can induce a range of symptoms depending on the virus and the host plant. Infected plants often show common features such as, leaf yellowing, leaf distortion and/or many other growth distortions. Stunting is common during viral infection and may affect the whole plant or certain parts of the plant more than others (reviewed by Hull, 2001). This is generally due to a reduction in leaf size rather than a reduction in the number of leaves that develop. Reductions in the total yield of fruit is common as well as a reduction in the quality of the fruit produced which again is of major economic significance e.g. with Plum pox virus (PPV; Németh, 1994; Dunez and Susic, 1988). One of the most frequent and most obvious effects of virus infection is the development of mosaic patterns in infected leaves. Vein banding can vary in its severity, with some plants showing very faint banding and others much more striking emphasis to the vein pattern. This feature of virus infection can often persist as a major symptom of the disease. Symptoms of disease are often similar to those produced by a wide range of physical, chemical and biological agents, such as, hormone treatment and temperature changes, which indicates that it is changes within the host plant that leads to the physical symptoms of disease. It is thought that virus infection activates genetically pre-programmed responses in the host plant (Covey *et al.*, 2000; Johal *et al.*, 1995) resulting in the development of specific symptoms.

Plants can show a variety of responses to pathogens and in the case of susceptible plants they usually develop the symptoms of disease. An additional host response to viral infection is sometimes characterized by the initial development of the typical symptoms associated with the infection, followed by a recovery of the host plant (Chellappan *et al.*, 2004). This recovery is due to inactivation of the virus by a host response reminiscent of cosuppression of transgenes and posttranscriptional gene silencing (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al Kaff *et al.*, 1988). It was demonstrated that when Kohlrabi (*Brassica oleracea gongylodes*) was infected with CaMV it initially developed systemic symptoms but within weeks it had recovered through a loss of the virus. At 14 or 15 days after infection, a few days before the amelioration of the plant symptoms, there was a sudden transition in the composition of viral DNAs (Al Kaff and Covey, 1995). Reverse transcription products were lost, whereas supercoiled DNA was amplified. These changes are consistent with a sudden arrest of viral replication by gene silencing. To escape the gene silencing defences of plants, viruses have developed a counter defence strategy. Many plant viruses encode suppressors of gene silencing (Moissard and Voinnet, 2004; Chapman *et al.*, 2004; Voinnet *et al.*, 1999), as do some animal and insect viruses (Li *et al.*, 2002). For example the CMV 2b protein of Cucumber mosaic virus (CMV; Ji and Ding, 2001), HC-PRO of Potyviruses (Brigneti *et al.*, 1998; Zhao *et al.*, 2005) and P19 of tombusviruses (Silhavy *et al.*, 2002; Qui *et al.*, 2002) are associated with the enhancement of viral pathogenicity and virus accumulation.

Movement (1.3)

To induce a disease, a virus has to be able to spread from the site of initial infection throughout much of the rest of the plant and replicate. Over recent years the understanding of how viruses move within plants has greatly increased, and has led to the identification and characterization of virus encoded proteins that are involved in virus movement from cell to cell, known as movement proteins or MPs, as well as other factors that control movement. It was first suggested by Samuel (1934) that virus movement could be divided into two different components, cell to cell movement and long distance movement. Cell to cell movement usually refers to the movement from the initially infected cell or cells, usually epidermal or mesophyll cells, to the vascular bundle. Once a virus arrives at the vascular tissue long distance movement begins and the virus is transported through the vasculature, usually through the phloem sieve tubes to the rest of the plant. Then further cell to cell movement establishes systemic infection. In order for plant viruses to move from cell to cell within their hosts they have had to overcome the barrier that the cell wall creates. To do this, plant viruses, have evolved to move between cells directly through intercellular connections, the plasmodesmata (Hull, 1989; Lucas and Gilbertson, 1994). Plasmodesmata are cytoplasmic connections between adjacent cells and they play important roles in intercellular transport, cell to cell communication, cell differentiation, plant growth and development (Gunning and Robards, 1976; Boevink and Oparka, 2005). The smallest physical dimension of a known plant

virus is larger than 10 nm (Gibbs, 1976) whilst a recent high-resolution electron microscopic study established that the most obvious transport channels within plasmodesmata of tobacco leaves are physically about 2.5 nm in diameter (Ding *et al.*, 1992). Therefore, plant virus particles are too large to be able to pass through unmodified plasmodesmata, so it is believed that this transport likely occurs by an active mechanism mediated by specific viral movement proteins (Oparka, 2004; Oparka and Roberts, 2001).

The first proposed movement protein was identified by Nishiguchi and colleagues (1978) who showed that the temperature sensitivity in the capacity of Tomato mosaic virus (ToMV) to move through its host was not due to any effect on replication and was subsequently mapped to the 30 KB pp gene. This then led to the identification of the 30-kDa movement protein of tobamoviruses (Deom *et al.*, 1987), and since then, to the identification of MPs in many other plant viruses. It is thought that different virus MPs act in different ways to facilitate the spread and movement of the virus through the host plant (Carrington *et al.*, 1996).

Four virus families including the caulimoviridae produce tubules that extend through the plasmodesmata or cell walls. In the case of the caulimoviridae it has been shown that these tubules contain virus particles. Carrington *et al.* (1996) suggested that MPs localize to the plasmodesmata where they induce the removal of the desmotubule and assemble into tubules that extend unidirectionally into the adjacent cell. Virions that have been assembled in the

cytoplasm are then escorted to the tubules through interactions with their MP and subsequently transported to the adjacent cell (Perbal *et al.*, 1993; Storms *et al.*, 1995). Other MPs act as chaperone molecules, which bind to the viral nucleic acid and form an extended and unfolded protein-nucleic acid complex that targets modified plasmodesmata (Carrington *et al.*, 1996). This is thought to be how Tobacco mosaic virus (TMV) MP functions (Citovsky, 1999). TMV MP interacts with the plasmodesmata and modifies the gating properties and therefore directly facilitates the movement of viruses through the plasmodesmata (Ding *et al.*, 1992; Wolf *et al.*, 1989). It is thought that MPs also facilitate the active transport of genomes from cell to cell rather than merely increasing the Size Exclusion Limit (SEL) (Citovsky *et al.*, 1990; Citovsky and Zambryski, 1991). BL1 MP of bipartite geminiviruses and the product of ORF 2 of potato virus X are also believed to act in this way (Carrington *et al.*, 1996).

A final group of plant viruses exploit a unique triplet of proteins known as the triple gene block (TGB) of movement proteins to facilitate virus spread and establish a systemic infection in the host plant (Waigmann *et al.*, 2004; Hull, 2001). The three proteins work together to transport the virus RNA from the initial infected cell, through surrounding cells to the vascular system for translocation throughout the plant (Morozov and Solovyev, 2003). Although some information is available on the relative roles of the TGB proteins, the molecular details of the processes are not well understood. A combination of molecular and cell biology tools have been used to try to dissect the mechanisms involved (Lough *et al.*, 1998; Torrance *et al.*, 2006; Tamai and Meshi, 2001).

The phloem of higher plants is an essential pathway for the movement of photoassimilates from mature, exporting tissues often referred to as sources, to immature, developing tissues that exert a carbon demand and therefore act as sinks (Gifford and Evans, 1981; Roberts *et al.*, 1997). Phloem transport and unloading within intact plants has been confirmed through the use of confocal laser scanning microscopy (Oparka *et al.*, 1994; Wright *et al.*, 1996). It has been shown that plant viruses also have the ability to move over long distances in the phloem (Maule, 1991; Leisner and Turgeon, 1993; Roberts *et al.*, 1997). This process of long-distance transport is less well understood than that of cell to cell transport and has very different requirements from those of cell to cell movement (Ryabov *et al.*, 1999). Minor veins are generally sheathed by bundle sheath cells and contain various cell types including vascular parenchyma cells, companion cells and enucleate sieve elements (Nelson and van Bel, 1998). Therefore, transport of a virus to and within vascular tissue implies movement from mesophyll cells to bundle sheath cells, from bundle sheath cells to vascular parenchyma and companion cells, and entry into sieve elements (Ryabov *et al.*, 1999). It is unclear how or where these viruses exit the phloem, but it could be in the reverse order to that described. It has been shown, that coat protein (CP) is essential for efficient long-distance movement of most plant viruses (Petty and Jackson, 1990). However, CP is not required for cell to cell movement of TMV but it is essential for long-distance movement (Ryabov *et al.*, 1999). In the few cases where the CP gene is partially or entirely dispensable for systemic spread, the time required for systemic spread may be greatly increased in its absence (Scholthof *et al.*, 1995). Many viruses also encode

proteins that provide additional functions required for systemic spread (Ryabov *et al.*, 1999). Tobacco bushy stunt virus (TBSV) encodes two structural proteins, p19 and p22, both of which are necessary for systemic infection. Cell to cell movement functions are provided by p22, whereas p19 promotes long-distance transport in a host specific manner (Scholthof *et al.*, 1995). CMV also encodes a protein, 2b, that promotes host specific long-distance movement (Ding *et al.*, 1995; Ji and Ding, 2001) and potyvirus HC-Pro protein provides functions required for both long-distance movement and efficient genome replication (Klein *et al.*, 1994; Cronin *et al.*, 1995). CMV 2b, the HC-Pro of potyviruses and P19 of TBSV are all suppressers of gene silencing, thus implies a link between long distance movement and silencing.

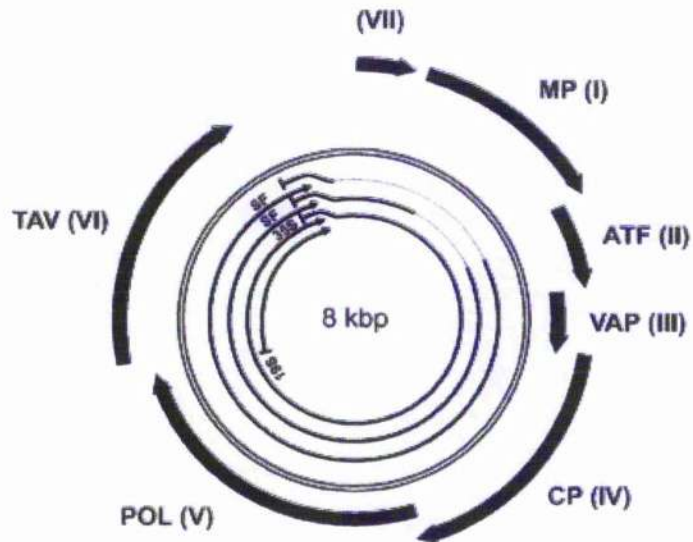
There is also some limited genetic evidence that MPs perform specific long-distance movement functions in addition to their roles in cell to cell movement (Fenczik *et al.*, 1995; Wang *et al.*, 1998). For example, several alanine-scanning mutants of Red clover necrotic mosaic virus (RCNMV) with substitutions affecting the MP exhibit host-specific defects in long-distance transport (Carrington *et al.*, 1996). Additionally, many viruses encode replication proteins that appear to have specific roles in long-distance transport (Weiland and Edwards, 1996; Ding *et al.*, 1995; Treynor *et al.*, 1991; Nelson *et al.*, 1993).

Cauliflower mosaic virus as a model system (1.4)

A useful model system for showing the interaction between a higher plant and a compatible viral pathogen is Cauliflower mosaic virus and the plant *Arabidopsis*. CaMV is often used as a model system as a large amount of information is known about this virus, and it is convenient to use as there are many isolates available that range in pathogenicity (Al-Kaff and Covey, 1995).

CaMV is a member of the Caulimoviridae family, which is the only family of plant viruses with dsDNA genomes. Family Caulimoviridae contains all of the plant viruses that replicate through reverse transcription. All have a circular double-stranded DNA genome with gaps or discontinuities at specific sites, and asymmetric transcription gives rise to RNA, which is longer than the genome (Hull, 2001; Haas *et al.*, 2002). This RNA molecule is both the template for reverse transcription and the mRNA for at least some of the gene products. There are six genera within this family and these fall into two groups, the caulimoviruses and the badnaviruses.

The CaMV virus comprises (T=7) icosahedral particles containing an 8 Kb circular double stranded DNA genome and has been found to encode six potentially eight genes which are closely spaced but with very little overlap except for the possible gene VIII (see **Figure 1.1**; Haas *et al.*, 2002; Hull, 2001; Cheng *et al.*, 1992).






Key:  Open Reading Frames
 DNA
 Transcripts - 35S RNA, 19S RNA and two spliced forms (SF)

Fig.1.1 Schematic diagram of the CaMV genome showing the seven ORFs and their associated functions. Movement Protein (MP), Aphid Transmission Factor (ATF), Capsid Protein (CP), Transactivator Domain (TAV). Adapted from Ryabova *et al.*, (2004).

The functions or roles of the products produced by these genes have been determined (see **Table 1.1**). CaMV is a member of the pararetrovirus family (Haas *et al.*, 2002). Pararetroviruses have two phases to their replication cycle, a nuclear phase where the viral DNA is transcribed by host DNA-dependent RNA polymerase II; and a cytoplasmic phase where the RNA product of transcription is reverse-transcribed by virus encoded RNA-dependent DNA polymerase or reverse transcriptase (RT) to give DNA.

During replication of CaMV, the dsDNA of the virus particle moves to the cell nucleus, where the overlapping nucleotides at the discontinuities are removed, and the gaps covalently closed to form a fully closed dsDNA (Covcy *et al.*, 1990). The covalently closed DNA associates with host histones to form a minichromosome that is used as a template by the host DNA dependent RNA polymerase II to transcribe two RNAs, 19S and 35S. The two polyadenylated RNA species then migrate to the cytoplasm for the next phase of replication. The 19S RNA is translated in large amounts to produce the protein P6 and the 35S mRNA is reverse transcribed to produce a negative DNA strand and once this is complete a positive DNA strand is synthesised. These RNAs are translated by the cellular machinery following two unconventional strategies, ribosomal shunt and termination-reinitiation (reviewed by Rayabova *et al.*, 2002). Virus particles together with viral proteins accumulate in inclusion bodies composed of P6, the product of CaMV ORF VI, which will be discussed later. Virus particles may be taken up by aphids, used to reinfect the nucleus or transported to other cells.

Gene	Product Size (KDa)	Role/Function
I	37	Movement of virus
II	19	Aphid transmission
III	14	Capsid associated protein within virus particle, aphid transmission
IV	57	Precursor of the 42 kDa protein subunit of the icosahedral shell
V	79	Viral reverse transcriptase gene
VI	58	Major protein of viroplasms, significant role in disease induction, symptom expression and in controlling host range. Also functions in trans to activate translation of other viral genes.
VII/VIII	—	Not present in other Caulimoviruses. Function unknown.

Table 1.1 Details of the CaMV open reading frames including protein size and function.

CaMV infects plants of the Cruciferae family, including the Brassicae species and Arabidopsis, often inducing symptoms such as vein clearing, chlorosis and stunting. CaMV infected Arabidopsis seedlings develop mosaics and stunting symptoms, although the character and severity of these symptoms varies, and is dependent on the genetic background of both the host and the CaMV isolate used (Cecchini *et al.*, 1998). A number of CaMV isolates (>20) varying in severity from very mild to very severe are available for studies into the infection process. Cecchini *et al.*, (1998) characterised the pathogenic interactions between variants of CaMV and Arabidopsis, and combinations thought to be useful in molecular genetic studies were identified. When a single Arabidopsis ecotype was infected with a range of CaMV isolates a variety of symptom types were seen. For example, isolate Bari-1 was asymptomatic in ecotype Col-0, and yet caused mild symptoms in ecotype Ler although the levels of virus that accumulated were similar in both ecotypes (Cecchini *et al.*, 1998). Therefore, the symptoms of viral disease in plants must be dependent on specific host loci, which presumably influence symptom expression independently of levels of virus accumulation within the plant (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002). Lee *et al.*, (1996) reported differences in symptom expression in Arabidopsis ecotypes infected with Tobacco ringspot nepovirus (TRSV) that appeared to be independent of virus accumulation. This suggests that it is specific interactions between host and virus gene products that contribute to symptom expression, not necessarily the amount of virus present. Growth conditions and environments can also affect symptom development, with light being an important factor (Cecchini *et al.*, 1998;

Cecchini *et al.*, 2002). Leisner *et al.*, (1992) demonstrated that if the development of early-flowering plants is retarded by growth under sub-optimal conditions, inoculated plants become more susceptible to CaMV and systemic infections more widespread.

A role for the P1 protein has been identified in CaMV cell to cell movement (Thomas *et al.*, 1993; Haas *et al.*, 2002). P1 has been found to be localised to cell walls and specifically to plasmodesmata. It forms tubules through the plasmodesmata, allowing CaMV particles to move from one cell to another (Perbal *et al.*, 1993) There is also significant amino acid homology between the gene I protein and the P30 movement protein of TMV. It has been demonstrated that a central domain of P1 is required for targeting the protein to the cell periphery (Huang *et al.*, 2001), whereas most of the protein, except for the C-terminal is required for tubule formation (Thomas and Maule, 1999). P1 has also been found to contain an RNA-binding domain that partially overlaps the sequence involved in its localization to the cell periphery (Citovsky *et al.*, 1991; Thomas and Maule, 1995).

CaMV is transported systemically through the phloem where it moves with the flow of photoassimilates (Leisner *et al.*, 1992; Love *et al.*, 2005). Therefore systemic CaMV infections spread from source leaves to sink leaves. Sink-source relationships change during plant development, as does the pattern of virus

movement. Further evidence for virus movement being dependent on the movement of photoassimilates is that CaMV does not invade leaves that have already gone through the sink to source transition but follows the movement of photoassimilates from source to sink (Leisner *et al.*, 1992). However, in contrast to photoassimilates, CaMV is restricted from developing leaves at a much earlier stage of development (Leisner *et al.*, 1992). Therefore, during development, the region of the plant that is accessible to systemic infection from any given source leaf is progressively reduced. Plants with an early flowering phenotype were reported to show reduced susceptibility to CaMV (Leisner and Howell, 1992) and this is known as 'developmental resistance'. Cecchini *et al.*, (2002) have subsequently shown that mutations that delay flowering enhanced symptom development although virus titres were unaffected. Arabidopsis plants have such a rapid life cycle that even small alterations in environmental conditions can have a profound effect on the apparent susceptibility to systemic CaMV infections.

The product of ORF II P2 is an 18 KDa protein that is involved in the aphid transmission of CaMV but which is dispensable for virus replication (Armour *et al.*, 1983). Several experiments have demonstrated a role for P2 in aphid-mediated transmission of virus particles and P2 is therefore considered to be the aphid transmission factor (ATF). This conclusion was reinforced by the observation that some non-aphid transmissible strains of CaMV contain a mutation that maps to P2, such as Campbell and CM1841 (Haas *et al.*, 2002). P2

is also a major component of the electron-lucent inclusion bodies formed in infected cells; these also contain P3 as well as scattered virions (Druckler *et al.*, 2002). However, P2 is not associated with the viroplasms/inclusion bodies composed of P6, suggesting that after its synthesis P2 is rapidly released and accumulates to form electron-lucent inclusion bodies. P2 self assembles into highly organised paracrystalline filaments in plants (Blanc *et al.*, 1993) via coiled-coil structures involving the C-terminal region (Hebrard *et al.*, 2001).

This region of P2 also interacts with CaMV P3 protein (Leh *et al.*, 1999), so that the formation of P2-P2 and P2-P3 complexes which must somehow be regulated during the viral cycle, play an essential role in the interaction between virions and the aphid stylet. P3 is the 15KDa product of ORF III and has been shown to be closely associated with purified CaMV particles (Dautel *et al.*, 1994). It is associated with the capsid but no structural role has been found. An 11 KDa product resulting from the cleavage of P3 at its C-terminus by a cysteine proteinase is frequently detected when extracts from CaMV infected plants are incubated (Guidasci *et al.*, 1992). P3 has also been shown to play a pivotal role in the formation of a CaMV complex transmissible by aphids (Leh *et al.*, 2001).

The product of ORF IV protein P4, (approximately 56 KDa), is the precursor of the capsid proteins (CP) and corresponds to the gag protein found in retroviruses (Haas *et al.*, 2002). The acidic N- and C-terminals are removed post translationally by the viral aspartic proteinase encoded by ORF V, and by cellular proteases to produce a set of polypeptides ranging in size from 37 to 44

KD (Torruella *et al.*, 1989). These polypeptides possess a zinc finger motif at their C-terminus, which is conserved in all pararetroviruses and in the nucleocapsid proteins of retroviruses. This zinc finger motif is of particular importance, since mutations abolish not only the interaction with RNA but also CaMV infectivity (Guerra-Peraza *et al.*, 2000). Close to their N-terminus the capsid proteins possess a nuclear localization signal; (NLS) which is exposed on the surface of mature virions. Mutations in this region can completely abolish infectivity (Leclerc *et al.*, 1999). Karises *et al.*, (2002) have shown that the acidic N-terminus inhibits nuclear targeting, making it likely that this inhibition acts as a control making sure that the CP precursor is used for virus assembly in the cytoplasm, and that only mature virions reach the nuclear envelope.

The ORF V encodes a polypeptide (P5), of 78 KDa in size. Homology between ORF V of CaMV and the pol gene products of various animal retroviruses indicates that P5 is involved in the replication of CaMV DNA by reverse transcription (Hass *et al.*, 2002). P5 contains an aspartic proteinase and a reverse transcriptase domain in the N-terminal region. However unlike the pol gene from retroviruses it does not contain an integrase motif. The proteinase, 18 KDa is released from P5 by self-cleavage (Torruella *et al.*, 1989). The reverse transcriptase uses tRNA as a primer for the synthesis of (-) DNA strand from the pre genomic RNA. RNase H digests the RNA template except for a few purine rich sequences, which remain, annealed to the (-) DNA strand and then act as primers for the synthesis of the (+) DNA strand (Haas *et al.*, 2002).

ORF VI encodes a multifunctional 60-66 KD polypeptide (P6) that is essential for CaMV infection. At a cellular level, a prominent cytopathological effect of CaMV infection is the appearance of viral inclusion bodies or viroplasms in the cytoplasm of infected cells (Covey and Hull, 1981). The major inclusion body matrix protein is P6, translated from the 19S RNA. The primary role that has been identified for P6 is modification of the host translation machinery to facilitate the translation of the polycistronic CaMV 35S RNA (Rothnie *et al.*, 1994; Park *et al.*, 2001). This function has been designated the translational transactivator (TAV) function (Bonneville *et al.*, 1989; Scholthof *et al.*, 1992), which is mediated by physical interactions between an internal region, including the minimal sequence of P6 required for transactivation, the initiation factor eIF3 (Park *et al.*, 2001) and the ribosomal proteins L13 (Bureau *et al.*, 2004), L18 (Leh *et al.*, 2000), and L24 (Park *et al.*, 2001). It has since shown that as well as being required for the expression of other CaMV genes, P6 is also involved in host range and symptom determination (Daubert *et al.*, 1984; Baughman *et al.*, 1988; Agma *et al.*, 2002). Construction of recombinants between CaMV variants showing different pathological characters led to the conclusion that the gene VI region contains pathogenic determinants, including those that regulate host specificity and symptom type and severity (Wintermantel *et al.*, 1993; Daubert *et al.*, 1990). *In vitro* mutagenesis of P6 suggests that the pathogenicity determinants map to the N-terminal region and may be distant from the TAV and interaction domains (Kobayashi and Hohn, 2003). Further evidence implicating gene VI as an important CaMV pathogenic determinant comes from experiments

in which P6 has been expressed as a transgene in plants in the absence of virus infection.

Expression of P6 in both nonhost, *Nicotiana tabacum*, and host plants, *Arabidopsis* leads to the development of symptom-like leaf mosaics, mottling, chlorosis and necrotic spots (Baughman *et al.*, 1988; Goldberg *et al.*, 1991; Cecchini *et al.*, 1997; Zijlstra and Hohn, 1996) and in *Arabidopsis* P6 transgenics, cells contained empty inclusion bodies, characteristic of infected plants (Cecchini *et al.*, 1997). The symptom character was dependent on the level of P6 expression, and on the isolate from which the P6 originated (Cecchini *et al.*, 1997). Geri *et al.* (1999) performed differential display polymerase chain reactions to identify changes in gene expression in P6 transgenic *Arabidopsis*. They identified changes in the abundance of more than 30 host genes, (either up or down-regulated), and they noted that the altered gene expression patterns were broadly similar to those observed in CaMV-infected *Arabidopsis*.

Plant Hormones and Responses to Virus Infection (1.5)

Plant hormones are signal molecules, and changes in their concentration and quantity regulate a whole range of developmental processes, many of which involve interactions with environmental factors. Virus infection can result in the alteration of physiological, biochemical, and metabolic processes within the plant (Fraser, 1987). The alterations in plant growth can be regarded as cytopathic effects of virus infection (Wenham and Fraser, 1990), which may be

manifested in alterations of plant hormone metabolism. During virus infection, the symptoms that develop may include those that reflect changes in the biosynthesis and metabolism of plant hormones (reviewed by Pennazio and Roggero, 1996). Early investigations into the effects of virus infection on plant hormones showed varied results, but pointed towards a role in plant pathogen interactions. However, since the 1970s, little new work has been done on the role of classical plant hormones such as gibberellic acid (GA), abscisic acid (ABA), auxin and cytokinin in virus infection. Past investigations that tried to establish the role these hormones play in virus infection relied mainly on techniques such as bioassays and local lesion assays, where crude infected sap from hormone-treated material was inoculated onto a secondary host and local lesion production recorded (Jameson and Clarke, 2002; Fraser and Whenham, 1987). Attempts were made to measure hormone levels in infected plants using techniques that were only semi-quantitative and often gave rise to inaccurate and inconsistent results. For example, Rajagopal (1977) showed a decrease in abscisic acid (ABA) concentration in TMV-infected tobacco in the early stages of virus infection whereas Keller and Lutge (1991) showed that infected plants had a higher ABA content (Jameson, 2002). Only a limited number of studies of GA content following virus infection have been carried out and the majority of these reported a decrease in GA activity in virus-infected tissues. Ben-Tal and Marco (1980) reported a significant reduction in GA concentration in cucumber mosaic virus (CMV) - infected cucumber. More recently Zhang *et al.*, (1997) used a GA based ELISA to confirm earlier studies reporting a decrease in GAs in diseased compared with healthy tissue. Kuriger and Agrios (1977) demonstrated a decline

in cytokinin activity in root exudates of cowpea infected with tobacco ringspot virus (TRSV), whereas Sziraki *et al.*, (1980) reported that systemic infection by CMV caused an increase in cytokinin content in leaves of tobacco (Pennazio and Roggero, 1996). The inconsistency of these results has made it impossible to define the role of these hormones or even determine if they are involved at all.

More specifically, the plant hormones ethylene, jasmonic acid (JA) and salicylic acid (SA) have been shown to be important in plant defence responses against a variety of plant pathogens (for review see Dong, 1998; Raymond and Farmer, 1998) and a number of investigations have been carried out to establish the role of these hormones in controlling plant defence mechanisms during virus infection (see for example Knoester *et al.*, 2001; Penninckx *et al.*, 1998; Niki *et al.*, 1998; O'Donnell *et al.*, 2003; Delaney *et al.* 1994).

Ethylene (1.5.1)

Ethylene is an endogenous plant growth regulator that affects many aspects of plant growth and development (reviewed by Stepanova and Ecker, 2000).

Ethylene is among the best-characterised plant hormones and participates in processes such as fruit ripening, inhibition of stem and root elongation, senescence, promotion of seed germination and flowering, and sex determination. Over the past few years understanding of ethylene's role in these processes, and the ethylene-signalling pathway have greatly increased. The major components of the ethylene-signalling pathway have been identified and

characterized through the use of genetic studies in *Arabidopsis*. This began with the identification of mutants defective in the ethylene triple response.

Arabidopsis seedlings grown for three days in the dark under continuous exposure to ethylene exhibit a phenotype that is known as the "triple response" (Ecker, 1995). This phenotype includes thick, short roots and hypocotyls, and an exaggerated curvature of the apical hook. Screens for mutants unable to generate the triple response when treated with ethylene have been used to identify ethylene insensitive mutants and those that show the triple response in the absence of ethylene are either ethylene overproducing or constitutive-signalling mutants (Bleecker *et al.*, 1988).

