Host Responses to Infection by Cauliflower Mosaic Virus

By

Andrew J Love

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"Something should remind us once more that the great things in this universe are the things that we never see."

Dr. Martin Luther King Jr.

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.

Andrew J Love

October 2002

Dedication

To my father, mother and sister.

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Abbreviations

ADP : Adenosine diphosphate

AlMV : *Alfalfa mosaic virus*

AOS : Allene oxide synthase

AOX : Alternative oxidase

ATP : Adenosine triphosphate

BPMV : *Bean pod mottle virus*

CaMV : *Cauliflower mosaic virus*

CC : Companion cell

CGMMV : *Cucumber green mottle mosaic virus*

CIRV : Carnation Italian ringspot virus

CMV : *Cucumber mosaic virus*

CP : Coat protein

CPMV : *Cowpea mosaic virus*

DAB : 3, 3'-diaminobenzidine

dCTP : Deoxycytosine 5'-triphosphate

DD-PCR : Differential Display PCR

dpi : Days post inoculation

EDTA : Ethylenediaminetetra-acetic acid

G6PDH : Glucose 6 phosphate dehydrogenase

GFP : Green Fluorescent Protein

GLM : General Linear Model

GST : Glutathione-S-transferase

GUS : β-glucuronidase

HR : Hypersensitive Response

HXK : Hexokinase

IAA : Indol-3-acetic acid

ISR : Induced systemic resistance

JA : Jasmonic acid

LTP : Lipid Transfer Protein

NADP : Nicotinamide adenine dinucleoside phosphate

NADPH : Nicotinamide adenine dinucleoside phosphate (reduced)

NADP-ME : NADP+ dependent malic acid enzyme

OSRMV : *Oilseed rape mosaic virus*

PCR : Polymerase Chain Reaction

PD : Plasmodesmata

PGI : Phosphoglucose isomerase

PLRV : *Potato leaf roll virus*

PR : Pathogenesis related protein

PTGS : Post Transcriptional Gene Silencing

PVX : *Potato virus X*

PVY : *Potato virus Y*

RBCS : Ribulose bisphosphate carboxylase/oxygenase small subunit

RCMV-O : *Red clover mottle virus strain O*

RdRp : RNA dependent RNA polymerase

ROI : Reactive oxygen intermediates

SA : Salicylic acid

SAR : Systemic Acquired Resistance

SC DNA : Super coiled DNA

SE : Sieve element

SEL : Size Exclusion Limit

SHAM : Salicylhydroxamic acid

SOD : Superoxide dismutase

SYNV : *Sonchus yellow net virus*

TAV : *Tomato aspermy virus*

TBRV : *Tomato black ring virus*

TEV : *Tobacco etch virus*

TMV : *Tobacco mosaic virus*

TSWV : *Tomato spotted wilt virus*

TYMV : *Turnip yellow mosaic virus*

WClMV : *White clover mosaic virus*

Summary

The role of soluble sugars in the development of symptoms in compatible virus infection was examined in turnip and several *Arabidopsis thaliana* lines infected with *Cauliflower mosaic virus* CaMV.

In turnip (*Brassica campestris* cv. Just Right) infected with four CaMV isolates that induced symptoms of differing severity, there were changes in levels of free soluble sugars in infected plants, but the changes showed no direct correlation with either the level of virus replication or symptom severity. This implies that in CaMV-infected turnip, changes in sugar levels probably do not act directly as elicitors of symptom development.

CaMV infection in wildtype and putative *Arabidopsis* sugar signalling mutants had little effect on levels of free soluble sugar during symptom development. Again this observation does not support a role for changes in sugar levels in potentiating the development of symptoms. However on infection, the mutants developed a range of unusual symptom phenotypes and showed differences in level of virus accumulation, when compared with wildtype plants. This suggested a possible link between sugar signalling pathways and symptom development and viral pathogenesis.

Soluble sugars have been reported to act as signalling molecules in defence responses. To test this the expression of three PR protein genes, which are reported to be modulatable by sugars and the SA defence pathway, were analysed in infected *Arabidopsis*. In infected plants, the expression of these genes was greatly elevated, whereas uninfected plants had undetectable levels of expression. Since sugar levels were unaffected during virus infection this

suggests that sugars are probably not involved in the invocation of PR defence genes.

The temporal and spatial activation of three defence pathways was investigated using defence marker gene promoter::Luciferase reporter plants. *Arabidopsis thaliana GST1*::Luciferase was used in the analysis of the oxidative burst, *Arabidopsis thaliana PDF1.2*::Luciferase for the JA/ethylene pathway and tobacco *PR-1*::Luciferase for the SA pathway. Systemic activation of the JA/ethylene pathway and oxidative burst was detectable two hours after virus inoculation, and continued until 5 dpi. In contrast activation of the SA-mediated defence pathway was first detected 8 days after infection, continuing strongly until at least 19 dpi. The timing of the SA pathway activation coincided with a second burst of increased *AtGST1*::Luciferase. However plants stained for H_2O_2, a product of the oxidative burst, demonstrated that the oxidative burst occurred only in the very early stages of virus infection (3.5 hours- 4 days after inoculation). This suggested that the second phase of *GST1*::Luciferase activity is perhaps regulated by other pathways, in the absence of the oxidative burst.

The invocation of defence responses during compatible interactions has often been overlooked since the pathogen appears to replicate and move freely in the plant. These results provide strong evidence for a co-ordinated activation of defence pathways in response to infection by a compatible virus pathogen.

Chapter 1

Introduction (1.1)

When plants are infected with a virus, they often develop symptoms such as vein clearing, stunting, leaf malformation and chlorosis. These symptoms are common to many groups of unrelated plant viruses. Generally the appearance of symptoms is associated with the spread of virus systemically from the site of infection to more distal areas of the plant. Infections in which viruses are able to replicate and move systemically have been called compatible infections. In contrast, particular virus- host combinations may arise which activate plant defence responses, resulting in the localisation of the invading pathogen and prevention of systemic spread. These interactions have been classified as incompatible infections (Hunt and Ryals, 1996; Bol *et al.,* 1990; Bol *et al.,* 1996). Viruses cannot be designated compatible or incompatible, it is only the plant-virus interactions that can be labelled in such a fashion.

The leaf distortions, chlorosis and stunting associated with compatible plant virus interactions can lead to a reduced yield and quality, resulting in crop loss. These crop losses are particularly prevalent in tropical areas of the world. For example, rice is afflicted by the rice tungro bacilliform-spherical virus complex, which every year produces losses of around $1.5 billion in SE Asia (Hull, 2002). In an effort to stem the proliferation of compatible plant virus diseases, various approaches have been taken. These have focused on the breeding of resistant or immune cultivars by classic genetic procedures (Valleau, 1946; Duffus, 1987), the control of vectors, the production of seed (Kimble *et al.,* 1975; Carroll, 1983) and vegetative propagates (Walkey, 1985; Walkey *et al.,* 1985; Hansen *et al.,* 1985;

Toyoda *et al.*, 1985) that are free of virus and the production of transgenic plants carrying viral genes that confer resistance to specific viruses (Lomonossoff, 1995; Prins and Goldbach, 1996; Palukaitis and Zaitlin, 1997). These measures have had some success in reducing crop losses due to virus disease. However, a continued and sustained effort is required to prevent these losses from becoming more and more extensive. Natural resistance genes against some viruses, such as PLRV (*Potato leaf roll virus*) resistance in Solanaceous species (Barker and Waterhouse, 1999) and *Potato virus X* (PVX) resistance in potato (Bendahmane *et al.*, 1997; Bendahmane *et al.*, 1999) have been found. However, for many viruses suitable resistance genes have not been discovered and there is great variability in the protection offered by these genes. For example, the success of *RX1*-mediated resistance against *Potato virus X* is isolate dependent (Bendahmane *et al.*, 1995). Thus, protection can be limited to just one viral strain or only several viruses. Therefore an integrated and multidisciplinary approach is needed not only to control virus transmission but also to understand the mechanism by which symptoms arise, allowing the development of appropriate methods for symptom amelioration or design of resistance strategies.

Virus movement (1.2)

Systemic spread of virus involves firstly cell-cell movement from the initial point of infection and secondly long-distance movement via the vascular system. For most plant viruses, cell-cell movement is facilitated by the action of a movement protein. Movement proteins are generally divided into two overlapping classes, one which facilitates the creation of mobile viral nucleoprotein complexes, and the other

which allows the virus to pass into neighbouring cells by increasing the size exclusion limit (SEL) of the plasmodesmata.

One class of movement protein has several nucleic acid binding domains, specific for viral nucleic acids. Subsequently nucleoprotein complexes can pass through the plasmodesmata into neighbouring cells (Fujiwara *et al.*, 1993). Viruses such as tobamoviruses (Citovsky *et al.*, 1990), potexviruses (Lough *et al.*, 2000) and possibly caulimoviruses (Thomas and Maule, 1995) use this strategy.

Cytological studies have revealed that some plant virus movement proteins (MP) can promote the formation of tubular structures that traverse cell walls by modifying plasmodesmata. This property of the movement protein is common to many virus families such as comoviruses (Van Lent *et al.*, 1990), nepoviruses (Wieczorek *et al.*, 1993), tospoviruses (Storms *et al.*, 1998) and caulimoviruses (Perbal *et al.*, 1993). It is thought that the MP induced tubule formation enables the movement of virus particles through the modified "propped open" plasmodesmata. These MP-induced tubules are composed of microtubules and actin filaments, the major components of the cellular cytoskeleton. In normal uninfected cells the cytoskeleton dynamically grows and shrinks, however in cells that express the *Cauliflower mosaic virus* (CaMV) MP neither the formation nor the maintenance of tubules requires the normal assembly/disassembly of the cytoskeleton (Huang *et al.*, 2000). This suggests that the CaMV MP can stabilise the cytoskeleton, making a rigid and defined framework, which would be more effective for virus transport than if the cytoskeleton were allowed to change its size and position via assembly/disassembly. Although similar strategies are used by many virus families,

some make no use of the host cytoskeleton to establish tubules. For example, during *Cowpea mosaic virus* (CPMV) infection of cowpea, desmotubules in the plasmodesmata are replaced with virion containing tubules (Kasteel, 1999). However these tubules are thought to be composed entirely of the 48 kDa moiety of the CPMV MP, there being no evidence for host protein involvement (Kasteel, 1999). It is thought that this modification could increase the SEL of the plasmodesmata.

After viral MPs facilitate cell-cell spread, viruses move through the vascular system to distal parts of the plant. Generally speaking, different virus types tend to follow prescribed pathways and preferential routes that are common to many viruses (Roberts *et al.,* 1997; Ding *et al.,* 1998). Transport of viruses occurs symplastically via the phloem and is facilitated by the bulk flow of photoassimilates through minor and major leaf veins in source leaves to developing sink tissues (Oparka *et al.,* 2000; Leisner *et al.,* 1993).

Recently it has been demonstrated using immunocytochemistry and Green Fluorescent Protein (GFP) tagged *Cucumber mosaic virus* (CMV) movement proteins, that MPs are able to enter minor vein sieve elements (SE) from infected companion cells (CC). In other studies involving tobamovirus and potyvirus infections in the Solanaceae and Fabaceae, virus accumulation in minor veins was shown to spread from phloem parenchyma cells that abut the sieve elements (Ding *et al.,* 1998). Thus virus could enter the SE via CC or cells closely neighbouring the SE in minor leaf veins. Studies using GFP tagged *Tobacco mosaic virus* (TMV) have confirmed this, and shown that minor and major vein classes in source leaves can

equally support viral long-distance movement (Cheng *et al.*, 2000). However, work by Leisner *et al.* (1992) has shown that CaMV can utilise minor veins more frequently than major veins.

Although the initial stages of cell-cell movement prior to long distance movement may involve nucleic acid complexes and/or intact virions (Blackman *et al.*, 1998; Ding *et al.*, 1997; Gilbertson *et al.*, 1996; Nelson and van Bel, 1998; Ryabov *et al.*, 1998; Santa Cruz, 1999), encapsidated viruses appear to be the functional long-distance movement complex (Blackman *et al.*, 1998; Gilbertson *et al.*, 1996; Nelson and van Bel., 1998; Simon- Buelo *et al.*, 1999; Vaewhongs *et al.*, 1995). For some viruses it has been reported that mutations or deletions in the coat protein can abolish functional long distance movement. This implies that encapsidation of the viral nucleic acid by the coat protein is required for long-distance movement. For example, although TMV can move cell-cell without its CP, the CP is thought to be required for the long-distance movement, suggesting that TMV phloem movement involves virions (Carrington *et al.*, 1996, Gilbertson *et al.*, 1996; Nelson *et al.*, 1998). In addition, analysis of sieve tube exudate has confirmed that *Cucumber green mottle mosaic virus* (CGMMV), which is very closely related to TMV, travels in an encapsidated manner (Simon- Buelo *et al.*, 1999).

Some viruses such as *Groundnut rosette virus* do not produce a CP, but can still move long-distance via the phloem. It was found that the protein product of ORF3 of this virus was responsible for facilitating long-distance movement. Since this protein product can enable CP deficient TMV to move long-distance, this

suggests that encapsidation is not an absolute requirement for long-distance movement (Ryabov *et al.*, 1999).

Although it is likely that a large number of viruses move in an encapsidated form, it has been discovered that the location of encapsidation can vary between viruses. For example, in CMV infected *Nicotiana clevlandii*, virions have not been detected in mesophyll plasmodesmata nor in the plasmodesmata that connect the SE and CC (Blackman *et al.*, 1998). However, CMV was found to assemble at the parietal layer of the SE, prior to long distance movement (Blackman *et al.*, 1998). In contrast, other spherical viruses have been detected in an encapsidated form in plasmodesmata joining the SE to the CC (Murant *et al.*, 1979), suggesting considerable plasticity of plasmodesmata in accomodating viral trafficking.

Some viruses actively facilitate their exit into sink tissues, although the mechanism of exit from the SE-CC complex remains unknown. Recent work has tried to identify regions of viral deposition and replication in the sink tissues, so that future research can focus on viral exit mechanisms. Cheng *et al.* (2000) have suggested that developmental transition in sink tissues produces increased viral accumulation in the older base of the sink leaf and less in the younger apical parts. They also showed that major veins in sink leaves or in the sink region of transition leaves allowed successful virus amplification in phloem associated cells. There are several ways in which viruses can move from the SE-CC complex and invade sink tissues. Since most viruses move in an encapsidated form it is possible that viral disassembly occurs within the SE, prior to movement of the viral nucleic acid into the sink CC. For this to happen, it is likely that viral movement proteins may be

required. A second possibility is that the SEL of sink tissue plasmodemata linking SE and CC, in common with the majority of plasmodesmata (Fisher *et al.*, 2000; Oparka *et al.*, 1999; Wang *et al.*, 1994), is considerably larger than in source tissues, allowing intact virions to pass freely between SE and CC. In the CC, disassembly could occur followed by re-initiation of the viral replication and movement cycles. It was later shown that in potato infected with *Potato virus X* (PVX), the large SEL of plasmodesmata in sink tissues was not enough to permit successful movement of the virus. It was found that the viral MP was needed to modify the plasmodesmata in sink tissues still further for virus infection to progress (Oparka *et al.*, 1999; Santa Cruz, 1999). The MP may be exported from infected companion cells in source tissues and exit into the post phloem pathway of sink tissues. Such a strategy could increase the SEL of plasmodesmata in sink tissues and prime the sink tissue for the subsequent export and transport of virus. In contrast viruses such as the luteovirus produce phloem-limited infections (Taliansky and Barker, 1999), whereby virions are unable to ingress into sink tissue from the phloem, but are able to replicate in phloem-associated cells. In phloem limited viruses, restriction of virus movement into sinks usually occurs outside the SE-CC complex (Ding *et al.*, 1998, Nelson *et al.*, 1998; Sudarshana *et al.*, 1998), which suggests that the limiting steps in tissue invasion lie in cell to cell post-phloem movement rather than the ability to escape the SE-CC complex.

Once sink leaves mature into source leaves they will become reservoirs of virus, initiating infection of emergent sink tissues. During systemic infection, very mature source leaves can avoid virus ingression. Leisner *et al.* (1993) has suggested

that in CaMV infection of *Arabidopsis* this may be due to the very low probability of virus particles managing to move counter-stream to the bulk flow of photoassimilates (which move from sources to sinks). Also Maule *et al.* (1989) directly inoculated mature turnip leaves and found that they could support CaMV replication but that the virus movement was restricted, perhaps because the plasmodesmata had reached a stage in development which made them more resistant to modification by MP. This supports the idea that successful phloem movement and cell- cell movement are both required for successful long distance movement. These viral movement pathways are continually changing in the plant since development produces transitions from sinks to source, resulting in alterations in photoassimilate bulk flow and plasmodesmatal structure.

Throughout the process of systemic compatible virus movement there is the progressive development of symptoms. The plant skeleton hybridisation technique, in which *Arabidopsis* plant skeletons were probed with CaMV viral DNA, has not only confirmed the viral movement via the phloem but it has also shown that patterns of symptoms closely corresponds to the pattern of hybridisation (Leisner *et al.*, 1992). The skeleton hybridisation of early flowering ecotypes has shown that speed of growth and alteration of photoassimilate partitioning directly relates to the efficiency of virus movement throughout the plant (Leisner *et al.*, 1992). Therefore it seems quite apparent that the rate of plant development is closely matched to the kinetics of CaMV movement and the pattern of symptom development (Leisner *et al.*, 1993).