Over twelve genes have now been implicated in the ethylene signalling pathway and their order of action carefully determined. **Figure 1.2** shows a current model of the ethylene signal transduction pathway. In *Arabidopsis* a family of integral membrane receptors perceive ethylene. This family consists of at least five members, Ethylene Triple Response 1 (ETR1) and Ethylene Receptor 2 (ETR2), Ethylene Insensitive 4 (EIN4), and Ethylene Response Sensor 1 and 2 (ERS1 and ERS2) (Chang *et al.*, 1993; Hua *et al.*, 1995; Sakai *et al.*, 1998). Ethylene binds to these receptors via a copper cofactor (Rodriguez *et al.*, 1999) and studies have suggested that binding of the hormone inactivates the receptors (Hua and Meyerowitz, 1998). The receptors are predicted to be functionally active histidine-kinases that activate a Raf-like serine/threonine kinase, Constitutive Triple Response 1 (CTR1) which is also a negative regulator of the ethylene pathway and a MAPKK (Keiber *et al.*, 1993). Other components of the

pathway such as, EIN2, EIN3, EIN5 and EIN6 are thought to be positive regulators of the ethylene response (Roman *et al.*, 1995). Epistasis studies have now revealed that ETR1, ETR2 and EIN4 along with the homologs ERS1 and ERS2, work upstream of CTR1, whereas EIN2, EIN3, EIN5 and EIN6 appear to work downstream of CTR1. Ethylene Insensitive 3, EIN3, is believed to work downstream of EIN2 and serve as a transcription factor that, in response to ethylene binds to specific sequences in the promoters of target genes and activates their transcription. Ethylene Response Factor 1 (ERF1) is a positive regulator of ethylene signalling and is the only known target of EIN3 (Solano *et al.*, 1998; Chao *et al.*, 1997). In Arabidopsis EIN3 binds to the GCC-box in the promoters of several ethylene responsive genes (Solano *et al.*, 1998), including some defence related genes. Given the range of responses to ethylene, it seems that some ethylene-related phenotypes result from the activation of some genes and the repression of others

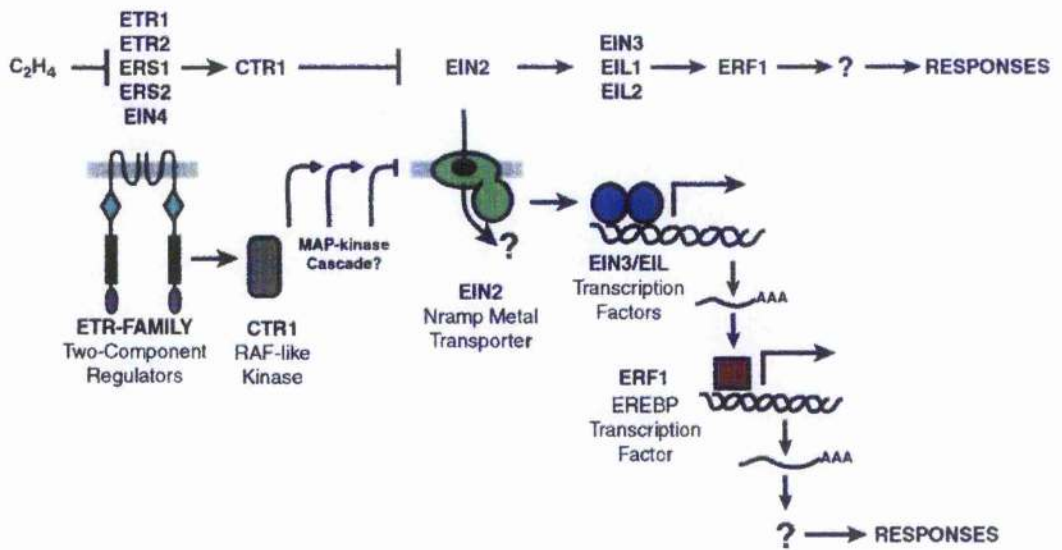


Fig 1.2 Ethylene signalling pathway. Diagram from: Bleecker (2000).

As well as elucidation of the ethylene signalling pathway, it has been shown that ethylene plays a central role in plant disease resistance and symptom development (reviewed by van Loon *et al.*, 2006; Knoester *et al.*, 2001; Thomma *et al.*, 1999; Geraats *et al.*, 2002). Ethylene gas is frequently synthesised during both incompatible and compatible host-pathogen interactions (Johnson and Ecker, 1998) and is thought to be a part of the plant defence mechanism against the spread of pathogens. In the past few years it has been demonstrated that a functional ethylene-signalling pathway is required for resistance against some but not all plant pathogens (Stepanova and Ecker, 2000). This was illustrated by Knoester *et al.*, (1998) who made use of transgenic tobacco plants transformed with a dominant-negative mutant allele (*etr1-1*) of the Arabidopsis ethylene receptor gene *ETR1*. The transgenic plants with a disrupted ethylene response were more susceptible than wild-type plants to normally non-pathogenic soil-borne *Pythium spp*, whereas their level of resistance to TMV was unaffected.

It has also been shown that ethylene production appears to be increased by virus infection and that it has been associated with the development of necrotic or chlorotic lesions, correlated with the suppression of hypocotyl elongation or associated with other growth abnormalities. The majority of the work has focused on systems in which a hypersensitive response occurs but Chaudhry *et al.*, (1998) specifically showed enhanced ethylene production in leaves of tobacco systemically infected with CMV. Love *et al.*, (2005) reported that CaMV infection stimulated the expression of ethylene responsive markers in Arabidopsis and have shown that two ethylene insensitive

mutants show reduced susceptibility to CaMV infection. The use of biosynthetic inhibitors, ethylene action inhibitors, or hypobaric conditions have all been shown to relieve virus-induced symptoms, including reducing lesion size (Ohtsubo *et al.*, 1999) delaying chlorotic lesion development (Marco and Levy, 1979), and increasing hypocotyl elongation and preventing virus-induced epinasty, all of which support causative role of ethylene in the development of these symptoms.

Although it is evident that ethylene plays a specific role in plant disease resistance, its effect on plant-pathogen interactions is pleiotropic and depends on the host and pathogen species and conditions tested. For example, ethylene overproduction in tobacco resulted in enhanced lesion formation upon TMV infection (Ohtsubo *et al.*, 1999). However, ethylene insensitivity had no effect on the hypersensitive response or the overall resistance of Arabidopsis plants to Turnip crinkle virus (TCV) infection (Kachroo *et al.*, 2000). Takahashi *et al.*, (2002) demonstrated through the use of Arabidopsis plants carrying the RCY1 resistance gene but defective in SA, JA and ethylene signalling that there is a requirement for ethylene and SA signalling in mounting a resistance response to CMV-Y.

Jasmonic Acid (1.5.2)

Jasmonic acid is a plant growth regulator derived from oxygenated fatty acids via the octadecanoid pathway and characterised by a pentacyclic ring structure

(reviewed by Schaller *et al.*, 2005). Regulated changes in the relative levels of this hormone and other forms of jasmonates contribute to the control of metabolic, developmental and defensive processes in plants. Jasmonates have been shown to be involved in fruit ripening, production of viable pollen, root growth, response to wounding and abiotic stress as well as defence against insects and pathogens. Increases in JA in response to pathogen or insect attack occur both locally and systemically (Schaller *et al.*, 2005). Reduced Potato virus M (PMV) content in potato meristems was correlated to increased jasmonic acid levels (Ravnikar *et al.*, 1990). Measurement of JA with radioimmunoassay showed that JA and its amino acid conjugates occur at different concentrations in virus-free and in PVM infected potato plants. They found that JA, in addition to a previously reported effect on senescence, increases the number of meristems which developed in plants *in vitro* and increases the proportion of mature virus-free potato plants (*Solanum tuberosum* L. cv. Vesna) completely infected with PMV.

A role for JA in plant defence was first shown by Farmer and Ryan (1990) who demonstrated the induction of proteinase inhibitors by methyl jasmonate (MeJA) and JA as part of the defence response against herbivorous insects. JA is now considered to be one of the key compounds in plant defence reactions to both biotic and abiotic stimuli. Jasmonates have been shown to be active inducers of antimicrobial phytoalexins (Gundlach *et al.*, 1992) and to induce defence gene expression for enhanced resistance against insect predators and pathogens (Blee *et al.*, 2002; reviewed by Reymond and Farmer, 1998). The regulation of insect-

and pathogen-defence-gene expression is affected in mutants that fail to respond to JA like *coil*, *jar1* and *jin1* mutants in Arabidopsis (Berger *et al.*, 1996; Staswick *et al.*, 2002; Lorenzo *et al.*, 2004) or the *jai1* mutant in tomato (Li *et al.*, 2004). It has been demonstrated that the production of jasmonates leads to the induction of many defence related genes including PDF1.2 a plant defensin (Pennickx *et al.*, 1998).

Salicylic Acid (1.5.3)

Salicylic acid (SA) is a central signalling intermediate in plant defence (Dempsey *et al.*, 1999). SA accumulates in plant tissues following a hypersensitive response (HR), a resistance response most often characterised by programmed host cell death at the point of attempted entry by the pathogen (Heath, 2000). The occurrence of HR is usually associated with an enhancement in resistance to a broad spectrum of pathogens throughout the plant called systemic acquired resistance (SAR; Dempsey *et al.*, 1999). This state of enhanced resistance can also be induced by treatment of plants that have not undergone an IIR with SA or its synthetic analogues (Dempsey *et al.*, 1999; Murphy *et al.*, 2001). SA is known to induce expression of many of the pathogenesis-related (PR) proteins involved in plant defence as well as limiting the replication and movement of viruses throughout the plant (Murphy and Carr, 2002; Hooft van Hujsduijn *et al.*, 1986; Naylor *et al.*, 1998). In recent years another layer of SA-regulated defence has been identified which is based upon RNA silencing although this link is not fully established (reviewed by Singh *et al.*, 2004). RNA silencing is a sequence specific RNA degradation process,

affecting all highly homologous sequences in which foreign, over-expressed or aberrant RNA molecules are targeted for destruction in a sequence specific manner (Baulcombe, 2001; Voinnet, 2001). This mechanism can contribute to resistance to viruses, and it has been demonstrated that a number of plant and animal viruses encode silencing suppressor proteins (Li *et al.*, 2002; Vance and Vaucheret, 2001). The possibility that a functional connection exists between SA-induced resistance to viruses and RNA silencing came from the discovery that a tobacco gene encoding an RNA-dependent RNA polymerase (RdRp) was induced by SA (Xie *et al.*, 2001). This is significant as RdRps from host plants can play important roles in the induction of RNA silencing (Ahlquist, 2002). Also the CMV protein 2b affects both RNA-silencing and SA-dependent defence responses (Ji and Ding, 2001)

Reactive oxygen species (ROS) are often produced during the early stages of a plant resistance response (Love *et al.*, 2005; Wojtaszek, 1997; Lamb and Dixon, 1997; Alvarez *et al.*, 1998). Production of ROS can significantly alter the redox balance within cells responding to the virus and it has become widely accepted that cellular redox or changes in redox state can influence the activity of protein molecules that participate in defence signalling, e.g. PR gene induction (Pastori *et al.*, 2003, Koch *et al.*, 1998). One of the ways in which a plant can regulate ROS generation while maintaining electron flow into the respiratory chain is through the activation of the Alternative Oxidase (AOX; Maxwell *et al.*, 1999).

It is clear that SA plays an essential role in plant resistance to virus infection but it will not be discussed further as it is not directly relevant to the main aims of the thesis.

Integration of Viral Defence pathways (1.5.4)

There is a growing body of literature that reports that JA, SA and ethylene defence signalling pathways do not function independently (Reymond and Farmer, 1998; Dong, 1998). It is thought that they are involved in a complex signalling network in which the different pathways influence each other through both positive and negative regulatory interactions.

Several studies have shown positive interactions between JA and ethylene signalling pathways (Reymond and Farmer, 1998; Dong, 1998). Both JA and ethylene signalling are required for the expression of the defence related gene *PDF1.2* in response to infection by *Alternaria brassicicola*, and for the expression of *PDF1.2*, *HEL* and *CHIIB* in response to treatment with *Erwinia carotovora* culture filtrates (Penninckx *et al.*, 1996). Furthermore, when both are applied exogenously to plant tissue, JA and ethylene appear to function synergistically to induce *PDF1.2*, *HEL* and *CHIB* in Arabidopsis.

There is a limited amount of data that suggests both positive and negative regulatory interactions between ethylene and SA. For example, SA and ethylene may function together to co-ordinately induce several defence related genes (Manners *et al.*, 2000), whilst the basal level of PR-1 mRNA appears to be

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There is a limited amount of data that suggests both positive and negative regulatory interactions between ethylene and SA. For example, SA and ethylene may function together to co-ordinately induce several defence related genes (Manners *et al.*, 2000), whilst the basal level of PR-i mRNA appears to be

significantly elevated in *ein2* mutant plants (Lawton *et al.*, 1994). This contradictory evidence may reflect the complexity of the interactions between ethylene and SA signalling pathways.

Interactions between SA and JA also appear to be highly complex, and as is the case with ethylene and SA there is evidence for both positive and negative regulatory interaction between these pathways (Doares *et al.*, 1995a; Doares *et al.*, 1995b). Several studies have provided evidence for an antagonistic effect of SA on JA signalling in *Arabidopsis*. The *eds4* and *pad4* mutants, which are impaired in SA accumulation, exhibit enhanced responses to inducers of JA-dependent gene expression (Gupta *et al.*, 2000). There is also growing evidence for an antagonistic effect of JA on SA signalling. Studies in tobacco have shown that JA inhibits the expression of SA dependent genes (Ohashi *et al.*, 1998).

Cross talk between signalling pathways is believed to be an important factor in allowing plants to respond effectively to a wide range of pathogens and allowing them to fine tune their response and to ensure that the right combination of defences are deployed against specific pathogens.

Auxin (1.6)

The relationship between auxin and virus diseases was first investigated by Grieve (1936) who studied the role of IAA in stunting in tomato plants infected with Tomato spotted wilt virus (TSWV). Grieve used the techniques employed by Went (1926) to compare the effects of diseased and healthy sap upon the inward curvature induced by auxin on split pea stems. In four experiments, such curvatures were observed upon the stems placed in healthy sap with auxin but were absent from those treated with virus infected sap and the hormone. This seemed to show an inactivation of the growth regulator by the virus, but a further number of similar experiments did not indicate any difference between the two kinds of treatment. Another more conclusive method of study involved the injection of various amounts of auxin into healthy, and into diseased tomato plants. Adventitious roots appeared upon the stem in the majority of healthy plants, but did not form upon the diseased tomatoes, unless the plant appeared to have thrown off the disease. Later, Baldacci (1953) suggested that IAA was liberated in virus-infected potato and that it was responsible for the resultant symptoms and distortions (Pennazio and Roggero, 1996). Most reports to date indicate a reduction in auxin activity and/or concentration in diseased plants, generally associated with stunting (Fraser and Whenham, 1982; Pennazio and Roggero, 1996). Smith *et al.*, (1968) found reduced auxin activity in bean, sugarbeet and tomato plants when infected with beet curly top geminivirus (BCTV). However, a substantial increase in auxin activity has sometimes been observed in some cases showing severe symptoms (Jameson *et al.*, 2002). In

both TSW- and TMV- infected tomato plants, Jones (1956) found auxin activities higher than in healthy control plants, although the former were visibly stunted (Fraser and Whenham, 1982).

Other evidence suggests that wound, pathogen and auxin perception may share conserved signal transduction pathways that cross-talk at some point. For example expression of a pathogen-induced gene can be mimicked by auxin insensitivity. The *CEVI-1* gene is found in tomato plants and its expression is up-regulated during the course of compatible viral infections with citrus exocortis viroid (CEV; Gadea *et al.*, 1996) but not in incompatible interactions or by the application of signalling molecules such as SA, ethylene or JA (Mayda *et al.*, 2000). The homologous Arabidopsis mutants, called *detachment* (*dht*) mutants that are deregulated in the control of expression of transgenic *CEVI-1* have been identified. From initial analysis of two of these mutants, *dht2* and *dht23*, both the activation of *CEVI-1* gene expression and an auxin-resistant phenotype occurred (Mayda *et al.*, 2000). These results seem to imply that during systemic infections with viruses, auxin homeostasis is one of the components participating in the regulation of the overall defence response. It has also been observed that elicitor induced expression of some of the defence-related genes in tobacco protoplasts are negatively regulated by auxin (Jouanneau *et al.*, 1991). In tobacco, preparation of protoplasts without auxin appeared sufficient to induce a spontaneous defence reaction and it was also found that the accumulation of PR proteins was maximal when tobacco and soybean protoplasts were prepared and further cultured in the absence of auxin (Jouanneau *et al.*, 1991).

More recently, Navarro *et al.*, (2005) have shown that a microRNA, which negatively regulates messenger RNAs for the F-box auxin receptors *TIR1*, *AFB2* and *AFB3* and subsequently represses auxin signalling, can lead to resistance to *Pseudomonas syringae*. This implicates auxin in disease susceptibility and miRNA-mediated suppression of auxin signalling in resistance.

Unfortunately, the mechanisms leading to changes in auxin level in virus-infected plants are still unknown. Therefore, no clear correlation between changes in auxin levels and distribution and impaired plant growth and development has been found. To do so more accurate quantitation of hormone levels and distribution along with molecular genetic approaches are needed.

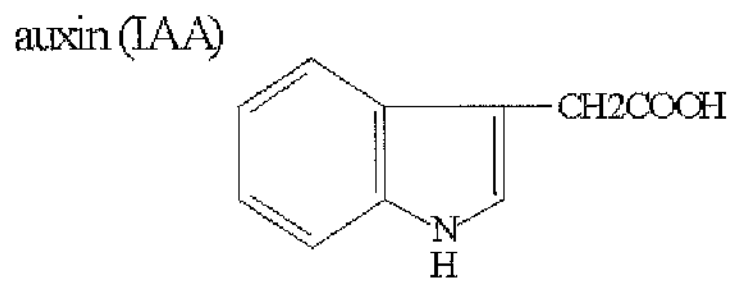


Fig 1.3 Chemical structure of Indole-3-acetic acid (IAA)

The main form of auxin in higher plants is indole-3-acetic acid (IAA), which is found both free and as conjugates within the plant (see **Figure 1.3**). Auxin is thought to regulate a variety of responses in plants including rates of cell expansion, rates of cell division and establishment and maintenance of pattern formation during growth and development (Hobbie *et al.*, 1998). The role of auxin in cell elongation has been demonstrated by studies on hypocotyl elongation. For example, when auxin is added to isolated stem segments and coleoptiles it is able to induce elongation (Collet *et al.*, 2000). A role for auxin in elaborating the embryonic axis has been suggested as polarity defects can be induced in embryos by blocking normal auxin movement (Hadfi *et al.*, 1998).

Auxin has been found to be unique among plant hormones as it has its own dedicated transport system. Auxin is transported in a polar fashion from the shoot apex down the stem towards the roots, largely through cells associated with the vasculature. This transport system has been termed Polar Auxin Transport (PAT) and has been found to occur in a basipetal (apex to base) direction within the aerial tissues of plants and in two distinct streams within root tissues, both basipetal and acropetal (base to tip) directions. Rubery and Sheldrake (1974) and Raven (1975) proposed within their chemiosmotic hypothesis that the polarity of IAA transport reflects the asymmetric subcellular distribution of auxin efflux and possibly influx carrier proteins within specialised transport cells (Rubery and Sheldrake, 1974; Raven, 1975).

Knowledge about PAT has been advanced through the identification of Arabidopsis mutants that have defective auxin responses. The use of suitable inhibitors of auxin response has been very useful in identification of the carrier complexes that mediate auxin transport (Bennett *et al.*, 1998). The majority of this work has focused on the efflux carrier as work on the influx carrier has been held back by the lack of suitable inhibitors. Morris *et al.*, (1991) suggested that the efflux carrier consists of at least three polypeptides, which include a transmembrane carrier protein, NPA binding protein and a third labile component.

The *PINI* (PIN Formed I) Arabidopsis gene has been proposed to encode a component of the efflux carrier complex based first on its mutant phenotype, which can be phenocopied by culturing wild-type seedlings in the presence of the efflux inhibitor NPA, and second, on the significantly reduced rates of polar auxin transport within mutant inflorescence tissues (Okada *et al.*, 1991). *PINI* encodes a membrane-localised protein, prompting the suggestion that it may represent the transmembrane component of the efflux carrier. *AtPINI* shows similarity to some bacterial transporters and it has been shown that disruptions in this gene affect PAT, patterning in the embryo and vascular development (Swarup *et al.*, 2001). Petrasek *et al.*, (2006) have shown recently that PINs mediate auxin efflux from mammalian and yeast cells without needing additional plant-specific factors. Conditional gain-of-function alleles and quantitative measurements of auxin accumulation in Arabidopsis and tobacco cultured cells have revealed that the action of PINs in auxin efflux is rate-limiting, specific to

auxins, and sensitive to auxin transport inhibitors. Therefore suggesting a direct involvement of PINs in catalyzing cellular auxin efflux.

The most likely candidates for additional auxin transport proteins are the plant orthologs of mammalian multidrug resistance/P-glycoproteins (MDR/PGPs). In mammalian systems, MDR/PGPs function in ATP-dependent movement of hydrophobic substrates, and there is evidence that a subset of the 21-member PGP-type ATP-binding cassette ABC transporter family play a similar role in auxin efflux in plants. Defective PGP1 and PGP19 result in decreased auxin transport and reduced growth phenotypes in Arabidopsis, maize and sorghum. PGP1 has been shown to mediate the efflux of auxin from Arabidopsis protoplasts and heterologous systems such as yeast and HeLa cells (Geisler *et al.*, 2005) It has also been shown that PGPs interact with PIN and that although they characterise coordinated, independent auxin transport they also function interactively in a tissue-specific manner (Blakeslee *et al.*, 2005). It has been shown that PGP1 and PGP19 colocalize with PIN1 in the shoot apex in Arabidopsis and with PIN1 and PIN2 in root tissues. Specific PGP–PIN interactions were seen in yeast two-hybrid and coimmunoprecipitation assays and PIN–PGP interactions appeared to enhance transport activity and, to a greater extent, substrate/inhibitor specificities when coexpressed in heterologous systems. It is likely that PGPs mediate the ATP-dependent transport of IAA in conjunction with PIN proteins, which offer directionality and substrate specificity.

Another component of the auxin efflux carrier was thought to be the product of the *TIR3* (Transport Inhibitor Response 3) gene. Ruegger *et al.* (1997) described the *tir3* mutant, which exhibits a reduced rate of polar auxin transport. Studies also observed that the mutant had significantly reduced levels of NPA binding which led to the suggestion that the *TIR3* gene may encode or regulate the activity of the NPA binding protein associated with the efflux carrier (Ruegger *et al.*, 1997). However, Gil *et al.*, (2001) have demonstrated that *tir3-1* is allelic to the *doc1-1* mutation. The *DOC1* (Dark Over expressing *CAB 1*) gene is a putative component of the light signal transduction pathways (Li *et al.*, 1994). The *DOC1* locus has been recently renamed as *BIG* (Gil *et al.*, 2001) and is predicted to encode a large protein (560 KD) with significant similarity to the *Drosophila* protein Calossin/Pushover. Calossin is believed to be involved in the control of synaptic transmission at the neuromuscular junction, in a process that requires the synaptic vesicle cycle (Xu *et al.*, 1998). Like synaptic transmission, the asymmetric distribution of auxin efflux carriers at the plasma membrane depends on targeted vesicle transport (Steinmann *et al.*, 1999) and recent work has shown that *BIG* is essential for proper positioning of the auxin efflux carrier, *PIN1*, at the plasma membrane (Paciorek *et al.*, 2005). Therefore *TIR3* is essential for either the synthesis, localization, or the function of the NPA binding site (Gil *et al.*, 2001) but is not a component of the carrier itself.

The influx carrier was first described by Rubery and co-workers who observed a saturatable component for auxin uptake within suspension cell cultures and stem segments (Rubery and Sheldrake, 1974; Davies and Sheldrake, 1978). More recently, molecular genetic studies in *Arabidopsis* (Swarup *et al.*, 2001) have identified putative auxin influx carrier components encoded by the *AUX1* gene sequence. The *Arabidopsis AUX1* gene was isolated using a gene tagging approach and found to encode a highly hydrophobic polypeptide featuring up to 11 membrane-spanning domains. Colinearity between *AUX1* and a family of sequences that encode plant amino acid permeases (Frommer *et al.*, 1993) was found, suggesting that they may share a common ancestry and domain structure. Plant amino acid permeases facilitate the uptake of amino acids into plant cells; Bennett *et al.* (1996) have suggested that *AUX1* may therefore perform a similar transport role, facilitating the movement of the amino acid-like signalling molecule, IAA.

Carrier mediated uptake requires a proton motive force because the influx carrier functions electrogenically, transporting one proton with every protonated IAA molecule (see **Figure 1.4**). As a weak acid, IAA is rapidly protonated in the apoplastic space, then taken up by the plant cell either by diffusion or via the auxin influx carrier. Once within the plant cytosol (pH 7.0) the dissociated IAA⁻ is removed by a distinct auxin efflux carrier (Delbarre *et al.*, 1996). There is also growing evidence in *Arabidopsis* for a non-PAT mode of auxin redistribution from auxin source tissues such as young leaves to the roots via the phloem

(Swarup *et al.*, 2001). It has been found from Mass Spectrometry (MS) measurements that a significant proportion of root apical IAA is unloaded by AUX1 from a phloem source (Swarup *et al.*, 2001). AUX1 localises to the protophloem cell poles, therefore revealing a novel apoplastic transport pathway for delivering phloem borne IAA to the root apex.

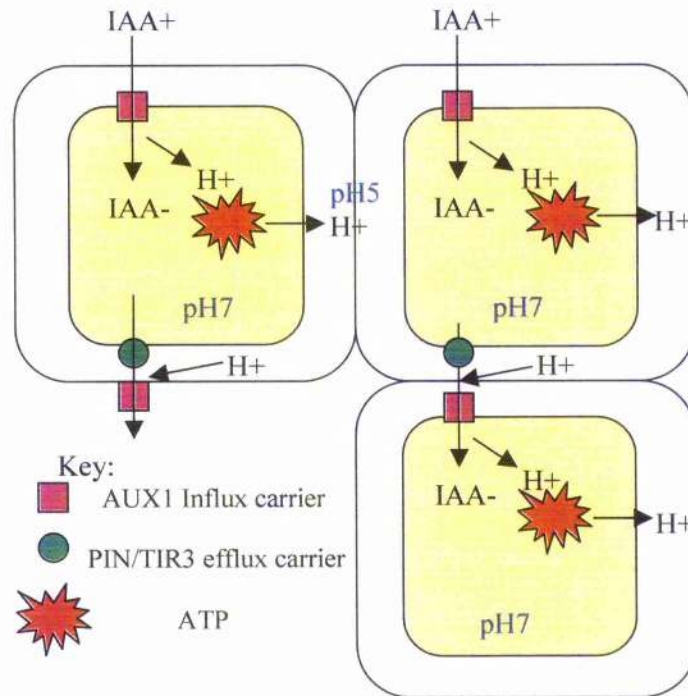


Fig 1.4 Model of cellular auxin transport. IAA moves into cells via the *AUX1* influx carrier and exits through the efflux carrier. The efflux carrier consists of three components including *PIN1* and *TIR3*.

Other work on the auxin-signalling pathway has demonstrated that in wild-type *Arabidopsis* plants auxin sensitivity depends on the correct functioning of targeted protein degradation (Gray *et al.*, 2000; del Pozo and Estelle, 2000). Wild-type auxin signalling depends on the function of a ubiquitin ligase complex of the SCF type (see **Figure 1.5**). SCF complexes are found in a range of eukaryotes and consist of an SKP1 homologue, a Cullin/CDC53 homologue, an F-box protein and an RBX1/ROC1 homologue (Leyser, 2001). The F-box protein determines which proteins are degraded in the proteasome and consequently SCF complexes are considered to be the hub of the degradative machinery. *Arabidopsis* mutants that are defective in several components of this system have been recovered and found to share a variety of morphological phenotypes that reflect their reduced sensitivity to auxin. The first gene to be identified in this way was Auxin Resistant 1 (*AXR1*), which encodes a protein with similarity to the amino terminal half of ubiquitin activating enzyme E1. E1 catalyses the first step in the pathway leading to the conjugation of ubiquitin (UBQ) to specific target proteins. The UBQ is then passed to ubiquitin conjugating enzyme E2 and from there to target proteins, often requiring the action of a ubiquitin-protein ligase E3. It is the E3 enzyme that confers target specificity, and in the case of auxin regulated protein degradation is encoded by the *TIR1* gene. *TIR1* encodes an F-box protein similar to the yeast proteins Cdc4p and Grr1p and mutations in this gene result in an auxin resistant phenotype quantitatively similar to that of *axr1* but less severe (Ruegger *et al.*, 1997; Leyser, 2001). *TIR1* interacts with plant orthologs of Skp1 (*ASK1* and

ASK2) and a cullin protein to form SCF^{TIR1}. The proteins targeted for degradation by this SCF^{TIR1} complex are members of the AUX/IAA family of transcriptional repressor proteins, of which there are 29 in Arabidopsis. AUX/IAAs can dimerize with and repress the activity of, transcriptional activators of the auxin response factor (ARF) family of DNA binding proteins. Thus the degradation of AUX/IAAs leads to the derepression of ARF-mediated transcription. Therefore the interaction between the AUX/IAA and the SCF^{TIR1} complex is central to auxin biology. More recently, results have shown that auxin binds directly to the SCF^{TIR1} complex, indicating that TIR1 is the auxin receptor for this response.