Symptoms (1.3)

The first symptoms to develop are often local lesions. These develop following cell-to-cell spread, and virus multiplication in a relatively few cells around the site of inoculation. In compatible infections the lesions are often of a chlorotic nature, although with some host-virus interactions the lesions may accumulate more chlorophyll than the surrounding tissue (Hull, 2002). Lesions may resemble pinpoint patches, as seen in potyvirus infected *Chenopodium* (Fry, 1954), or may be chlorotic spots surrounded by concentric rings of chlorosis (Hull, 2002). These are the primary symptoms of virus infection and typically affect the infected leaf. With time, the virus spreads from the infected leaf into sink tissues and the stem apex. Virions located close to the stem apex are able to move into primordial leaf cells that emerge from the apex and develop into systemically infected leaves carrying virus in most of their cells (Matthews, 1991). By using fluorescent antibody techniques and electron microscopy, this has been shown to occur with CaMV-turnip interactions (D.S. Turner and S. N. Covey unpublished observations). However, most viruses are unable to invade the apical meristem (Mori *et al.,* 1982). Systemic movement through the plant results in plant-wide symptoms that are economically damaging.

A common symptom of systemic virus infection is reduced plant size, and this can be seen even in latent infections, where more overt symptoms are absent. Stunting can affect all parts of the plant and can involve a reduction in the size of leaves, flowers, fruits, roots and a shortening of petioles and internodes (Hull, 2002; Kimura *et al.,* 1987). Some virus infections may affect some parts of the plant more

than others, for example little cherry disease only affects the size of the fruits (Hull, 2002). Stunting can also lower the size and number of fruits that crop plants can bear (Hampton, 1975; Way *et al.*, 1963), and is therefore economically an important aspect of virus disease. Virus infection may in some instances delay the initiation of growth in spring (Gilmer *et al.*, 1970) and prevent normal germination of seed harvested from infected plants (Walkey *et al.*, 1985).

Developmental abnormalities may arise, often in combination with stunting. For example there is a suppression of the leaf blade in the leaves of CMV and TMV infected tomatoes (Francki *et al.*, 1980). In other diseases the leaf blade may be rolled or curled upwards or downwards, and in some cases there may be raised blisters or crinkling producing further deformation (Hull, 2002). The later production of distorted fruits and flowers can be attributed to this uneven growth and development, although this might not occur for all virus diseases (Hull, 2002).

In addition to stunting and malformation, one of the most noticeable systemic symptoms is the development of altered patterns of colouration throughout the whole plant. In many cases the differences in colouration manifest themselves as mosaics on the leaves, whereby cellular patches of varying levels of chlorosis and dark greening juxtapose one another. In TMV infections in tobacco the mosaics are formed by patches of only two shades, whereas multiple shades are present in the *Turnip yellow mosaic virus* (TYMV)- Chinese cabbage (*Brassica campestris* ssp. *pekinensis* group var. *cephalata*) interaction (Hull, 2002). In some interactions the colouration between patches is so indistinct that mosaics are not visible. Variation in mosaics is also dependent on leaf age. In more mature leaves the mosaic pattern can

be absent, possibly reflecting limitation of virus spread (Hull, 2002). Older leaves with mosaics have a large number of small patches, in contrast to younger leaves that have fewer but larger mosaics. Primarily the mosaic pattern is laid down at a very early stage of leaf development and may remain unchanged, except for general enlargement (Hull, 2002). Colouration symptoms may be preceded by a yellowing of the leaf veins, which may subsequently become more severe to produce a vein banding effect, a major feature of some viral infections.

In the latter stages of infection stunted and/or malformed flowers and fruits may develop. The mosaic pattern seen on the systemically infected plant may also be present on the flowers, as streaks or sectors of tissue with a different colour from normal. Depending on the virus and host, the colour differences may be due to hyper or a hypo pigmentation. Such differences can be seen in potyvirus-Stock (*Mathiola bicornis*) and CMV-Gladiolus (*Gladiolus capitatus*) interactions (Hull, 2002). Fruits formed on plants showing mosaics on the leaves may display mottling and rings, although stunting and deformations may be absent on the fruits.

Some plant-virus host interactions result in the production of yellowing but not mosaics, which may be localised to just a few regions of the leaves, as in the case of Strawberry yellow edge disease (Hull, 2002), or more widespread throughout the plant (Weideman *et al.*, 1975).

Symptoms have been associated with a programmed expression of host genes (Cecchini *et al.*, 1998; Wang *et al.*, 1995). In such a co-ordinated process, virus and host genetic backgrounds determine the outcome of symptom development (Cecchini *et al.*, 1998). Additional research has suggested that this genetic

programmed response is more important for symptom development than the metabolic burden imposed on the host by viral replication (Cecchini *et al.,* 1998). However metabolic perturbations do occur during virus infection (Hull, 2002), which are probably dependent on the programmed response enabled by host plant and viral genetic make up. It is reasonable to assume that during compatible infection, the programmed gene expression and changes in metabolism are linked. Such complex interactions which may lead to compatible symptoms, will be discussed in more detail below.

Virus accumulation and symptoms (1.4)

The diversion of metabolites into virus production might induce disease symptoms by making host cells deficient in some respect. It might be expected that the severity of symptoms correlate with the level of virus accumulation. For example *Bean pod mottle virus* (BPMV) infections have fluctuating viral replication states and corresponding increases and reductions in symptoms (Gillaspie and Bancroft, 1965). In 1986 Fraser *et al.* showed that for 26 TMV isolates in tobacco there was a correlation between the severity of symptoms and the level of virus accumulation. In contrast, tomato-TMV interactions were also examined and it was found that no correlation between levels of virus accumulation and symptom severity existed (Fraser *et al.,* 1986). In other cases, related strains of the same virus may multiply to similar levels in a particular host, yet cause marked differences in symptom severity. For example the CaMV Braunsweig isolate causes very severe symptoms in *Arabidopsis* Col gl1, often leading to death, but very mild symptoms in turnip

(Cecchini *et al.*, 1998). In comparison the Yug-3 isolate produces no symptoms in *Arabidopsis* Col gl1, but severe symptoms in turnip (*Brassica campestris*; Cecchini *et al.*, 1998). The same virus isolate may produce different symptoms in different hosts, despite the fact that viral accumulations are the same. For example CaMV Bari-1 virus levels were similar between *Arabidopsis* Col gl1 and Ler gl1 ecotypes, although Ler gl1 developed normal symptoms whereas Col gl1 was asymptomatic (Cecchini *et al.*, 1998). Similar findings were reported for *Oilseed rape mosaic virus* (OSRMV) infections in 116 *Arabidopsis* ecotypes (Martin *et al.*, 1997). The genetic background of the virus and host seem to be more important than the level of virus replication in determining the symptom severity. These observations gave rise to the concept of a programmed response (Cecchini *et al.*, 1998). In particular host–virus combinations the levels of accumulation are similar in symptomatic and asymptomatic plants, suggesting that metabolic drain is not a likely primary cause of symptoms. It has also been reported that the amount of virus formed may be quite small relative to the reduction in macromolecules (such as amino acids, chlorophyll and host proteins; Crosbie and Matthews, 1974b; Hull, 2002), which suggests that in some cases tertiary effects on the metabolism are more significant for symptom induction than the direct drainage of resources to support virus replication.

Virus induced changes in photosynthesis and carbohydrate metabolism (1.5)

Symptoms may lead to or be caused by temporal and spatial perturbations in cellular biochemistry and physiology at the whole plant level. Attempts have been

made to characterise metabolic perturbations to see if an association could be correlated with symptom development, but these have not been conclusive. Recently there has been a return to the problem using the more established tools of biochemistry and molecular biology.

Reduced photosynthesis rates have been reported during virus infection (Christor *et al.*, 2001; Funayama *et al.*, 1997; Rahoutei *et al.*, 2000). In contrast, other viral infections have been shown to have no effect, or even stimulate the rate of photosynthesis (Magyarosy *et al.*, 1973; Smith and Neales, 1977). However, these results appear to be dependent on the virus type (Mayarosy *et al.*, 1973), host and protocols (Bedbrook and Matthews, 1973). Many virus groups have been implicated in reducing photosynthesis rates, such as tobamoviruses (Balachandran *et al.*, 1997; Rahoutei *et al.*, 2000), luteoviruses (Jensen, 1972; Malmstrom and Field, 1997), closteroviruses (Clover *et al.*, 1999; Hall and Loomis, 1972; Swiech *et al.*, 2001) and cucomoviruses (Hunter and Peat, 2001; Tesci *et al.*, 1996). Photosynthesis rates can be affected by a reduction in pigmentation and changes in the chloroplast function, which has been seen in regions where the virus multiplies (Naidu *et al.*, 1986). Ultrastructural and morphological differences have been detected in the chloroplasts of virus infected plants when compared with uninfected plants (Hull, 2002). It has been reported that *Sonchus yellow net virus* (SYNV) and *Eggplant mottled dwarf virus* infections in various tobacco hosts induce structural changes in the chloroplast (Ismail *et al.*, 1987) and lead to reductions in the amount of thylakoid membrane (Askeer *et al.*, 1993).

The effect of virus infection on photosynthesis provides further support for the concept of a programmed response, in which virus and host participate. For example, SYNV infection of *Nicotiana glutinosa* and *Nicotiana edwardsoni*, which are closely related, resulted in a lower photosynthetic capacity (Askeer *et al.*, in press). However in *N. glutinosa*, lowered photosynthesis rates were attributed to a loss of reaction centres and light harvesting complexes. In contrast infection of *N. edwardsoni* did not lower gross photosynthesis rates, but the reduced photosynthetic capacity arose from the reduction of chlorophyll a/b binding light harvesting complexes.

During virus infection, chlorophyll loss (Funayama-Noguchi, 2001; Osmond *et al.*, 1998; Tecsi *et al.*, 1996), and biochemical changes in the chloroplast are primarily involved in lowering rates of photosynthesis. Leaf crinkling might also affect the rate of photosynthesis due to reduced stomatal opening (Hall and Loomis, 1972).

Stunting, crinkling and pigment loss are not uniform throughout the virus infected plant. This implies that some areas of the infected plant are more able to fix carbon and distribute photoassimilate than others. This might lead to perturbations with regard to sugar production and carbohydrate partitioning. It has been reported that starch accumulates in the leaves of virus infected plants (Lindner *et al.*, 1959; Israel and Ross, 1967; Cohen and Loebentein., 1975; Roberts *et al.*, 1982; Tomenius *et al.*, 1982), and this may deprive growing parts of the plant of some newly fixed carbon (Holmes *et al.*, 1984), leading to stunting. In *Red clover mottle virus strain O* (RCMV-O)-infected pea plants, atypical large starch grains accumulate in the

chloroplast (Tomenius and Oxelfelt, 1982). Although RCMV-O infects plants systemically, symptoms include necrosis at the actively growing apex. This necrosis effectively removes the sink, allowing sugar to accumulate and maybe responsible for the subsequent increase in starch levels (Tecsi *et al.,* 1992). In other cases, such as CMV infection of marrow plants, soluble sugars accumulate, while levels of starch decrease (Tecsi *et al.,* 1994). Here, CMV infection increased starch hydrolase and lowered ADP-glucose pyrophosphorylase activities. This might be expected to increase the conversion of starches into soluble sugars, such as glucose, fructose and sucrose. The inhibition of starch accumulation and/or starch degradation is probably a consequence of the increased demand for soluble sugars, required to maintain the respiration rate and support viral replication. Interestingly photosynthesis rates were not significantly affected in this case.

Sugar signalling and compatible plant-virus interactions (1.6)

Sugars constitute the primary respiratory substrate for most cells. Organisms must therefore, be able to sense their presence and respond appropriately. The yeast, *Saccharomyces cerevisiae*, has been the primary model system used to explore how sugar is sensed and what responses are activated after sensing. It has been reported that in yeast, sugars can affect enzyme levels by altering mRNA translation rates (Vallari *et al.,* 1992), transcript stability (Federoff *et al.,* 1983; Lombardo *et al.,* 1992) or the level of protein degradation (Holzer, 1976). It has been shown that such events are initiated after a signal(s) produced from hexose phosphorylation leads to the activation of transcription factors (Trumbly, 1992; Gancedo, 1992; Ronne, 1995;

Zimmermann and Scheel, 1977). These transcription factors are able to modulate the expression of various genes in a manner that is dependent on the amount of sugar, and its rate of phosphorylation (Lutifiyya and Johnston, 1996; De Vit *et al.,* 1997).

In higher plants it has also been reported that sugars may affect the expression of genes encoding many enzymes. It is thought that in plants, sugar signalling mechanisms similar to those in yeast are responsible for these changes. Research using non-metabolizable sugar analogues (Graham *et al.,* 1994; Jang and Sheen, 1994; Prata *et al.,* 1997) and the discovery of yeast sugar signalling homologues in higher plants (Alderson *et al.,* 1991; Halford and Hardie, 1998; Le Guen *et al.,* 1992; Park *et al.,* 1993) strongly supports this suggestion. Also, the complementation of yeast mutated in sugar metabolism by plant homologues has suggested that these genes perform similar sugar signalling functions in plants as they do in yeast (Alderson *et al.,* 1991; Muranaka *et al.,* 1994).

In both yeast and higher plants it has been shown that a large number of sugar modulated enzymes are involved in regulating sugar metabolism and partitioning (Pego, 2000). Therefore, sugars can actively influence their own production, metabolism and storage. In higher plants, sugars move from areas of high concentration (fully expanded and photosynthesising leaves) to regions of deficit (actively growing tissues; Pego, 2000). In tissues that are a source of sugars, excess hexoses may induce the expression of sucrose synthase, and at the same time repress the expression of invertases (Koch *et al.,* 1992; Koch and Nolte, 1995). Increased levels of sucrose synthase lead to excess glucose and fructose being converted into sucrose. Sucrose, which is the main form in which sugars are

transported, moves into the phloem and is translocated to regions in the plant that are deficient in sugars (sinks for photosynthate). In source tissues the repression of invertase by hexoses is critical, since cleavage of sucrose into less mobile forms would significantly reduce the export capabilities (Von Schaewen *et al.*, 1990). In sink tissues the deficit in hexoses such as glucose fails to repress invertase expression, which results in increased cleavage of sucrose into glucose and fructose. Glucose and fructose can subsequently be utilised for growth.

In plants, the partitioning of sugars between sinks and sources is constantly changing as a result of developmental and/or environmental influences (Pego, 2000). These changes may result in sugar partitioning perturbations, which may manifest themselves as unusual sugar deficits or surpluses. It is thought that in the plant these perturbations are short lived, since sugars can influence the expression of a plethora of genes that can finely tune the supply of sugar to its requirement. In regions of the plant where sugar accumulation outweighs its catabolism and transport, various sugar storage strategies are employed. Elevated sugar levels have been reported to induce many starch-related enzymes such as starch synthase (Visser *et al.*, 1991). This would subsequently result in the conversion of soluble sugars into starch grains. Excess carbon may also be stored as amino acids and proteins, and a number of genes for storage proteins have been shown to be induced by high sugar levels (Sadka *et al.*, 1994; Mason *et al.*, 1992). A fall in sugar levels would result in the mobilisation and utilisation of the stored carbon sources.

In addition to directly influencing sugar metabolism and partitioning, elevated sugar levels may affect upstream events such as sugar production. For

example, surplus sugars are known to inhibit photosynthesis by repressing the expression of many genes, encoding proteins such as chlorophyll a/b binding protein, (Dijkwel *et al.,* 1996), Rubisco small subunit (Cheng *et al.,* 1992) and plastocyanin (Dijkwel *et al.,* 1996). High levels of sugar accumulation may also result in chlorophyll loss (Von Schaewen *et al.,* 1990). In parallel with controlling the rate of sugar storage and production, sugars may also affect other metabolic pathways. It has been reported that sugars may repress the utilisation of other energy reserves such as lipids, whereas low sugar levels encourages mobilisation of lipid reserves (Graham *et al.,* 1994; McLaughlin and Smith, 1994).

The sugar-mediated gene regulation of sugar, protein, lipid metabolism and photosynthetic activity is highly co-ordinated. This enables the plant to respond finely to developmental and extraneous environmental changes. In times of sugar deficit, there will be an increase in photosynthesis and mobilisation of energy reserves, whereas valuable resources need not be committed to these processes if carbohydrate supplies are already sufficient. When sugar is abundant, storage prepares the plant for times of sugar deficiency.

In addition, sugar-mediated regulation of gene expression has been implicated in processes as diverse as wounding, pigmentation and defence (Jonhson and Ryan, 1990; Kim *et al.,* 1991; Tsukaya *et al.,* 1991; Miao and Graynor, 1993; Brugliera *et al.,* 1994; Moalem-Beno *et al.,* 1994; Murray *et al.,* 1994; Takeuchi *et al.,* 1994; Herbers *et al.,* 1995, 1996), flowering (Blazquez *et al.,* 1998), mannitol metabolism (Prata *et al.,* 1997), sugar transport (Hilgarth *et al.,* 1991; Chiou and

Bush, 1998), nutrient uptake (Mito *et al.*, 1996), cell cytoskeleton synthesis (Sheu *et al.*, 1994) and respiration (Hilgrath *et al.*, 1991; Krapp and Stitt, 1994).

It is possible that in infected plants, perturbations in sugar partitioning may affect sugar-regulated gene expression, resulting in alterations of chloroplast protein complexes. In transgenic tobacco plants in which sugars are forced to accumulate in leaves by over-expressing a yeast invertase, a mosaic phenotype and stunting ensue, which are reminiscent of a compatible virus infection (Von Schaewen *et al.*, 1990). Alterations in sugar concentrations are a plausible mechanism for the elicitation of symptoms. Possibly, increases in sugar levels in localised regions of the leaf may lead to chlorosis. Neighbouring cells may produce more green pigments to compensate for the loss in carbon fixation, resulting in mosaics.