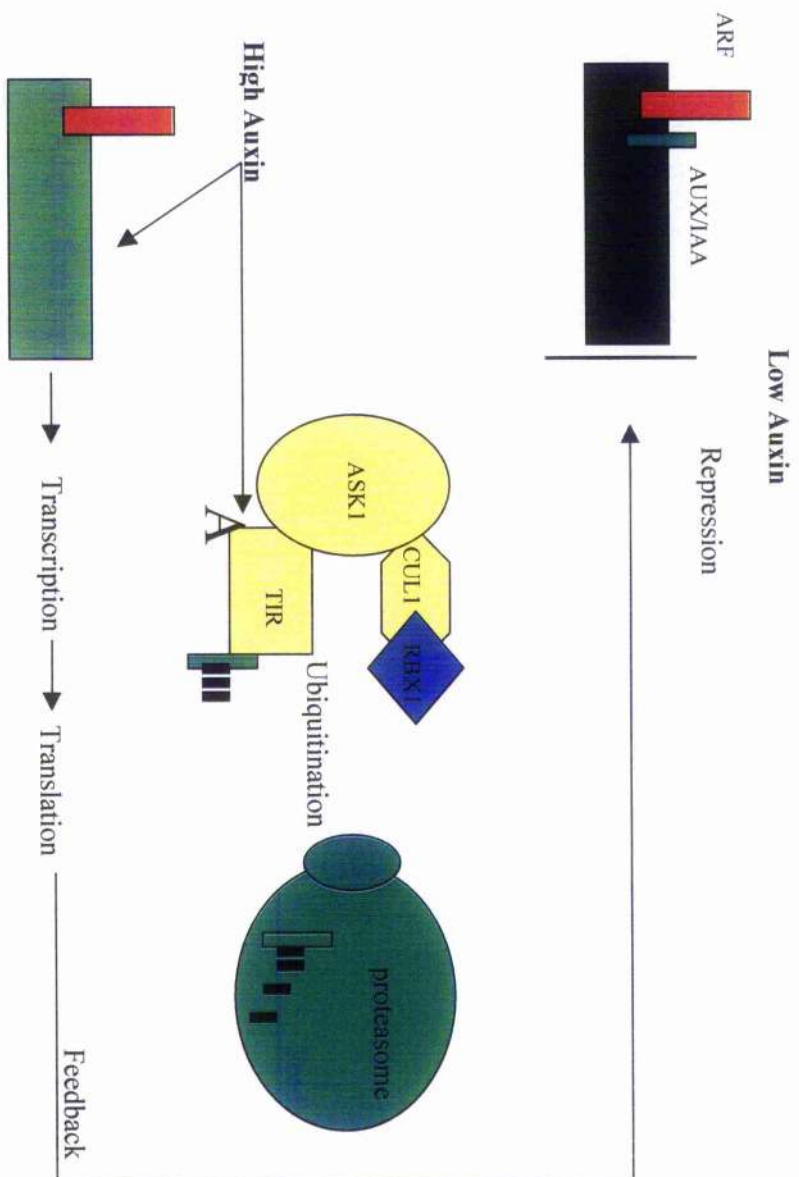


Fig 1.5 Auxin regulated gene expression through targeted degradation of the AUX/IAA transcription factors by SCF-type ubiquitination and degradation in the proteasome. Adapted from Napier, (2005).

Further upstream, the site of auxin perception is still being debated (Hertel, 1995). The most likely candidate for an auxin receptor is Auxin Binding Protein 1 (ABP1). *ABP1* was first identified because of its ability to bind specifically to auxin and there is good evidence to suggest that it regulates downstream auxin responses (Hobbie *et al.*, 1998; Leyser, 2001). Over expression of *ABP1* in transgenic cell lines and plants can alter auxin sensitivity in a variety of responses, including cell expansion and guard cell potassium currents. More recently an insertion in the *ABP1* gene from *Arabidopsis* has been identified, allowing analysis of the null mutant phenotype. Plants that are homozygous for this insertion die in the early globular stage of embryogenesis, clearly demonstrating the importance of this gene. However, it is thought that there are multiple sites of auxin perception and so it is possible that *ABP1* is not the only receptor. Other possible receptors are the auxin efflux carriers themselves and as mentioned previously TIR1 (Napier, 2005). TIR1 functions as the F-box component of the SCF complex. Which regulates targeted protein degradation. Work from two separate groups, has shown that TIR1 binds auxin directly and therefore the auxin receptor for this degradation pathway (Kepinski and Leyser, 2005; Dharmasiri *et al.*, 2005). It has been demonstrated that TIR1 after purification retains the ability to interact with Aux/IAA substrates in an auxin dependent manner, that IAA binding correlates with TIR1 abundance and TIR1 produced in the *Xenopus* oocyte expression system exhibits saturable, high affinity auxin binding. These studies provide the receptors for auxin-mediated

gene activation and, as such, receptors for most of the large array of responses controlled by auxin. However, there are also responses that TIR1 signalling cannot account for. It is likely that rapid responses and those responses initiated at the cell surface are not TIR1 mediated. It is possible that these are mediated by ABPI.

Integration of the Auxin Signalling Pathway (1.7)

Interactions between signal transduction pathways furnish a rapid and efficient tuning mechanism for optimising non-cognitive behaviour in response to various combinations of stimuli. These connections between the pathways are termed cross-talk (Genoud and Metraux, 1999). Shared signal transduction pathways might mean that fewer elements are needed for overall hormone signalling.

As a non-cell autonomous signalling mechanism, polar auxin transport is a frequent target for other signalling pathways to regulate plant development. Mutant screens that were originally designed to isolate lesions within auxin signal transduction very often identified mutants with altered responses to multiple hormones (Ephritikhine *et al.*, 1999; Tiryaki and Stawsick, 2002). For example mutants screened for ability to grow on inhibitory concentrations of ethylene were subsequently identified as alleles of *aux1* (Pickett *et al.*, 1990). *tir3-1* mutant which was originally identified from a screen for plants with altered responses to auxin transport inhibitors has now been demonstrated to be the *doc1-1* mutant and has roles in vesicle trafficking and in the light signal

transduction pathways (Gil *et al.*, 2001). This highlights the occurrence of "cross-talk" between auxin and other signalling pathways (Swarup *et al.*, 2001).

Auxin appears to interact with many of the other plant hormones. Of these interactions, the cross-talk between auxin and ethylene and auxin and JA are probably the most significant in terms of response to virus infection, since both, ethylene and JA have known roles in plant defence. Auxin and ethylene co-ordinately regulate several developmental programs in plants. For example, both are required to regulate apical hook formation (Raz and Ecker, 1999; Lehman *et al.*, 1996), root hair differentiation, root hair elongation (Pitts *et al.*, 1998), root hair growth, and hypocotyl phototropism (Harper *et al.*, 2000). They have been described to interact at the level of ethylene biosynthesis (Swarup *et al.*, 2002). Given that auxin stimulates the production of ethylene, it is often unclear whether developmental effects attributed to auxin are due to auxin, ethylene, or synergistic interaction between both classes of phytohormones. Auxin and JA cross-talk studies appear to show that jasmonic acid inhibits auxin-regulated elongation in etiolated oat coleoptiles (Swarup *et al.*, 2002).

Other hormones whose signalling pathways appear to interact with auxin include ABA, cytokinin and GA. For example, ABA and auxin have been observed to interact antagonistically to regulate stomatal aperture (Eckert and Kaldenhoff, 2000). Auxin and GA have been described to co-ordinately regulate several developmental programs in plants including pea stem elongation (Ross and O'Neill, 2001) and parthenocarpy (Ikeda *et al.*, 1999). It appears that auxin

and GA regulate each other's biosynthesis (Ross *et al.*, 2000). Finally, auxin and cytokinin appear to co-ordinately regulate shoot cell proliferation by controlling the expression of the interacting cell cycle regulating components, *cdc2* and *cycD3* (John *et al.*, 1993). They frequently control plant development by regulating each other's abundance. It is thought that auxin and auxin conjugates may play a role in regulating the metabolism of cytokinin conjugates. However, little is known about how auxin and cytokinin interact at the signal transduction level (Swarup *et al.*, 2002).

Aims (1.8)

Auxin has long been suspected of involvement in plant responses to virus infection (see Pennazio and Roggero, 1996). However previous investigations have not produced any definitive evidence as to its role. This has been partly due to the limited nature of the experimental techniques available at the time when the majority of the early work was undertaken. The main problem with these approaches was that although such bioassays are able to show gross differences in auxin levels when comparing infected and uninfected plants, they do not provide any link to the causes of these differences. Also many of these techniques are only semi-quantitative, it can be difficult to distinguish IAA from inactive auxin analogues. In order to investigate the role that auxin plays in response to viral infection it is necessary to use better quantitative techniques to accurately measure the hormone levels, and more importantly to take advantage of the availability of mutants with lesions in auxin transport or responses.

The aims of this thesis are to establish whether auxin has a role to play during compatible plant-virus infection, to examine what this role is, and to determine how this fits in with the existing knowledge of plant virus-interactions.

Chapter Two

Chapter 2: Materials and Methods

Seed Stocks and Virus Isolates (2.1)

Arabidopsis thaliana variants were obtained from a local collection and contained the genotypes detailed below in **Table2.0**.

Seed Stock	Supplier	References
Col-0	Nottingham Arabidopsis Stock Centre (NASC)	www.arabidopsis.info
Ler <i>gll</i>	NASC	www.arabidopsis.info
Ws	NASC	www.arabidopsis.info
<i>Aux1-100</i>	Alan Marchant	Bennett <i>et al.</i> , 1996
<i>Aux1-7</i>	Alan Marchant	Pickett <i>et al.</i> , 1990
<i>Aux1-22</i>	Malcolm Bennett	Marchant and Bennett, 1998
<i>Tir1-1</i>	Malcolm Bennett	Ruegger <i>et al.</i> , 1998
<i>Tir2-2</i>	Malcolm Bennett	www.arabidopsis.info
<i>Tir3-1</i>	Malcolm Bennett	Ruegger <i>et al.</i> , 1997
A7	Joel Milner	Love <i>et al.</i> , 1998
B6	Joel Milner	Love <i>et al.</i> , 1998
D4-2	J. Schoelz	Schoelz <i>et al.</i> , 2003
LAX2::GUS	Malcolm Bennett	Parry <i>et al.</i> , 2001

Table2.1 List of genotypes used in studies with details of the original supplier and relevant references.

Plant growth conditions (2.2)

Soil preparation (2.2.1)

Peat based potting compost (Levington Horticulture Ltd.) was autoclaved for 1 hour at 121°C in order to eliminate insect and fungal pests. After cooling, a solution of Intercept systemic insecticide (Levington Horticulture Ltd.) at 0.2gL⁻¹ was used to moisten the soil prior to its use.

Arabidopsis (2.2.2)

Arabidopsis seeds were scattered onto soil filled trays, covered with cling film and left in darkness at 4°C for 2-4 days. Stratification promoted synchronised growth and development. Trays were then maintained at 21 ± 1°C under 10 hours daylength. Osram “warm white” fluorescent tubes provided light at an intensity of 120µ E/m²/s. These conditions have been shown to be optimal for the development of symptoms after virus inoculation (Cecchini *et al.*, 1998).

Virus isolates (2.3)

Virus stocks (2.3.1)

CaMV isolate Cabb B-JI (Delseny *et al.*, 1983) was originally provided by Dr. Simon Covey at the John Innes Centre (JIC) in Norwich. This isolate is described in Al-Kaff and Covey (1994, 1995) and in the VIDE database (<http://biology.anu.edu.au/Groups/MES/vidc/>). CaMV isolate CM4-184 can also be found on the VIDE database and is described by (Howarth *et al.*, 1981).

Production of virus stocks (2.3.2)

The virus stocks were maintained as desiccated leaf tissue from infected plants. Working virus solution was derived from the stocks via propagation in turnip and purification according to Hull *et al.*, (1976) and Gardner and Shepherd (1980). Approximately 1 cm² of desiccated tissue was ground up in 300 µl dH₂O, centrifuged and the supernatant removed. Celite was added to the supernatant and 10 µl was rubbed on to the first true leaf of each turnip plant at the two true leaf stage of development. After 3-4 weeks, at which time symptoms had developed (vein clearing, chlorosis mosaics and stunting), leaf material was taken from strongly symptomatic turnip plants and was frozen at -80°C.

The infected turnip tissue to be used was washed and wiped and ground up in 2-4-ml/g ice cold grinding buffer (0.5M KH₂PO₄, pH 7.2 and 0.75% (w/v) sodium sulphite) in a chilled blender. Urea was added to 1M along with 10 % (v/v) Triton X-100 to a final concentration of 2.5%. The mixture was then covered and left to extract overnight while gently stirring at 4°C.

The next day the ground up tissue was centrifuged at 5,000x g for 10 minutes at 4°C. The supernatant was then strained through 4 layers of muslin and spun at 25,000x g for 2 hours at 4°C in a Sorvall T865 rotor. The virus pellet was re-suspended in dH₂O and shaken at 4°C for a further 1-2 hours. The suspension

was then centrifuged at 7,000x g for 10 minutes at 4°C. The supernatant was decanted and spun down at 45,000 rpm in a Sorvall T865 rotor for 1 hour at 4°C.

The virus pellet was then re-suspended in TE buffer and stored in aliquots at –80°C. The concentration of virus in the aliquots was estimated spectrophotometrically at 260nm and 320nm (Gardner and Shepherd, 1980). The viability of each virus preparation was tested by inoculating turnip (Section 2.3.3) with 1µg of virus per plant and observing symptoms.

Virus infection (2.3.3)

Prior to inoculation, Arabidopsis seedlings were thinned to a density of 2 to 3 plants per cell. Plants at the 2-true leaf stage were mechanically inoculated by rubbing 2µl (100ng) of purified virus on one side of the first emergent true leaves (leaf 1 or 2) using celite as an abrasive. Mock-inoculated plants were inoculated with autoclaved water and celite.

Assessment of Symptom Response (2.4)

Digital photographs were taken using a Nikon Finepix S1 Pro digital camera fitted with a 28-135 mm lens. For assessment of symptoms photographs were taken of trays of plants and at higher magnification of individual plants for one control and two infected plants from each mutant line (Cecchini *et al.*, 2002).

Quantification of Virus Accumulation (2.5)

DNA Extraction (2.5.1)

DNA extraction was carried out using the Nucleospin Plant Kit (BD Biosciences, UK) as per the manufacturer's instructions. As the DNA was to be used in virus titre studies it was necessary to include a Proteinase K step in the protocol to allow the extraction of DNA from virus capsids (Cecchini *et al.*, 2002). This is an important step as CaMV DNA can be found within infected tissue in both encapsidated and unencapsidated forms. Sixteen μ l of Proteinase K (20mg/ml stock solution) was added to the samples, which were then shaken, before being left to incubate at room temperature for 3 hours. An extra centrifugation step was also added before the elution of the DNA. Samples were centrifuged for an additional 3 minutes at 11,000 \times g in a fresh collection tube to ensure that all traces of ethanol from the previous step were removed.

DNA gel electrophoresis (2.5.2)

DNA samples were separated by gel electrophoresis on 1.0% agarose gel in 0.5x TBE buffer (Sambrook and Russell, (2001); Appendix 1.17) as described in Sambrook and Russell, (2001), section 5.10. Samples were mixed with a loading dye (#161-0767, BIO-RAD, UK), 1 part dye to 5 parts sample, then loaded into the wells on the set gel. A 100bp ladder (#170-8352, BIO-RAD, UK) was also loaded onto the gel as a marker. Ethidium bromide-stained gels were photographed under UV illumination using a BIO-RAD gel documentation system.

Quantification of dsDNA using the PicoGreen Quantification Kit (2.5.3)

DNA samples extracted from both wild-type and mutant Arabidopsis samples were quantified using the PicoGreen dsDNA Quantification Reagents and Kits (Molecular Probes Europe BV, The Netherlands) as stated in the manufacturers' instructions. 1 µl of sample DNA was used in the protocol along with the low-range standard curve.

Real time QPCR with DNA using SybrGreen (2.5.4)

Real time quantitative PCR was carried out using the Stratagene MX4000 thermocycler and Stratagene Brilliant SYBR Green QPCR Master Mix (#600548 Stratagene, California, USA) as described by Love *et al* (2005). Firstly the experimental reaction was prepared. A master mix was made containing the sense and anti-sense primers for the gene of interest, water and SYBR Green 1 dye. The volumes of each, per sample were as follows:

- 12.5 µl 2x SYBR Green 1
- 0.5 µl Sense primer (10 pmol/µl)
- 0.5 µl Anti-sense primer (10 pmol/µl)
- 9.5 µl PCR-grade water

The master mix was then dispensed into each of the appropriate wells followed by 2 µl of the experimental DNA. As well as the samples to be assayed, standards were also prepared. For viral DNA quantification, a standard stock of DNA extracted from Col-0 plants with known virus concentration was used for all experiments (Love *et al* 2005). This was serially diluted to give aliquots

containing from 100pg to 0.032pg. A no template control was also included to check for contamination of the reagents. The reactions were gently mixed and centrifuged briefly to remove any bubbles that could interfere with the fluorescence detection. The reactions were placed into the MX4000 instrument and the appropriate PCR program run. Depending on the primer sets used the temperature and cycle number was adjusted to provide optimal conditions. For viral DNA the PCR conditions were 10 min at 95°, then 40 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C. The CaMV BJI 1 to 7 primers (5' TACGCCAACTTCGACTCTCA^{3'}; 5' TGCTCGCTTTGGGTATTTTC^{3'}) amplify a 141 bp sequence across the ORF VII/ ORF I boundary and the rDNA control, the primers (5' CGTGATCGA TGAATGCTACC^{3'}; 5' GGGGTTTGTGTCACGTATTA^{3'}) amplify 199 bp of sequence in the Arabidopsis 18S rRNA gene (Love *et al.*, 2005). Because the SYBR Green 1 dye is light sensitive solutions containing the dye were stored in the dark until use.

To correct for any possible errors in estimating DNA concentrations in the samples, part of the gene encoding the Arabidopsis 18S rRNA was used as an internal reference standard (Love *et al* 2005). For each sample, the concentrations of CaMV DNA and 18S rDNA were estimated using the software provided by Stratagene. Final CaMV DNA concentrations were then expressed in arbitrary units, normalized to the amount of 18S rDNA in the sample

Northern Analysis (2.6)

RNA Extraction for Gene Expression (2.6.1)

The extraction of RNA from plant tissue was carried out using TRI Reagent, (Sigma-Aldrich, Inc, #T9424, UK) as outlined in the manufacturers instructions. An extra wash with ethanol was added to ensure that the RNA was clean enough to be used in subsequent RT-QPCR reactions. All samples were stored at -80°C until required for further analysis.

Removal of DNA from RNA Samples (2.6.2)

Contaminating DNA was removed from RNA preparations using the DNA-free kit supplied by Ambion (Europe) Ltd as per the manufacturer's guidelines.

RNA Gel Electrophoresis (2.6.3)

Before the sample can be run on a gel the gel tank, gel tray and the combs to be used should be wiped with a dilute NaOH solution. RNA samples are run on a 1.3% formaldehyde gel. The agarose was heated with water until it dissolved fully and then allowed to cool to around 60°C , then in the fume hood formaldehyde and 10x MOPS were added and the solution swirled gently to mix. This was then poured into the gel tray and allowed to set, which can take at least 45 minutes.

The RNA samples were heated at 65°C for 10 minutes before being mixed with loading dye (1 part dye to 5 parts RNA) and loaded onto the gel. The running buffer was 1xMOPS and the gels are run at 50V.

Quantitative Slot-Blot Hybridisation (2.6.4)

The slot-blots were set up using DNase treated RNA extracted using the TRI Reagent method (2.6.1). The first stage of this protocol is sample preparation. A loading buffer was prepared as follows:

660 µl formamide

210 µl 37% (w/v) formaldehyde

130 µl 10x MOPS buffer (Sambrook and Russell, 2001; Appendix 1.18)

A series of microcentrifuge tubes were set up and to these the RNA samples and the loading buffer was added in the ratio 1 part RNA to 3 parts loading buffer. The samples were heated at 65°C for approximately 10-15 minutes to denature the RNA. They were then removed from the heat and placed immediately on ice. Once this stage had been reached the samples could be left on ice for as long as necessary until required.

The second stage of this protocol was to assemble the slot-blot manifold that holds the nylon membrane in place and also allows the samples to be drawn through the membrane using a small vacuum. The manifold consisted of 2 pieces

of 3MM paper and 1 layer of membrane. The manifold should only be handled while wearing gloves to avoid contamination.

Once assembled the samples were loaded. Taking one sample at a time 200ul of 20x SSPE was added and gently mixed before being loaded straight into the well. This was repeated until all of the samples had been loaded.

The manifold was then allowed to sit for 30 minutes or so to allow the samples to go through the membrane. After this time a pipette pump was used to apply a small vacuum to the manifold to draw any remaining samples onto the membrane. Then the manifold was dismantled, the membrane was wrapped in 3MM paper and left to air dry. When dry a cross-linker was used to fix the RNA onto the membrane, which was subsequently stored at 4°C until required.

Hybridization and Detection (2.7)

Prehybridization/Hybridization of Membrane (2.7.1)

The membranes were prehybridized in Northern Max Prehybridization/Hybridisation buffer (#8677, Ambion, Inc, USA). This was accomplished by placing the membranes in the NorthernMax buffer (20ml of buffer per hybridization tube) for a minimum of 2 hours at 42°C. Following prehybridization, the radiolabelled probe was denatured by boiling at 100°C and then combined with the buffer. The membranes were then left to hybridize overnight at 42°C.

Quantification of autoradiographs (2.7.2)

The total amount of nucleic acid loaded onto each lane was quantified by pixel analysis of digitally reversed images of fluorescence (Cecchini *et al* 2002), using QuantiScan (Biosoft, Cambridge, UK). Autoradiographs were scanned on a flatbed scanner, digitised and hybridisation was quantified by pixel analysis using QuantiScan (Cecchini *et al* 2002). To correct for any lane-to-lane variations in the quantity of RNA loaded, for each lane, the value of hybridising RNA was divided by the value for total RNA. RNA expression levels (in arbitrary units) are thus presented relative to the amounts of total RNA. Statistical analysis was carried out by ANOVA using Minitab version 13.20 (Minitab Inc., Pennsylvania, USA).

DNA Probes (2.8)

PCR (2.8.1)

DNA fragments required as probes were amplified from wild-type DNA using the HotStar Taq PCR Master Mix Kit (Qiagen, Crawley, UK) as detailed in the accompanying handbook. Primers were designed using the Primer 3 website (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences are shown in **Table 2.2**.

Probe	Sense	Antisense
GH3	GCACTTATGAACCCGTTGT	GTGGAAGCACCTTGTGAG
SAUR_e	GCTTAAACAATGGCAGAAAA	AGGAAGTAACGGACGAGATT
SAG21	AGATTTCTTGGGTCCAGAT	AAACCAAACGCTTCTCTATTT

Table 2.2 Sense and Antisense primer sequences as used in RT-QPCR and Northern Hybridisation.

Cloning (2.8.2)

PCR products to be used as probes in gene expression studies were cloned into *E. coli* cells using the TOPO TA Cloning Kit and OneShot TOP10 competent cells (# K4500-01, Invitrogen) The procedure was performed according to the protocols supplied with One Shot TOP10 cells.

Bacterial culture and plasmid isolation (2.8.3)

The *E. coli* glycerol stocks containing the vectors were grown in LB broth containing 50 µg/ml ampicillin according to Sambrook and Russell (2001); A2.2, A2.6. Bacteria were pelleted by centrifugation at 4000 rpm for 5 mins in a microcentrifuge, and re-suspended in STE (Sambrook and Russell, (2001) A1.22). Plasmids were extracted using the QIAprep Spin Miniprep Kit Protocol (Qiagen, West Sussex, UK). The last step of the protocol was modified, whereby the EB buffer was diluted 1 in 4 and heated to 70°C before addition to the column.

Liquid and Solid Bacterial Media (2.8.4)

Luria-Bertani Medium (LB) was prepared as outlined in Sambrook and Russell, (2001) Appendix 2.2, and for solid media agar was added, 15g/L as detailed in Sambrook and Russell, (2001) Appendix 2.5.

Purification of PCR fragments (2.8.5)

PCR fragments were separated by gel electrophoresis according to the protocols described by Sambrook and Russell, (2001) Section 5.14-5.17. The ethidium bromide-stained bands were visualized using a 302 nm UV illuminator, and the desired fragments were identified by comparing against 100bp and 1kbp ladders (BIO-RAD, UK). The appropriate bands were excised and the fragments were purified using a QIAprep Spin Miniprep Kit (# 27104, Qiagen, Crawley, UK) as instructed in the manufacturers' handbook.

Radiolabelling of Probes (2.8.6)

Purified DNA fragment were labelled with 50 μCi ^{32}P -dCTP (3000Ci/mMol; Amersham, UK) using the Rediprime II Random Prime Labelling System (#RPN 1634, Amersham Biosciences, Buckinghamshire, UK) as described in the manufacturers' guidelines. The fragments were purified using a NICK Column (Pharmacia Biotech). The purified radiolabelled probe was boiled at 100°C for 10 minutes before adding to the filters that had already been pre-hybridised (2.7.1).

RT-QPCR (2.9)

cDNA Synthesis (2.9.1)

RT-QPCR was carried out according to Love *et al* (2005). The reverse transcriptase step was performed using AMV Reverse Transcriptase (#M5101 Promega Corporation, USA). Each sample contained:

1ng DNase treated RNA

1.25µl Oligo(dT)15 Primer (#C1101, Promega Corporation, USA)

PCR-grade water to give total of 15.9 µl.

This was then heated for 10 minutes at 70°C then immediately placed on ice. To each tube 9.1 µl of master mix (see Pg 64) was added and the tubes were placed back into the PCR machine (PTC) for 30 cycles under the following PCR conditions:

Master Mix: 1 µl AMV (10U/ µl)	Program: 48 °C for 45 minutes
0.625 µl RNasin (40U/ µl)	95 °C for 5 minutes
1 µl dNTPs (25mM)	4 °C forever
5 µl AMV Buffer (5x)	
1.5 µl PCR-grade water	

Reverse transcriptase Real-Time Quantitative PCR (2.9.2)

After cDNA synthesis the contents of each reaction was used as template in real time quantitative PCR (2.5.4). This was carried out as described previously (2.5.4) except that the standards used were plasmid DNA. Plasmids contained cDNAs for the following auxin-responsive gene, SAUR_e (see Section 2.8.2).

Primers used were as follows:

SAUR_e 5' CGATTGTGACTCGGTGTGAG 3'

5' CCAGGAAGTAACGGACGAGA 3'

ACT2 5' CTAAGCTCTCAAGATCAAAGGCTTA 3'

5' ACTAAAACGCAAAACGAAAGCGGTT 3'

Plant Growth Assays (2.10)**Seed sterilization (2.10.1)**

A 1% Tween 20 solution was prepared and one PRESEPT tablet (Coventry Chemicals Ltd., UK) was added to 35ml distilled water in a beaker and allowed to dissolve. At this point 4-5 drops of 1% Tween 20 were also added. Once fully dissolved, 5ml of this solution was added to 45ml 95% ethanol. This was mixed and 1ml added to tubes containing 10-15mg of the seeds to be sterilized. The seeds were left to soak for approximately 5-10 minutes and then shaken gently and left for a further 6-7 minutes.

Seed sterilization was carried out in a laminar flow hood.

The solution was poured off each of the tubes of seeds, and replaced with 1ml of 95% ethanol. The tubes were shaken gently and the ethanol poured off. This was repeated a total of three times and once completed, the tubes were covered with nescofilm, leaving an air gap, and left to dry in the hood overnight (2-3 hours minimum).

Plant Growth Media (Solid) (2.10.2)

1/2 Murashigi and Skoog (MS) with 0.8% agar was prepared.

IAA Root Growth Assays (2.10.3)

Sterilized seeds for the following genotypes, Col-0, WS, aux1-100, aux1-7, MDR1, A7, B6 and ARC 1A, 1C, 2C and 2E, were sprinkled onto 1/2 MS plates and the plates sealed. The plants were left for 4 days in the cold room and then transferred to the growth room where they were incubated vertically for 5 days.

Half MS plates containing various concentrations (0nM, 1nM, 5nM, 10nM, 50nM and 100nM in 100% Analysis Grade Ethanol) of Indole-3-acetic acid (IAA) were prepared (Pickett *et al.*, 1990). Seedlings from the initial MS plates were transferred to the IAA containing plates and arranged in a line. Plants were selected of roughly the same size, and a line was marked on the plate where the roots ended. The plates were sealed and incubated vertically in the growth room for 5 days. The roots were then measured from the previous line onwards and any root branching taken into account. The measurements were done using

TIBA Growth Assay (2.10.4)

The responses of various *Arabidopsis* auxin mutant and CaMV gene VI transgenic lines to the auxin efflux inhibitor 2, 3, 5-triiodobenzoic acid (TIBA) were assayed. Sterilized seed (2.10.1) for each of the various lines were sprinkled onto the surface of ½ MS plates containing TIBA at concentrations ranging from 0µM to 35µM. The plates were sealed and stored in the dark at 4°C for 1-2 days before being transferred to a short day growth room with a photoperiod of 10 h or less, and grown at 20°C. The plants were visually assessed at 16 days and 24 days after being sown (2.4); also at each of these time points duplicate tissue samples were harvested (the contents of one plate). The samples were frozen in liquid N₂, ground to a fine powder and RNA extracted (2.6.1). Each sample was then analysed using RT-QPCR.