Recent studies have focussed on how specific viral components may affect sugar partitioning, photosynthesis and other gene expressions. Expression of TMV MP, which is known to alter the plasmodesmatal size exclusion limit (Wolf *et al.*, 1989) in transgenic tobacco, increased the concentration of sugars. This was attributed to a retarded export (Olesinski *et al.*, 1996). This effect may be independent of plasmodesmatal dilation. Balachandran *et al.* (1995) created transgenic tobacco that expressed a MP that had C-terminal deletions. These did not show plasmodesmatal dilation but sugar partitioning was still affected. Similar experiments were carried out by Herbers *et al.* (1997) on the MP of the *Potato leafroll virus* (PLRV) in tobacco. Transgenic tobacco expressing the MP of PLRV developed stunting, bleached regions and "inflated" plasmodesmata. These plants showed 9-fold increases in sugar levels and 3-fold increases in starch levels.

Analysis of the ratios of starch to sugars showed that the tobacco PLRV movement protein transgenics tended to favour sugar accumulation in place of starch, when compared to wildtype plants. It was anticipated that one effect of increased sugar levels would be to repress the level of photosynthesis-related gene expression, and this seemed to be the case (Herbers *et al.*, 1997). The authors also suggested that the stunting seen in the transgenics and particularly the high increase in accumulation of carbohydrates in source leaves, was due to limited export of sucrose, the main form in which sugars are transported through the plant. Thus, functional plasmodesmata are required for sucrose loading, and viral MP can modify plasmodesmatal sugar translocation processes and give rise to a symptom like phenotype. This suggests that the virus-induced alterations in carbohydrate metabolism might play a significant role in the development of symptoms.

High sugar levels have also been reported to cause an increase in defense related PR-protein transcripts, and hexoses have been shown to play some role in the incompatible response (Herbers *et al.*, 1995; Herbers *et al.*, 1996). For example, in sugar accumulating transgenic tobacco infected with PVY, viral titre and spread were reduced compared to wildtype. This suggests that sugar-induced defence mechanisms may protect against viral invasion (Herbers *et al.*, 1996).

Defence, compatibility and the incompatible response (1.7)

Much of the research carried out on plant diseases has focussed on the mechanisms employed by the plant to set up an incompatible response. Although it

is compatible interactions that result in the development of economically damaging symptoms, these are less well studied.

Incompatible interactions are associated with the activation of an array of defense responses that serve to prevent pathogen replication and/or movement. After the presence of a pathogen has been sensed, an oxidative burst may be rapidly initiated, involving an increase in the level of reactive oxygen intermediates (Baker *et al.*, 1995; Lamb *et al.*, 1997). The oxidative burst is characterised by the production of superoxide radicals, H_2O_2 and hydroxyradicals via successive one-electron reductions of molecular oxygen. High levels of these reactive oxygen intermediates (ROIs) are thought to impede pathogen ingression by promoting cell necrosis, damaging pathogens, increasing lignification and potentiating downstream defence responses. In association with the oxidative burst, crosslinking of cell wall proteins and production of anti-microbial compounds also occurs (Hammond-Kossack *et al.*, 1996; Yang *et al.*, 1997). Later the host plant can induce callus tissue build up and cell death around the initially infected cell, producing visible necrotic lesions that are often referred to as a hypersensitive response (HR) (Hammond-Kossack *et al.*, 1996; Dangl *et al.*, 1996). In parallel with the development of local necrotic lesions, there is a localised increase in the expression of PR proteins (Kombrink *et al.*, 1997). These PR proteins are subsequently expressed in the distal portions of the plant, concurrent with the development of a long lasting, broad-based resistance known as systemic acquired resistance, or SAR (Ryals *et al.*, 1996).

Salicylic acid (SA) is a crucial signalling molecule involved in the development of these local and systemic disease resistances (Dempsey *et al.*, 1999;

Shah *et al.*, 1999). This has been shown by the susceptibility of SA deficient *NahG* transgenic *Arabidopsis* to a variety of pathogens which would not normally induce disease (Gaffney *et al.*, 1993; Delaney *et al.*, 1994). *NahG* is a bacterial gene from *Pseudomonas putida* encoding a salicylate hydroxylase, which converts salicylic acid to catechol. Catechol is not involved in the development of SAR and is thought not to inhibit the growth of pathogens (Gaffney *et al.*, 1993; Delaney *et al.*, 1994).

It has been shown that the oxidative burst can work upstream of SA, and in some cases can induce SA accumulation (Bi *et al.*, 1995; Neuenschwander *et al.*, 1995; Chamnongpol *et al.*, 1995, 1998). Conversly, SA can potentiate the increase of reactive oxygen intermediates (ROI) in the cell (Chen *et al.*, 1995; Conrath *et al.*, 1995; Wendehenne *et al.*, 1998). Clearly the interactions between SA and ROIs are complex and at present relatively undefined. However, it is known that SA is responsible for the up-regulation of PR defence genes. As SA accumulates it activates the NPR1 protein, an interaction that was identified through plant mutant studies. NPR1 bears a limited homology to IκBa and has ankyrin repeats (Cao *et al.*, 1997), which suggests that it is involved in protein-protein interactions (Shah *et al.*, 1997; Cao *et al.*, 1997; Ryals *et al.*, 1997). These protein interactions were analysed using a yeast two-hybrid system (Zhou *et al.*, 2000). It was found that activated NPR1 can interact with a family of bZIP transcription factors which can subsequently bind to PR gene promoter elements (Lebel *et al.*, 1998). The interaction of transcription factors with the ankyrin repeat domain of NPR1 was essential for the induction of SA responsive genes. This suggests that members of

this transcription factor family transduce the SA signal through NPR1 to the activation of SA-inducible defence genes.

Activation of the SA-mediated pathway results in the downstream activation of a whole array of PR protein defence genes, such as *PR-1*, *PR-2* and *PR-5* (Uknes *et al.*, 1992; Gorlach *et al.*, 1996). PR proteins are believed to be primarily anti-fungal and can be found in the extracellular space and in vacuoles (Kitajima *et al.*, 1999). The fourteen SA-inducible PR protein family members have various attributes (although not all are characterised), which will be summarised using PR-1, PR-2 and PR-5 as examples. Members of the PR-1 group are proteins with a molecular weight of about 15kDa, which have potent anti-fungal activity at the micromolar level against a number of plant pathogenic fungi (Niderman *et al.*, 1995). However, the mechanisms of action against fungi are unknown. The anti-fungal activities of the 22kDa PR-5 proteins are also unknown. However, based on amino acid homology studies it has been suggested that PR-5 may have limited glucanase activity (Trudel *et al.*, 1998), could induce alterations in the permeability of fungal cells (Roberts, 1990) or may perturb the regulation of fungal cell wall assembly (Yun *et al.*, 1997; Yun *et al.*, 1998). In contrast the mode action of the ~33kDa PR-2 protein family have been well studied. PR-2 proteins have (1,3)β-glucanase activity (Jach *et al.*, 1995; Stintzi *et al.*, 1993) that can hydrolyze the (1,3)β-glucan present in fungal cell walls.

In recent years it has been demonstrated that the expression of some SA associated PR proteins, such as PR-2 and PR-5 may occur in an SA independent manner (Nawrath *et al.*, 1999; Glazebrook *et al.*, 1996; Rogers and Ausubel, 1997).

In these cases it is thought that both the SA pathway, and other upstream pathways, may regulate the expression of PR-2 and PR-5 (Nawrath *et al.,* 1999). A possible SA independent regulation of these genes could be mediated by soluble sugars. For example, Herbers *et al.* (1996a) have shown that the accumulation of sugars in tobacco leaf discs can result in the SA-independent induction of PR proteins such as PR-2 and PR-1. In contrast, Herbers *et al.* (1996b) later reported that in transgenic tobacco plants over-expressing invertase, the accumulation of sugars in the apoplast produced an SA-dependent increase in the expression of PAR-1, PR-1b and PR-Q. These results suggest that SA-dependent and independent pathways may be modulated by the level of soluble sugars.

In addition to the SA and sugar associated pathways, different defence genes may be activated by another distinct set of pathways that are dependent on both ethylene and jasmonate (Dong, 1998; Pieterse *et al.,* 1999). Jasmonic acid (JA) is a plant wound response regulator, with a role that is now known to include defence and resistance against certain pathogens (Reymond and Farmer, 1998). On the other hand, ethylene has been shown to control disease symptom expression rather than absolute resistance (Thomma *et al.,* 1999; Penninckx *et al.,* 1996). For example, mutations in the ethylene response regulator *EIN2* causes enhanced susceptibility of *Arabidopsis* to the necrotrophic fungus *Botrytis cinerea* (Thomma *et al.,* 1999). Recent research has implicated ethylene in some JA related defence pathways, a role that may be modulated by the type of host and pathogen interaction.

Ethylene together with JA, was first implicated in disease resistance after elevated ethylene, JA and plant defence gene expressions were detected in

Arabidopsis infected with the necrotic fungus *Alternaria brassicicola* (Penninckx *et al.*, 1996). During this infection, the plant defensin *PDF1.2* was strongly expressed. PDF1.2 is a member of the defensin family that consists of 3-5 kDa cysteine rich basic anti-fungal peptides, which are found in many plant species. PDF1.2 has potent anti-fungal properties against *A. brassicicola in vitro* (Penninckx *et al.*, 1996, 1998). Its expression can be induced by the exogenous applications of JA and ethylene, but not SA (Penninckx *et al.*, 1996). Subsequent studies on JA and ethylene signalling/response mutants have shown that functional JA and ethylene pathways are required for PDF1.2 induction (Thomma *et al.*, 1998). *npr1* mutants and *NahG* transgenic lines were unaffected in *PDF1.2* expression or resistance to *A. brassicicola*; thus establishing the JA/ethylene pathogen resistance pathway as distinct and independent from SA (Thomma *et al.*, 1998). Interestingly, mutations in the ethylene-signalling component *EIN2* abolished *PDF1.2* expression, but did not affect resistance to *A. brassicicola* (Thomma *et al.*, 1999). This suggests that sometimes JA can mediate resistance without ethylene, and that *PDF1.2* is not so critical for resistance against *A. brassicicola* in *Arabidopsis*. The functional JA/ethylene pathway probably activates a variety of defence genes, with *PDF1.2* being the best known marker for activation of this pathway.

JA and ethylene have also been implicated in induced systemic resistance (ISR). This novel response has been shown to be activated in the presence of several non-pathogens such as *P. fluorescens*, and once activated, can systemically protect the plant against a whole range of pathogens (Pieterse *et al.*, 1998). Details of the defence responses induced in this pathway are not known, but neither *PDF1.2* nor

PR-1 are believed to be involved. Although *PR-1* is not expressed, *NPR1* (which has been placed downstream of SA in the SAR pathway) is also involved in ISR (Cao *et al.*, 1994; Delany *et al.*, 1995; Pieterse *et al.*, 1998). Since *NPR1* is involved in the transcription of the SAR related *PR-1*, and probably ISR components, it follows that some molecular switch may determine what particular genes are expressed via NPR1. Therefore there is cross talk between the ISR and SAR pathways.

It has been reported that SA may impinge on both ISR and JA/ethylene pathways by modulating the level of JA production. For example SA can inhibit the rate-limiting enzyme of JA biosynthesis (Pena-Cortes *et al.*, 1993). However, SA can increase the transcript and protein levels for allene oxide synthase (AOS), a key enzyme in JA biosynthesis (Laudert *et al.*, 1998). It is possible that the increase and decrease in JA caused by SA may serve to finely regulate defence responses. This has strongly been suggested by Clarke *et al.*, (1998) who reported that the JA/ ethylene dependent *PDF1.2* expression can be reduced by increased levels of SA. It is possible that this change in expression may be dependent on other mechanisms. For example it has been reported by Shah *et al.* (1999) that a switch may regulate cross talk between the SA and JA/ethylene mediated signal transduction pathways. Since the SA pathway has close associations with the oxidative burst and can interact with the JA/ethylene pathway, it is possible that ROI formation may indirectly impinge on the JA/ethylene pathway. However, recent evidence suggests that the oxidative burst may directly influence the JA/ethylene pathway. For example, it has been reported that the build up of ROIs can induce the expression of *PDF1.2* in a SA independent manner (Mitter *et al.*, 1998). Therefore, these defence

pathways are tightly integrated, and are selectively induced depending on the pathogen. With regard to incompatible virus infections, the SA pathway has been extensively linked to resistance mechanisms, with no direct evidence to suggest involvement of the JA/ethylene pathways. However, since such extensive cross-talk takes place between different defence pathways, it is possible that elements of the JA/ethylene pathways may still participate in anti-viral strategies. It is possible that events involved in the initial recognition of the pathogen may determine what defence responses are activated.

Plant perception of pathogens involves the interaction between the products of resistance genes in the host and an elicitor(s) in the pathogen. Genetic resistance to TMV infection is well characterized in tobacco and involves the product of a dominant resistance gene N (Holmes, 1938; Baker *et al.,* 1997, 1995; Dinesh-Kumar *et al.,* 1995). Initiation of the resistance response is proposed to involve interaction of the N gene product with the virus-encoded replicase (Padgett and Beachy, 1993; Padgett *et al.,* 1997; Erickson *et al.,* 1999). A second source of recognition between the plant cell and TMV involves the interaction of the host N9 gene product with nascent viral coat protein synthesized within the cell (Culver *et al.,* 1994; Taraporewala and Culver, 1996; Erickson *et al.,* 1999). After interaction between host and viral components, an incompatible interaction may be induced with the subsequent elicitation of the HR/SAR defence response (Baker *et al.,* 1997). Work on TMV has recently shown that ROIs are also induced in TMV-tobacco gene-for-gene incompatible interactions (Allan *et al.,* 2001). Immediately after TMV inoculation there is a rapid increase in ROI accumulation; this is sometimes referred

to as Phase 1. Later, a more sustained oxidative burst is initiated (Phase 2) which correlates with increased resistance. Interestingly, it was found that compatible TMV-tobacco infections elicited the phase 1 burst but not phase 2. Induction of ROIs by TMV manifests a degree of specificity since CMV, which is unrelated to the tobamoviruses, is fully compatible in tobacco yet failed to elicit either the phase 1 or phase 2 ROI burst (Allan *et al.*, 2001). This evidence strongly suggests that interactions thought of as being compatible may actually elicit some defence responses; however, in these interactions there is not the subsequent development of necrotic lesions, pathogen localisations, HR or SAR that can be seen in incompatible responses. This suggests that the pathogen can induce a subset of possible responses to infection, with different pathogens eliciting a different array of events.

Although SA levels increase during incompatible TMV infections of tobacco and there is subsequent induction of PR genes (Murphy *et al.*, 1999; Chaerle *et al.*, 1999), PR proteins may not be important in virus resistance (Cutt *et al.*, 1989; Linthorst *et al.*, 1989). In fact, many of the PR proteins have chitinase or lysozyme activity and appear to be directed against bacterial and fungal pathogens (Dann *et al.*, 1996; Hammel *et al.*, 1997). These antimicrobial activities are also shared by the JA/ethylene induced plant defensins, which have not been shown to be elicited during incompatible virus infection (Thomma *et al.*, 2002). Salicylic acid is essential for localisation of the virus to the vicinity of the necrotic lesions, and for the establishment of systemic acquired resistance. However, viable TMV can be isolated from living cells surrounding necrotic lesions (Matthews, 1991). Thus, programmed

cell death of virus-infected cells or PR gene inductions may not be the exclusive mechanism by which SA mediates resistance to viruses.

It has been proposed that the plant defence signal transduction pathways separate downstream of SA into two branches (Murphy *et al.*, 1999). One branch leads to the induction of resistance to viruses and is salicylhydroxamic acid (SHAM) sensitive, whereas the other pathway (SHAM insensitive) leads to the induction of PR proteins and fungal and bacterial resistance. The SHAM sensitive alternative oxidase (AOX) pathway is a mechanism for siphoning off excess electrons from the cytochrome pathway in mitochondria, and recent evidence suggests that AOX might be involved in defence against viruses. For example resistance to viruses is promoted or inhibited by inducers or inhibitors of AOX activity (Chivasa *et al.*, 1998). Also AOX protein and transcript levels are elevated in plants expressing resistance to viruses (Chivasa *et al.*, 1997; Chivasa *et al.*, 1998; Lennon *et al.*, 1997). Although AOX is a likely component of SHAM sensitive virus resistance, SHAM may also affect other enzyme systems which could restrict virus invasion (Murphy *et al.*, 1999). Therefore, all other SHAM sensitive biochemical pathways cannot be discounted from playing a role in virus resistance (Ordog *et al.*, 2002). This supports the view of defence signal transduction as a web of converging and branching signalling pathways, and is dependent on the particular host and pathogen. The involvement of defence pathways with compatible interactions are still poorly understood and may vary according to host and pathogen.

SA, JA and ACC (a precursor of ethylene) have been exogenously applied to compatibly infected plants to try and determine the effect on viral titre and the

pathological outcome. Clarke *et al.* (1998) found that JA, SA and ethylene reduced viral titre dramatically, and increased PR defence gene expression in the normally compatible *Phaseolus vulgaris* and *White clover mosaic virus* (WClMV) interaction. In comparison untreated WClMV infected *P. vulgaris* developed compatible symptoms and had a much lower level of defence gene induction. This again suggests that induction of PR proteins are not sufficient to prevent viral spread. However, exogenous application of JA, SA and ethylene may facilitate the activation of additional defence pathways and prevent viral proliferation. Later work by Clarke *et al.* (2000b) has shown that application of JA, SA and ACC can lower the population of WClMV dsRNA replication intermediates in *P. vulgaris*, suggesting a direct effect on virus replication. In *Arabidopsis Nah G* transgenic plants infected with CaMV, virus levels were 4-fold greater than in non-transgenic plants (Laird, J., Geri, C., Guintini, P., unpublished). This suggests that SA-dependent signalling pathways may play a role in limiting the accumulation of CaMV in wildtype *Arabidopsis*. In contrast, some ethylene and JA-response mutants accumulate lower levels of CaMV during infection (Cecchini, E. and Barrett, S. J., unpublished). This implicates both the SA and JA/ethylene defence pathways in compatible CaMV infections of *Arabidopsis*, again emphasising the complex crosstalk between defence signalling pathways.

Other phytohormones may also participate in defence pathways against virus infection. In 1982 Fraser reported that exogenous application of ABA to tobacco (*Nicotiana tabacum*) leaves prior to TMV inoculation resulted in a significant decline in lesion size and number. Fraser *et al.* (1989) subsequently demonstrated

that TMV-resistant tomato plants had higher endogenous ABA content than susceptible plants. In contrast Clarke *et al.* (1998) reported that exogenous ABA and GA had no effect on viral titre in the WCIMV–*P. vulgaris* interaction. Again, activation of defence strategies by phytohormones may be dependent on host plant and pathogen. Phytohormones are clearly involved in various plant defence strategies, however it has also been suggested that plant hormones play a role in the production of compatible symptoms like stunting and leaf crinkling (Hull, 2002). This is discussed in greater detail below.

Phytohormone perturbations induced by virus infection (1.8)

Many symptoms of virus infection such as stunting, internode shortening and leaf distortions resemble hormonally induced disturbances. It has long been suspected that hormonal changes may be integral to virus-induced symptom expression. Normal plant development involves intricate spatial and temporal hormonal control, which is regulated via the expression of multiple gene systems (Barendse and Peters, 1995). Early studies in virus infected plants reported changes in the levels of some phytohormones during systemic infection; however such studies were qualitative and could not consider the complexity of hormonal interactions. For example, decreases in auxin levels have been reported in TMV-infected tobacco (Rajagopal, 1977), however increases were found in tomatoes stunted by TMV (Jones, 1956). Russell and Rimmins (1971) did not detect significant differences in endogenous auxin activity in stunted barley plants infected with BYDV. They did demonstrate, however, that stunting correlated with a

decrease in endogenous gibberellins. Although differences in the concentration of particular hormones occur during compatible virus infections (even although symptoms are the same), this could be explained by the fact that different hormones can have overlapping and often conflicting functions which are dependent on the host type and developmental stage.

Phytohormone and carbohydrate metabolism are tightly co-ordinated processes in the plant. Characterisation of sugar mutants has revealed that many are perturbed with regard to phytohormone metabolism and response. For example, the *prl1* mutant shows increased sensitivity not only to sugars, but also to ABA, ethylene, cytokinin and auxin (Nemeth *et al.*, 1998). Analysis of putative sugar-sensing mutants such as *sun6* (sucrose insensitive; Huijser *et al.*, 2000) and *gin6* (glucose insensitive; Arenas-Huertero *et al.*, 2000), have shown them to be allelic to the ABA insensitive mutant *abi4* (Laby *et al.*, 2000). This suggests that various sugar and phytohormone pathways may interact throughout the lifecycle of the plant. It is reasonable to assume that virus infection may impinge upon either pathway and affect interactions between them.

Symptoms and virus genes (1.9)

In addition to analysing the physiology of infected plants, a genetic approach has been taken which examines the effects of viral genes *in planta*, viral replication and the effect of infection on host gene expression. This genetic approach has created a greater understanding of how virus and plant behave during infection, and has highlighted possible pathways that may play a role in symptom development.

Genetic studies have been able to attribute symptom phenotypes to individual viral genes. For example, some mutations in the 126kDa/183kDa protein of TMV resulted in a loss of ability to induce symptoms in host plants (Nishiguchi *et al.*, 1985). Mutations in TMV coat protein have been shown to affect the induction of chlorosis, leading to the placement of a second potential symptom determinant on to the TMV genome (Dawson *et al.*, 1988). Mutations at defined regions in the genome of other viruses such as CaMV (Daubert *et al.*, 1983; Daubert *et al.*, 1984) have also been used to identify symptom determinants. After mutagenesis the plants were infected and the symptoms were noted. This effectively identified regions of the CaMV genome that were involved in systemic spread, stunting, timing, host range, chlorosis and severity (Daubert *et al.*, 1983; Daubert *et al.*, 1984). A map reproduced from Covey (1991) showing regions of the CaMV genome involved in specific aspects of disease is displayed in Figure 1.

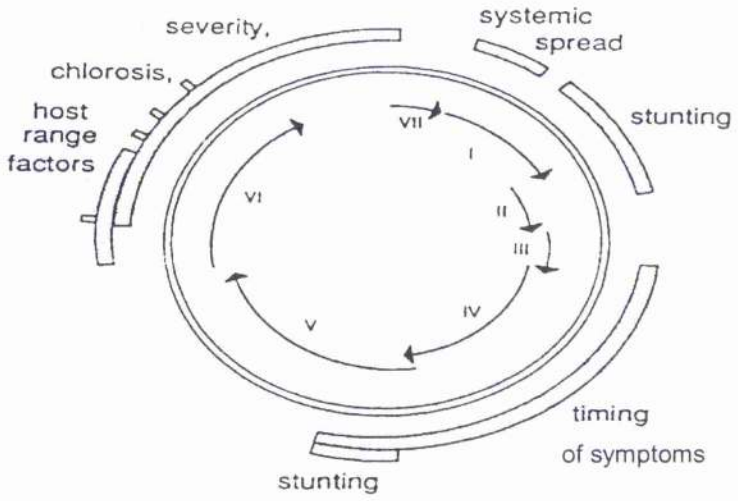


Figure 1 (Covey, 1991): Pathogenic determinants aligned to coding regions of the CaMV genome.
Gene I- movement
Gene II- aphid transmission
Gene III- coat protein
Gene IV- coat protein
Gene V- reverse transcriptase
Gene VI- translational activator

In addition to mutation studies, construction of hybrid viruses has identified and mapped the symptom determinants of numerous viral genomes. For example hybrids of CMV and *Tomato aspermy virus* (TAV) have revealed that the coat proteins of these viruses are major symptom determinants (Salanki *et al.*, 1997; Hellwald *et al.*, 2000). Hybrids created from mild (Bari-1) and severe (Cabb B-JI) isolates of CaMV have implicated gene VI in determining the degree of chlorosis (Stratford and Covey, 1989), as well as regulating host specificity (Wintermantel *et al.*, 1993), symptom severity and type (Daubert *et al.*, 1984; Daubert and Routh 1990; Anderson *et al.*, 1992). See Figure 1.

The expression of virus genes in transgenic plants can give rise to a symptom-like phenotype in the absence of infecting virus, and this approach has been used to map symptom determinants (Herbers *et al.*, 1997; Olesinski *et al.*, 1996). For example, Burgyan *et al.* (2000) has demonstrated that the necrotic response of *N. benthamiana* during *Cymbidium ringspot virus* (Cym-RSV) or *Carnation Italian ringspot virus* (CIRV) infection is dependent on the expression of both p35 and p19. It has been reported by Cecchini *et al.* (1997) that a symptom determinant taken from different isolates of CaMV and expressed in *Arabidopsis*, produce very different symptom-like phenotypes. This study also reported that the severity of the phenotype was dependent on the origin of the gene VI (producer of P6) symptom determinant and its level of expression. P6 derived from different isolates can have similar accumulations *in planta* but yield different symptoms, this supports the concept of a programmed response by the host (Cecchini *et al.*, 1997).

Viral induced effects on host gene expression (1.10)

Analysis of gene expression during compatible virus infection has identified a variety of genes whose expression is up or down-regulated. Several studies carried out have managed to correlate gene expression changes to regions in which the virus has proliferated. It has been reported that at the invasion front of pea seed borne mosaic potyvirus, that several genes, including those encoding HSP70 and polyubiquitin, appeared to be co-ordinately up regulated (Wang *et al.*, 1995; Aranda *et al.*, 1996). However other genes like those for lipoxygenase and heat shock cognate proteins were down regulated. It is possible that such a co-ordinated alteration of gene expression is common to many virus types, as has been suggested by Escaler *et al.* (2000). These authors reported that infection of pea embryonic tissues by a range of unrelated viruses produced similar spatial and temporal alterations in the expression of HSP70 and LIPOXYGENASE1 (LOX1). It was found that for these viruses HSP70 was up-regulated at the expanding lesion edge, but LOX1 was down-regulated. In a different study Havelda *et al.* (2000) reported that *Cucumber mosaic virus* (CMV) infection of *Curcubita pepo* resulted in an upregulation of HSP70 and $NADP^+$- dependent malic acid enzyme (NADP-ME) in a band of uninfected cells ahead of the infection front. However, these genes were down regulated in the interior of the lesion in which the virus had already invaded. This down regulation appears to be part of a general suppression of host gene expression, possibly involving degradation of host mRNAs (Aranda *et al.*, 1998).

A GUS reporter- Lipid Transfer Protein (LTP) promoter system in *Arabidopsis* was used to measure the effect of CaMV infection on the defence

related LTP (Sohal *et al.*, 1999). It was reported that up-regulation of LTP expression strongly correlated with the appearance of local lesions and the systemic spread of virus. LTP::GUS crosses with transgenic *Arabidopsis* expressing gene VI confirmed that P6 may be a major elicitor of LTP expression.

Differential display PCR (DD-PCR) was used to identify differences in gene expression in CaMV infected and gene VI transgenic *Arabidopsis* (Geri *et al.*, 1999). The authors reported that possibly several hundred genes were affected in expression, of which 30 genes were identified. Many of the up- and down-regulated genes which were identified from the *Arabidopsis* database, have also been reported as being stress or pathogen inducible. These included a putative defence related phenol sulphotransferase (Lacomme and Roby, 1996) whose expression falls during infection, and an *Arabidopsis MYB* gene whose expression increases. Interestingly *MYB* genes in other plants have previously been reported to be up-regulated in the incompatible response (Yang and Klessig, 1996). This could suggest that some defence reponses are activated during CaMV infection.

These studies indicate that the expression of many genes is modulated by virus infection. This is also dependent on temporal and spatial factors (i.e time required for virus spread and symptom elicitation).

Post-transcriptional gene silencing (1.11)

It has been reported that under certain circumstances plants are able to repress the expression of transgenes, retroelements and viruses (Hull, 2002). This gene silencing was initially discovered in transgenic plants which express wildtype

or mutated viral sequences, which were designed to interfere with the normal functioning of the target virus. It was unexpectedly found that in many cases protection was most effective in transgenic plants in which the transgene protein was undetectable. For example PLRV coat protein transgenic potato plants showed high levels of resistance to the virus, but did not contain detectable levels of the coat protein (Kawchuck *et al.,* 1991). In transgenic plants for the *Tomato spotted wilt virus* (TSWV) N protein gene, the highest levels of resistance to TSWV were found in plants accumulating the lowest levels of transgene transcript (Pang *et al.,* 1993). Similar findings were later reported by Mueller *et al.* (1995), who demonstrated that transgenic tobacco lines expressing lower levels of PVX replicase gene were more resistant to PVX infection, than the high expressing lines. This suggested a mechanism in which both transgene and homologous virus sequences are targeted.

Reductions in the level of transcripts in many cases are not due to a suppression of transgene transcription rates. For example Lindbo *et al.* (1993) reported that tobacco transformed with TEV coat protein was resistant to TEV and that the resistance mechanism did not affect transgene transcription rates. This suggested that the resistance mechanism employs RNA degradation, a process which is highly nucleotide specific (Lindbo *et al.,* 1993). English *et al.* (1996) inoculated GUS transgenic tobacco plants with modified PVX viruses, containing either GUS or GFP. It was found that low GUS expressing plants were more resistant to both PVX and GUS expression in the PVX:GUS construct. However, the low GUS expressing lines were not resistant to the PVX:GFP constructs. The high GUS expressing lines were susceptible to both PVX: GUS and PVX:GFP. This suggested

that silencing mechanisms operate against the whole RNA in which a target sequence is located (English *et al.,* 1996). The post transcriptional gene silencing (PTGS) in most cases, is thought to target sense RNA (Baulcombe, 1996b). Waterhouse *et al.* (1998) have reported that co-expression of sense and antisense RNA leads to gene silencing, which suggests that antisense RNA may be involved. Covey *et al.* (2000) has reported that deadenylation of sense RNA species may be a second mechanism in which targetting for degradation may be achieved.

Once the particular RNA has been tagged for degradation, a host RNA dependent RNA polymerase (RdRp) binds and produces a dsRNA (Cogoni *et al.,* 1999; Lindbo *et al.,* 1993; Dalmay *et al.,* 2000). The dsRNA can subsequently be attacked and degraded by a host RNase (Bass, 2000) into small ~21bp fragments, which may act as mobile potentiation signals for the maintenance of gene silencing (Voinnet *et al.,* 1998; Palauqui *et al.,* 1997). It is also possible that these degradation products can lead to an increase in the methylation of target viral or transgene DNA (Finnegan *et al.,* 2001), resulting in the production of aberrant ssRNA or hairpin RNA species which are readily degraded by PTGS-mediated processes.

Gene silencing may account for the recovery seen in some virus infections. Recovery refers to the amelioration of symptoms and is often seen in emergent leaves of the systemically infected plant (Hull, 2002). This phenomenon has been seen in nepovirus (Wingard, 1928; Lister and Murant, 1967), tobamovirus (Cadman and Harrison, 1959) and caulimovirus (Al Kaff and Covey, 1995) infected plants. In recovered tissues, viruses can still be detected, as in the case of *Alphalpha mosaic virus* (AlMV) infected *N. tabacum* (Gibbs and Tinsley, 1961; Ross, 1941). However

it has been shown in numerous instances that recovered tissues have significantly less virus than the systemically infected tissues (Covey and Turner, 1991). Recovered tissues are resistant to reinoculation by the virus, and experiments with PVX and *Tomato black ring virus* (TBRV) in transgenic *N. clevelandii* suggests that viral replicative RNA intermediates are suppressed in recovered tissues (Ratcliffe *et al.*, 1997). Reductions in viral RNA, but increases in super coiled DNA, were detected in the ameliorated tissues of a less susceptible host (*Brassica napus*) infected with mild CaMV isolates (Covey *et al.*, 2000). In the recovered tissues high levels of non-polyadenylated viral RNA were detected and run off analyses revealed that there was no change in CaMV transcriptional activity (Covey *et al.*, 2000). This supports the suggestion that recovery may be due to PTGS.

Despite the fact that gene silencing mechanisms operate in a very broad manner, some viruses are still able to spread and multiply in plant hosts. One reason for this that many viruses possess mechanisms that enable them to overcome or repress such host responses. By inserting various components of 16 viruses in several PVX vectors and then infecting the host plant, Voinnet *et al.* (1999) demonstrated that the components of 12 of the viruses suppressed PTGS. Subsequent studies have revealed that suppression of gene silencing can be mediated by protein products of several viruses, such as CMV and TEV (Beclin *et al.*, 1998; Lucy *et al.*, 2000). Voinnet *et al.* (1999) have reported that there was no common feature between these proteins except that they are frequently regarded as symptom determinants. Although there is no common homology between the particular viral PTGS repressors, the mechanisms may operate in a similar manner. It was found

that gene silencing of viral replication was suppressed by the HC-Pro gene of TEV (Pruss *et al.*, 1997; Anandalakshmi *et al.*, 1998; Kasschau and Carrington, 1998). Further work on potyviral HC-Pro revealed that it inhibited the accumulation of the small RNAs and reduced the level of cytosine methylation (Llave *et al.*, 2000). Mallory *et al.* (2001) subsequently reported that HC-Pro may prevent plants from responding to the mobile silencing signal. The PTGS suppression induced by a particular virus is dependent on the host. For example, the 2b gene of TAV in a TMV vector can suppress PTGS in *N. benthamiana*, but induces HR in *N. tabacum* (Li *et al.*, 1999).

Some viruses such as PVX and TMV appear not to have a specific suppression mechanism but may avoid PTGS responses by very rapid replication and spread, or by compartmentalisation.

CaMV as a tool for studying compatible interactions (1.12)

CaMV is a suitable virus for studying compatible interactions, for several reasons.

1. CaMV can systemically infect *Arabidopsis*, facilitating studies which take advantage of the genetic background of the host.
2. It has an 8kbp DNA genome from which recombinants can be easily made.
3. There are more than 50 isolates available, which induce symptoms of different severity and character.
4. The entire genetic sequence of several isolates have been determined and symptom determinants mapped.

In turnip and *Arabidopsis* infected with CaMV, the symptoms are first visible at approximately 9 days post-inoculation (9 dpi), with pin-prick chlorotic local lesions on the inoculated leaf. After systemic spread, which follows the preferential route through the phloem, systemic symptoms develop. Over the next two to three weeks, virus infected plants develop vein clearing in which leaf lamina tissue at the border of the veins suffers chlorophyll loss. Over time the chlorotic regions broaden until they coalesce, producing leaves with complete chlorosis. Before complete chlorosis, green islands of leaf tissue (between the chlorotic vein borders) hyper-accumulate chlorophyll and appear a darker green than that of uninfected leaves. During later stages of infection severe stunting and leaf crinkling, especially in newly emerging leaves, is observed. Infected *Arabidopsis* plants also have a tendency to flower early (Cecchini *et al.*, 1998).

Once in the cell, the virus particles are targeted to the nucleus where they uncoat, liberating the genomic DNA and the methionyl tRNA replication primer (refer to Figure 2, an adaptation from Covey *et al.*, 1991). The virus DNA becomes supercoiled, perhaps via a host topoisomerase, and associates with histones to assemble a transcriptionally active minichromosome (Figure 2, page 39, stages 1-3).