Analysis of Virus Movement (2.11)

Virus movement was followed using a recombinant CaMV engineered to express GFP as a free protein (Turner and Covey, unpublished). CaMV-GFP, which expresses GFP as a free protein in the cytosol, was constructed by substituting ORFII of Cabb B-JI with a modified M-GFP5 (Chalfie *et al.*, 1994). The full length CaMV-GFP DNA sequence was inserted into pBluescript using the unique *SalI* site at nucleotide 4831 of the Cabb B-JI sequence, and was maintained and propagated as plasmid DNA. For infections, plasmid DNA containing the CaMV-GFP sequence was digested with *SalI* to release the insert.

Biolistic Particle Delivery System (2.11.1)

Because CaMV-GFP shows poor infectivity when manually inoculated onto Arabidopsis plants were infected by particle bombardment using a protocol developed by V. Laval (Laval *et al.*, submitted). This was carried out using the PDS-1000/IIe particle gun (BIO-RAD, UK) as per the manufacturers' instructions. The bombardment chamber itself and all of the holders, rupture disks, stop screens and carriers were cleaned with 90% ethanol and all procedures were carried out in a laminar flow hood.

Twenty-five μl of sterile water was added to a microcentrifuge tube and placed on ice along with a supply of 100 % ethanol. The components required for sample preparations were removed from the freezer and allowed to thaw completely ready for use. A tube containing 40mg BIO-RAD submicron gold particles (0.6 μm) was sonicated for 1 minute in a sonicator bath (Grant Instruments Ltd, Cambridge, UK). Then 25 μl of gold was added to the tube of sterile water and sonicated again for 1 minute. The sample was taken back to the hood and either 5 μl sterile water was added (control samples) or 4 μl sterile water and 1 μl CaMV: GFP DNA (118 $\mu\text{g}/\text{ml}$) was added. Virus samples contained 0.118 $\mu\text{g}/\mu\text{l}$. It is important at this stage to keep the virus and water away from the gold until they have been mixed. To this mixture 50 μl 2.5M CaCl₂, 20 μl 0.1M Spermidine was added to the lid of the tube and mixed together. Once mixed the tube was capped and lightly vortexed. The tube was left on the bench for 3 minutes, lying on its side then centrifuged for 3 seconds at 13,000 rpm. The supernatant was removed and the pellet re-suspended in 150 μl

of chilled 100% ethanol. The suspension was centrifuged again for 3 seconds at 13,000 rpm. The supernatant was removed and re-suspended in 85µl of chilled 100% ethanol. The samples were then placed on ice until required.

The holders were placed in a petri dish and using forceps, the carrier disks were placed into the holders. A red plastic cup, which is provided with the chamber, was used to fit the disks neatly into the holders. The gold/virus DNA or gold only samples were vortexed briefly, 8µl was placed into the centre of the carrier disks, and allowed to dry. A new rupture disk was placed into the holder and screwed into the chamber and a stop screen was positioned into the bottom of the bombardment platform. Once dried, the carrier disk was inserted into its holder and the whole platform slotted into the chamber. Cells (40 x 40 mm) each containing two plants at the eight leaf stage were then placed in the centre of the chamber underneath the platform and a vacuum of approximately 28 psi is applied. When ready, the fire button was pressed until the disk was ruptured.

Following bombardment, the plants were sprayed with water to help recovery and placed to one side.

Confocal Laser Scanning Microscopy (2.11.2)

Visualisation of GFP from CaMV:: GFP plants was achieved using the LSM 510 laser scanning microscope (Carl Zeiss Ltd., UK). Prior to using the microscope the tissue samples were prepared and mounted onto slides. Two lines of adhesive were placed down the width of the glass slide and allowed to air dry. The tissue to be examined was harvested and de-gassed using a syringe. The tissue was then mounted onto the slide, covered with a cover slip and a small amount of water added under the cover slip. The slide was then viewed under the microscope with the Argon/2 laser at the following settings:

NFT 545nm , BP 560-615

HFT 488 nm

Excitation 10 % at 488 nm

Laser Intensity 75%

Scan Speed 1.60 μ S

Frame size 1024 x 1024

	Channel 2	Channel D
Pinhole	2.95	n/a
Gain	756	190
Amp Offset	-0.029	-0.03
Amp Gain	1	1
BP	505-530	

Western Blot Analysis of Proteins (2.12)

Analysis of proteins was carried out essentially as described by Sambrook and Russell, (2001), Appendix 9.28.

Sample preparation (2.12.1)

Plant tissue samples were ground to a fine powder in liquid nitrogen. Then 200 μ l solubilization buffer (detailed below) was added and the samples were ground on ice and then vortexed. The solubilized tissue centrifuged for 20 minutes at 13,000 rpm and 2 °C and the supernatant collected.

Solubilization buffer: 1.25ml 1M Tris pH 7.5

1.5ml 5M NaCl

0.25ml 1M MgCl₂

5ml Glycerol

42ml H₂O

Assay of Protein Concentration (2.12.2)

Protein concentration was assayed according to the procedure of Bradford (1976) according to the manufacturers protocol, using a BIO-RAD DC Protein Assay, kit (BIORAD, USA).

SDS-electrophoresis Gel (2.12.3)

Proteins were separated prior to Western Blotting by electrophoresis on SDS gels as described by Sambrook and Russell, (2001), Appendix 8.42. The 10% SDS-PAGE resolving gel contained 1.875ml acrylamide solution (40%; 37.5:1 acrylamide: N'N' methylene bisacrylamide) the 5.5% stacking gel contained 250ul acrylamide solution (40%; 37.5:1 acrylamide: N'N' methylene bisacrylamide). The resolving gel was poured first and then the stacking gel and left to set. To run the gel 10µl of protein (see section 2.12.1) was added to 10µl of 2x SDS loading buffer (detailed below). The protein was then denatured by heating at 100 °C for 5 minutes, and then centrifuged at 13,000 for 5 minutes. The samples were then loaded onto the gel and electrophoresed at 200 V for 50 – 60 minutes.

2x SDS loading buffer: 125mM Tris-HCL pH6.8

4% SDS

20% Glycerol

10% β-mercaptoethanol

0.008% bromophenol blue

Protein Transfer (2.12.4)

The stacking gel was cut off and the stacking gel incubated with 1x Transfer Buffer (200ml 10 x Transfer Buffer (61g Tris, 278g Glycine, 2L dH₂O, pH 8.3)

400ml Methanol and 1400 dH₂O) for 5 to 10 minutes. The nitrocellulose membrane, sponge, and Whatman paper were soaked in 1x Transfer Buffer to remove air bubbles and then the transfer sandwich was assembled in a BIO-RAD mini-protean[®] 3 Cell (BIO-RAD, USA). The transfer sandwich was then submerged in the transfer tank filled with transfer buffer in the cold room and left for 50 minutes at 100 volts under agitation.

Immunological Detection of Proteins (2.12.5)

Immune detection was carried out using a rabbit anti-P6 polyclonal antibody (a kind gift from Dr J Schoelz, Missouri, USA). The antibody was raised against P6 from CaMV isolate W260 (Yu *et al.*, 2003). The membrane was blocked prior to the addition of the primary antibody in a blocking buffer (8g dry skimmed milk in TB1 (50mM Tris and 1% Tween 20, pH 8.0) + 0.1% Triton X-100) for 1 hour. The milk was then removed and fresh buffer added along with a 1/2000 dilution of antiserum. This was left to incubate at room temperature for 1-2 hours. The primary antibody was then removed and the membrane washed for 5 minutes in 1x TBS. TT ((150mM NaCl and 10mM Tris HCl pH 7.5) + 0.2% Triton and 0.05% Tween) twice and then washed once in 1x TBS (150mM NaCl and 10mM Tris HCl pH 7.5).

A 1/5000 dilution of the secondary, anti-rabbit antibody was added to fresh blocking buffer and allowed to incubate for 1 hour. After this time the buffer/

antibody solution was removed and the membrane washed four times in 1x TBS.TT for 5 minutes each time followed by a single wash in 1x TBS. The membrane was then exposed briefly with autoradiography film in the dark room and then developed.

The protein-antibody complexes were detected using ECL Plus western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) as described in the manufacturers' instructions.

Genetic Crossing of Arabidopsis (2.13)

Crosses were carried out using a standard procedure adapted from Weigel and Glazebrook, 2002 (*Arabidopsis: A Laboratory manual*). In order to carry out cross fertilization between Arabidopsis mutant and transgenic lines, plants were grown under long day conditions in a greenhouse until the inflorescences had developed and the flowers were at a stage where they were not open but not tightly closed, just before the white petals became visible. Any flowers that were too old or young were removed from the inflorescence leaving approximately 2-6 flowers for pollination. Each flower was carefully stripped back using forceps to leave the intact ovary. Pollen was obtained from a donor plant and gently rubbed onto the top of the exposed ovary. This was repeated at least twice to ensure proper pollination. The ovary was then covered and labelled to show which cross had been performed. Once all of the desired crosses had been carried out the plants were returned to the greenhouse and allowed to develop until the siliques had reached maturity at which point the seeds were harvested.

Chapter Three

The effect of auxin on CaMV infection within *Arabidopsis thaliana*

Introduction (3.1)

The type and severity of the symptoms associated with disease are dependent on both the virus isolate and the host plant involved. The severity of symptoms is not simply dependent on the virus titre within the infected plant, but on a number of factors. Environmental conditions, such as light intensity and temperature (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002; Lee *et al.*, 1996), can influence the symptom response of a host plant, as can changes in the levels and distribution of plant hormones within the plants (Qiu *et al.*, 1997; Cecchini *et al.*, 2002). Interactions between components of the virus and host genomes lead to the induction of a set of pre-programmed responses within the host (Covey *et al.*, 2000; Johal *et al.*, 1995). Symptoms can differ widely between hosts but common features of systemic virus infections include chlorosis, leaf malformations, mosaic patterns, stunting and a reduction in yield. The first response to be observed is often the development of local lesions at the site of initial infection. These lesions may appear as chlorotic patches on the inoculated tissue. The virus then moves into the surrounding tissue and spreads through the vasculature to establish a systemic infection. It is the symptoms of a systemic infection, such as stunting and chlorosis that are the root cause of economic losses (Hull R. 2001).

It has been suggested that the physical symptoms that develop during infection are a result of virus-induced alterations in physiological, biochemical and metabolic processes within the plant (Reviewed by Pennazio and Roggero, 1996). Changes in the levels and distribution of plant hormones within infected plants are likely to be, at least in part, responsible for these responses. It is well known that the hormones ethylene, JA and SA have important roles in plant defence, against a number of different pathogens (Grant and Lamb, 2006).

However, much less is known about the roles, if any, that the 'classical' plant hormones, such as ABA, GA, cytokinin and auxin have in this process.

Historically, studies attempting to establish the functions of these hormones have been linked by the lack of accurate, sensitive techniques to assay the levels and distribution of these hormones and have therefore resulted in misleading and inconsistent results (Jameson, 2002).

In recent years, studies carried out to establish the roles of these hormones in response to viruses and other pathogens have been limited, and clear roles during infection have yet to be identified. In the past there has been suggestive evidence to indicate that auxin might be involved in the symptom response (Reviewed by Pennazio and Roggero, 1996). The symptoms observed during infection are often reminiscent of those observed in *Arabidopsis* mutants with defects in auxin signalling and transport. For example, auxin plays a key role in regulation of cell elongation and division (Bennett et al., 1998), and mutants with defects affecting these processes often show reduced growth and stunting reminiscent of the symptoms in virus infected plants.

Auxin signalling and transport is an area of research that has received considerable attention in recent years, and our understanding of the subject has been advanced greatly through the identification of *Arabidopsis* mutants that have defective auxin responses (Leyser, 1997). These mutants are now a valuable tool for studying the effects of a variety of different biotic and abiotic stresses on auxin signalling and transport.

Preliminary studies (Cecchini and Milner, personal communication) had pointed to a possible role for auxin in the response to CaMV infection. Infection studies using auxin mutants, *aux1* (Pickett *et al.*, 1990), *aux2* (Timpte *et al.*, 1994) and *aux4* (Hobbie and Estelle, 1995) suggested that *aux1* alleles showed changes in the symptom response to some isolates of CaMV. *aux2* and *aux4* were indistinguishable from the wild-type. This pointed to a possible involvement of auxin signalling during virus infection. The first objective of this work was to identify auxin mutants that showed alterations in response to CaMV infection compared to wild-type and to establish the effect of the mutation on virus replication and/or spread within the host plant. For example, how is virus accumulation affected? Do defects in auxin signalling and transport affect the movement and spread of the virus and ultimately its ability to establish systemic infection within a host?

Results (3.2)

Symptom Phenotypes and Virus Accumulation After Infection With CaMV in *Arabidopsis* mutants (3.2.1)

Auxin signalling and transport mutants and the corresponding wild-type ecotypes were manually inoculated with two isolates of CaMV, CM4-184, which causes moderate symptoms in *Arabidopsis* and Cabb B-II, which causes very severe symptoms (Cecchini *et al.*, 1998). Infections were carried out with two different virus isolates because preliminary studies (Cecchini and Milner, unpublished) had suggested that, depending on the mutant phenotype, differences in symptom response might be easier to identify with one or other of a severe or mild isolate (Cecchini *et al.*, 2002; Geri *et al.*, 2004). The symptom response of each of the mutant lines and wild-type plants were then followed on a day to day basis by visual inspection, and plants were photographed at regular intervals from the development of symptoms, typically 12 dpi, through until 24 dpi (days post infection).

***AUX1* Mutant Alleles (3.2.1.2)**

The first mutants to be studied were alleles of the *AUX1* gene since preliminary studies had suggested differences between *aux1* mutants and wild-type in response to CaMV infection (Cecchini and Milner, unpublished). The *Arabidopsis AUX1* gene has been identified as a member of the auxin amino acid permease (AAP) family of proton driven transporters. Plant amino acid permeases facilitate the uptake of amino acids into plant cells; Bennett *et al.*

(1996) suggested that *AUX1* performed a similar transport role, facilitating the movement of the amino acid-like signalling molecule, IAA.

Approximately fifty mutant alleles of *AUX1* have been identified. It is important to use more than one allele when using a genetic approach since allele-specific differences can be eliminated. Three alleles were chosen for this work, *aux1-100*, *aux1-7* and *aux1-22* (See **Table 3.1**) along with their corresponding wild-type lines, Columbia (Col-0) and Wassilewskija (WS). One of these alleles, *aux1-100* produces no AUX1 protein, the other two are weak alleles in which a modified protein is produced.

Allele	Type of Mutation	Description
<i>aux1-100</i>	Deletion (T-DNA insertion)	No protein produced. Resistant to ethylene and auxin; agravitropic root phenotype and no obvious aerial phenotype. (Bennett <i>et al.</i> , 1996)
<i>aux1-7</i>	Point (G → A)	Single substitution, leading to Gly → Asp at position 459. Produces full length protein but with single amino acid substitution. Aerial portions of plant similar in appearance to wild-type; slight increase in root elongation and altered geotropic response; resistant to ethylene and auxin. (Marchant and Bennett, 1998)
<i>aux1-22</i>	Point (T → A)	Single nucleotide change at splice junction, leading to a deletion of 34 bp in the cDNA. Smaller protein than wild-type produced. Auxin and ethylene resistant roots; agravitropic roots. Leaves show slight curling. (Marchant and Bennett, 1998)

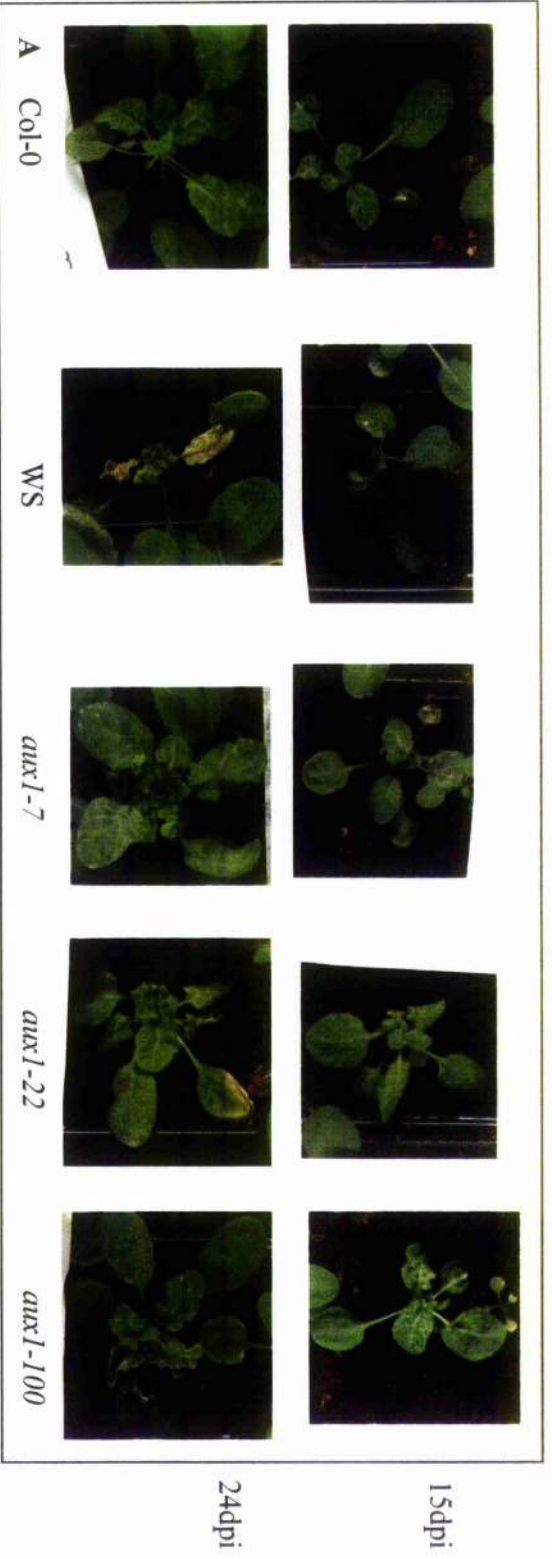
Table 3.1 Alleles of the AUX1 influx carrier.

The typical symptoms (stunting, chlorosis, vein clearing, mosaics) associated with CaMV infection were observed in all genotypes (see **Figure 3.1**). As expected the less severe isolate CM4-184 induced slightly milder symptoms than with the severe isolate Cabb B-JI. These consisted primarily of stunting, leaf malformation, vein clearing, patches of chlorosis and the development of mosaic patterns. In CM4-184-inoculated wild-type plants, Col-0 and WS, local lesions were first identified at 11 dpi and systemic symptoms were first visible at 15 dpi for all three of the *aux1* mutant alleles and wild-types. Local lesions developed much earlier in the Cabb B-JI inoculated plants, typically from around 7 dpi in Col-0, WS and all three *aux1* alleles. Systemic symptoms were also observed earlier and appeared much more severe than those associated with CM4-184. The first mild systemic symptoms (e.g. vein clearing, chlorosis) were noted at 9 dpi and continued to increase in severity through until 28 dpi. At this point symptoms were so severe that the plants were very severely stunted, with nearly all of the leaf tissue being chlorotic. However, although slight differences were observed between the two isolates of the virus at early timepoints, in contrast to the preliminary results of Cecchini and Milner, there were no differences in the severity or quality of the symptoms that developed between the mutants and their corresponding wild-types.

Fig 3.1 Symptom response of *aux1* alleles and the corresponding wild-types, Col-0 and WS, to CaMV infection.

(A) Symptom response of the *aux1* mutant alleles and wild-types inoculated with CaMV isolate CM4-184.

(B) Symptom response of the *aux1* mutant alleles and wild-types inoculated with CaMV isolate Cabb B-JI.



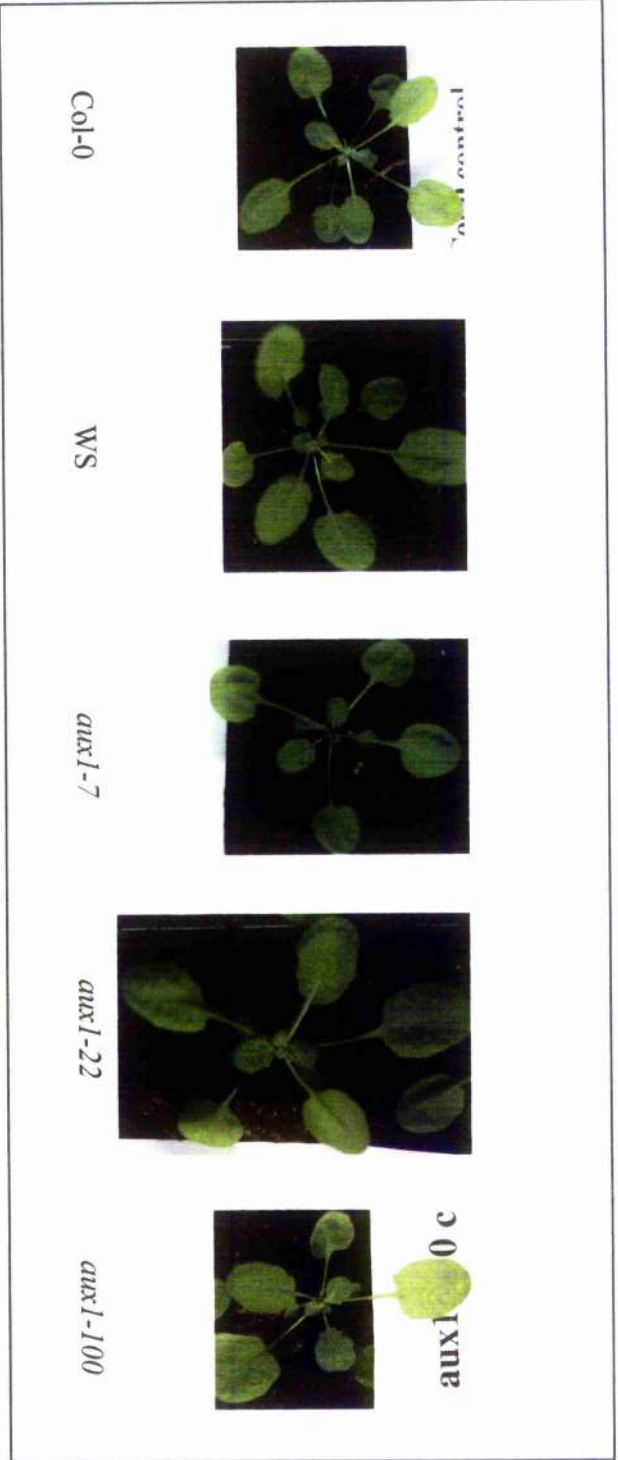


Fig 3.2 Uninfected control Arabidopsis wild-type and aux1 mutant alleles.

Although no differences in symptom severity or timing were observed, symptom responses in CaMV infected *Arabidopsis* are not necessarily directly related to virus titres within infected plants (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002). Therefore to examine what effect if any, changes in auxin signalling and transport might have on virus levels, virus accumulation was compared in wild-type and mutants. Triplicate tissue samples were harvested from Cabb B-JI - inoculated and also mock inoculated control plants at 3 day intervals from 0 dpi until 28 dpi. Each sample was produced from a pool of three individual plants. Total DNA was extracted (see Materials and Methods 2.5.1) from infected plants and virus DNA was quantified using quantitative real-time PCR (See Materials and Methods 2.5.4). DNA was extracted using a Nucleospin Kit but the procedure was modified by the addition of Proteinase K. This results in release of virus DNA from assembled virus particles (Cecchini *et al.*, 2002). The virus DNA was quantified as a proportion of the total DNA extracted from the samples. Genomic DNA encoding part of the 18S ribosomal RNA was used as an internal standard and levels of virus DNA were normalized to levels of this ribosomal DNA. The QPCR data was analysed statistically using the General Linear Model (GLM) with Bonferroni analysis. Results are shown in **Figure 3.3**.

Virus levels were extremely low during the early stages of infection, from 0 dpi through to 8dpi, in all of the *aux1* alleles and their corresponding wild-types, Col-0 and WS. From 12 dpi onwards the virus levels began to rise rapidly until 21 dpi or 24 dpi ($p < 0.05$). At 28 dpi a significant decrease ($p < 0.05$) in virus was

observed in all of the lines except Col-0. *aux1-7* and *aux1-22* consistently showed slightly more virus accumulation than the wild-type Col-0. However, ANOVA indicates that these differences are not significant ($p>0.05$). *aux1-100* also closely followed the pattern of virus accumulation shown by its wild-type WS. Again ANOVA indicated that virus accumulation in *aux1-100* and WS are not significantly different ($p>0.05$).

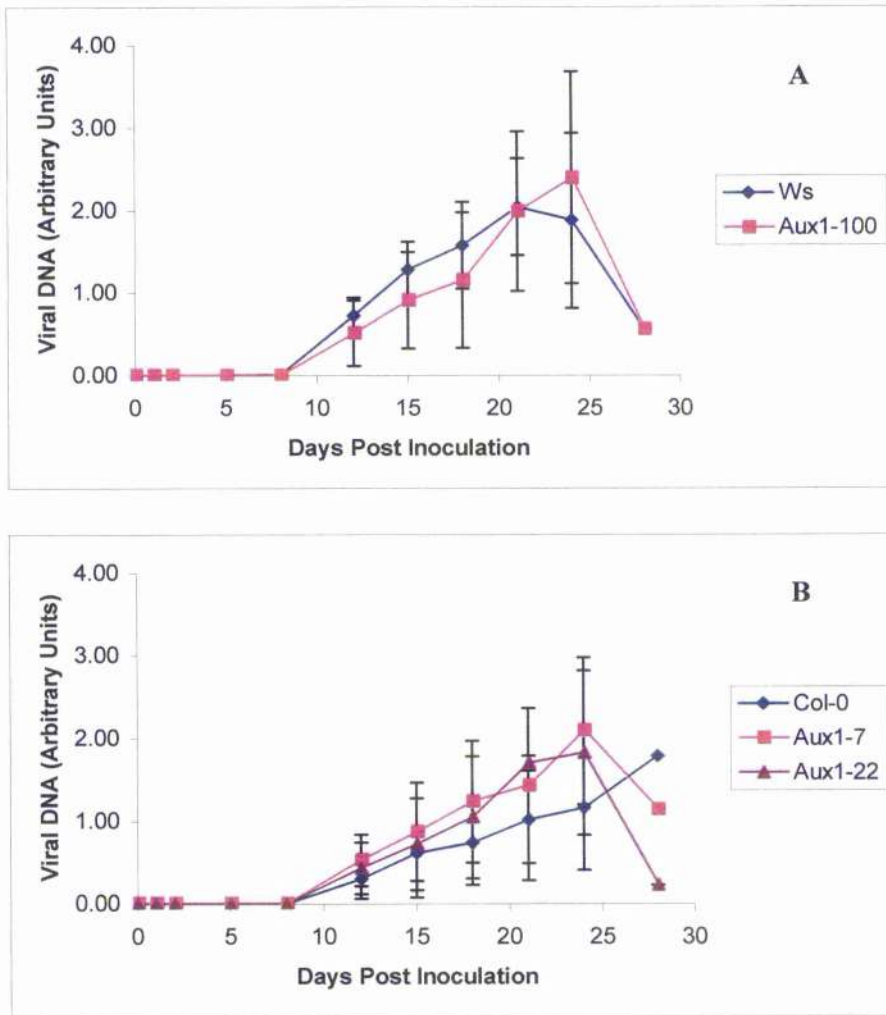


Fig 3.3 CaMV DNA Accumulation in auxin mutants and wild-type Arabidopsis. Levels of virus DNA in arbitrary units were normalized to levels of 18S ribosomal DNA. A value of 1.00 is equivalent to an average of 1400 molecules of CaMV per cell (Love *et al.*, 2005). Samples were taken at three day intervals from 0 dpi until 28 dpi. Error bars show standard errors (n=3).
 (A) Levels of CaMV DNA in *aux1-100* and the corresponding wild-type WS.
 (B) Levels of CaMV (Cabb B-JI) DNA in *aux1-7*, *aux1-22* and the corresponding wild-type Col-0.

Transport Inhibitor Response Mutants (3.2.1.3)

Three further auxin mutants were examined. These included the Transport Inhibitor Response mutants (TIR), which were identified from a screen for plants with altered responses to transport inhibitors (Ruegger *et al.*, 1997). The responses to CaMV infection of three mutants, *tir1-1*, *tir2-2* and *tir3-1* were examined (See **Table 3.2**). Plants were inoculated with CaMV CM4-184 and Cabb B-JI, the symptom responses were assessed and virus titres quantified as described previously for the *aux1* alleles.

Allele	Type of Mutation	Description	Type
<i>tir1-1</i>	Point (G→A at +440)	Semi dominant mutation; resistant to auxin inhibition of root elongation, deficient in auxin regulated growth processes, encodes a protein that contains an F-box domain and leucine rich repeats. Aerial phenotype is similar to wild-type.	Auxin signalling. Component of SCF-type ubiquitination complex. Auxin receptor. (Ruegger <i>et al.</i> , 1998; Kepinski and Leysner, 2005)
<i>tir2-2</i>	Unknown	Similar phenotype to <i>tir1</i> .	Auxin Signalling, not mapped.
<i>tir3-1</i>	Point (T→A at +7331)	Variety of morphological defects. Both auxin and NPA binding activity are dramatically reduced.	Essential for the synthesis, localization or function of the NPA binding site. Encodes BIG protein with homologies to Calossin/Pushover from <i>Drosophila</i> . Involved in protein trafficking (Gil <i>et al.</i> , 2001).

Table 3.2 Transport Inhibitor Response mutants used in infection studies.

Two of the three mutant lines, *tir1-1* and *tir2-2* developed the typical symptoms associated with CaMV- infection of their corresponding wild-type (Col-0; see **Figure 3.5**). In CM4-184 inoculated plants, local lesions appeared at approximately 12 dpi with systemic symptoms first observed at 14 or 15 dpi. However the *tir3-1* mutant, showed a consistent delay of 3 days in the appearance of both local and systemic symptoms compared to Col-0 (see **Figure 3.4, A**). When plants were inoculated with Cabb B-JI, symptoms appeared at a much earlier stage of infection than with CM4-184. In *tir1-1*, *tir2-2* and Col-0 local lesions were observed as early as 7 dpi, with systemic symptoms appearing after 10 dpi. The symptoms associated with Cabb B-JI infection were more severe than those associated with CM4-184, and by the end of the timecourse infected plants were very chlorotic and stunted. However, like with CM4-184, *tir3-1* plants inoculated with Cabb B-JI showed a delay of approximately 3 days in the onset of both local lesions and systemic symptoms compared to wild-type (See **Figure 3.4, B**). Symptoms were also much less severe in the *tir3-1* mutant. This consistent delay suggests that the *tir3-1* mutation may be affecting either the movement and/or replication of the virus.

Fig 3.4 Symptom response of *tir* mutants and the corresponding wild-type, Col-0, to CaMV infection.

(A) Symptom response of the *tir* mutants and wild-type inoculated with CaMV isolate CM4-184.

(B) Symptom response of the *tir* mutants and wild-type inoculated with CaMV isolate Cabb B-JI.

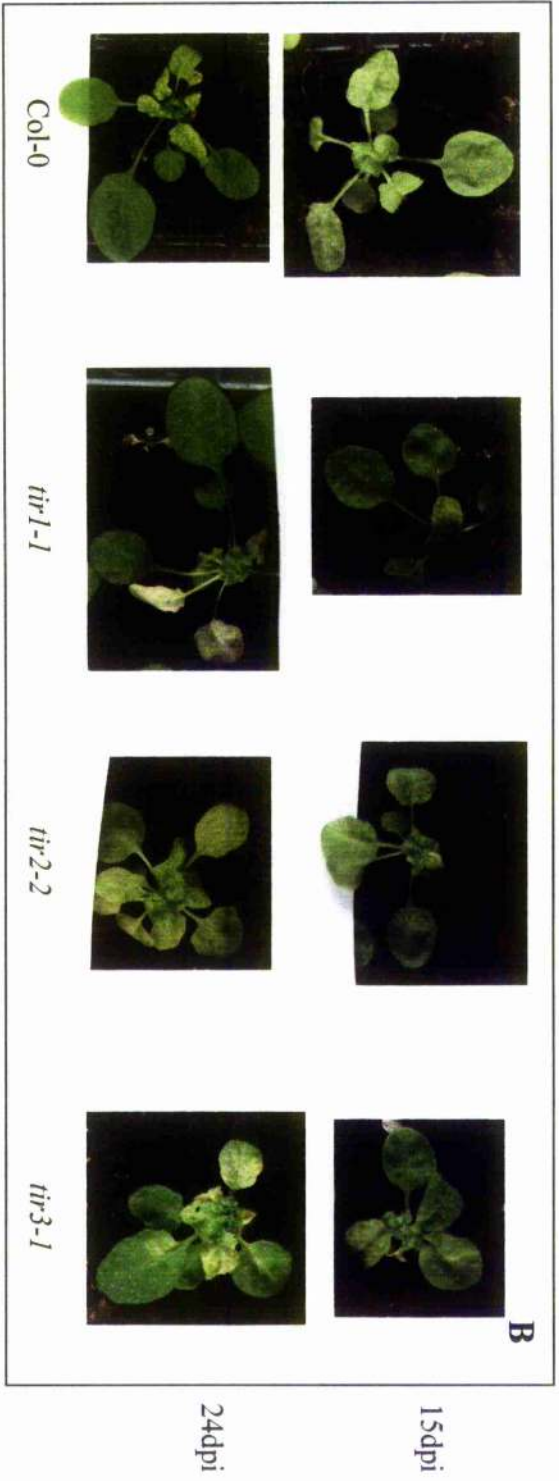
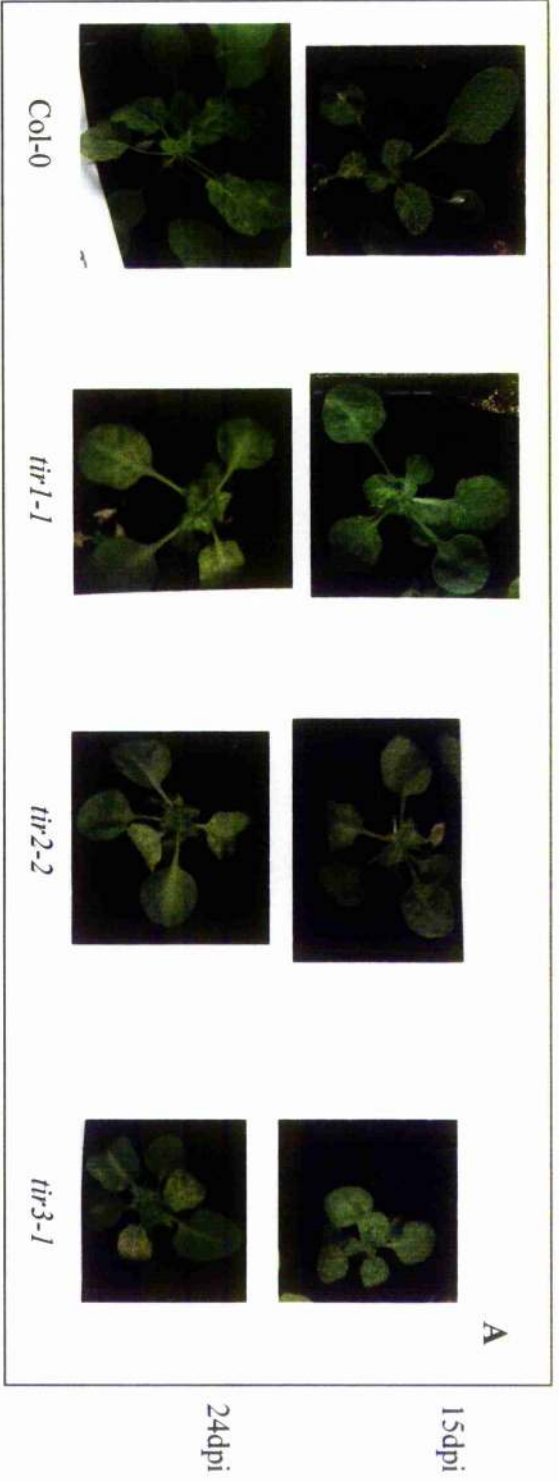




Fig 3.4 Uninfected control Arabidopsis wild-type and tir mutant plants.

To determine if virus levels were altered in any of the mutants, virus levels were compared with wild-type (see **Figure 3.6**). All plants contained almost undetectable levels at the early timepoints, 0 dpi through to 8 dpi, but from 12 dpi onwards the virus levels increased in all genotypes. As was observed with the *aux1* alleles, virus levels decreased after 24 dpi (ANOVA $p < 0.05$). *tir1-1* virus levels appeared consistently higher than wild-type; ANOVA identified the p value to be 0.073. Although this is greater than the conventionally accepted figure of $p = 0.05$, there is still a 93% chance that differences in virus levels in *tir1-1* compared to wild-type, are an authentic consequence of the mutation. There were also no significant differences in virus DNA levels between Col-0 and the *tir2-2* mutants ($p > 0.05$). In contrast, *tir3-1* showed a consistent delay in virus accumulation and ANOVA identified *tir3-1* virus levels as being significantly lower than either Col-0 or *tir1-1* ($p < 0.05$). Therefore in *tir3-1*, virus accumulation is delayed compared to wild-type and virus titres throughout the infection are lower. This delay in virus accumulation most likely corresponds with the delay seen in the development of symptoms in the *tir3-1* mutants. Thus mutations to a gene with a role in auxin efflux, impinge significantly on virus infection.

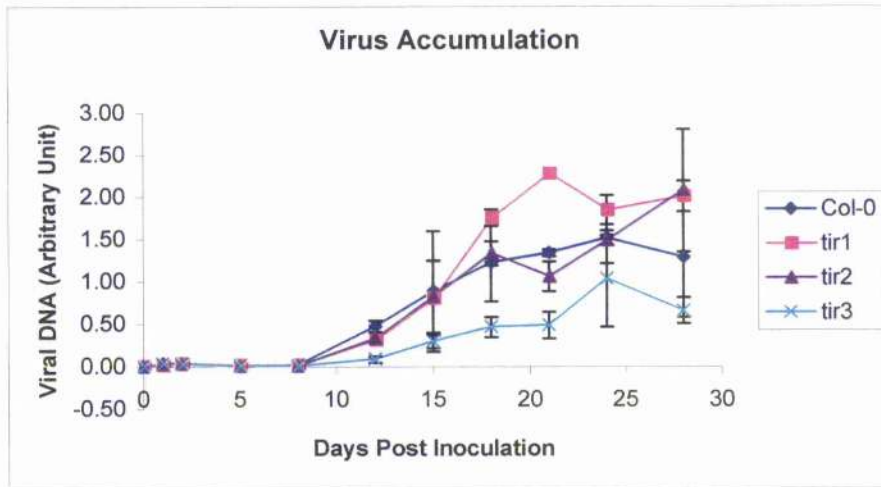


Fig 3.6 Levels of CaMV (Cabb B-JI) DNA present in mutant and wild-type. Levels of virus DNA in arbitrary units were normalized to levels of 18S ribosomal DNA. A value of 1.00 is equivalent to an average of 1400 molecules of CaMV per cell. Samples were taken at three day intervals from 0 dpi until 28 dpi. Error bars show standard errors (n=3). The symbols for each genotype are indicated in the figure legend.

Impact of Auxin Signalling on the Movement and Spread of CaMV (3.2.3)

tir3-1 shows a very significant reduction/delay in virus accumulation; this could be a result of reduced replication at a cellular level, reduced cell to cell movement, or a reduction in long distance movement. To distinguish between these possibilities I used a CaMV recombinant in which ORFII, encoding the aphid transmission helper protein, has been replaced with the gene for modified GFP and which expresses GFP as a free protein (Turner and Covey, unpublished). Spread of virus through the infected plants was visualized by confocal microscopy. Manual inoculation of plants with CaMV-GFP has proved unsuccessful in previous studies (Love *et al.*, 2006, unpublished); therefore infection was achieved by bombardment with gold particles coated in virus DNA (See Materials and Methods 2.11.1). *aux1-100*, *aux1-7*, *tir1-1*, *tir3-1* and the corresponding wild-types, Col-0 and WS were bombarded with virus DNA using the BioRad PDS 1000 Biolistic Particle Delivery System. Plants were examined from 12 dpi through until 28 dpi. Representative inoculated, expanded and newly emerging leaves (five leaves) were taken and examined for each timepoint. Samples were viewed at two different wavelengths, 505-530nm and 560-615nm, the latter allows discrimination between genuine GFP fluorescence linked to the virus and autofluorescence (a potential consequence of cell death). Results are shown in **Figures 3.7, 3.8, and 3.9.**

Local lesions were first observed as chlorotic spots on the bombarded leaves at 12 dpi for the wild-types, Col-0 and WS. With time, the lesions grew in size and the plants developed systemic symptoms, including chlorosis, stunting and vein

clearing. GFP fluorescence corresponded with the visual symptoms. Local lesions comprising patches of fluorescent cells about 20-30 cells across were first visible at 12 dpi and then grew in both size and number (See **Figure 3.7 A and D**). By 15 dpi, GFP could be seen to spread into the vascular system and appeared to follow the pattern of vein clearing (see **Figure 3.7 B and E**). Both of the wild-types showed systemic spread of GFP by 18 dpi (see **Figure 3.7 C and F**). GFP fluorescence was visible in the expanding and emerging leaves from 18 dpi onwards, and from this point on, the amount of tissue showing GFP fluorescence increased greatly. GFP fluorescence in systemically infected leaves closely followed the pattern of symptom expression and was generally linked with chlorotic regions.

In *aux1-100*, *aux1-7*, *tir1-1* and *tir2-2* the patterns of GFP expression were indistinguishable from the corresponding wild-type (see **Figure 3.8**). However, in the *tir3-1* mutant visual assessment did not identify any development of chlorotic local lesions earlier than 21 dpi (see **Figure 3.9 E**). Although wild-type plants typically showed 4-6 fluorescent lesions per leaf as early as 12 dpi, in *tir3-1* even by 18dpi there were fewer than 2 lesions per leaf. At 21 dpi, these were significantly smaller than those observed for Col-0 plants at 12dpi, typically half the diameter of wild-type lesions (see **Figure 3.9 A and E**). Both vascular spread, and growth of the lesions were also consistently delayed in this mutant. These results show clearly that in *tir3-1* both systemic and cell to cell movement of the virus is impaired, perhaps as a result of reduced replication of the virus.

All samples were examined at higher magnifications but differences between the mutants were not observed. GFP was generally confined to areas of the leaf showing chlorotic symptoms and cells examined within these regions appeared the same, with fluorescence observed in the cell boundary and the nucleus.

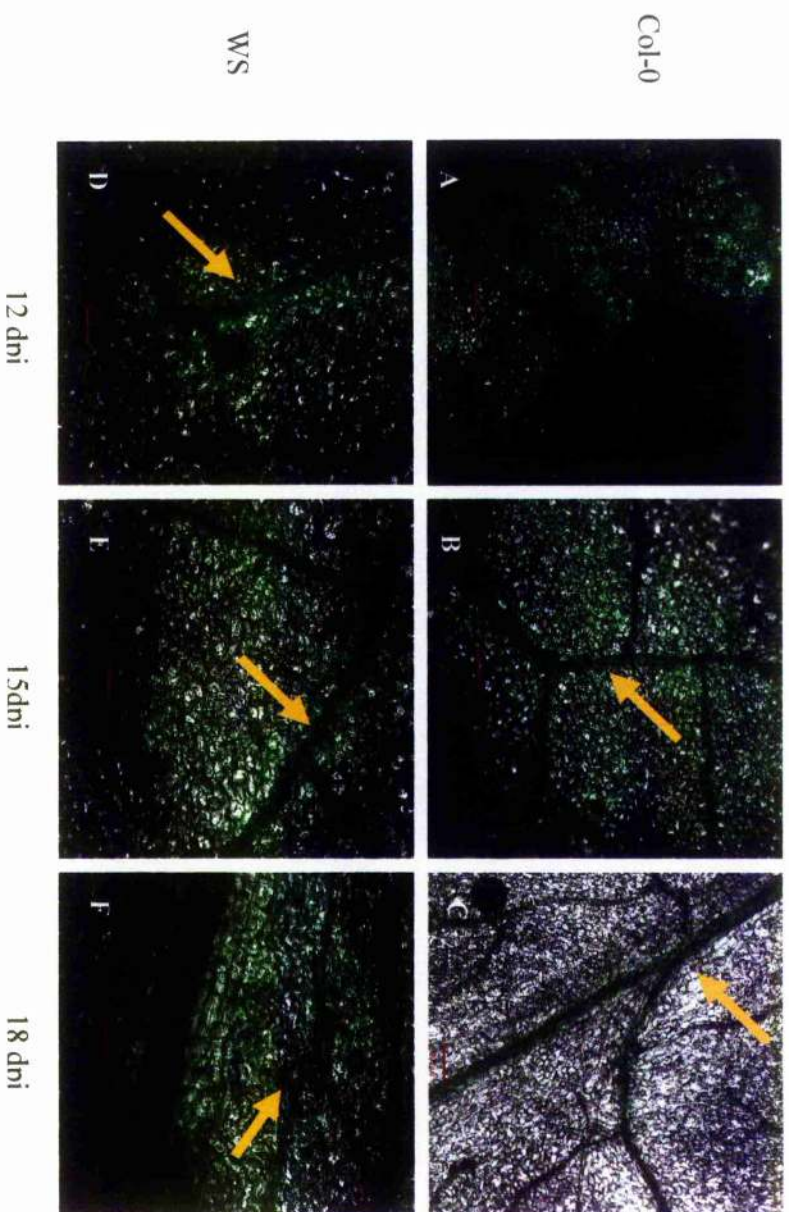


Fig 3.7 Confocal microscope images from wild type, Col-0 and WS infected with CaMV-GFP. All images are at 5x magnification. The excitation wavelength was 488nm and the emission wavelength was 505-530 for GFP. Arrows indicate areas of virus related GFP fluorescence. Yellow Arrows indicate infected vasculature. Scale bars equal 200µm

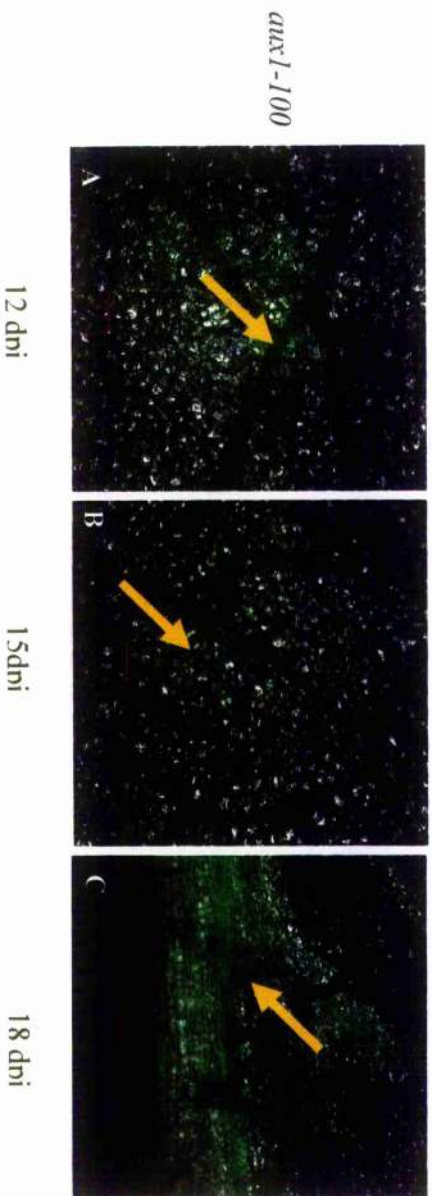


Fig 3.8 Confocal microscope images from *aux1-100* infected with CaMV-GFP. All images are at 5x magnification. The excitation wavelength was 488nm and the emission wavelength was 505-530 for GFP. Arrows indicate areas of virus related GFP fluorescence. Yellow arrows indicate infected vasculature. Scale bars equal 200 μ m

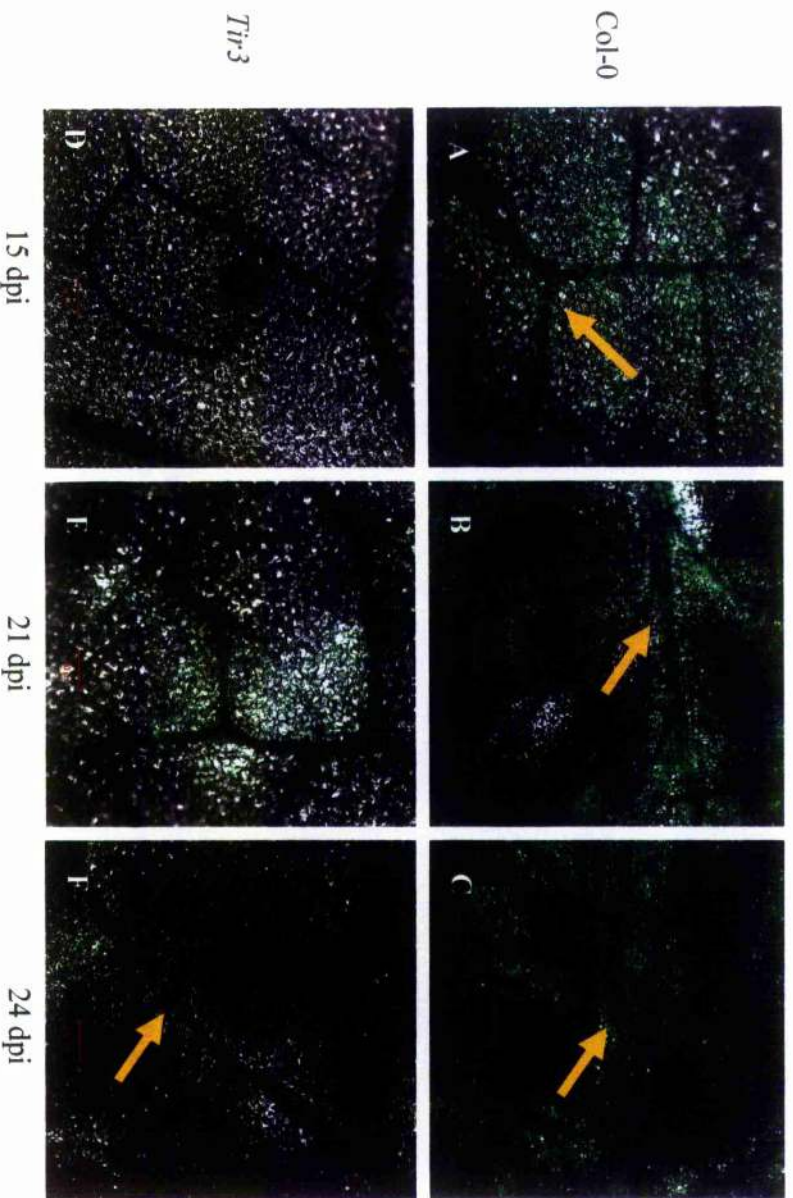


Fig 3.9 Confocal microscope images from plants infected with CaMV-GFP. All images are at 5x magnification. The excitation wavelength was 488nm and the emission wavelength was 505-530 for GFP. Arrows indicate areas of virus related GFP fluorescence. Yellow arrows indicate infected vasculature. Scale bars equal 200µm

Summary (3.3)

These results demonstrate a link between at least one component of the auxin signalling and transport pathway and CaMV infection. A significant difference in symptom response, virus accumulation and spread between a mutant *tir3*, which is affected in auxin efflux was observed compared to wild-type. None of the other mutants examined showed any significant alterations in their response compared to the corresponding wild-types. Although, it is important to note that differences in response to infection occur between the wild-types Col-0 and WS. WS appears to have a less stunted phenotype and develops systemic symptoms slightly earlier than that of Col-0. In *tir3-1*, virus accumulation and spread were reduced and delayed. This delay corresponded with the delay already observed in the development of symptoms and the two are presumably related. Two other mutants, *cpr5* (constitutive expression of PR genes 5) identified from a screen for constitutive expression of systemic acquired resistance (SAR) and *etr1* (ethylene response 1) also show reduced virus accumulation. However in these, unlike in *tir3-1*, this is largely a result of reduced long-distance virus movement (Love *et al.*, 2006, unpublished). The *aux1* alleles and *tir1-1* and *tir2-2* did not show any significant differences compared to wild-type. Since other mutations affecting PAT (*aux1*) or auxin signalling (*tir1*, *tir2*) did not significantly affect CaMV accumulation, the role of *TIR3* may be fairly specific. The role of *TIR3* in vesicle trafficking may be of importance and account for the auxin responses observed, rather than a direct involvement of auxin. This could help explain why the other auxin mutants failed to show any differences in response to infection.

Chapter Four

The effect of CaMV Infection on Virus Induced Gene expression

Introduction (4.1)

Response to virus infection within host plants induces alterations in the expression patterns of a variety of host genes involved in a wide range of processes within the host plant. For example, Whitham *et al.*, (2003) demonstrated that infection by five different RNA viruses elicited co-ordinated changes in gene expression in Arabidopsis plants. These included alterations in the expression of genes associated with distinct defence and stress responses. Geri *et al.*, (1999) showed through the use of differential display PCR that the expressions of various Arabidopsis genes were either up or down regulated by CaMV infection and also by transgenic expression of the viral pathogenicity determinant P6. Microarray investigations have identified a variety of Arabidopsis genes altered in response to virus infection (www.geneinvestigator.ethz.ch).

Preliminary data was obtained from a full genome Affymetrix ATH1 microarray investigating the effects on gene expression of CaMV infection using RNA extracted from CaMV- infected Col-0 plants (Laval V. and Milner J., unpublished). Approximately 1100 genes from Arabidopsis were identified that showed greater than 3-fold changes in expression and of these, seven genes identified from the annotation as being auxin responsive were highly up regulated in infected plants. These results provide preliminary evidence that CaMV infection induces changes in auxin levels, transport or perception (see

Table 4.1). However, the microarray studies provided no direct correlation between the symptoms or changes in host gene expression during infection, and the auxin levels, concentration and/or distribution. To do so more quantitative and accurate techniques along with a molecular genetic approach are required.

The objective of the work described in this chapter was to examine the effect that CaMV infection has on auxin regulated gene expression and auxin levels during infection.

Control	Infected	Infected/Control	Description
3.4	268.2	79	Auxin-responsive - like protein Nt-gh3 deduced protein, <i>Nicotiana tabacum</i> , EMBL:AF123503 GH3-like
42.4	1025	24	Putative protein auxin-regulated gene, <i>Vigna radiata</i> SAUR ^e
105.1	2007.6	19	Coded for by <i>A. thaliana</i> cDNA T46835 similar to several small proteins (~100 aa) that are induced by heat, auxin, ethylene and wounding such as <i>Phaseolus aureus</i> indole-3-acetic acid induced protein ARG (SW:32292); supported by full-length cDNA: Ceres:23194. SAQ21
19.4	124.8	6	Auxin conjugate hydrolase (ILL5) identical to auxin conjugate hydrolase [<i>Arabidopsis thaliana</i>] (ILL5) GI:5725649; contains nonconsensus AT acceptor splice site at exon3
22.6	76.3	3	Auxin-induced protein IAA5, putative similar to auxin-induced protein IAA5 GI:972913 from <i>Arabidopsis thaliana</i>
75.2	229.2	3	Auxin-inducible gene (IAA2) identical to auxin-inducible gene (IAA2) GB:AF027157 [<i>Arabidopsis thaliana</i>] (Plant Physiol. 115, 1730 (1997))
23.3	69.7	3	Unknown protein contains similarity to auxin-induced protein GB:P33082 from [Glycine max]

Table 4.1. Details of 7 genes from an Affymetrix ATH1 full genome chip which showed greater than 3-fold increases in expression levels and annotated as being auxin responsive.

Results (4.2)

Expression patterns of auxin responsive genes during CaMV infection

(4.2.1)

To further analyse how the expression of putative auxin-responsive genes was affected by CaMV infection, a full timecourse of expression of the three most highly up-regulated genes identified from the microarrays was carried out (See **Table 4.2**). Wild-type Arabidopsis and the auxin mutants discussed in Chapter 3 were infected with the severe isolate of CaMV, Cabb B-JI. The *aux1* and *tir* mutants were used as it was thought that if auxin transport or signalling is involved during infection then disruption in these pathways should lead to altered responses to infection. Tissue samples were taken in triplicate during the infection process at timepoints from 0 dpi through until 28 dpi. Total RNA was extracted from each of the samples (see Materials and Methods, 2.6.1) and the expression levels quantified using quantitative slot-blot hybridisation.

Gene	Description	Reference
SAG21 (At4g02380)	Encodes late embryogenesis abundant protein, mRNA levels are elevated in response to various stresses including cold, and reactive oxygen species.	Weaver <i>et al</i> (1998)
SAUR_e (At4g34750)	Auxin-induced protein, contains similarity to IAA induced protein ARG7 from <i>Phaseolus aureus</i> .	Reddy <i>et al</i> (2002)
GH3-like (At5g13320)	auxin-responsive-like protein, Nt-gh3 deduced protein, <i>Nicotiana tabacum</i> .	Hagen <i>et al.</i> , (1991) Guilfoyle <i>et al.</i> , (1993)

Table 4.2 Auxin responsive genes identified from microarray investigations as being highly up-regulated in response to CaMV infection.

Total RNA was extracted and treated with DNase to remove any contamination with genomic DNA. The RNA was then added directly onto the membrane by vacuum blotting and once dry, stained with toluidine blue stain to allow the total amount of RNA loaded to be visualised and quantified (See Materials and Methods 2.6.4). The stained membrane was scanned using a flatbed scanner and the RNA quantified using QuantiScan as described by Cecchini *et al.*, (2002). The filters were then probed with the gene of choice (See Materials and Methods 2.7) and any radiolabelled probe that hybridised was detected using a phosphorimager. Bands were quantified using QuantiScan and the results normalised against initial RNA quantities (Cecchini *et al.*, 2002).