The chromosome has two promoter sites, one for a subgenomic RNA, the 19S RNA, and another for a genome length RNA, the 35S RNA (Covey *et al.*, 1981). The two transcripts are exported to the cytoplasm after transcription (Figure 2, stages 4-6). The 19S RNA functions as the messenger for the 58kDa P6 protein. This is a multifunctional protein which can act as a translational transactivator of the polycistronic 35S RNA (Bonneville *et al.*, 1989; Gowda *et al.*, 1989), enabling the

tandemly arranged genes to be translated via an unusual mechanism (Figure 2, stages 7-8).

The 35S RNA has a 600 nucleotide leader sequence that can fold into a large stem and loop structure. It has been shown that the leader region is inhibitory to the translation of the downstream genes (Baughman and Howell, 1988). It is thought that P6 can stabilise this leader sequence by tightly binding very small non-coding ORFs, enabling downstream migration of the ribosomes. The individual coding regions of 35S RNA are translated using a novel "relay race" model, first proposed by Dixon and Hohn (1984). In this model, a ribosome binds first to the 5' end of the RNA and translates to the first termination codon. After the first protein has been produced, the ribosome does not leave the RNA, but reinitiates protein synthesis at the nearest downstream AUG. It is thought that P6 can stabilise the interaction between the ribosome and the RNA, preventing the ribosome from leaving the RNA once a stop codon has been reached (Ryabova *et al.*, 2002; Hohn *et al.*, 2001). As well as facilitating translation of the 35S RNA, P6 also builds up in the cell to form electron-dense inclusion bodies, which are large protein aggregates in which are imbedded virus particles. The function of these inclusion bodies is unclear.

The 35S RNA can also be targeted by the methionyl tRNA replication primer which was initially liberated as the virus is uncoated. It is thought that this selects 35S RNA for reverse transcription. Reverse transcription results in the production of new ds DNA viral genomes (Figure 2, stage 9). The 79kDa P5, a product from 35S RNA translation, transcribes the 35S RNA targetted by the tRNA replication primer (Pfeiffer *et al.*, 1983; Menissier *et al.*, 1984; Gordon *et al.*, 1988). P5 not only has

reverse transcriptase capability, but it also has a functional aspartate protease activity at its N-terminus, which may cleave several CaMV primary translation products (Torruella *et al.*, 1989).

One of the translation products from the 35S RNA is the P1 movement protein, a 37 kDa protein implicated in modifying the plasmodesmata, which facilitates cell-cell movement (Maule *et al.*, 1991; Figure 2, stage 14). Such properties were initially ascribed to P1 as it is localised to the plasmodesmata, as shown by immunogold cytochemistry (Albrecht *et al.*, 1988; Linstead *et al.*, 1988), and P1 has homology to other viral movement proteins (Linstead *et al.*, 1988; Hull *et al.*, 1986; Hull and Covey, 1985). The observation that CaMV genomes with deletions in gene 1 were able to replicate in single cells but were unable to move from cell to cell is further evidence that P1 functions as a MP (Thomas *et al.*, 1993). These authors have shown that P1 has a nucleic acid binding domain with a very high affinity for ssRNA. This suggests that P1 not only has the capability to deliver virus particles via a tubule based system, but that P1 could alter the SEL of plasmodesmata and form complexes with the genome length 35S RNA which could be carried to other cells. These two putative forms of CaMV virus movement via fully formed virions and 35S RNA are perhaps operational at different stages of the multiplication cycle or in different tissues (Thomas *et al.*, 1995).

The P3 and P4 proteins are also translation products of the 35S RNA. Mesnard *et al.* (1990) proposed that the 15 kDa P3 is a non-sequence specific DNA binding protein that is part of the icosahedral virus structure along with P4. The P4 product is a 57 kDa precursor of the 42 kDa protein subunit of the virus shell

(Franck *et al.*, 1980; Daubert *et al.*, 1982). The 42 kDa protein is assumed to be derived from the 57 kDa molecule via proteolysis after formation of the virus shell (Hahn and Shepherd, 1982). Subsequently it is phosphorylated on serine and threonine residues (Menissier de Murcia *et al.*, 1986; Martinez-Izquierdo and Hohn, 1987) and then glycosylated (Du Plessis and Smith, 1981).

After shell formation and encapsidation of the newly synthesised genomic DNA (Figure 2, stages 10-13) the viral progeny may become embedded in electron-lucent inclusion bodies. These bodies are created via the accumulations of the 18 kDa P2 product from the 35S RNA. It is thought that P2 aggregates acquire virus particles and facilitates their uptake by aphid vectors (Schmidt *et al.*, 1994; Blanc *et al.*, 1996; Espinoza *et al.*, 1991; Woolston *et al.*, 1987). Virus particles are also found in electron-dense P6 inclusion bodies (Figure 2, stage 15-16). Their function in infection is not fully understood, however they could be sites of virion synthesis or bodies regulating free virion levels in cells (Covey and Turner, 1991).

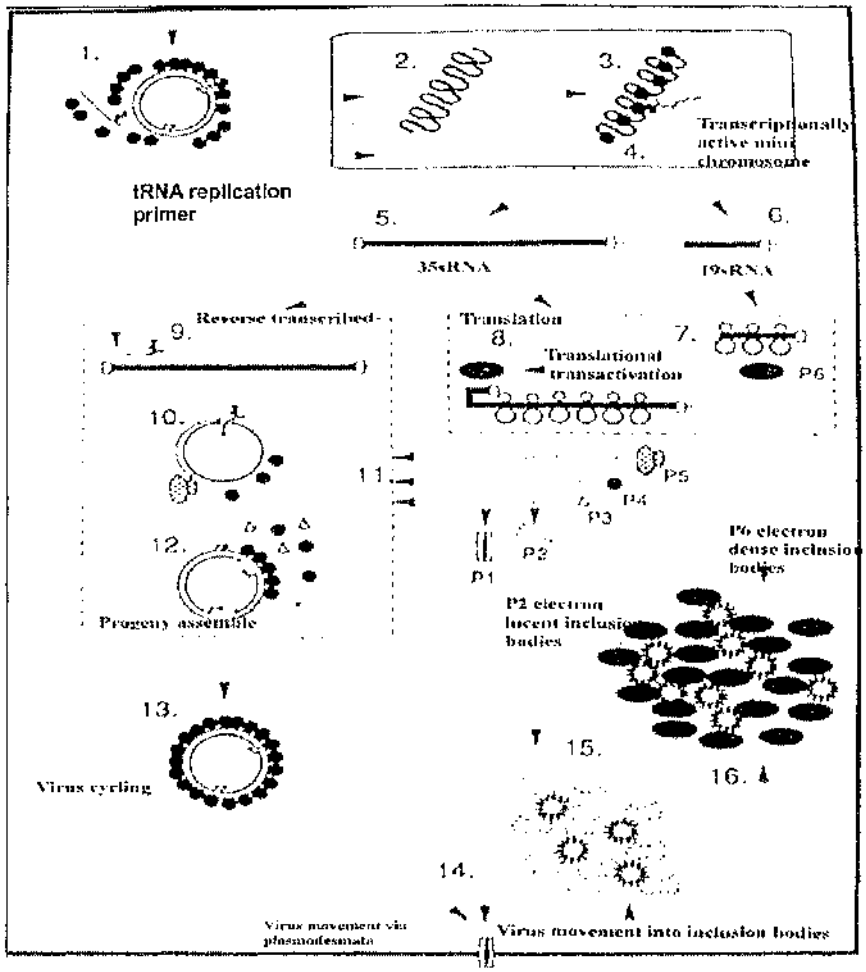


Figure 2 (from Covey, 1991): Replication cycle of CaMV.

1-3 Virus uncoats and forms transcriptionally active minichromosome in cell nucleus.

4-6 19S and 35S RNA transcription products migrate into cytoplasm.

7-8 19S RNA product (P6) transactivates the translation of 35SRNA or forms inclusion bodies. 35S RNA translation products are structural components of the virus (P3 and P4), can reverse transcribe 35S RNA (P5) or facilitate virus movement (P1) and aphid uptake (P2).

9 35S RNA is reverse transcribed by P5 after priming by a tRNA replication primer.

10-12 Virus particles assemble.

13-16 Virus particle move from cell-cell or can be held in P6 and P2 inclusion bodies.

Project Aims (1.13)

The objectives of this Ph.D project were:

1. To examine the possible correlation between changes in carbohydrate accumulation and symptom severity in turnip and Arabidopsis during compatible CaMV infection.
2. To use Arabidopsis sugar signalling/uptake mutants to probe the role of sugar signalling as an effector of symptoms.
3. To analyse the interaction between sugar signalling and defence gene induction during CaMV infection.
4. If such defence genes are activated as a result of compatible virus infection, then the further aim was to characterise their temporal and spacial induction.

Chapter 2

Plant seed stocks (2.1)

Turnip seed (*Brassica campestris* cv. Just Right) was provided by Dr. Simon Covey, John Innes Centre (Tompkins, 1937). *Arabidopsis thaliana* seed stocks are summarised in Table 1 below.

Seed stocks	Description	Supplier / reference
Col-0		Nottingham *Arabidopsis* Stock Centre (NASC) (http://nasc.nott.ac.uk/)
Ler-0		NASC (http://nasc.nott.ac.uk/)
WS		NASC (http://nasc.nott.ac.uk/)
NahG (Ler-0/Col-0)	Transgenic plant expressing a Salicylate degrading enzyme from *Pseudomonas putida*.	Syngenta Seeds, Cambridge, UK (Lawton *et al.*, 1995).
ein2-1 (Col-0)	Ethylmethanesulphonate (EMS) generated ethylene signalling intermediate mutant, does not show ethylene triple response.	NASC (Guzman and Ecker, 1990)
etr1-1 (Col-0)	EMS generated ethylene receptor mutant, does not show ethylene triple response.	NASC (Bleecker *et al.*, 1988)
cai 160 (WS)	EMS generated putative sugar signalling/uptake mutant, shows altered response to 100mM sucrose and 0.1mM nitrogen.	Prof. Ian A Graham, University of York (Graham, I. A., and Martin, T., unpublished).
cai 171 (Col-0)	EMS generated putative sugar signalling/uptake mutant, shows altered response to 100mM sucrose and 0.1mM nitrogen.	Prof. Ian A Graham, University of York (Graham, I. A., and Martin, T., unpublished).
cai 181 (Col-0)	EMS generated putative sugar signalling/uptake mutant, shows altered response to 100mM sucrose and 0.1mM nitrogen.	Prof. Ian A Graham, University of York (Graham, I. A., and Martin, T., unpublished).
PR-1::Luc (Ler-0)	Transgenic plant containing tobacco PR-1 promoter coupled to a luciferase reporter gene.	Dr. Gary Loake, Edinburgh University (Loake, G., and Yun, B. W., unpublished).
AtGST1::Luc (Ler-0)	Transgenic plant containing *Arabidopsis* GST promoter coupled to a luciferase reporter gene.	Dr. Gary Loake, Edinburgh University (Grant, *et al.*, 2000).
PDF1.2::Luc (Ler-0)	Transgenic plant containing *Arabidopsis* PDF1.2 promoter coupled to a luciferase reporter gene.	Dr. Gary Loake, Edinburgh University (Loake, G., and Yun, B. W., unpublished).

The PR-1::Luc and PDF1.2::Luc *Arabidopsis* lines (Loake, G., and Yun, B. W., unpublished) were both produced in the laboratory of Dr. Gary Loake at ICMB, Edinburgh University. *Arabidopsis Landsberg erecta* were transformed with a construct containing the 903bp PR1a promoter from tobacco (Payne et al., 1988) fused to the firefly luciferase (Luc) marker gene (pSP-Luc+ Vector; Promega, Chilworth, Southamptom) and the *ocs* terminator from *Agrobacterium tumifaciens* (Figure 3).

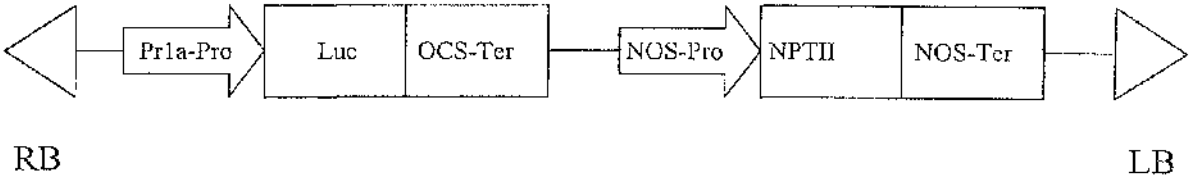


Figure 3: The transformation cassette used to produce PR-1::Luc transgenic plants. LB- Left border, Luc- luciferase gene, NPTII- neomycin phosphotransferase (encoding kanamycin resistance), NOS- nopaline synthase, OCS- octapine synthase, Pro-promoter, RB- right border, Ter- terminator.

A similar expression cassette was used to make PDF1.2::Luc lines, except that an *Arabidopsis* PDF1.2 promoter (Accession gi: 17380797) was used in place of the tobacco PR-1 promoter.

Plant growth conditions for virus infection (2.2)

Soil preparation (2.2.1)

A peat based potting compost (Levington Horticulture Ltd.) was autoclaved for 1 hour at 120°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 3 days. Stratification promoted synchronised growth and development. Trays were then maintained at 21°C±1°C under 10 hours daylength. Osram "warm white" fluorescent tubes provided light at an intensity of $120\mu mol/m^2/s$. These conditions have been shown to be optimal for the development of symptoms after virus inoculation (Cecchini *et al.*, 1998).

Turnip (2.2.3)

Five turnip "Just Right" seeds were pushed 1cm into the surface of each soil filled 15cm diameter pot. Turnip were grown under similar light and temperature intensities to those of *Arabidopsis*, but daylengths were 16 hours.

Virus isolates (2.3)

Virus stocks (2.3.1)

CaMV isolates Aust (Al-Kaff and Covey, 1994), Baji-31 (McCallum, 1994; Cecchini *et al.*, 1998), Bari-1 (Delseny *et al.*, 1983; Stratford *et al.*, 1988) and Cabb B-JI (Delseny *et al.*, 1983) were originally provided by Dr. Simon Covey at the John Innes Centre (JIC) in Norwich. These isolates are described in Al-Kaff and Covey (1994, 1995) and in the VIDE database (http://image.fs.uidaho.edu/vide/html).

Propagation and maintenance of virus stocks (2.3.2)

The virus stocks were maintained as dessicated 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 $1cm^2$ of desiccated tissue was ground up in 300µl dH_2O, centrifuged and the supernatant removed. Celite was added to the supernatant and 10µl was pipetted on to the first true leaf of each turnip plant at the two true leaf stage of development. The treated leaf was rubbed with a glass rod. After 3-4 weeks, at which time symptoms had developed, leaf material was taken from strongly symptomatic turnip plants and was frozen at –80°C.

In a chilled blender, a solution of 0.5M KH_2PO_4, pH 7.2 and 0.75% (w/v) sodium sulphite was added to the frozen leaf material in the ratio of 3ml per gram of leaf tissue. The material was ground, urea was added to 1M and 10% (v/v) Triton X-100 was added to a final concentration of 2.5%. This mixture was covered and left overnight on a shaker at 4°C.

The mixture was centrifuged at 5,000 g for 10 minutes at 4°C. The supernatant was then strained through 4 layers of muslin and centrifuged for 2 hours at 25,000 rpm in a Sorvall T865 rotor. The pellet was resuspended in 2 ml of dH_2O with a pipette and by mixing on an angled shaker at 4°C for 2 hours. The suspension was then centrifuged at 7,000 g for 10 minutes at 4°C. The supernatant was centrifuged at 45,000 rpm in a Sorvall T865 rotor for 1 hour at 4°C. The virus pellet was resuspended in dH_2O and stored in aliquots at –80C. Virus concentration was estimated spectrophotometrically (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 inoculation (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 with a glass rod on to one side of the first emergent true leaf, using celite as an abrasive. Mock-inoculated plants were inoculated with autoclaved water and celite. Turnip plants were inoculated in a similar manner and stage of development to *Arabidopsis* (the two true leaf stage), except that 1µg of purified virus in a volume of 10µl was used for each inoculated leaf.

Northern analysis (2.4)

RNA extraction (2.4.1)

Fully expanded and unexpanded leaves (referred to as source and sink) were dissected from infected plants using scissors. Triplicate samples were taken from 12 plants, with each sample pooled from the tissues of 4 plants. Subsequently total RNA was extracted following the PureScript Kit (Flowgen, Shenstone, Staffs, UK) protocol for 10 mg of plant tissue.

RNA electrophoresis and blotting (2.4.2)

RNA samples were separated by gel electrophoresis according to Sambrook and Russell (2001). Approximately 2µg of total RNA per well was loaded into a 36 well 1.4% agarose-formaldehyde gel. Ethidium bromide stained gels were photographed under UV illumination using a BioGene gel documentation system (BV2040). RNA was transferred to Hybond N membrane

(Amersham, Bucks, England) according to Sambrook and Russell (2001) and fixed under UV (Syngene Synoptics Ltd).

DNA dotblotting for estimating viral DNA accumulation (2.5)

Total DNA was extracted from tissue using a Puregene kit (Flowgen, Shenstone, Staffs, UK). Leaves were ground in extraction buffer according to the manufacturer's protocol. To release encapsidated CaMV DNA from virions, proteinase K was added to a concentration of 250μg/ml and the extracts incubated at room temperature for 3 hours (Covey *et al.*, 1998). DNA was then purified according to the manufacturer's protocol. DNA was assayed spectrophotometrically on a Perkin Elmer Lambda 800 spectrophotometer. Based on these measurements appropriate dilutions were made for slot blotting. For each sample, 100ng, 10ng and 1 ng of DNA in 250μl dH_2O were denatured for 10 minutes at 100°C and then placed on ice. These were loaded into the wells of a slotblot manifold (Schleicher and Schuell Ltd.) which sandwiches Hybond N membrane. Subsequently the filter was left to air dry and the DNA was fixed in a Syngene UV crosslinker. To confirm equal loading, the membranes were stained in methylene blue according to Sambrook and Russell (2001). Filters were then hybridised as described in Section 2.6.1.