The results are shown in **Figures 4.1, 4.2 and 4.3**, because *aux1-100* is in a WS background and *aux1-7* and *aux1-22* are in a Col-0 background these have been

shown separately. In Col-0 and WS (see **Figures 4.1**) levels of expression were very low or undetectable for all three genes during the early stages of infection, 0dpi to 8dpi, after 8dpi levels increased rapidly. All three *aux1* alleles and the corresponding wild-types (Col-0 and WS) showed an increase in the expression of the selected auxin responsive genes over the duration of the timecourse, which was concomitant with virus accumulation. Both *aux1-7* and *aux1-22* showed patterns of expression (see **Figure 4.2**) similar to that of their wild-type, Col-0 for all three genes examined. ANOVA of the data indicated that there were no significant differences ($p > 0.05$) between levels of expression of the three genes between the mutants and wild-types. *aux1-100* also showed early low level expression followed by a rapid increase in transcript levels from 8 dpi onwards but from 12 dpi on transcript levels were greatly reduced in comparison with the wild-type Ws (see **Figure 4.3**). For all three genes studied, *GH3*-like, *SAUR_e* and *SAG21*, ANOVA indicated a significant reduction ($p < 0.05$) in transcript levels in *aux1-100* infected plants compared to the corresponding wild-type.

These results show that CaMV infection is accompanied by an increase in the transcript levels of three of the auxin responsive genes and confirm the microarray results indicating that the expression of these auxin-responsive genes is greatly stimulated by infection. This virus dependent rise in transcript levels is significantly lower in *aux1-100*, a complete null mutant that does not produce

any functional protein, but not in *aux1-7* and *aux1-22* which contain mutations and produce altered proteins with partial function.

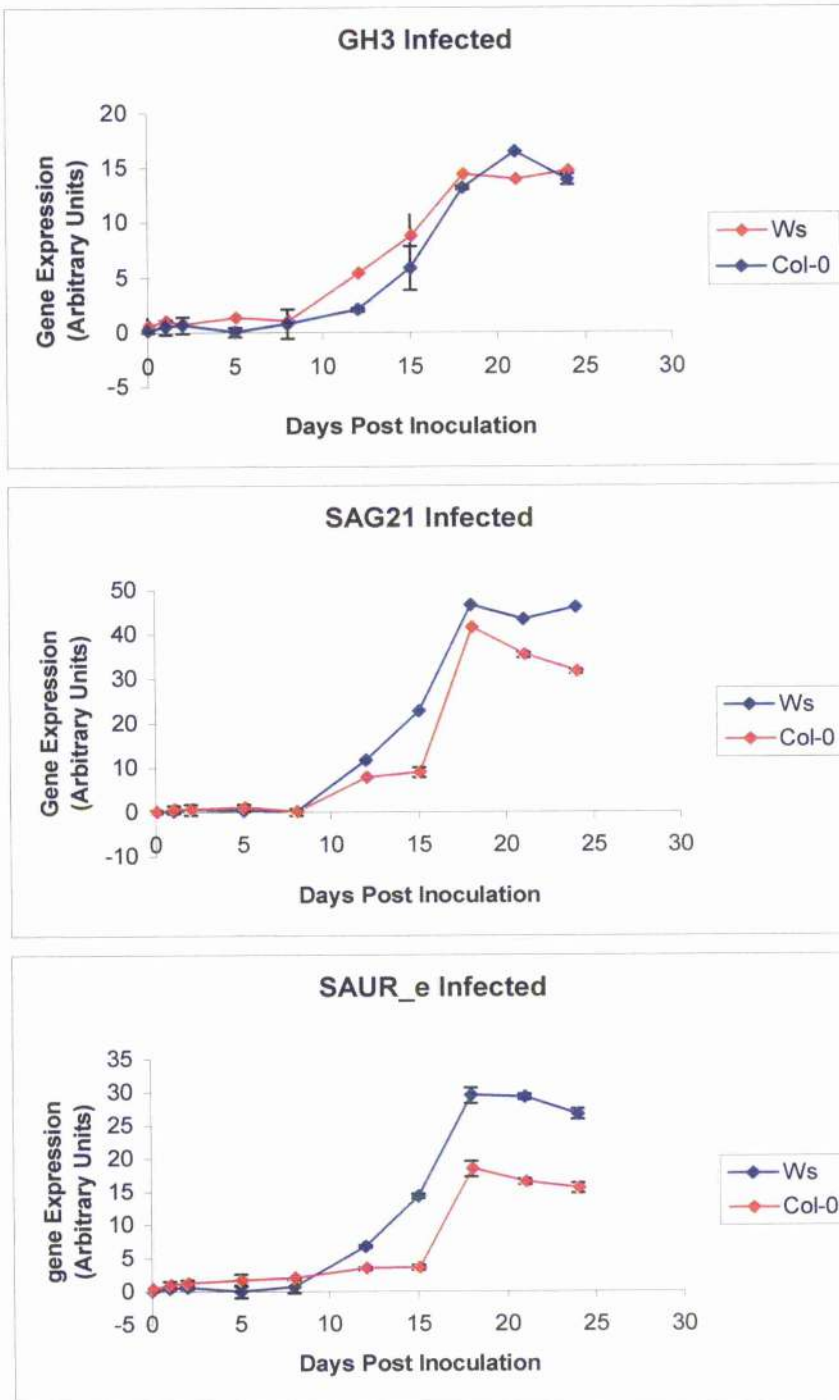


Fig 4.1 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in wild-type Col-0 and WS as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

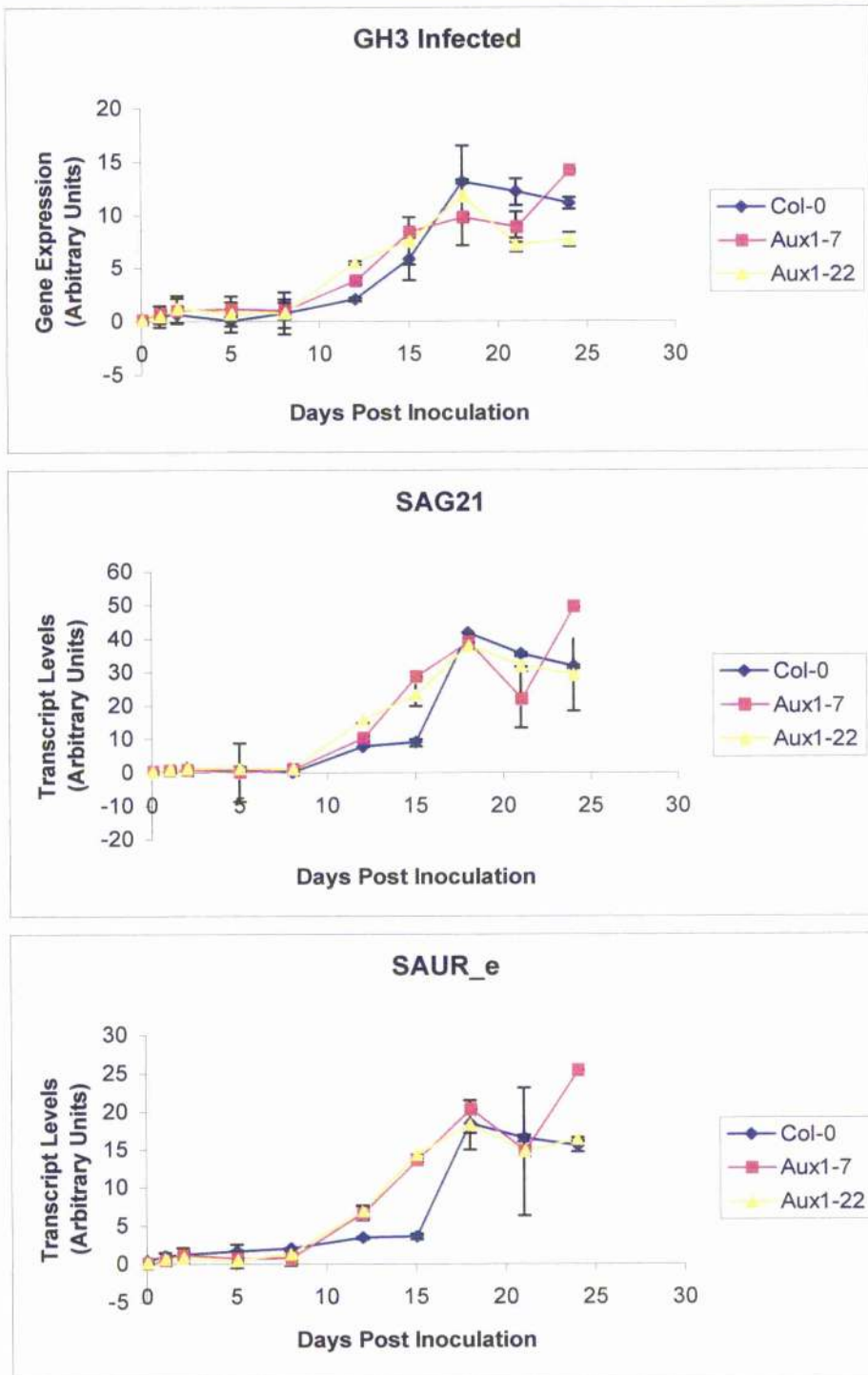


Fig 4.2 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in *aux1-7* and *aux1-22* and the respective wild-type Col-0 as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

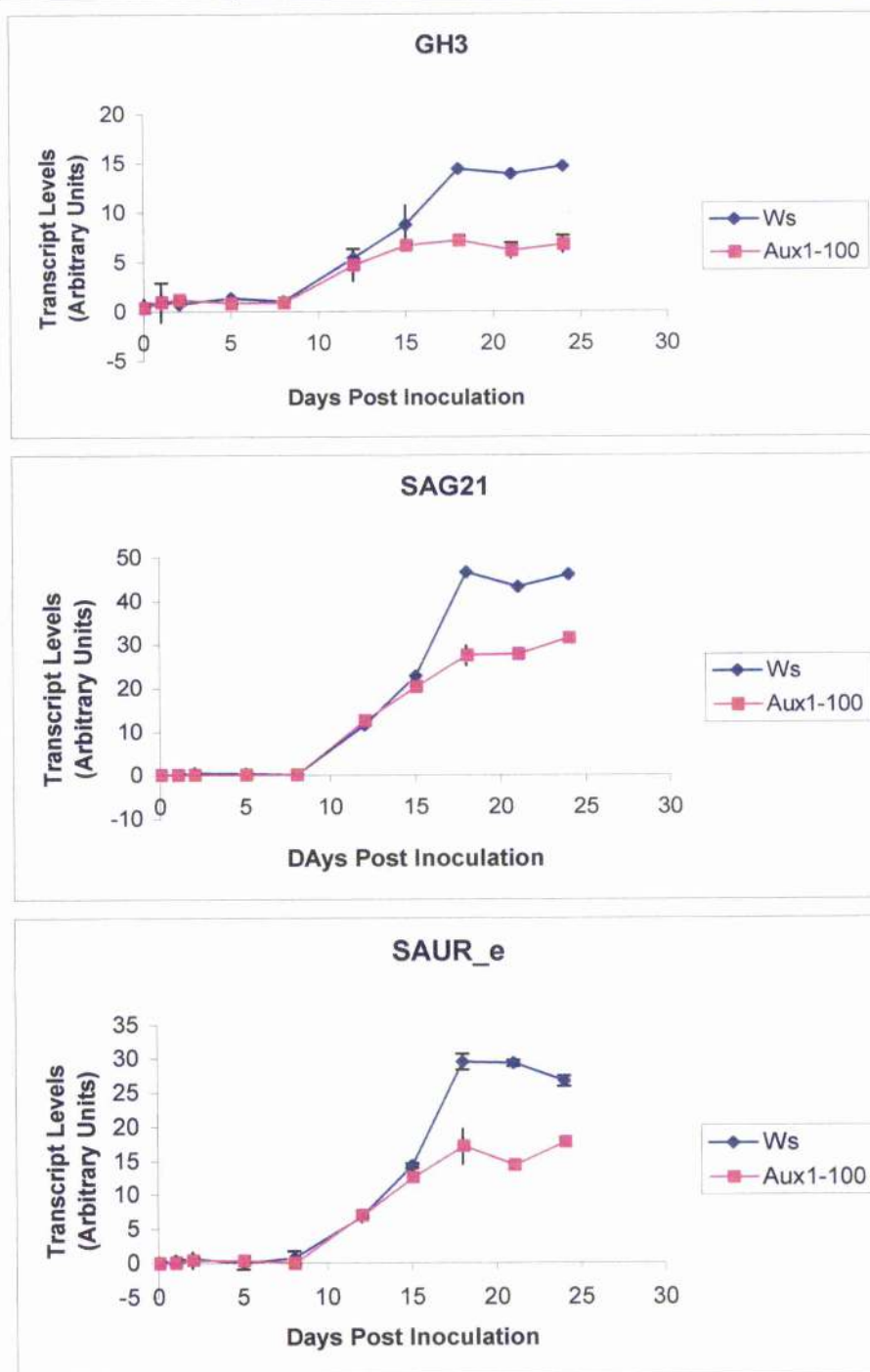


Fig.4.3 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in *aux1-100* and the respective wild-type WS as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

Expressions of these three genes were then examined in the three *tir* mutant lines, *tir1-1*, *tir2-2* and *tir3-1* (see **Figure 4.4**). *tir1-1* and *tir2-2* showed a similar pattern of expression to Col-0 for all three genes, *SAG21*, *GH3*-like and *SAUR_e*, and were not found to be significantly different throughout the course of the infection from the wild-type. In *tir3-1*, *SAG21* and *GH3* levels were similar to those of Col-0 and ANOVA showed that there were no significant differences ($p > 0.05$). However, in *tir3-1* the virus dependent increase in *SAUR_e* transcript levels was lower than in wild-type. Indeed, in *tir3-1* levels of *SAUR_e* transcripts at 21 dpi were not significantly greater from those at 0dpi. These results suggest that *TIR3* may be required for virus-induced up regulation of *SAUR_e* but not *SAG21* or *GH3*.

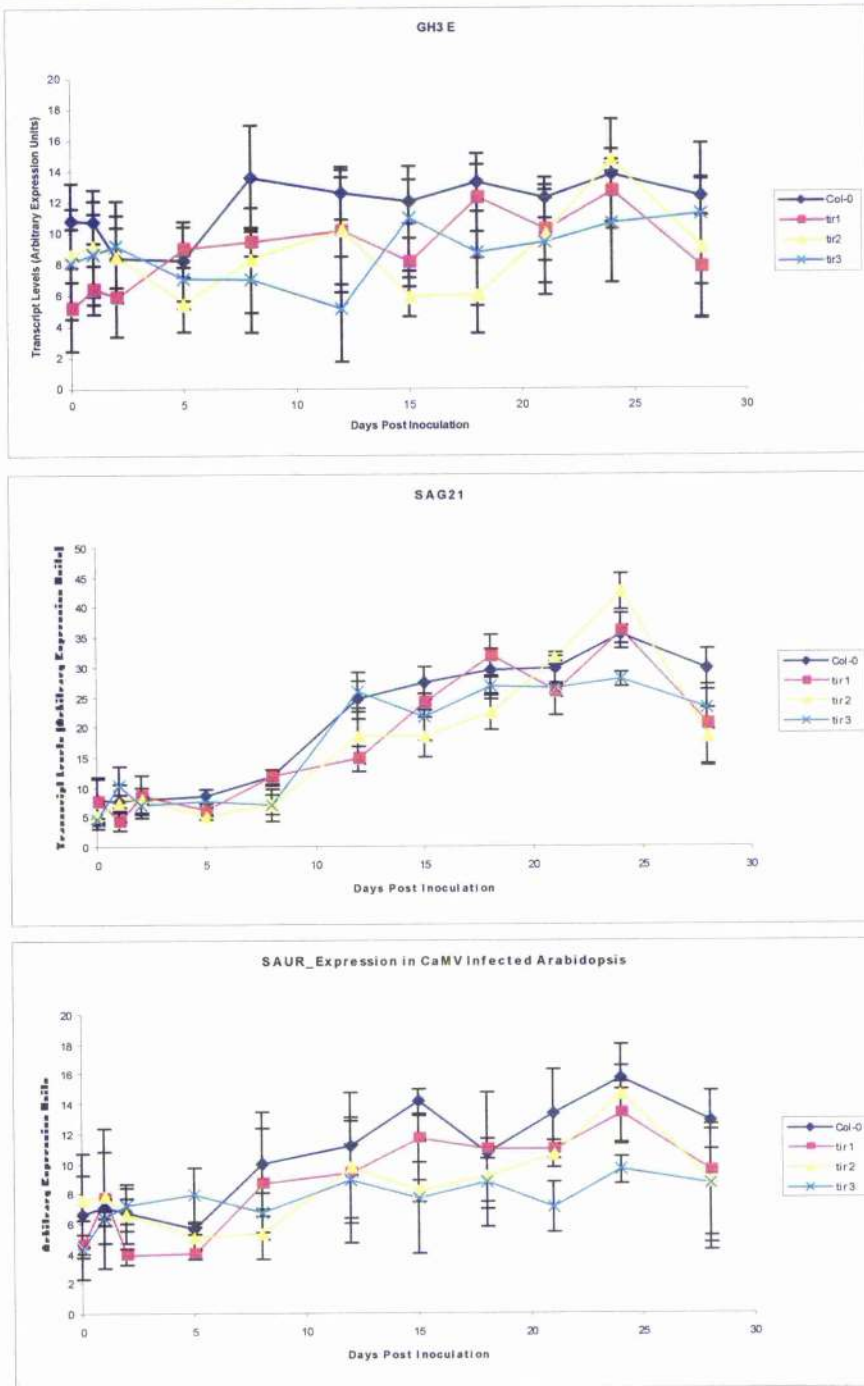


Fig 4.4 Virus induced expression of auxin responsive genes *GH3*, *SAG21* and *SAUR_e* in *tir1-1*, *tir2-2* and *tir3-1* and the respective wild-type Col-0 as determined by quantitative slot-blot hybridisation. Error bars indicate standard errors (n=3).

The effect of CaMV on the Expression Of Further Auxin Regulated Genes using Reporter Promoter Fusions (4.2.2)

To examine further the effect of CaMV on the expression patterns of auxin-responsive genes, transgenic plants containing an auxin-responsive promoter fused with a GUS reporter gene were examined. *LAX2* is an *AUX1* analogue that is involved in the loading of auxin via the phloem, and expression of *LAX2* is auxin responsive (Bennett, unpublished; Parry *et al.*, 2001). This gene is expressed mainly in the shoot tissue, and as this is the principle site of virus replication it should provide a useful marker for any dependent auxin response. Plants were infected with Cabb B-11 at the two true leaf stage (see Materials and Methods 2.3.3) harvested at suitable times after inoculation and stained for GUS activity. To distinguish between virus and wounding responses mock-inoculated plants were used as a control. Results are shown in **Figure 4.5**.

In mock-inoculated plants at the very early stages after mock inoculation, GUS staining was limited to the apical region in the centre of the seedling; no GUS stain was seen in the surrounding shoot tissue. By 14 dpi, at which point the infection had progressed and symptoms had begun to appear in virus-inoculated plants, GUS staining could be seen in the surrounding leaf tissue. The area of GUS staining continued to expand and by 28 dpi the mock inoculated samples showed extensive staining of the rosette leaves. Virus-infected plants showed a similar pattern of staining to the mock-inoculated plants at the early stages of the infection process; GUS stain was limited to the apex and no GUS was observed in the surrounding leaf tissue. As the infection progressed and symptoms became

visible, GUS staining remained confined to the plant apex, in contrast to the expanding area of stain in mock-inoculated plants. Even by 28 dpi GUS was still confined to the apex whereas mock-inoculated controls showed extensive staining at the same stage. These results demonstrate that in uninfected plants there is a developmental increase in *LAX2* expression. This effect, which may reflect changes in auxin distribution or signalling, appears to be suppressed by CaMV infection since the expansion of the stained area was absent in virus-infected plants.

DR5 is a synthetic auxin response element, consisting of seven tandem repeats of the AuxRE TGTCTC motif (Mattsson *et al.*, 2003; Ulmasov *et al.*, 1997).

DR5::Reporter fusions have been used as a tool to trace changes in auxin distribution (Mattsson *et al.*, 2003). Plants were either mock inoculated or inoculated with CaMV isolate Cabb B-JI, and samples examined at timepoints from 0 dpi through to 28 dpi. With DR5::GUS both mock inoculated and CaMV infected samples showed uniform GUS staining throughout the leaf tissue at all timepoints studied (see **Figure 4.6**). No obvious changes were observed in either the intensity or distribution of GUS, nor were there any differences between mock-inoculated and virus-inoculated plants. These results suggest either that auxin levels are unaffected by virus infection or that this reporter does not provide a reliable indication of changes in auxin levels.

Confocal microscopy was used to try to visualise alteration in auxin levels using a DR5::GFP construct. Plants containing DR5::GFP were inoculated with CaMV

Cabb B-II, controls were mock inoculated with water, and the aerial tissue removed and examined over a timecourse, from 0 dpi to 28 dpi. However, no GFP fluorescence could be seen in any of the shoot tissue examined. Data for this is not shown.

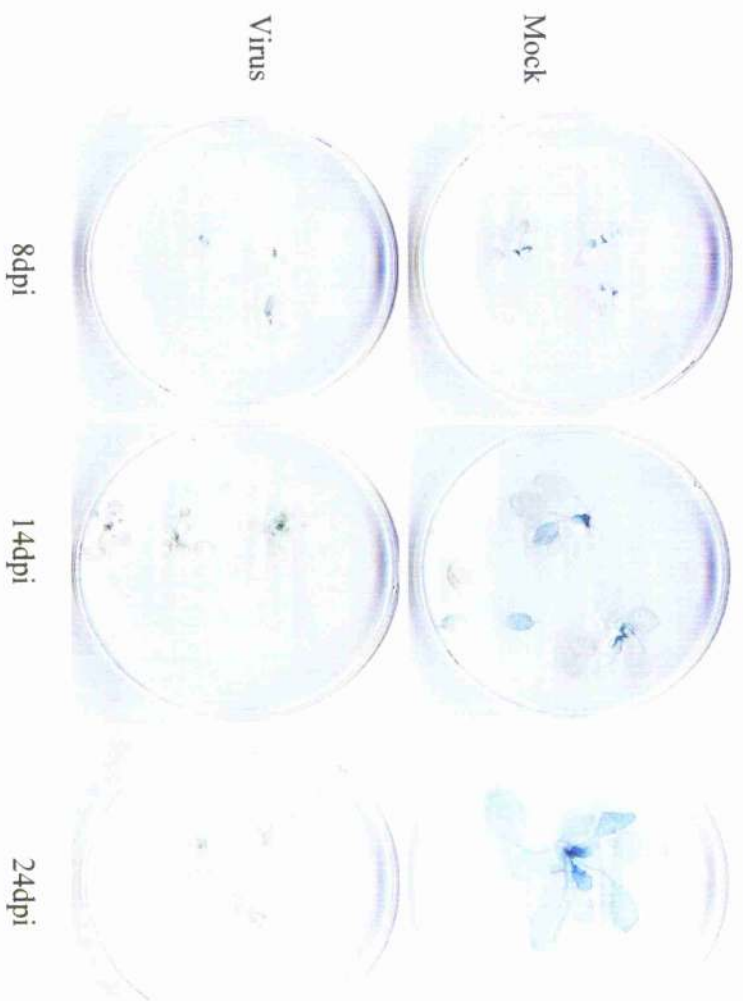


Fig. 4.5 GUS reporter driven by LAX2 promoter. Activity in infected and non-infected LAX2::GUS plants over a timecourse of 24 dpi.

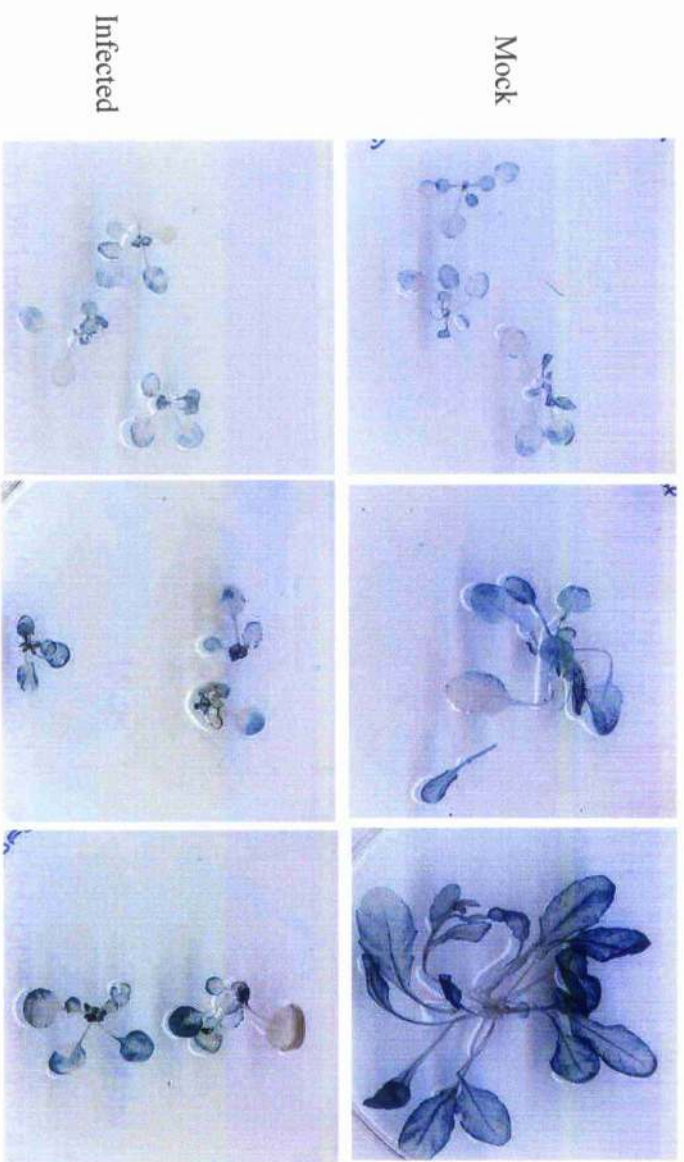


Fig. 4.6 GUS reporter driven by DR5 promoter. Activity in infected and non-infected DR5::GUS plants over a timecourse of 24 dpi.

IAA Analysis and Quantification (4.2.3)

Expression studies on three auxin responsive genes has shown them to be highly up-regulated in response to virus infection. These results suggest that infection may result in alterations in auxin levels that correspond to increased virus titre and spread. Further evidence for changes in auxin levels are provided by the pattern of expression in *LAX2::GUS* reporter lines, although the results with DR5 driven reporters does not support this. To test whether auxin levels are affected by infection, IAA levels were assayed directly. Total free IAA was quantified by GC-MS in samples consisting of pools of plant tissue from approximately 10-12 plants. Plants were infected with CaMV- Cabb B-JI and then harvested over a timecourse from 0 dpi through until 24 dpi. The entire aerial portion of the plant was removed and ground to a fine powder in liquid nitrogen. Of this ground tissue, approximately 20 mg of frozen tissue was taken and the free IAA extracted and purified using an SPE-based purification method. Once purified IAA was quantified using gas chromatography-mass spectrometry as described in Edlund *et al.*, (1995).

Results are shown in **Figure 4.7, A and B**. In both infected and uninfected plants, levels of free IAA showed no significant changes over the duration of the infection. IAA levels were slightly higher during the early timepoints, 0dpi to 5dpi, followed by a reduction, which continued until the end of the timecourse but these differences are not significant ($p > 0.05$). There were also no significant differences between *aux1-100* and wild-type ($p > 0.05$; see **Figure 4.7 B**). These results indicate that there are no gross changes in auxin levels either as a result

of development or infection. However by harvesting of the entire aerial portion of the plant, any differences in auxin distribution would not have been apparent. This suggests that virus is not affecting the overall amount of auxin within the plants and so alterations in transcript levels of the auxin-responsive genes do not correspond to changes in auxin levels although it could be affecting the distribution of auxin.

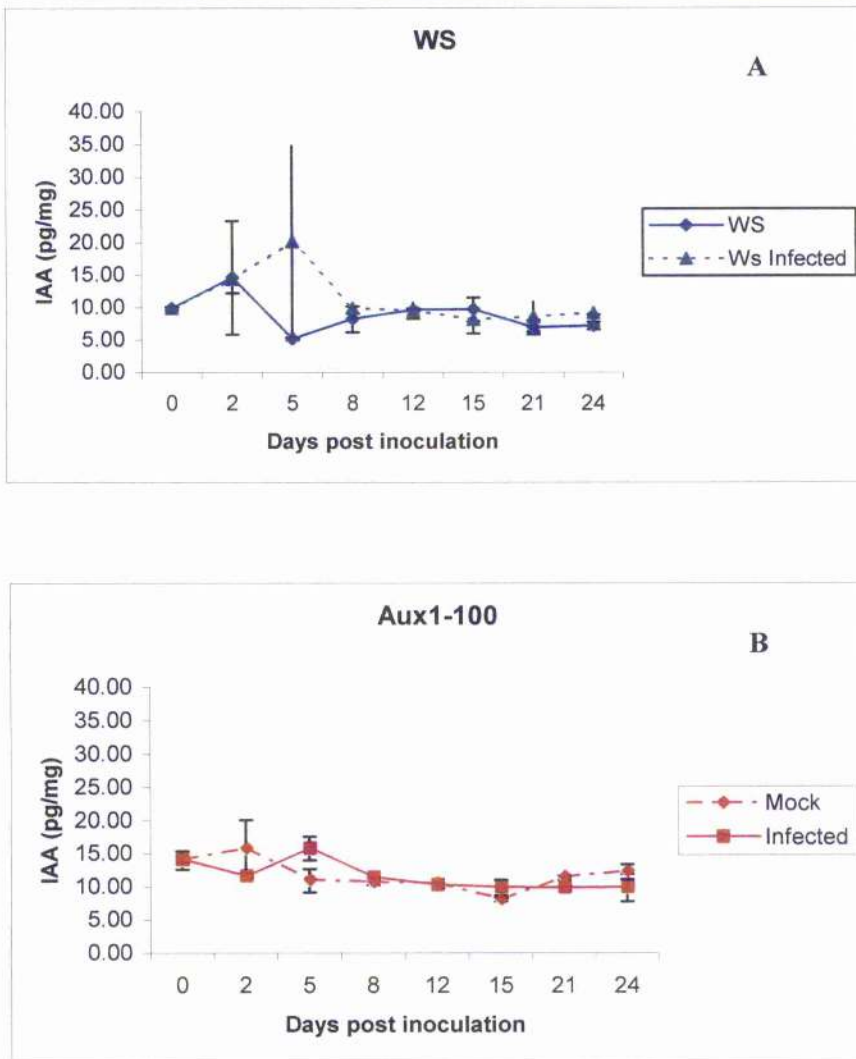


Fig 4.7. Quantification of free IAA from both infected and mock-inoculated plants. Samples were collected from 0 dpi at three day intervals until 24 dpi and IAA extracted and quantified using HPLC techniques. Results are shown with standard errors (n=3)

(A) IAA levels in CaMV infected and mock inoculated wild-type WS.

(B) IAA levels in CaMV infected and mock inoculated *aux1-100*.

Summary (4.3)

The main objective of the work described in this chapter was to define the effect of CaMV infection on auxin regulated responses within the host plant.

Microarray studies had identified seven auxin responsive genes that were highly up-regulated in response to infection and here a more complete analysis of the pattern of expression of the three most strongly up-regulated candidate genes was carried out. In three independent experiments virus infection stimulated an increase, up to 8-fold, in the level of *SAG21*, *GH3* and *SAUR_e* transcripts.

Transcript levels increased from 8 dpi, this corresponds to the increase in virus titre in these plants (Love *et al.*, 2005). This increase in transcripts was significantly reduced, by about 2-fold, but not abolished in *aux1-100* mutants when compared to WS for all three genes. In contrast the weak alleles *aux1-7* and *aux1-22* showed little difference from wild-type, Col-0. *aux1-7* and *aux1-22* both produce proteins with partial function whereas *aux1-100* is a null mutant and does not produce a functional protein. The significant reduction in responsiveness for all three genes in the *aux1-100* mutant suggests that polar auxin transport facilitates the virus dependent response.

Three transport inhibitor response mutants were compared to wild-type, Col-0. In *tir1-1* and *tir2-2* infection with CaMV levels of *SAG21*, *GH3* and *SAUR_e* transcripts were similar to wild-type. However, *tir3-1* showed a significant reduction ($p < 0.05$) in virus dependent *SAUR_e* expression compared with the wild-type, Col-0. The *TIR3* gene product is involved in auxin efflux (Ruegger *et*

al., 1997), whereas *tir1* and *tir2* are affected in auxin signaling. Again this result points to a possible role for polar auxin transport in the virus induced response.

Work from Bennett (unpublished) has shown that *LAX2* expression is auxin-responsive and that the gene is expressed mainly in the shoot tissue. It is an *AUX1* homologue and is believed to be responsible for the loading of IAA into the phloem. Developmental expression of GUS driven by the *LAX2* promoter was almost completely suppressed in infected plants, and GUS expression did not spread into the rosette leaf tissue, but remained confined to the apex. These results provide more evidence to suggest that CaMV infection is affecting auxin regulated gene expression and suggest that some aspect of auxin signaling or transport is affected. However GUS expression driven by the synthetic auxin responsive element DR5 failed to show any alterations in auxin distribution in response to either development or virus infection. This suggests that either auxin is unaffected, or that the DR5::GUS reporter line is not a reliable indicator of auxin levels and distribution. Another explanation for the inconsistencies between these two studies could be due to the expression pattern of the two constructs. *LAX2* is expressed in the shoot tissue at points where loading of IAA into the phloem occurs and so will presumably only respond to local changes in auxin concentration or distribution. Whereas DR5 is expressed throughout the plant and therefore it is possible that any small local changes in auxin observed when we look only at *LAX2*::GUS are not observed when we look at DR5::GUS as this is responding to changes in auxin throughout the plant. It might therefore

be useful to examine these plants at a cellular level to see if there are changes in auxin response between cells that could not be seen during this investigation.

DR5 is useful as a global marker of auxin response whereas LAX2 is an effective local marker.

Previous studies have tried to establish the effect that virus infection has on the levels of free IAA within plants and to link the physical symptoms observed during infection to these changes (reviewed by Pennazio and Roggero, 1996).

The results have been inconsistent and different studies have shown both increases and decreases in auxin levels depending on the virus and host studied.

Here, to establish if CaMV infection was affecting auxin levels within symptomatic plants, GC-MS was used to quantify the levels of free IAA within

both infected and mock inoculated plants. No differences in overall IAA levels between infected and mock inoculated plants were identified either in wild-type or *aux1-100*. This result illustrates the difficulties in establishing interactions between virus infection and auxin signalling by measuring auxin levels in tissue samples. Auxin distribution could be affected but the changes too subtle to be observed with the techniques used here. For example, auxin levels could be altered in areas of chlorosis compared to unaffected areas, between different tissue types or even between cells. Results using auxin-responsive reporters are contradictory. LAX2::GUS suggests a virus dependent change in auxin response, whereas no such change was identified with DR5::GUS. LAX2 is a naturally occurring auxin responsive gene and so the promoter could be responding to

other developmental cues within the plant, whereas DR5 which is completely synthetic will presumably not respond to anything other than auxin. These differences might account for the conflicting results from the two lines.

Chapter Five

Interaction Between CaMV Gene VI and Auxin Signalling and Transport

Introduction (5.1)

The work described in the previous chapters suggests that auxin signalling components are involved in the host response during CaMV infection. One possibility may be that CaMV gene products interact with components of auxin signalling or distribution. A possible candidate for this interaction is the CaMV protein P6. P6 functions as a translational transactivator, regulating the expression of other CaMV genes through modification of the host translation machinery (Reviewed in Chapter 1). As well as this role it is also the major genetic determinant of pathogenicity (Reviewed in Chapter 1). Recombinant virus genomes produced from CaMV isolates with different pathological characteristics have demonstrated that gene VI encodes pathogenicity determinants including those involved in host range and symptom determination (Rothnie *et al.*, 1994; Daubert *et al.*, 1990). Work from Hohn's group suggests that different domains may be involved in translational transactivation and pathogenicity (Kobayashi and Hohn, 2003; Kobayashi and Hohn, 2004). In the absence of virus infection in Arabidopsis and tobacco, P6 transgenic plants can show a symptom-like response (Cecchini *et al.*, 1997; Baughman *et al.*, 1988). P6 transgenic Arabidopsis plants show the symptoms commonly associated with CaMV infection, including vein clearing, chlorosis and stunting (Cecchini *et al.*, 1997). The type and severity of the symptoms observed correlated with the severity of the isolate from which the P6 was obtained and also to the levels of

P6 expression within the transgenic plant (Cecchini *et al.*, 1997). Most likely, during infection the host response is dependent not only on the levels of virus present but also on interactions between the CaMV and host-encoded components, triggering a set of pre-programmed responses. A likely candidate for this interaction is P6 as it appears to play a critical role in the host response. P6 transgenic *Arabidopsis* plants show many of the changes in gene expression that are detected in infected plants (Geri *et al.*, 1999). Therefore P6 appears to be accountable for these changes.

Previous studies have shown that P6 interacts with ethylene signalling during the infection process (Geri *et al.*, 2004), and P6 transgenic plants display an ethylene insensitive phenotype. P6 has also been shown to be a potent suppressor of SA-mediated defence responses (Love *et al.*, paper in preparation). Preliminary investigations into the effect of 1-naphthylphthalamic acid (NPA), an auxin inhibitor suggested that the symptom-like phenotype of P6 transgenics was ameliorated reversibly when grown on NPA, suggesting a link between auxin signalling and P6 (Cecchini and Milner, unpublished observation). The objective of the work described in this chapter was to establish whether CaMV P6 interacts with components of the auxin signalling and transport pathways, and if so whether this could account for the apparent auxin dependent responses reported in chapter 4. The P6 transgenic plants are a valuable resource with which to investigate this question.

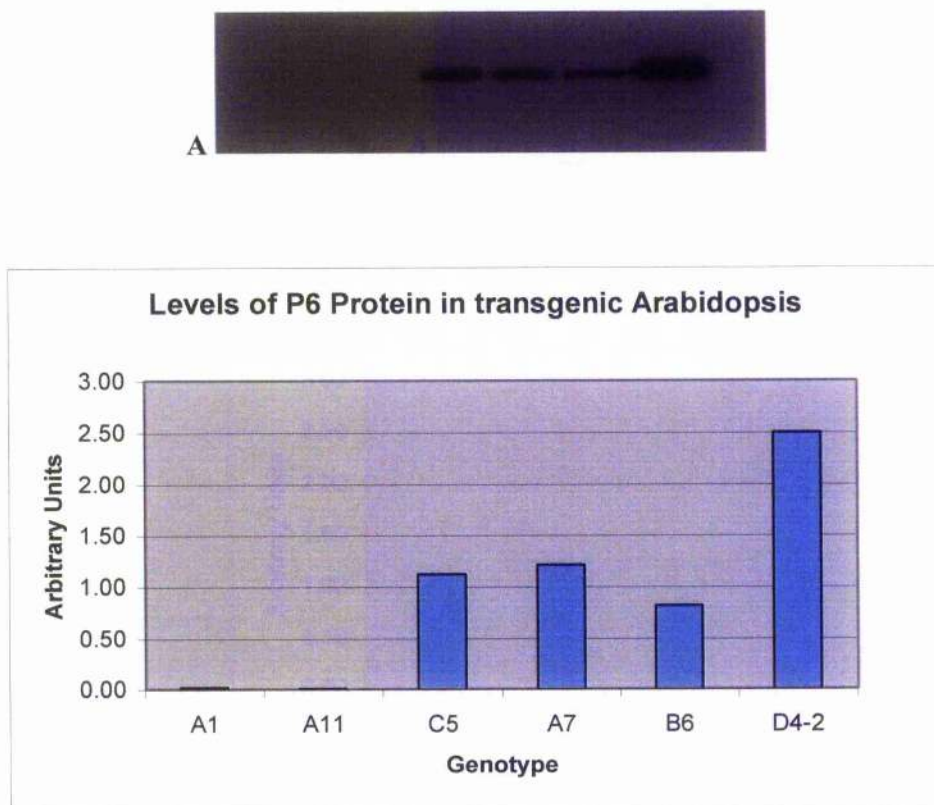
P6 Expression Levels in P6 Transgenic Arabidopsis (5.2.1)

Cecchini *et al.*, (1997) constructed more than 40 transgenic lines that express P6 from different CaMV isolates in an Arabidopsis *Ler-gll* background. Levels of P6 protein in these lines were originally assayed in T2 plants, because transgene expression can change from generation to generation as a result of silencing, protein levels were assayed in the T6 plants used for these studies. P6 protein levels were also assayed in line D4-2 (a gift from J. Schoelz). The D4-2 transgenic line is homozygous for gene VI from the D4 isolate of CaMV (Yu *et al.*, 2003). In contrast to Cabb B-JI the D4 strain of CaMV induces only mild symptoms in infected plants. Transgenic Arabidopsis expressing gene VI from D4 were almost symptomless and appeared similar to wild type. To establish directly the levels of P6 protein in the P6 transgenic Arabidopsis lines, western blotting was used to quantify the protein levels present in T6 plants. P6 protein was detected using a polyclonal antibody raised against P6 from CaMV strain W260 (Schoelz *et al.*, 1991). This antibody has been shown to cross react with P6 from a number of CaMV strains including D4 (Yu *et al.*, 2003), Cabb B-JI (Milner, unpublished data). Five independent transgenic lines were examined, A1, A11, C5, A7, B6 (which all containing a gene VI construct derived from Cabb B-JI; Cecchini *et al* 1997) and D4-2 (see Table 5.1). Protein was extracted from samples of each transgenic line (see Materials and Methods, 2.12), western blots carried out and probed and the protein levels quantified using Quantiscan (as described for RNA).

Transgenic Line	CaMV Isolate	Phenotype	P6 Transcript Levels (Cecchini <i>et al.</i> , 1997)
A7	Cabb B-JI	Stunted, chlorotic	High
B6	Cabb B-JI	Stunted, chlorotic	High
C5	Cabb B-JI	Stunted, chlorotic	Moderate
A1	Cabb B-JI	Nontransgenic phenotype	Low
A11	Cabb B-JI	Nontransgenic phenotype	Undetectable
D4-2	D4	Mild chlorosis, no stunting	High

Table 5.1 Phenotypes and P6 mRNA levels in T2 transgenic *Arabidopsis* plants expressing Cabb B-JI gene VI driven by the 35S promoter as determined by northern blots (Cecchini *et al.*, 1997).

The transgenic lines were tested in western blots using the polyclonal rabbit anti-P6 antibody. Two silenced lines, A1 and A11, were used as controls. These have been shown previously to have low levels of P6 mRNA (Cecchini *et al.*, 1997). Western blots confirmed this as P6 protein was undetectable in these lines. C5, B6 and A7 all contained significant levels of P6 protein. In contrast to T2 plants where C5 was reported to have only moderate P6 mRNA levels, P6 levels in C5 were similar to A7 and higher than in B6. In D4-2, which contains gene VI from the D4 isolate of the virus, signals in the Western blot were about twice that from A7. Both A7 and B6 show a strong phenotype, including stunting and chlorosis. The D4-2 transgenic line appears phenotypically wild type despite accumulating greater levels of P6 than the severely chlorotic and stunted A7 and B6. However, it is possible that differences in the cross reactivity of the antibody could account in part for the apparent high P6 levels in D4-2.



B

Fig 5.1 Quantification of P6 protein in P6 transgenic Arabidopsis plants. (A). Photograph of western blot showing P6 protein, detected using chemiluminescence (B) Quantification of levels of chemiluminescence from western blots using Quantiscan (as described for RNA).

Interaction Between CaMV Protein P6 and Auxin Signalling and Transport

(5.2.2)

P6 transgenics which express gene VI from the Cabb B-JI isolate of the virus were reported to show an ameliorated reversible phenotype when treated with NPA, an auxin analogue that can also act as an inhibitor of auxin transport (Cecchini and Milner, unpublished). However the levels of NPA used in these studies were very high. Therefore a different inhibitor of auxin efflux was tested. Plants were grown on a series of increasing concentrations of 2,3,5-triodobenzoic acid (TIBA), an inhibitor of carrier-mediated auxin efflux. TIBA may block auxin efflux by competitively binding to the auxin binding protein (Rubery, 19990; Muday and DeLong, 2001). The mechanism of action of this compound is not completely understood, but it may either directly block auxin movements or indirectly alter the cycling of auxin transport proteins to or from the plasma membrane (Muday and DeLong, 2001). Both the phenotypic response and the expression levels of an auxin responsive marker gene, *SAUR_e* were examined at 16 and 24 days after germination. The phenotypes were assessed visually and plants were photographed. Levels of *SAUR_e* transcripts were determined using RT-QPCR (See Materials and Methods, 2.9). *SAUR_e*, which is an auxin responsive gene, is a member of the small auxin up-regulated RNA protein family. Results in Chapter 4 found this gene to be highly up-regulated in response to CaMV infection. Three independent transgenic lines were used, A7 and B6, which express high levels of P6 from isolate Cabb B-JI and D4-2, which contains P6 from the D4 isolate. *aux1-100* was used as a control. Also tested were P6 lines, B2-3 (*cse-2*) and

B4-2 (*cse-1*). These lines, which carry a suppressor mutation, *cse* (CAMV P6 Suppressor-Ethylene Insensitive), were identified from a screen for mutants that suppressed the P6 phenotype of chlorosis and stunting. Seeds from the transgenic P6 line A7 were mutagenized by γ -irradiation and M2 seedlings were screened for mutants that showed a reduced P6 phenotype. Two lines, B2-3 and B4-2, that are allelic for recessive mutations at the same locus, appear considerably larger and greener than the A7 parental line but have been shown to contain an intact and transcriptionally active transgene. However these plants still show some stunting and chlorosis compared to non-transgenic Arabidopsis. The *cse* mutation does not suppress ethylene or SA insensitivity in P6 transgenics (Geri *et al.*, 2004).

Sensitivity of P6 transgenic plants to TIBA (5.2.2.1)

The transgenic lines together with the *aux1-100* control and wild types were germinated on plates containing TIBA at concentrations, ranging from 0 μ M to 35 μ M. Although Geldner *et al.*, 2003 has suggested that concentration above 25 μ m may cause cytoplasmic acidification other studies using TIBA to examine the effects of auxin transport on growth of the plant have used a range of concentrations up to 100 μ m (Chen *et al.*, 2007) or 200 μ m (Garcia-Gomez *et al.*, 1994). After 24 days of growth on the plates, phenotypes were assessed and the plants were photographed. The wild type plants, Col-0 and Ler *gll* developed normally at low concentrations of TIBA, 0 μ M and 15 μ M but were unable to grow successfully at concentrations greater than 20 μ m (see **Figure 5.2 and 5.3**). At concentrations above 20 μ M, the seedlings appeared stunted

and severely chlorotic. *aux1-100* plants were able to grow and develop normally at concentrations up to 30 μ M; however at 35 μ M plants were starting to show stunting and chlorosis. In contrast A7 transgenic plants did not show this sensitivity to TIBA and were able to grow successfully on concentrations of TIBA as high as 35 μ M. At this concentration the plants appeared similar in appearance to those grown on plates without TIBA added. The seedlings showed no additional chlorosis over and above that associated with the P6 phenotype. The B6 transgenic line was also able to tolerate TIBA concentration up to 35 μ M. However, it did not grow as successfully as A7 and at 35 μ M TIBA some plants showed the stunting and severe chlorosis observed in the wild type.

D4-2 also demonstrated enhanced TIBA resistance compared to wild type and plants were able to grow successfully at concentrations of 35 μ M without any visible stunting or chlorosis. D4-2 showed a strong TIBA-resistant phenotype that appears to be independent of the stunting and chlorosis phenotype induced by expression of P6 from Cabb B-J1 but not seen in the D4-2 transgenic line. To test this further the two suppressor lines B2-3 (*cse-2*) and B4-2 (*cse-2*) were tested. The A7 transgenics containing the suppressor mutation, B2-3 and B4-2, showed much reduced resistance to TIBA compared to A7 or B6, and were only slightly more resistant than the Ler *g11* wild type parent. B2-3 and B4-2 were unable to grow on concentration greater than 20 μ M; at higher concentrations the plants began to show severe chlorosis and stunted growth.

Fig 5.2 Growth of *Arabidopsis* P6 transgenics and *ler gl1* wild-type in response to various concentrations of the auxin efflux inhibitor TIBA.

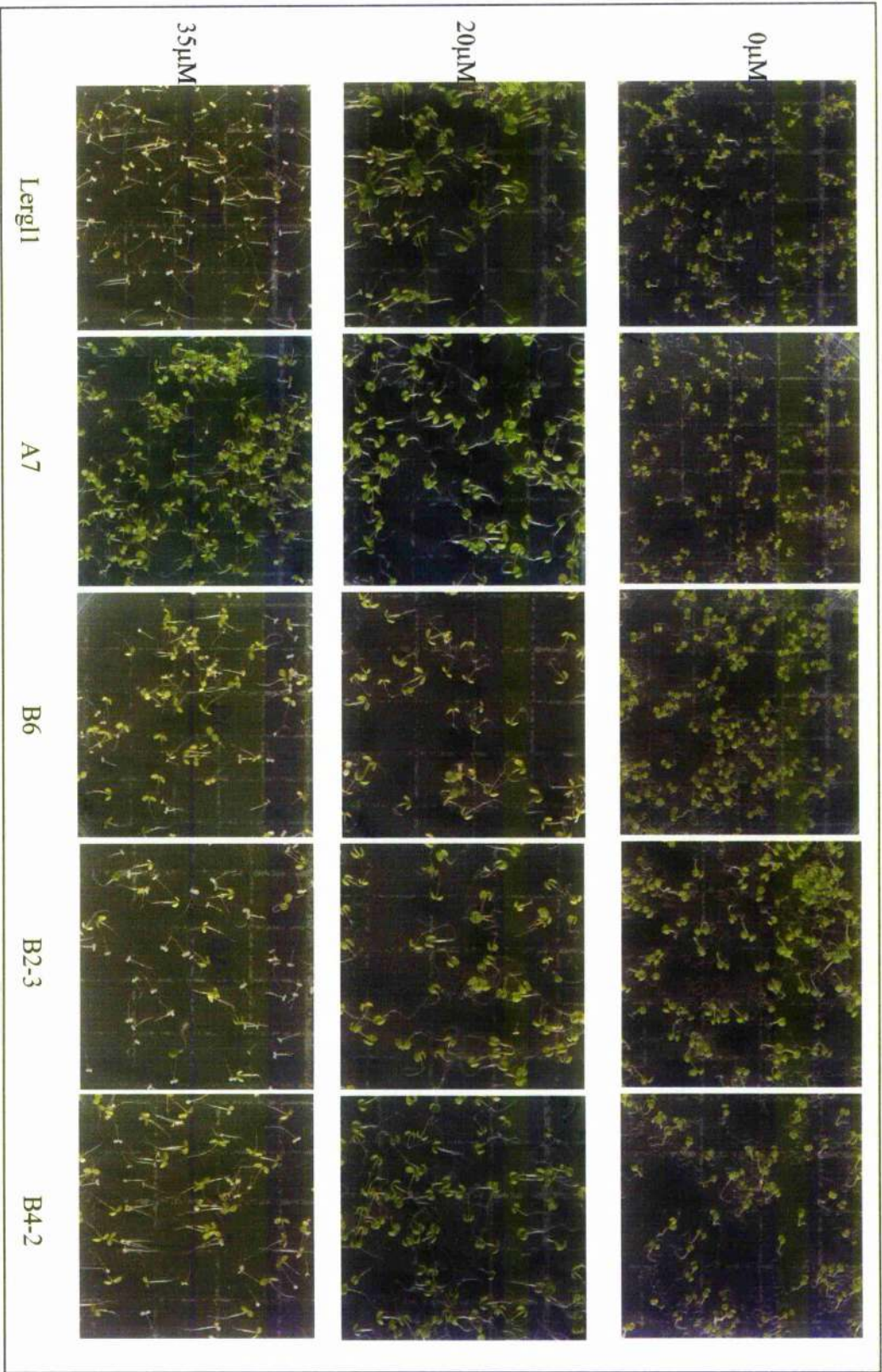
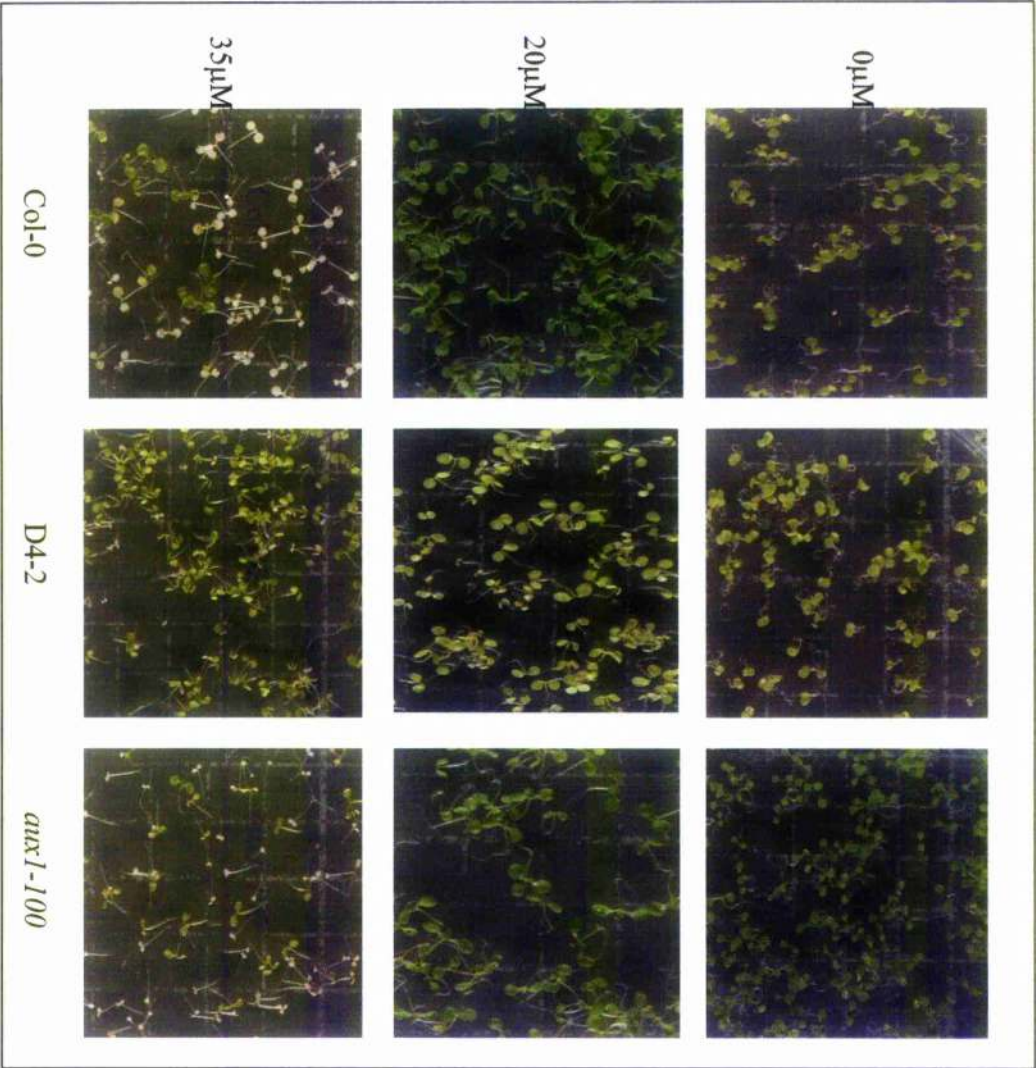


Fig 5.3 Growth of Arabidopsis P6 transgenics and Col-0 wild-type in response to various concentrations of the auxin efflux inhibitor TIBA.



Expression of Auxin Responsive Genes in P6-transgenic plants (5.2.2.2)

To further analyse the effect of TIBA on the P6 transgenic lines, the expression of an auxin responsive gene was investigated. Infection studies, described in Chapter 4, demonstrated that the auxin responsive gene *SAUR_e* was highly up-regulated in response to CaMV infection. This gene has also been identified as being TIBA-responsive from analysis of Genevestigator and NASC (Zimmermann *et al.*, 2004; Riken Group, 2004, unpublished; genevestigator chip # 113). Experiments carried out in triplicate where wild type seedlings were treated with TIBA found that expression of *SAUR_e* is reduced in response to TIBA treatment.

Plants were grown on plates containing increasing levels of TIBA, from 0-35 μ M, and tissue samples were taken at 16 and 24 days after germination. Levels of *SAUR_e* mRNA, were quantified in samples of each of the transgenic lines, *aux1-100* control, and wild types. RNA was extracted and DNase treated before being quantified. cDNA was synthesised and subsequently used in RT-QPCR reactions. *SAUR_e* expression was normalised against the levels of *ACT2* mRNA. *ACT2* has been found to be expressed constitutively in Arabidopsis, and makes a good internal reference standard for RT-QPCR (Laval *et al.*, 2002, Love *et al.*, 2005)

In the wild type plants, Col-0 and Ler, levels of *SAUR_e* transcripts were low at all concentrations of TIBA (see **Figure 5.4 and 5.5**). In contrast to the study reported in Genevestigator (Zimmermann *et al.*, 2004; Riken Group, 2004,

unpublished; genevestigator chip # 113), levels of *SAUR_e* transcripts showed no consistent decrease in plants germinated on TIBA. Also, transcript levels remained relatively constant between the two timepoints. Real time RT-PCR is an exquisitely sensitive technique and the levels of *SAUR_e* transcripts were very low relative to *ACT2* (equivalent to 10 or fewer copies of the mRNA per cell; see Love *et al* 2005, Laval *et al* 2002). Therefore the results carry a large amount of inherent variation and the wild type plants do not appear to show any consistent pattern of expression in response to TIBA. In contrast, in the absence of TIBA, A7 transgenic plants showed highly elevated levels of *SAUR_e* transcripts, about 30-fold higher than in the Ler *gll* wild type. Moreover *SAUR_e* transcripts appear to be TIBA responsive. Levels increased a further 2-3 fold when treated with levels of TIBA of 15 μ M or more. B6 plants also show high levels of *SAUR_e* transcripts compared to the wild type; however these were less elevated than in A7, approximately 10 fold higher than wild type. D4-2 demonstrated the highest *SAUR_e* transcript levels of all of the lines tested. In the absence of TIBA D4-2 showed a 6-fold increase in *SAUR_e* transcript levels compared to wild type.