Hybridisation, probes and autoradiography (2.6)

Hybridisation (2.6.1)

Membranes were placed in Techne roller hybridisation tubes (Techne, Staffs, UK) and covered with 15ml of Northern Max prehybridisation solution

(Ambion, Huntingdon, Cambridgeshire, UK) containing denatured sheared salmon sperm (Ambion, Huntingdon, Cambridgeshire, UK) to a final concentration of 100µg/ml. Prehybridisation was carried out at 42°C for 18 hours. The probe was boiled for 10 minutes, added to the filters and allowed to hybridise for 18 hours at 42°C. Subsequently, SSPE washes of different stringencies were used to remove the unspecifically hybridised Sambrook and Russell (2001). Membranes were immersed in 2 x SSPE wash at room temperature in a shaker for 1 hour. Next 1 x SSPE and 0.5 x SSPE washes were carried out, each for 0.5 hours at 55°C.

Autoradiography (2.6.2)

Filters were wrapped in clingfilm, placed in a cassette with an intensifying screen and overlaid with Kodak MXB autoradiograph film in a darkroom. The autoradiograph films were exposed for an appropriate period (12 hours to 3 days) at -70°C. The film was developed in an X-OMAT Kodak developer.

Quantification of autoradiographs (2.6.3)

The total amount of nucleic acid loaded onto each lane was quantified by pixel analysis of digitally reversed images of fluorescence, using Quantiscan for Windows (Biosoft, Cambridge, UK). Autoradiographs were scanned on a flatbed scanner (Hewlett Packard Scanjet 4470c), digitised and hybridisation was quantified by pixel analysis using Quantiscan for Windows. 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. Data

were analysed using Minitab version 13.20 (Minitab Inc., Pennsylvania, USA) and presented using Excel (Section 2.12).

Isolation of DNA fragments for use as probes (2.7)

Sources of probes (2.7.1)

Fragments used for probing were contained in vectors obtained from the sources shown in Table 2.

Fragment	Plasmid vector	Source
Gene VI (Cabb B-JI)	pUC-19	Cecchini *et al.*, 1997.
PR-1 (*Arabidopsis*)	pBluescript	Novartis, North Carolina, USA
PR-2 (*Arabidopsis*)	pBluescript	Novartis, North Carolina, USA
PR-5 (*Arabidopsis*)	pBluescript	Novartis, North Carolina, USA

Table 2: Sources of probes.

Bacterial culture and plasmid isolation (2.7.2)

The *E. coli* glycerol stocks containing the vectors were grown under Ampicillin selection according to Sambrook and Russell (2001). Bacteria were pelleted by centrifugation at 4000 rpm and resuspended in STE (Sambrook and Russell, 2001). 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.

PCR (2.7.3)

Probes were prepared by PCR in an air thermocycler (Rapid Cycler Idaho Technology, Idaho, USA) according to Sambrook and Russell (2001). Primers

were obtained from MWG Biotech (Milton Keynes, UK), dNTPs from Promega (Southampton, UK) and PCR buffers from Biogene (Cambs, UK).

Purification of PCR fragments (2.7.4)

PCR fragments were separated by gel electrophoresis according to the protocols described by Sambrook and Russell (2001). The ethidium bromide-stained gels were placed under a UV illuminator and PR-1 (750bp), PR-2 (1.2kb), PR-5 (1kb) and gene VI (750bp) fragments were identified by comparing against 100bp and 1kbp ladders (FMC, Surrey, UK). The appropriate bands were excised and the fragments were purified using a Quantum Prep Freeze-N-Squeeze Spin Column kit (Biorad, Herts, UK).

Quantifying the amount of purified PCR product (2.7.5)

To quantitate the amount of DNA after purification, 5µl of each fragment was electrophoresed along with DNA ladders of known concentration (50ng of DNA per band for each 5µl loaded on the gel; FMC, Surrey, UK). The ethidium bromide fluorescence of DNA fragments and ladders were quantified by pixel analysis of reversed gel images, using Quantiscan for Windows.

Radiolabelling (2.7.6)

Purified fragment DNA (100ng) was labelled using a High Prime kit (Roche Molecular Biochemicals, East Sussex, UK). The High Prime labelling mix contained 1U/µl Klenow polymerase, 0.125 mM dGTP, 0.125 mM dATP and dTTP in a stabilised reaction buffer. Four µl of the High Prime mix was added to a tube containing 25ng denatured DNA and 50μCi of $\alpha[^{32}P]$-dCTP (3000Ci/mMol; Amersham, UK). The final volume was brought to 20µl and the

labelling reaction progressed as outlined in the High Prime kit. The labelled fragments were subsequently purified using a NICK Column (Pharmacia Biotech). The NICK column contains G-50 Sephadex, which retains unincorporated radiolabelled dCTP, but allows newly synthesised labelled DNA of greater than 50bp to pass through. The purified radiolabelled probe was boiled at 100°C for 10 minutes before adding to the filters that were prehybridised (Section 2.6.1).

Starch and soluble sugar extraction and analysis (2.8)

Sugar extraction (2.8.1)

Arabidopsis tissue samples were taken from whole expanded and emergent leaves. Turnip tissues were harvested in a similar manner except that 1cm-diameter discs were removed from emergent and expanded leaves, with care being taken to avoid leaf veins. After determining the fresh weight of the tissue, it was stored at -80°C or kept in liquid nitrogen. Tissues were ground under liquid nitrogen, using a pestle specifically designed for 1.5ml eppendorf tubes (IBLS Workshop, Glasgow University). To each powdered sample 250µl 80% ethanol was added and then incubated for 1 hour at 70°C. After incubation, the tubes were centrifuged and the supernatants were removed and stored in fresh eppendorfs. This was repeated a further two times, with appropriate supernatants being combined. The tubes containing the tissue pellet were retained for starch analysis, whereas tubes containing 750µl of the ethanol extract were lyophilised in a Speed Vac Plus (Savant, model SC110A). After the extracts were dried they were re-dissolved in 50µl of 100 mM imidazole pH 6.9, 5mM $MgCl_2$ (buffer B). Sugar concentrations in the samples were determined according to Stitt *et al.*

(1989) as described below. All reagents used were obtained from Roche/Boehringer (East Sussex, UK).

Sugar analysis (2.8.2)

Free soluble sugars were analysed according to Stitt *et al.* 1989. Each well in a flat-bottomed 96 well microtitre plate (Costar) was filled with 195µl of cocktail composed of 19.1ml buffer B, 200µl 100mM ATP, 200µl 200mM NADP and 40µl G6PDH (40U).

Subsequently 5µl of each tissue extract was added to the wells, with the exception of three wells, which acted as blanks. Plates were incubated at 37°C. The absorbance at 340nm was measured in a Benchmark microplate reader (Bio-Rad), using wells containing buffer B rather than tissue extract as blanks. Where appropriate, sap samples were diluted in buffer B to give an A_{340} in the range 0.05-0.25. Subsequently, triplicate sugar standards were added to the plate (5mM D-glucose, D-fructose and sucrose) and the first A_{340} (A1) was measured and recorded. Afterwards, 1ul (0.3U) of hexokinase (HXK) in buffer B was added to each of the wells, shaken and incubated at 37°C for 15 minutes. Several A_{340} readings were taken during this period and the maximal level was recorded (A2). To each well, 1µl (0.3U) of phosphoglucose isomerase (PGI) in buffer B was added. The plate was shaken and incubated at 37°C for 15 minutes. The maximal reading was then taken (A3). Subsequently, 1µl (0.8U) of invertase in buffer B was added to each well. The plate was shaken and then incubated for 25 minutes. During this period, the maximal A_{340} was recorded (A4).

Each of the absorbances obtained was then used to determine an absorbance value representative of each sugar type. For example,

$A2-A1=\Delta A_{glucose}$, $A3-A2=\Delta A_{fructose}$ and $A4-A3=\Delta A_{sucrose}$. These values were then used in determining the carbohydrate concentrations, as described in Section 2.8.4.

Starch extraction and conversion into D-glucose (2.8.3)

Tissue pellets from the sugar extractions were briefly dried down in a Speed Vac Plus and used for starch analysis. Four hundred microlitres of 0.2M KOH was added to the dry pellet and incubated at 95°C for 2 hours on a heating block. After centrifugation the supernatant was transferred into a fresh eppendorf. Subsequently 70µl of 1M acetic acid was added to adjust the pH to 5.5, which was estimated using pH indicator paper. One hundred microlitres was removed and diluted 1:10 by adding dH_2O. Afterwards 3U/ml of amyloglucosidase and 0.8U/ml of α-amylase (Roche/Boehringer, East Sussex, UK) were added, and left to incubate overnight at 37°C. This process converts starch into a stoichiometrically equivalent amount of D-glucose, which was determined as described above (Stitt *et al.*, 1989).

Determining glucose, fructose and sucrose concentrations from absorbance (2.8.4)

Sugar concentrations were determined using the equation shown below (Stitt *et al.*, 1989).

$$c=[(V \times MW)/(e \times d \times v \times 1000)] \times \Delta A$$

c= concentration (g/L)

V= volume of fluid in each well (ml)

v= volume of extract added (ml)

MW= molecular weight of the analysed substance (g/mol)

d= light path (0.6cm)

e= absorption coefficient of NADPH at 340nm (6.3)

ΔA= absorbance of glucose ($\Delta A_{glucose}$), fructose ($\Delta A_{fructose}$) or sucrose ($\Delta A_{sucrose}$)

After determining the g/L of sugar in the sample, the amount of sugar in mg/gfw (gram fresh weight) of leaf tissue was calculated.

cai mutant screening (2.9)

Seed sterilisation (2.9.1)

One COVCLOR 1000 tablet (Coventry Chemicals Ltd.) was dissolved in 35 ml dH_2O, to which 3 drops of 1% (v/v) Tween 20 were added. Five ml of this stock solution was added to 45 ml of 95% drum ethanol. One ml of this sterilising fluid was added to an eppendorf containing 10-15 mg of *Arabidopsis* seeds, shaken and left for 15 minutes. In a laminar flow hood the fluid was removed and the seeds were then washed twice with 95% ethanol. Seeds were allowed to dry overnight under sterile conditions.

cai screening conditions (2.9.2)

cai mutants were screened on plates containing 0.1mM nitrogen and 100mM sucrose (Martin *et al.*, 2002). Controls were grown on 60mM nitrogen and 100mM sucrose plates. These media were made up according to Table 3.

The media was poured into 10 cm diameter petri dishes under sterile conditions, and approximately 200 sterilised seeds were scattered over the surface of each plate. The seeds were left to stratify for 4 days at 4°C in darkness. Seeds were germinated and grown under 24 hour light at an intensity of 120µmol/m^2/s (Osram "warm white" fluoresecent bulbs) and a temperature of

21°C. Phenotypes were examined after 8 days, the optimum time for differences in phenotype (Martin *et al.*, 2002).

Chemical	*Cai* media	Control media
H_3BO_3	100.275μM	100.275μM
$CoCl_2.6H_2O$	0.11μM	0.11μM
$CuSO_4.5H_2O$	0.10μM	0.10μM
$MnSO_4.H_2O$	131.9μM	131.9μM
$Na_2MoO_4.H2O$	1.0μM	1.0μM
KI	5μM	5μM
$ZnSO_4.7H_2O$	29.9μM	29.9μM
$CaCl_2$	2.99mM	2.99mM
KH_2PO_4	1.25mM	1.25mM
$MgSO_4.7H_2O$	1.30mM	1.30mM
NH_4NO_3	34.2μM	20.6mM
KNO_3	31.21μM	18.8mM
$FeSO_4.7H_2O$	100μM	100μM
$C_{10}H_{14}N_2O_8Na_2.2H_2O$ (disodium EDTA)	100.11μM	100.11μM
Sucrose	100mM	100mM
KCl	18.8mM	
Bacto agar	8g/L	8g/L
KOH	pH 5.6	pH 5.6

Table 3: Chemical composition of *cai* screening and control media.

Rescue media (2.9.3)

Seedlings rescued from the *cai* screen were transferred to plates containing ½ MS, 1% sucrose and 0.8% agar (pH 5.6-5.8). Transplanted seedlings were maintained in 10 hours day length at 100μE/m^2/s.

Luciferase Imaging (2.10)

Plants and growth conditions (2.10.1)

Transgenic plants were obtained from Dr Gary Loake, University of Edinburgh. Plants contained either *GST1* (Grant *et al.*, 2000) or *PDF1.2* promoters from *Arabidopsis*, or the *PR-1* promoter from tobacco coupled to luciferase reporter genes and transformed into *Arabidopsis* Ler-0 (Section 2.1).

Plants were grown and inoculated as described previously (Sections 2.2 and 2.3.3) except day length was 12 hours. An additional uninoculated control was included in the experiments since some of the Luc lines are wound responsive (Grant *et al.*, 2000)

D-Luciferin Treatment and Imaging (2.10.2)

Plants were lightly painted with a paint brush dipped in a solution containing 1mM luciferin (Promega), 0.01% (v/v) triton X-100 and 0.03% (v/v) Silwet (Union Carbide) in a 1mM sodium citrate buffer pH 5.8. Immediately after painting, the plants were placed in an ultra-low-light imaging camera system (EG+G Berthold Luminograph 980) and a series of brightfield images were taken and saved as TIFF files. With the plants remaining in the same position, the dark box was closed and the photon counting camera was set to record photon emission over 4 seconds. The software (EG+G Berthold Luminograph 980) produced an image that represented the location and intensity of photon emission. The image was recorded in pseudocolour and a logarithmic colour scale was used to represent the different levels of photon emission. These pseudocolour images were saved as TIFF files and were eventually overlaid onto

the corresponding brightfield. This gave pictures in which the distribution and intensity of luciferase activity could be located to specific regions in the plant.

Low levels of fluorescence were detected in plants not treated with luciferin solution. This fluorescence disappeared very rapidly (2 minutes). The endogenous luminescence is probably due to chlorophyll chemiluminescence (Abeles, 1986) and is typically seen in plants suddenly moved into darkness (Miller *et al.*, 1992). To avoid interference and possible distortion of the results, photon emissions were measured after background fluorescence disappeared.

Photon counting was carried out within 5 minutes of luciferin application and plants were not reused. This step was taken since it was reported by Jorda *et al.* (1999) that luciferin treatment can alter the expression of several defence related genes.

DAB Stain (2.11)

Arabidopsis seedlings or plants were excised just above soil level and placed into 50ml Nunc Falcon tubes. The plants were completely covered with a solution of 0.1% (w/v) 3, 3-diaminobenzidine and placed on an orbital shaker at 30 rpm for 18 hours. The DAB stain was poured off and the chlorophyll was removed by boiling the plants in 96% ethanol for 10 minutes (younger seedlings) or 40 minutes (older plants). Cleared plants were placed in petri dishes and covered with dH_2O, which enabled a more effective positioning of the test subject and reduced the anomalous surface glare created by patches of ethanol. Seedlings and larger plants were then photographed. DAB staining is an appropriate method for the visualisation of H_2O_2 increases *in planta* (Thordal-Christensen *et al.*, 1997). DAB is rapidly absorbed by the plant, and may then instantly and locally polymerise in the presence of H_2O_2 and peroxidase. The

polymerisation results in a very visible dark brown stain (Thordal- Christensen *et al.*, 1997).

Statistical Analysis (2.12)

The collation of data and production of graphs was carried out on Microsoft Excel 97 for PC. Banks of data were transferred from Excel into Minitab (Windows 13.20) and a multivariate ANOVA (Analysis of Variance) and Bonferroni procedure were both conducted at 95% confidence. The output from the multivariate ANOVA tells us the effect of single and multiple interacting factors on the data.

When conducting a multivariate ANOVA, it is often useful to use other statistical tools to determine what specific data points are different. The Bonferroni test uses a pairwise comparison of data points, whereby each data point is sequentially compared with all the rest. The output from such analysis shows the 95% confidence for each comparison. If the interval contains 0, then there is no significant difference between the groups. If 0 is not contained by the interval, then the groups are statistically significant. The results of the statistical analysis can be found in the appendices section of the thesis, presented in terms of confidence intervals.

Cecchini *et al.* (1998, 2002) have identified ecotype-specific differences in the response to CaMV infection. Therefore, throughout the statistical analysis, WS (*cai 160*) and Col-0 (*cai 171* and *cai 181*) backgrounds were treated as separate data sets to avoid the risk of introducing a type 1 error. Such errors increase the chance of researchers finding a statistical difference when none exists (the null hypothesis gets rejected).

Chapter 3

The effect of CaMV infection on carbohydrate levels in turnip

Introduction (3.1)

It is possible that in virus infected plants, the perturbations seen in sugar partitioning (Section 1.6) might affect sugar-regulated gene expression, in particular those encoding chloroplast protein complexes. In transgenic tobacco plants in which sugars are forced to accumulate by over-expressing a yeast invertase gene, a mosaic phenotype and stunting ensue, reminiscent of symptoms of a compatible virus infection (Von Schaewen *et al.*, 1990). Alterations in sugar concentrations are therefore a plausible mechanism for the elicitation of symptoms. Possibly, increases in sugar levels in localised regions of the leaf may lead to chlorosis. Subsequently, neighbouring cells may produce more green pigments to compensate for the loss in carbon fixation, resulting in mosaic formation.

In this chapter, the possible role of sugars in the development of symptoms is discussed. This was examined in turnips infected with four different CaMV isolates which induce symptoms ranging from very mild to very severe. The soluble sugar and virus levels in these plants was measured and statistically analysed at various stages of symptom development. By examining the interactions between symptom timing and severity with virus levels and sugar accumulation, this enabled the relationship between soluble sugars and symptoms to be assessed.