These results indicate that P6 stimulates the expression of the auxin responsive gene *SAUR_e*. This expression appears to be P6 dose dependent, and in P6 transgenic plants but not wild type, levels of *SAUR_e* transcripts are further stimulated 2-3-fold by TIBA treatment.

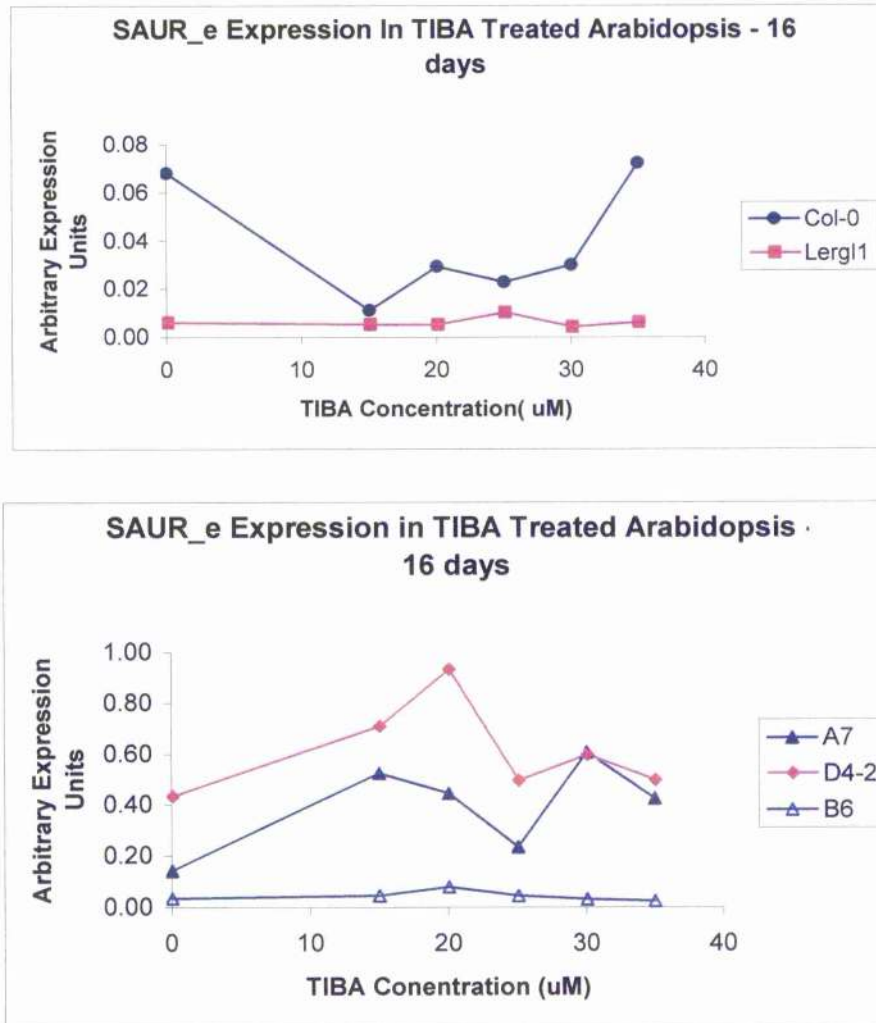


Fig 5.4 Transcript levels of the auxin responsive and TIBA responsive gene, *SAUR_e* in gene VI transgenics and wild types grown on TIBA concentrations ranging from 0 to 35 μ M. Levels are shown for 16 days after germination as quantified by RT-QPCR.

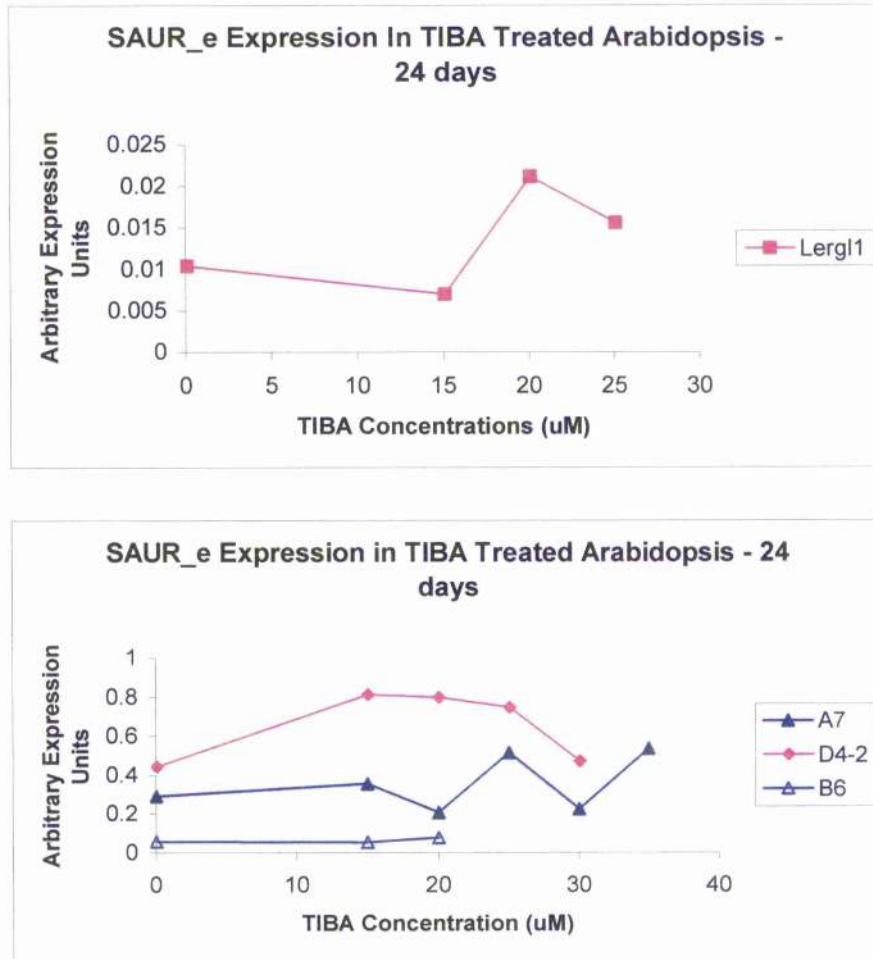


Fig 5.5 Transcript levels of the auxin responsive and TIBA responsive gene, *SAUR_e* in gene VI transgenics and wild types grown on TIBA concentrations ranging from 0 to 35 μ M. Levels are shown for 24 days after germination as quantified by RT-QPCR.

Genetic Analysis of the Interaction between P6 And Auxin (5.2.3)

To further characterize the interaction between P6 and auxin signalling and transport, and to try to identify at which point in the signalling pathway this interaction might occur, the P6 transgenic lines A7 and B6 were crossed with the auxin mutants, *aux1-100*, *aux1-7*, *tir1-1*, *tir2-2* and *tir3-1* (see Materials and Methods, 2.13). Controls were the P6 transgenics crossed with the wild types, Col-0 and WS. All of the auxin mutant alleles are recessive whilst 35S::gene VI is semi-dominant. Therefore, plants which are hemizygous for P6 are stunted and chlorotic but to a much lesser extent than those that have two copies of the transgene (Geri *et al.*, 2004). In crosses between P6 transgenics and wild type it was difficult to distinguish gene VI hemizygous plants from gene VI homozygous plants at the seedling stage; therefore the phenotypes were scored as though the 35S::Gene VI transgene was a dominant allele. Thus the expected segregation ratio of chlorotic:green plants is 3:1 (see **Table 5.2**).

For each cross, the F2 progeny from independent F1 plants were grown on soil and assessed visually. Wild type Col-0 when crossed with A7 produced F2 offspring that segregated phenotypically close to the expected ratio 3:1. On average 71.9 (+/-1.9) percent of the plants showed the chlorotic phenotype normally associated with P6. This was also the case for the F2 generation of the *aux1-7* and *aux1-100* crosses (see **Table 5.2**) where 80.2± 4.2% and 67.0 (+/- 7.4) percent respectively showed a pale chlorotic phenotype. Neither *tir1-1* nor *tir2-2* produced successful F2 lines for analysis. However, *tir3-1* when crossed

with A7 gave F2 progeny with an aberrant segregation ratio. Instead of the expected 25 percent (1:3) showing a dark green phenotype 42.4 (+/- 0.7) percent of the plants showed a dark green phenotype. ANOVA indicates that this differs significantly from the expected 25 percent ($p < 0.05$). Thus, the *tir3-1* mutation appears to be suppressing the P6 related chlorosis phenotype.

Cross	Number of Plants	Segregation Ratio (expected ratio 3:1)		Average (+/- Std deviation)	
		Pale Green	Dark green	Pale Green	Dark green
Col-0	102	70.6	29.4		
	56	73.2	26.8	71.9 (1.9)	24.1(1.9)
<i>aux1-7</i>	54	81.5	18.5		
	86	75.6	24.4	80.2 (4.2)	18.8 (4.2)
	61	83.6	16.4		
<i>aux1-100</i>	54	59.3	40.7		
	46	63.0	37.0	67.0 (7.4)	33.0 (7.4)
	46	69.6	30.4		
	46	76.0	24.0		
<i>tir3-1</i>	98	58.2	41.8		
	77	57.1	42.9	42.3 (0.7)	57.7 (0.7)

Table 5.2 Segregation ratios of the F2 generation of P6/auxin crosses. Plants scored as either showing wild type phenotype (dark green) or P6 phenotype (pale green). Each line represents the phenotypic segregation for the progeny of individual F1 plants. All crosses were carried out using A7 as the P6 transgenic parent.

Summary (5.3)

CaMV symptom determinant P6 has been shown to interact with both ethylene and SA signalling pathways in Arabidopsis (Geri *et al.*, 2004; Love *et al.*, in preparation). The results reported in this chapter demonstrate that P6 also shows an interaction with auxin signalling.

When the P6 transgenic lines were grown on plates containing different concentrations of the auxin efflux inhibitor TIBA, transgenic plants exhibited a strong TIBA resistant phenotype. Resistance to TIBA appeared to be P6 dose-dependent. D4-2, which contains the highest levels of P6 protein, was the most resistant to TIBA and B6 which contains the lowest levels of P6 was the least resistant. These results also indicate that this resistance to TIBA is independent of the stunting and chlorosis phenotype, as D4-2, which expresses P6 from a mild strain of CaMV and is wild type in appearance, was highly resistant to TIBA. Interestingly B2-3 and B4-2, which contain the A7 transgene together with two alleles of an unlinked mutation (*cse*) that suppresses P6 dependent chlorosis and stunting, reverted almost to wild type sensitivity to TIBA. Therefore in addition to suppressing the chlorotic dwarf phenotype the mutations at the *CSE* locus results in a suppression of P6-dependent TIBA resistance. In contrast the *cse* allele does not suppress either ethylene or SA signalling interactions (Geri *et al.*, 2004; Love *et al.*, in preparation). It may be significant that *cse* mutants show a leaf curling phenotype (Geri *et al.*, 2004) which is reminiscent of several auxin signalling, and transport mutations e.g. *mdr1*, and the *phot1phot2* double mutants

(Noh *et al.*, 2001; Sakamoto and Briggs, 2002). This provides further evidence for a role for CSE in auxin signalling.

Expression of P6 also radically altered the transcript levels of an auxin responsive gene *SAUR_e*, whose expression is up-regulated in response to CaMV infection (See Chapter 4). Transcript levels of the *SAUR_e* gene were highly elevated (up to 30-fold) in the P6 lines compared to the wild types. Levels of expression were P6 dose dependent. D4-2 showed the highest levels of both P6 protein and *SAUR_e* expression and B6 showed the lowest.

Interestingly, in contrast to the study reported in Genevestigator (Zimmermann *et al.*, 2004; Riken Group, 2004, unpublished; genevestigator chip # 113), *SAUR_e* transcript levels in wild type plants were low and showed no significant reduction in response to TIBA treatment. However, exposure of all three P6 transgenic lines to TIBA resulted in a further 2-3-fold increase in *SAUR_e* transcript levels. Therefore P6 was able to stimulate *SAUR_e* expression in the presence of TIBA.

Crosses between P6 transgenic lines A7 and B6 and the auxin signalling mutants provide further, albeit preliminary evidence for an interaction between P6 and auxin signalling. In one cross, *tir3-1* x A7 the F2 progeny segregated with an aberrant ratio. The increased proportion of green progeny in the F2 is consistent with a suppression of the chlorotic dwarf P6 phenotype in *tir3-1* homozygotes. *TIR3* maps to 2 cM on chromosome 3, and the gene VI transgene in A7 has been

roughly mapped to approximately 40 cM on the same chromosome (Laird and Milner, unpublished). Although both loci are located on chromosome 3 they are sufficiently far apart to behave as unlinked. If the *tir3-1* allele is indeed suppressing the P6-induced chlorosis and stunting phenotype, the predicted ratio of green: chlorotic seedlings should be 44:56 for two unlinked loci. This is very close to the observed ratio of 42:58. Therefore, although this evidence is preliminary, the segregation patterns of the F2 plants are consistent with an interaction between P6 and the product of the *TIR3* locus.

Therefore the results discussed above point to a specific role for *TIR3* in CaMV infection and suggest that it is somehow necessary for the development of systemic symptoms. An interaction between *TIR3* and the viral protein P6 which is known to be involved in symptom production is required. As no other components of the auxin transport and signalling pathway appeared to be involved it is likely that the auxin responses observed are as a consequence of this specific interaction. A possible mechanism is detailed in **Figure 5.6**.

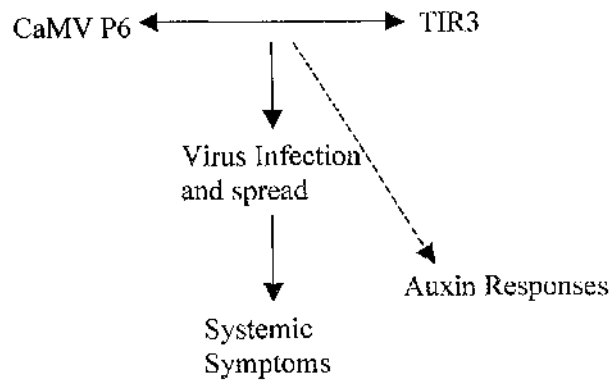


Fig 5.6 Possible mechanism showing the interaction of *TIR3* and CaMV P6 and the subsequent auxin responses that result from this interaction and the establishment of successful infection and systemic symptoms.

Chapter Six

General Discussion (6.1)

Auxin's Role in the Development of Compatible Virus Infection (6.1)

One of the main objectives of the work reported in this thesis was to assess the role of auxin in the host-virus interaction during compatible infections. To achieve this, a genetic approach was adopted and studies were carried out on a number of auxin signalling and transport mutant *Arabidopsis* lines. The initial aim was to assess the symptom response of both the wild-types and mutant lines when infected with CaMV and also to examine virus accumulation and spread within these plants. The second aim was to establish whether CaMV infection affected auxin signalling, transport or concentration and finally to determine mechanisms by which these responses occurred.

Historically, studying the role of auxin during virus infection involved the application of exogenous auxin compounds, or assays of auxin levels in infected plants (reviewed by Pennazio and Roggero, 1996). Many reports indicated that auxin treatment attenuated symptoms and also inhibited virus replication (reviewed by Fraser and Whetnam, 1982; Nichols, 1952; Kutsky and Rawlins, 1950). However, it is worth noting that these responses were often achieved through the application of cytotoxic quantities of auxin analogues. For example, van Loon *et al.*, (1990) demonstrated that IAA and NAA when applied exogenously slightly reduced whereas 2,4-D strongly increased TMV replication in tobacco plants. The majority of investigations measured virus multiplication by infectivity bioassay, assessing the number of local lesions produced on a suitably reacting second host. This is not accurate enough to be

able to discriminate between small differences in virus concentration (Fraser and Whenham, 1982). Also the treatment of plants with growth regulators may have an indirect influence on the specific infectivity of the virus. For these reasons an accurate direct measurement of virus replication and accumulation is required.

One effective way of dissecting the role of auxin in this process is to use a genetic approach. A large number of mutant alleles, carrying defects in various aspects of auxin signalling and transport, are available. These mutants provide an invaluable tool for investigating the role of auxin in many different processes within the plant. They can provide a link to the causes of the alterations in host response observed during infection.

If auxin is responsible for symptoms or other responses to infection we might expect that disrupting auxin signalling and transport would lead to alterations in responses to infection. However experiments described in Chapter 3, taken together with additional unpublished work in the Milner lab, indicate that such mutants in general, do not differ from wild-type in either symptom phenotype or virus accumulation. The auxin mutants *axr4* and *axr2* which have been examined previously (Cecchini and Milner, unpublished) do not show any obvious differences in response to virus infection compared to wild-type. This was also found to be the case for the three *aux1* alleles and the transport inhibitor response mutants *tir1-1* and *tir2-2* examined here. These results suggest that most mutations that affect polar auxin transport or signalling do not affect host-virus

interactions. Therefore, auxin signalling may not play a major role in the susceptibility of plants to infection. However, *tir1-1* was found to have a p-value of 0.0703 which although greater than 0.05 and therefore usually regarded as not significant still indicates that there is a 93% chance that the result is significantly different from the wild-type and could implicate *TIR1* in the infection process.

One exception to this was *tir3-1* mutant plants. Here, symptom development was very significantly delayed and virus titres were much lower (up to 3-fold) compared to wild-type. Visualization of the virus by CaMV::GFP fluorescence confirmed that in *tir3-1*, cell to cell spread of CaMV was greatly reduced and delayed and movement of the virus into the vascular system was also delayed. As only *tir3-1*, of all the mutants tested, showed an altered response to CaMV this suggests that *TIR3* may play a more specific role in the host-virus interaction. *tir3-1* was originally identified in a screen for plants with altered responses to auxin transport inhibitors. *TIR3* is essential for either the synthesis, localization, or the function of the NPA binding site (Gil *et al.*, 2001). Recent research has shown (Gil *et al.*, 2001) that *tir3-1* is allelic to the *doc1-1* mutation. The *DOC1* (Dark Overexpressing *CAB* 1) gene is a putative component of the light signal transduction pathways (Li *et al.*, 1994). Plants carrying mutations in this locus show overexpression of *CAB* genes in the dark (Li *et al.*, 1994). The locus has been recently renamed as *BIG* (Gil *et al.*, 2001) and is predicted to encode a large protein (560 KD) with significant similarity to the *Drosophila* protein Calossin/Pushover. Calossin is believed to be involved in the control of

synaptic transmission at the neuromuscular junction, in a process that requires the synaptic vesicle cycle (Xu *et al.*, 1998). Like synaptic transmission, the asymmetric distribution of auxin efflux carriers at the plasma membrane depends on targeted vesicle transport (Steinmann *et al.*, 1998) and recent work has shown that *BIG* is essential for proper positioning of the auxin efflux carrier, *PIN1*, at the plasma membrane (Paciorek *et al.*, 2005). *tir3* mutants also show defects in ethylene signalling and Kanyuka *et al.*, (2003) has suggested that rather than playing a *specific* role in auxin signalling, *BIG* may be involved in the control of vesicle transport and fusion, affecting a variety of signalling pathways. The results here all suggest that the *TIR3* locus is important for a successful CaMV infection to develop. Any interaction between auxin signalling and virus infection thus appears to be highly specific, and may not involve global changes in auxin levels and/or transport within the plant.

Fraser *et al.*, (1986) found a correlation between the level of symptoms and the level of virus accumulation in tobacco infected with TMV, although other studies have found that symptom severity may not be directly correlated to the level of virus accumulation within infected plants (Cecchini *et al.*, 1998; Fraser *et al.*, 1986). This suggests that several factors may influence symptom development and that these may vary, depending on the host and virus in question. Therefore, although a correlation between symptom response and virus titre was observed in the *tir3-1* mutant plants, the role of auxins might be more complex than simply altering symptom response through alterations in virus levels.

Auxin Responses are Altered by Compatible Virus Infection (6.1.2)

Auxin Responsive Gene Expression (6.1.2.1)

Virus infection not only elicits a symptom response within the host plant but can also lead to the altered expression of a number of host genes (Whitham *et al.*, 2003; CaMV infection, Geri *et al.*, 1999). These include defence-related genes but also a range of genes which function in the growth and development of the host. A preliminary microarray investigation using Arabidopsis full genome chips identified several auxin responsive genes whose expression increased more than 3-fold in infected Arabidopsis.

The results presented in chapter 4 confirm that for three selected genes at least, CaMV infection of wild-type Arabidopsis (Col-0 and WS) stimulates increased transcript levels concomitant with virus accumulation within the plants. All of the genotypes examined showed this virus induced up-regulation in gene expression. However in the *aux1-100* mutant alleles and for the *tir3-1* plants this up-regulation was reduced although not abolished. *aux1-100* demonstrated a significant decrease in this virus-induced up-regulation for all three of the genes examined whereas *tir3-1*, showed a significant reduction in virus induced up-regulation for the *SAUR_e* gene only.

The developmental expression of *LAX2*, an auxin-responsive *AUX1* homologue (Bennett, unpublished), was also examined. In reporter plants where GUS expression was driven by a *LAX2* promoter, virus infection reduced the developmentally regulated expression of the *LAX2* gene. One interpretation of

these results is that auxin levels or activity are reduced in response to infection. However *LAX2* expression is regulated developmentally as well as in response to auxin levels, so the results observed might be due to a combination of factors rather than to direct changes in auxin levels or distribution within the plants.

These results point to an involvement of auxin transport or signalling in the host response to infection. However, whether this is as a direct consequence of alterations in auxin levels is not clear, and as discussed below, direct measurement of auxin levels in infected plants did not reveal any obvious virus-dependent changes.

Effect of CaMV infection on free IAA levels (6.1.2.2)

Previous attempts to ascertain the effect of virus infection on auxin levels have relied on techniques which were only semi-quantitative and therefore results were often inconsistent and contradictory. For example, Smith *et al.*, (1968) found reduced auxin activity in bean, sugarbeet and tomato plants when infected with Beet curly top geminivirus (BCTV). A bioassay was used and sap from infected plants was examined and its ability to cause phototropic bending, an auxin regulated process was assessed. However, substantial increases in auxin activity have sometimes been reported in infected plants showing severe symptoms (Jameson *et al.*, 2002). In both TSW- and TMV- infected tomato plants, Jones (1956) found auxin activities were higher than in healthy control plants, although the former were visibly stunted (Fraser and Whenham, 1982).

One difficulty in interpreting the results of these studies is in differentiating between primary and secondary changes in hormone concentrations, in particular because of the extreme severity of symptoms caused by many viruses (Jameson and Clarke, 2002). Also, the symptoms and host responses associated with disease differ greatly depending on the virus, host plant and environmental conditions. Therefore the contradictory results gathered previously may well be due to a combination of different experimental systems and differences in the techniques employed.

In the work described in chapter 4, levels of free IAA in infected and uninfected plants were assayed directly using GC-MS. Auxin levels and distribution were also assayed indirectly using DR5::GUS reporter lines. Neither of these approaches revealed any significant changes in auxin levels as a result of virus infection, and the DR5::GUS reporter lines failed to show any changes in auxin distribution. It is possible that virus infection may induce subtle changes in IAA levels or distribution within infected plants that were not picked up by this study. However a second possibility is that the changes in expression of auxin responsive genes in infected plants do not occur as a consequence of alterations in auxin levels or distribution, but are indicative of disruption of the signalling pathways *downstream* of auxin.

P6 Interacts With Auxin Signalling and Transport Pathways (6.1.3)

If virus infection is interacting directly with downstream components of the auxin signal transduction pathways, then how might this be achieved? One

potential interactor encoded by the CaMV genome is P6, which has been shown to be a symptom determinant as well as a host range and pathogenicity determinant (Rothnie *et al.*, 1994; Bonneville *et al.*, 1989; Scholthof *et al.*, 1992). The direct involvement of P6 in symptom development has been demonstrated by the expression of P6 from a transgene, which in the absence of infection leads to the development of symptom-like leaf mosaics, chlorosis and mottling (Baughman *et al.*, 1988; Goldberg *et al.*, 1991; Cecchini *et al.*, 1997). P6 has previously been shown to alter responses to the plant hormones ethylene (Geri *et al.*, 2004) and SA. The work reported in this thesis demonstrates that P6 also affects auxin-signalling. P6 transgenics are able to tolerate high concentrations of the auxin efflux inhibitor TIBA and compared to wild-type non-transgenic plants contain greatly elevated levels of transcripts for the auxin responsive gene, *SAUR_e*. The function of *SAUR_e* is still unknown but it encodes a calmodulin binding protein and it has similarities to ARG7 an auxin induced gene.

Interestingly, in the presence of the P6 suppressor mutation, *cse* plants containing the P6 transgene from line A7, reverted to wild-type sensitivity to TIBA. In contrast, the presence of the *cse* allele does not suppress the P6-dependent ethylene and SA-insensitivity phenotypes (Geri *et al.*, 2004, Love *et al.*, in preparation). In the absence of the P6 transgene, plants homozygous for the *cse-1* and *cse-2* mutant alleles show a leaf distortion phenotype (Geri *et al.* 2004) reminiscent of two other mutants with lesions in auxin efflux, *mdr1* and *phot1 phot2* (Noh *et al.*, 2001; Sakamoto and Briggs, 2002), a weak ethylene-

insensitive phenotype (Geri *et al.*, 2004), but apparently wild-type responses to SA (Love *et al.*, in preparation). These observations suggest that the interactions between P6, its suppressor locus, and components of hormone response signalling pathways are complex and not necessarily equivalent.

The preliminary genetic analysis, carried out to identify the components of the auxin signalling pathway involved in the interaction with P6, found that *tir3-1* plants when crossed with A7 transgenic *Arabidopsis* produced F2 progeny with an aberrant segregation ratio. It would appear from these results that the *tir3-1* mutation may, like *cse*, be able to suppress the P6-associated chlorosis and stunting phenotype. *TIR3* is required for correct localization of the auxin efflux carrier *PIN1* and may also play a more general role in vesicle trafficking. The possibility that P6 might interact directly with *TIR3*, affecting trafficking and other auxin related responses is intriguing although at this stage still entirely speculative.

Conclusions (6.2)

These results highlight the inconsistency faced by researchers investigating the auxin response and help to explain why the work to date has been inconclusive as to the role of auxin in the infection process. CaMV-infected *Arabidopsis* plants show some of the responses normally attributable to changes in auxin levels or distribution; however it appears that these responses may reflect interactions between virus components and components of auxin-signalling pathways as opposed to alterations in auxin levels *per se*.

The *TIR3* locus appears to be important for virus infection. Indeed, the effects observed in response to CaMV infection might even be a consequence of a direct interaction between *TIR3* and CaMV P6. There is good evidence that auxin interacts or cross-talks with many of the other plant hormones (Swarup et al., 2001). This cross talk allows the plant to fine-tune its signalling so that a variety of specific responses can occur to a wide range of factors. It is possible that the role of *TIR3* in vesicle trafficking might not only affect auxin responses but also other hormone related responses triggered during infection. A role for ethylene during CaMV infection of *Arabidopsis* has already been identified (Geri *et al.*, 2004; Love *et al* 2005) and so an involvement for auxin indirectly in these responses cannot be ruled out.

Future Work (6.3)

In order to explain fully how auxin signalling interacts with CAMV during infection a number of additional studies are required. Firstly to establish whether the auxin responses that occur are due to alterations in auxin levels or distribution, auxin levels need to be examined further. Studies looking at differences in auxin levels between tissue types and even different cells along with more sensitive techniques which could pick up even subtle changes in auxin levels would be needed. The work carried out so far suggests that *TIR3* is important for infection with CaMV but it is still in doubt whether this is due to its role in auxin efflux as part of the carrier complex or due to its possible role in

vesicle trafficking. It would therefore be interesting to examine the responses of mutants with defects in the other components of the efflux carrier, for example with defects is *PIN*. The interaction between P6 and auxin also needs to be looked at in greater detail. The genetic analysis described was only preliminary and so would need to be repeated to confirm the interaction with *TIR3* and to help to clarify whether P6 directly interacts with the *TIR3* component of the auxin signalling pathway and that the alterations in auxin response are as a consequence of this. If P6 requires *TIR3* for trafficking it is possible that this component is also involved in the ethylene and SA response observed during infection. Investigation into the the role of *TIR3* in infection independent from the auxin response could offer insight in to the infection process.

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Appendices

Appendix 7.0

Disc 7.0 Statistics

Virus Accumulation (7.1)

7.1.1 Col-0, *aux1-7* and *aux1-22*

7.1.2 Ws and *aux1-100*

7.1.3 *tir* mutants and Col-0

Auxin Responsive Gene Expression (7.2)

7.2.1 Col-0, *aux1-7* and *aux1-22*

7.2.2 Ws and *aux1-100*

7.2.3 *tir* mutants and Col-0

Genetic Analysis (7.3)

7.3.1 *tir3-1* and Col-0 x A7