Results (3.2)

Turnip symptom phenotypes after infection with various CaMV isolates (3.2.1)

After viral inoculation with mild (Bari-1), intermediate (Baji-31) and severe isolates (Aust and Cabb B-JI), symptom development was observed every week (Section 2.3.3). Plants were photographed at 21 dpi, since this was the optimal time for symptom expression prior to senescence.

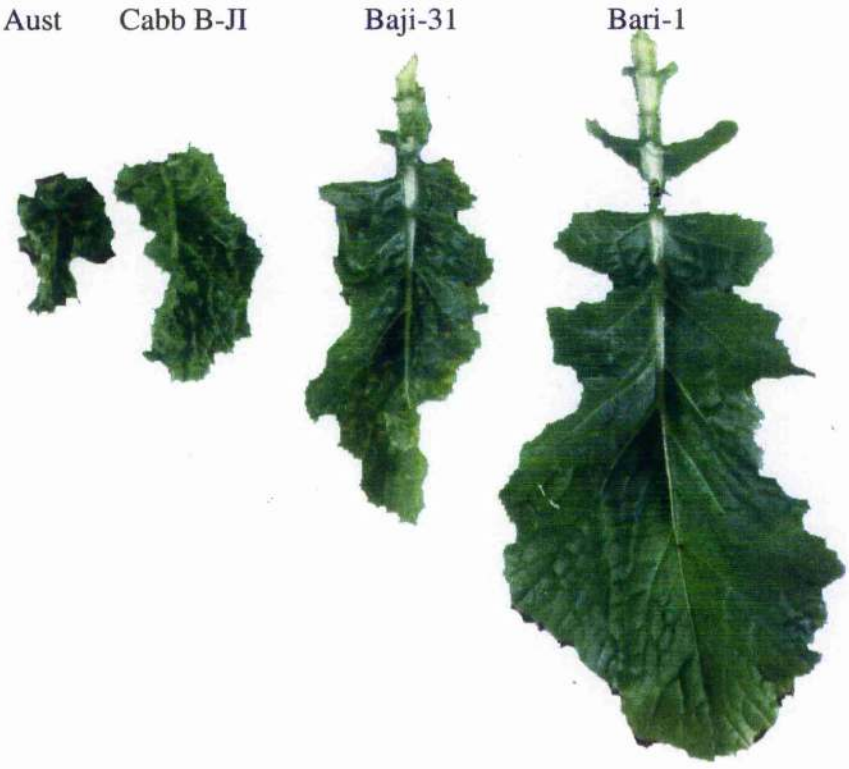
In all virus inoculated plants at 7 dpi, chlorotic local lesions appeared on the inoculated leaves. At 14 dpi mosaic patterning, mild stunting and leaf malformation were present in turnip inoculated with the severe isolates Aust and Cabb B-JI. In comparison Baji-31 induced no stunting or leaf malformation, however mosaics were present. At this time, no systemic symptoms were observed in Bari-1 infected plants.

Stunting and mosaic symptoms were very extensive in turnip inoculated with Aust or Cabb B-JI at 21 dpi (Figure 4). In contrast, stunting and leaf malformation were very mild in Baji-31, and non-existent in Bari-1-infected plants (Figure 4). During this period, Baji-31 infected plants developed a more extensive mosaic pattern, however Bari-1 infected plants displayed an unusual systemic dark green colouration. At 28 dpi the symptoms of virus infection were very severe in Aust infected plants and most plants died at 35 dpi. In contrast, although Cabb B-JI produced severe symptoms in turnip, the degree of stunting and malformation were consistently less than that seen in Aust infected plants. Baji-31 produced symptoms comparable to those seen in Cabb B-JI 7 days earlier, i.e. strong mosaic pattern and increased stunting. In comparison, Bari-1 infected plants had no stunting, were

similar in size to uninfected plants and had dark green leaves without any mosaics. These results are consistent with those reported previously (Cecchini *et al.*, 1997).

Figure 4: Symptom severities in turnip inoculated with different CaMV isolates at 21 dpi

Turnip plants were inoculated with either Aust, Cabb B-JI, Baji-31 and Bari-1 (Section 2.3.3). At 21 dpi the seventh leaf was excised from each virus inoculated turnip and photographed. Each picture below represents the symptoms that were observed in 5 or more plants.



Accumulation of different CaMV isolates in turnip (3.2.2)

It is possible that the different symptom severities observed between virus isolates might reflect differences in the rate of virus replication and accumulation. In order to measure virus accumulation, total DNA was extracted (Section 2.5) from duplicate samples taken from source and sink leaves of infected plants at 7, 14, 21 and 28 dpi. Levels of virus DNA were then assayed in dotblots by quantifying the levels of hybridisation (Sections 2.5 and 2.6). The results were analysed statistically using a multivariate Analysis of Variance (ANOVA) and Bonferroni test, as described in Section 2.13.

At 7 dpi virus was undetectable in source and sink leaves (Figures 5a and 5b). At this stage there appears to be little or no detectable virus spread from the inoculated leaf. At 14 dpi, in both source and sink leaves, virus levels were greatest in plants infected with the more severe isolates (Aust and B-JI), whereas milder isolates Bari-1 and Baji-31 had less accumulation (Figures 5a and 5b). In source leaves, virus levels of the two most severe isolates increased ($P<0.05$) up until 21dpi, after which a plateau was reached (Figure 5a). In comparison, for the milder isolates Bari-1 and Baji-31, levels reached their maximum accumulation at 14 dpi and then plateaued (Figure 5a). In sink leaves, all virus isolates reached their maximum levels at 14 dpi, after which levels decreased (Figure 5b).

All virus isolates accumulated to a greater extent in source leaves when compared with sink leaves. In order to further analyse the relationship between virus accumulation in source and sink leaves, the ratio of virus levels in source and sink leaves were examined.

The three most severe isolates (Aust, B-JI and Baji-31) had similar source/sink ratios ($P>0.05$; Figure 5c). In contrast Bari-1 had significant 6-fold elevations in the ratio of source/sink virus levels when compared with other isolates at 14 and 21 dpi ($P<0.05$). However, by 28 dpi all virus isolates had similar ratios (Figure 5c). This suggests that perhaps Bari-1 has reduced ability to move from source tissues into sink leaves, or may have significantly lower replication rates in sink tissues. It has been reported that with some mild virus isolates, gene silencing mechanisms may be activated, causing amelioration of symptoms and reduction in virus levels in sink tissues (Matthews, 2001; Al Kaff and Covey, 1995). However, the low levels of Bari-1 in sink tissues are probably not due to silencing mechanisms, since rather than recovery, symptoms became progressively more severe.

Generally, these results indicate that virus levels do not always correlate with the severity or type of symptoms that develop after infection. For example Bari-1 accumulated to similar levels as Baji-31, even although their symptom phenotypes were very different. These results confirm and extend those reported by Cecchini *et al.* (1998).

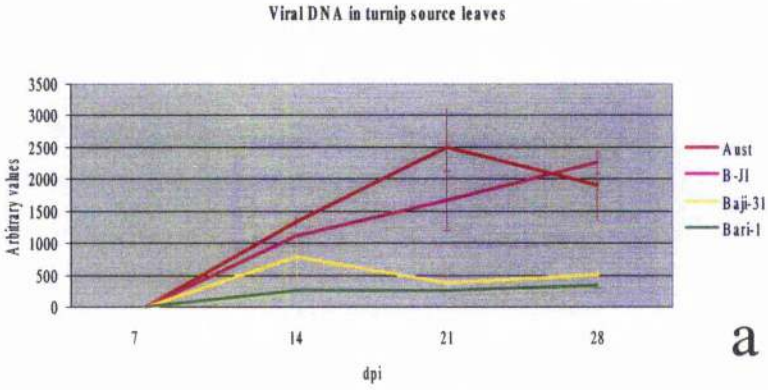
Figure 5: The accumulation of several CaMV isolates in turnip over 28 days

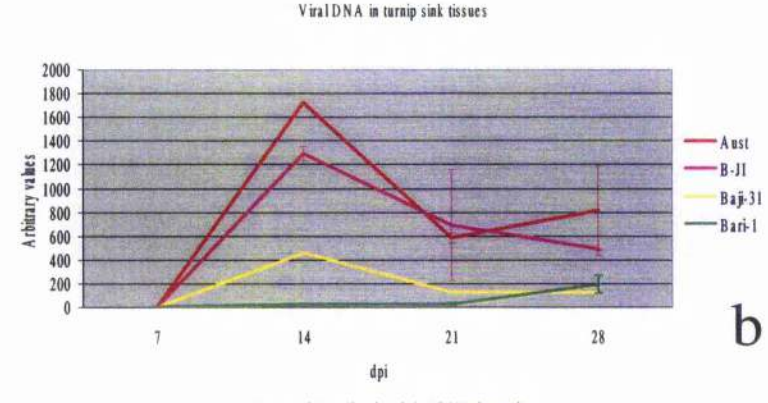
Turnips were infected at the two-true leaf stage of development with either Bari-1, Cabb B-JI, Baji-31 or Aust (Section 2.3.3). Total DNA was extracted (Section 2.5) from duplicate samples, each taken from the expanded (source) and emergent (sink) leaves of four infected plants at 7, 14, 21 and 28 dpi. Levels of virus DNA were then assayed in dotblots by quantifying the levels of hybridisation to a viral gene VI fragment (Section 2.5 and 2.6). Error bars represent the standard deviations of duplicate samples.

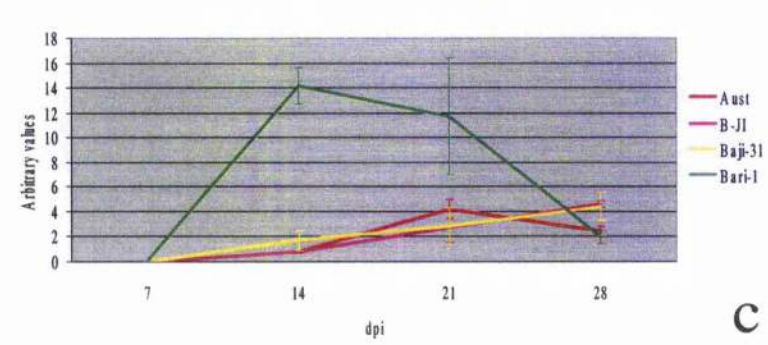
(a) Viral DNA levels in source leaves.

(b) Viral DNA levels in sink leaves.

(c) Ratio of viral DNA in source leaves compared with sink leaves.







Effect of CaMV infection on sugar levels in turnip (3.2.3)

To test the hypothesis that changes in levels of soluble sugars may be able to modulate aspects of symptom development, sugar levels in turnip infected with the four different virus isolates were measured at 7, 14, 21 and 28 dpi. At these time points, tissue samples were taken from source and sink leaves. Samples were taken in triplicate from 12 plants, each sample being derived from tissue pooled from 4 plants. This was carried out for both CaMV- inoculated and mock- inoculated samples. Levels of D-glucose, D-fructose and sucrose were analysed as described in Section 2.8. The data was analysed statistically using a multivariate Analysis of Variance (ANOVA) and Bonferroni test, as described in Section 2.13.

In uninfected plants, both source and sink leaves showed a steady increase in the level of total soluble sugars (D-glucose, D-fructose and sucrose) as plants became older (Figures 6a and 6b). Generally, in source leaves, sugar levels were not significantly different ($P>0.05$) between infected and uninfected plants (Figure 6a). However at 28 dpi, Aust-infected turnip had sugar levels 4-fold higher ($P<0.05$) than those of other isolates and uninfected plants (Figure 6a). In sink leaves there was little difference between sugar levels in uninfected plants and plants infected with Bari-1 and Baji -31 isolates (Figure 6b). In contrast, both Aust and Cabb B-JI infected plants had elevated levels (3-fold) of total soluble sugars that reached a maximum at 21 dpi (Figure 6b). At 28 dpi sugars decreased to levels that were similar to those in plants infected with other virus isolates and in uninfected plants (Figure 6b). The rise and then fall of sugar levels in sink leaves of Aust and Cabb B-

JI infected plants was statistically significant ($P<0.05$). Similar results were obtained in a second independent experiment (data not shown).

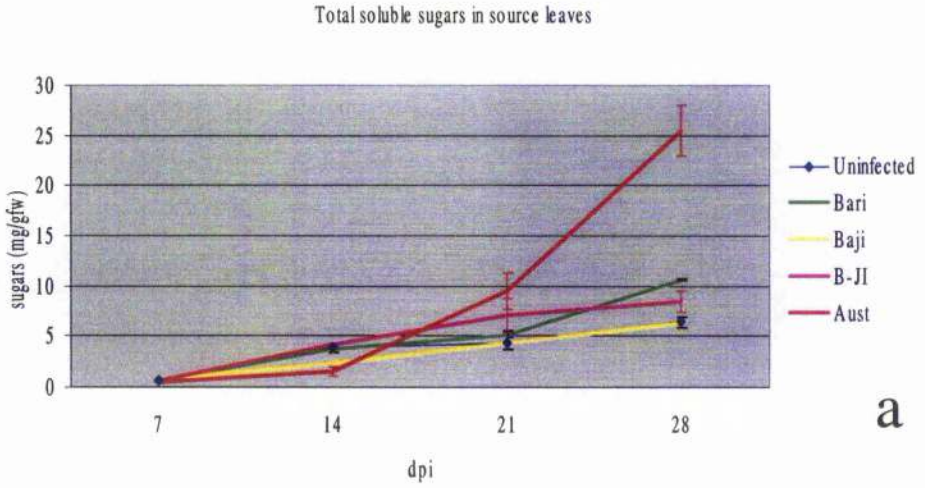
In source leaves at 14 dpi, just as systemic symptoms were appearing, sugar levels in turnips infected with all four isolates were similar. This implies that sugars might not be directly involved in modulating the development of symptoms. However at later stages of infection, it is possible that very severe symptoms might lead to perturbations in sugar levels. For example, the very severe stunting of sink tissues at 21 dpi in Aust and Cabb B-JI infected turnip coincided with a significant increase in the level of sugars. Since sugar levels at 14 dpi were similar in plants infected with virus isolates that have very different replication rates, it suggests that sugars are not affected by or have little affect on virus replication.

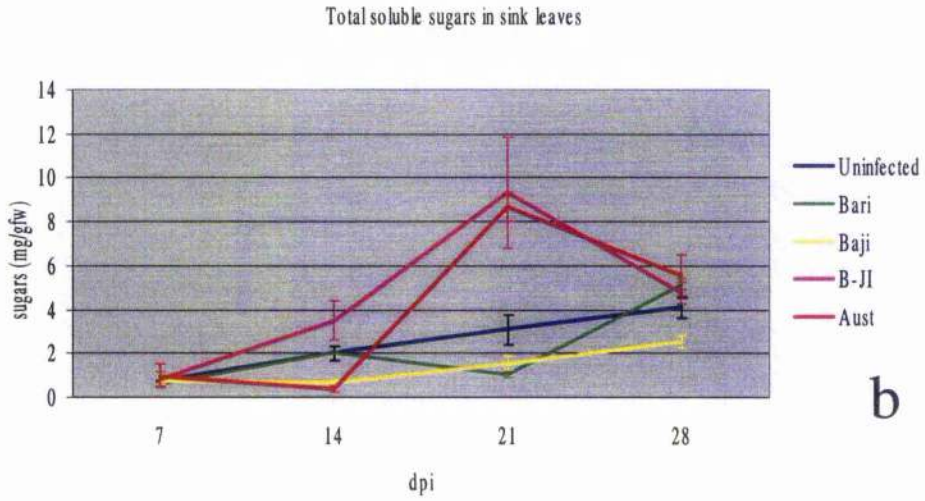
Figure 6: The accumulation of soluble sugars over 28 days in turnip infected with several CaMV isolates.

Turnips were infected at the two-true leaf stage of development with either Bari-1, Cabb B-JI, Baji-31 or Aust (Section 2.3.3). Uninfected plants were inoculated with water only. Total soluble sugars (D-glucose, D-fructose and sucrose) were extracted (Section 2.8.1) from triplicate samples, each taken from the expanded (source) and emergent (sink) leaves of four plants from each treatment at 7, 14, 21 and 28 dpi. Levels of total soluble sugar were quantitated by a linked enzyme assay (Section 2.8.2). Error bars represent the standard deviations of triplicate samples.

(a) Total soluble sugar levels in source leaves

(b) Total soluble sugar levels in sink leaves





Effect of CaMV infection on starch levels in turnip (3.2.4)

Although virus infection was associated with alterations in the levels of sugar, it is possible that the conversion of sugars into starch may have masked the extent of these changes. Therefore starch levels in infected and control plants were compared. The samples used for starch analysis were derived from the samples used for the sugar analysis (Materials and Methods Section 2.8.3). The results were statistially analysed using a multivariate Analysis of Variance (ANOVA) and Bonferroni test, as described in Section 2.13.

In uninfected plants, starch increased in source and sink leaves as time progressed (Figures 7a and 7b). Virus infection generally reduced the level of starch in source leaves when compared to the uninfected controls (Figure 7a). At 28 dpi, the source leaves of plants infected with the three most severe isolates had one quarter of the starch levels of uninfected controls. These reductions were statistically significant ($P<0.05$). In contrast, the mildest isolate Bari-1 induced only slight reductions in starch levels in source leaves when compared with the uninfected control (Figure 7a). In sink tissues up until 21 dpi, there was no significant difference ($P>0.05$) in the level of starch between uninfected and infected plants (Figure 7b). However by 28 dpi, starch levels in turnip infected with Cabb B-JI and Baji-31 isolates were significantly ($P<0.05$) lower than the other isolates and uninfected controls (Figure 7b).

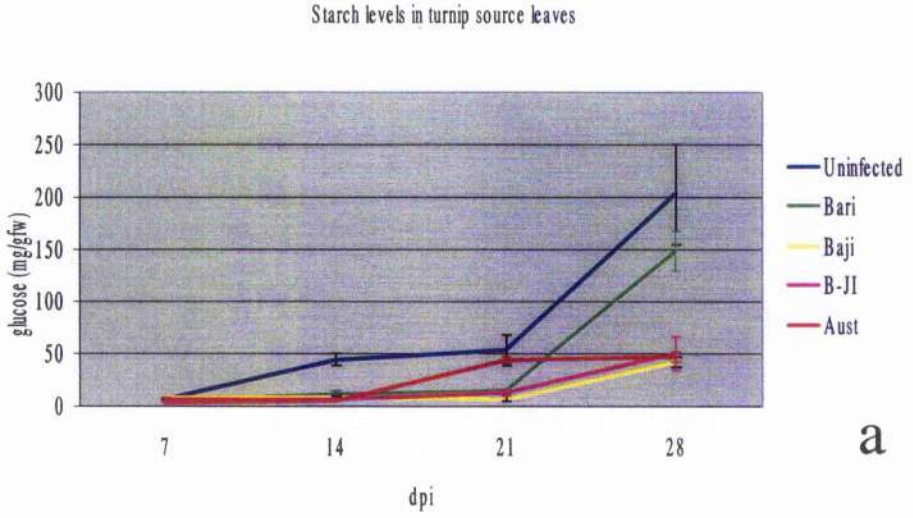
The effect of virus infection was generally to lower starch levels in source and sink leaves. However, despite the large differences in levels of virus accumulation, there was no significant difference in starch levels between plants

Figure 7: The accumulation of starch over 28 days in turnip infected with several CaMV isolates.

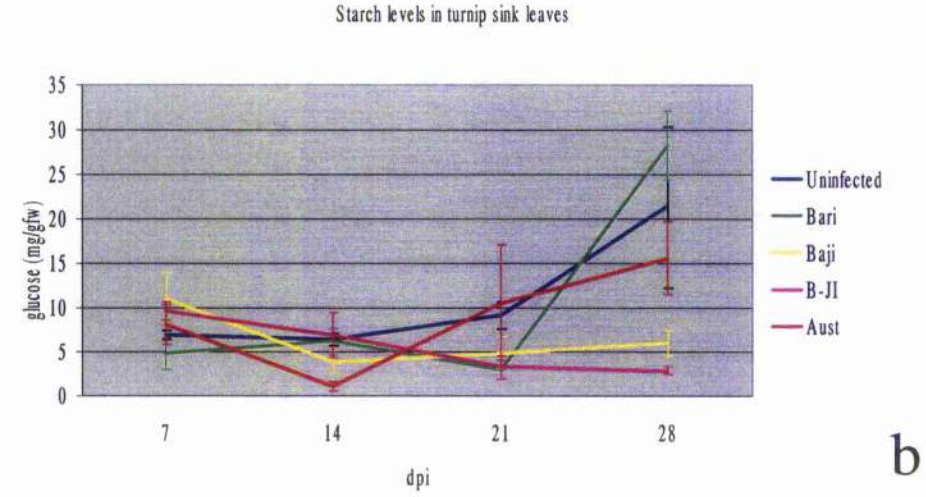
Turnips were infected at the two-true leaf stage of development with either Bari-1, Cabb B-JI, Baji-31 or Aust (Section 2.3.3). Uninfected plants were water inoculated. Starch accumulation was measured using the same samples which were previously used to measure total soluble sugars. Therefore starch was assayed (Section 2.8.3) from triplicate samples, each taken from the expanded (source) and emergent (sink) leaves of four plants from each treatment at 7, 14, 21 and 28 dpi. Error bars represent the standard deviations of triplicate samples.

(a) Starch accumulation in source leaves

(b) Starch accumulation in sink leaves



a



b

Effect of CaMV infection on sugar/starch ratios in turnip (3.2.5)

Clearly there are perturbations in both sugar and starch levels as a result of virus infection. It is possible that these perturbations may arise because infection may interfere with the interconversion of starch and sugars. In order to investigate this sugar/starch ratios were compared.

In source leaves of uninfected plants the sugar/starch ratios declined with time (Figure 8a); this may reflect increased starch accumulation (Figure 8a). In source leaves (Figure 8a), virus infection generally increased the ratio of sugar to starch compared with uninfected plants; these changes are probably a consequence of a decrease in the levels of starch (Figure 8a). When comparing amongst plants infected with different isolates, the sugar and starch ratios do not follow an observable pattern (Figure 8a). Therefore the interactions between sugar and starch are clearly very complex and difficult to dissect. However, in sink tissues this was slightly less problematic and therefore more information was retrieved.

In the sink leaves of uninfected plants, slight increases in sugar/starch ratios were observed with age (Figure 8b). In the sink tissues of plants infected with mild isolates Baji-31 and Bari-1, sugar/starch ratios were not significantly different ($P>0.05$) from those in uninfected plants (Figure 8b). In contrast, at 21 dpi in plants infected with severe isolates Cabb B-JI and Aust, ratios were significantly ($P>0.05$) elevated (6-fold and 2-fold respectively) when compared to plants infected with milder isolates and uninfected controls (Figure 8b). This increase is probably a consequence of the very elevated sugar levels that were detected in the sink leaves of Aust and Cabb B-JI infected plants at 21dpi (Figure 8b).

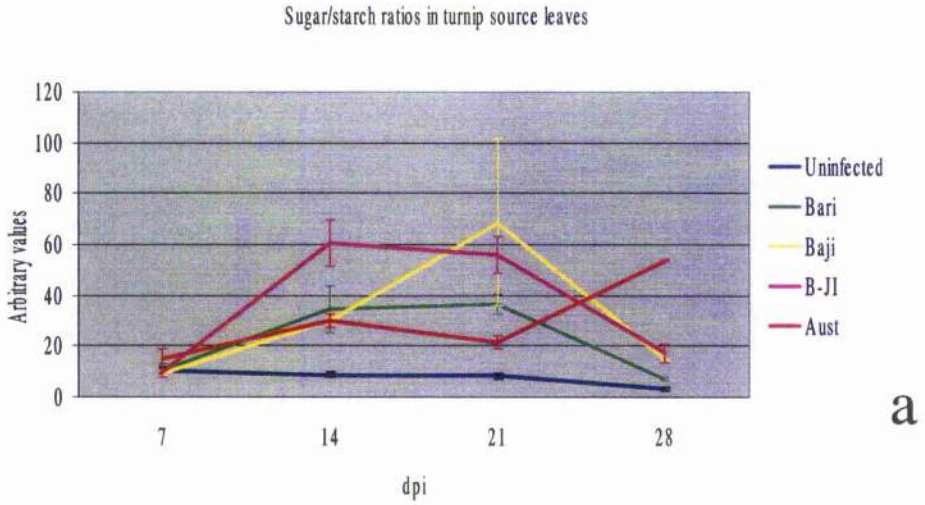
The sugar/starch ratio results are not easily interpreted. Interconversion of starches and soluble sugars involves a complex set of pathways, and is further complicated by the pleiotropic effects of virus infection.

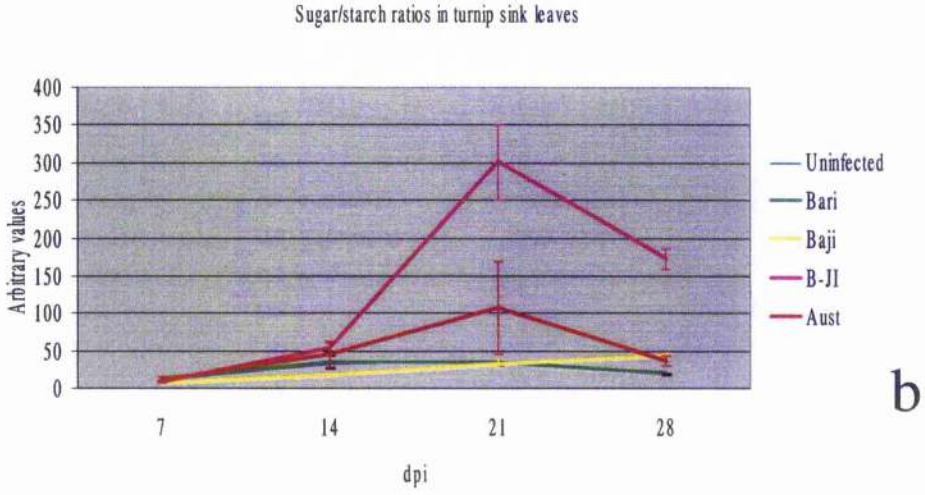
Figure 8: The ratio of sugar to starch over 28 days in turnip infected with several CaMV isolates.

The ratio of sugar/starch was determined in expanded (source) and emergent (sink) leaves of uninfected, Bari-1, Baji-31, Aust and Cabb B-JI infected plants at 7, 14, 21 and 28 dpi. Since sugar and starch levels were both derived from the same samples, the ratio of sugar to starch was calculated by dividing the appropriate sugar levels by the corresponding starch level. Error bars represent the standard deviation of triplicate data points.

(a) Sugar/ starch ratios in source leaves

(b) Sugar/ starch ratios in sink leaves





Summary (3.3)

In turnip, the most severe virus isolates accumulated to significantly higher levels than milder isolates. However, the levels of virus accumulation did not directly correlate with the severity or type of symptoms that developed. These results confirm and extend those reported by Cecchini *et al.*, (1998). This implies that there cannot be a simple causal correlation between the metabolic drain incurred in supporting virus replication and symptom development. Although levels of CaMV accumulation do not show a direct correlation with symptom severity, other factors, in particular sugar signalling may play a role. In turnip although levels of free soluble sugar became elevated in plants infected with severe CaMV isolates, this did not occur until much later than the appearance of symptoms. Also, although levels of virus accumulation differed between CaMV isolates, there was no obvious correlation between virus levels and sugar levels. This argues strongly against a simple model in which sugar levels directly potentiate symptom development. However, since virus infection can repress or up-regulate host genes, the converse, that altered sugar levels could be a consequence of symptom development is possible. For example, in the later stages of infection (21 dpi), at which time sink tissues infected with severe CaMV isolates (Aust and Cabb B-JI) had become severely stunted, transient increases in levels of free soluble sugars were observed. This build up of sugars could be a consequence of the cessation of growth, imported sugars no longer being required to fuel leaf expansion. This increase was a transient effect, possibly with excess sugars being sensed and redistributed to other areas in the plant. Thus, although there is no evidence to indicate that sugars potentiate

symptom development in turnip, the converse that symptoms may have an impact on sugar levels remains a distinct possibility.

Sugars form the metabolic precursor of starch, and levels of starch were significantly reduced as a result of infection in turnip. However, there was no obvious correlation between virus levels and changes in starch accumulation. These reductions in starch do not appear to be merely a direct consequence of the metabolic burden imposed on the plant by virus replication. In addition, the changes in starch levels did not correlate with either symptom development or severity. This implies that starch is not directly involved in the elicitation of symptoms.

Chapter 4

The interactions between carbohydrates and symptoms in *Arabidopsis*

Introduction (4.1)

Analysis of sugar levels in CaMV-infected turnip has demonstrated that photosynthate accumulation and partitioning is greatly altered in infected compared to uninfected plants. This suggests that perhaps sugar signalling mechanisms are altered during infection. Further investigation of this hypothesis is limited by the non-availability of sugar sensing mutants in turnip. However, several putative sugar-sensing mutant *Arabidopsis thaliana* lines are available. These mutants are a potentially valuable tool, enabling us to further probe the impact of sugar signalling (rather than sugar accumulation) on virus induced symptom development.

Arabidopsis thaliana putative *ca*rbohydrate *i*nsensitive (*cai*) mutants were first identified by screening EMS mutagenised populations for altered response to low nitrogen and high sucrose (I. A. Graham and T. Martin, unpublished; Boxall, S., 1999; Martin *et al.*, 2002). It is believed that under high sucrose and low nitrogen, plants reduce the accumulation of sugars by utilising them as carbon skeletons to produce amino acids (Stitt and Krapp 1999). However, simultaneously lowering the nitrogen levels adversely affects the removal of sugars by this route, and the sugar concentrations build up (I. A. Graham and T. Martin, unpublished; Boxall, S., 1999; Stitt and Krapp 1999; Martin *et al.*, 2002). As sugar concentrations increase, wild type plants develop chlorosis, accumulate anthocyanins and rapidly die (Plate 1). However two mutants, *cai 160* and *181*, which survived for much longer under these conditions, remained green with only small amounts of visible anthocyanins on the

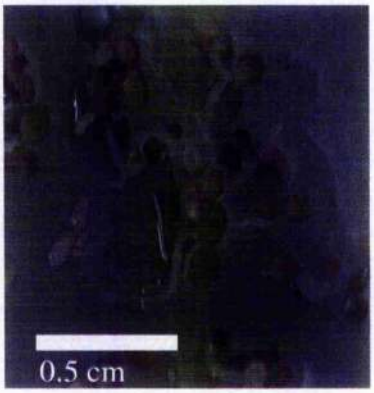
underside and edge of the leaf (Plate 1). Another mutant, *cai 171,* grew very well on the high sucrose low nitrogen medium but was light green in colour with no visible accumulation of anthocyanins (Plate 1). It was proposed that these altered responses to high sucrose might be a result of lesions in the sugar signalling and response pathways. By analysing the response of these mutants to CaMV infection, it was hoped that the involvement of sugar signalling in symptom development could be assessed.

Plate 1: Differences in phenotype between *cai* mutant and wild type *Arabidopsis*, when screened on high sucrose and low nitrogen selection media

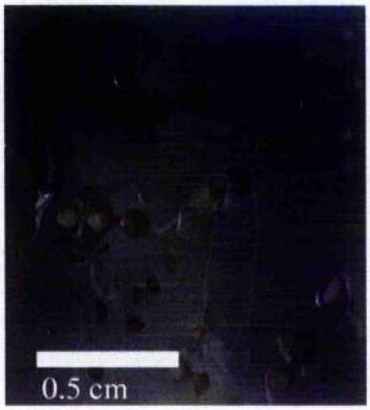
Around 200 seeds of WS, Col-0, *cai 171*, *181* and *160* were sown on 100mM sucrose and low nitrogen (Section 2.9) agar media. Eight days after germination differences in phenotype between wildtype and mutant plants were compared (*cai 181* and *171* were compared with Col-0, *cai 160* was compared with WS) and photographed. As an additional control, wild type Col-0 and WS were grown on standard MS media, which contains levels of nitrogen and sucrose that are appropriate for normal growth (Section 2.9)

Wild type (Col-0 and WS)

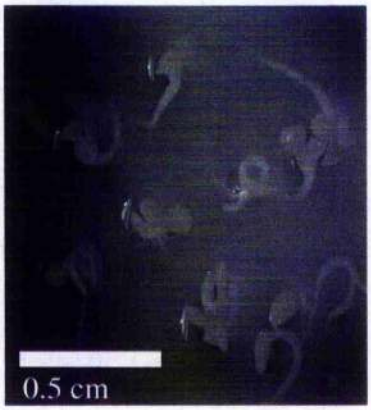
cai 160

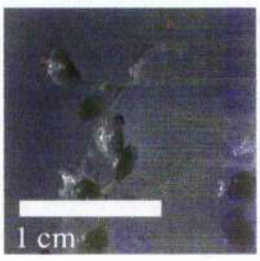


cai 181



cai 171





Wild types Col-0 and WS grown on standard MS media.

Results (4.2)

cai mutant symptom phenotypes (4.2.1)

In order to analyse the effect of infection on these mutants, batches of 50 *cai 160*, *cai 171*, *cai 181*, wild types Col-0 and WS *Arabidopsis* (*cai 171* and *cai 181* are in a Col-0 background and *cai 160* is in a WS background) were grown in soil under the conditions normally used for virus infection. At the two true leaf stage of development plants were inoculated with CaMV (Cabb B-JI) or H_2O (Section 2.2.3). At 21 dpi the symptoms were scored and plants photographed.

At 21 dpi, mock inoculated *cai* mutants and their respective background controls were similar in appearance (Plate 2). Although all virus infected plants developed systemic symptoms, some differences were observed between *cai* mutants and their respective wild type backgrounds. The wild types WS and Col-0 displayed typical systemic symptoms such as stunting, leaf crinkling and chlorosis (Plate 2). Symptoms in *cai 160* were indistinguishable from infected WS (Plate 2). In contrast, *cai 171*, compared to infected Col-0, showed much reduced crinkling and stunting, and had more fully expanded leaves (Plate 2). *cai 181* consistently developed more severe stunting and leaf malformation than wild type controls, it also developed extensive and unusual pink tinged chlorotic regions (Plate 2).

Plate 2: *cai* mutants infected with CaMV after 3 weeks

Approximately 500 plants of each wild type and *cai* mutant were infected at the two true leaf stage of development with Cabb B-JI (Section 2.3.3). Three weeks after inoculation the symptom phenotypes between each of the plants was compared and photographed (*cai 181* and *171* were compared to Col-0, *cai 160* was compared to WS). The plants shown below are representative of the phenotype observed in each group. All uninfected plants were identical to uninfected Col-0, and both wild types showed identical symptoms.

Uninfected Col-0

Col-0

cai 160

cai 171

cai 181

Two of these *cai* mutants show unusual and interesting responses to virus infection. However, Graham *et al.*, (unpublished) reported that occasionally, small numbers of *cai 160* and *181* seedlings on the sugar screen media showed a wild type phenotype. It was initially thought that these were genetic revertants and that the mutations were unstable. However, when the revertants were quickly rescued and used to produce seed, their progeny produced the typical *cai* phenotype. This suggested that the incomplete penetrance of the *cai* phenotype might be epigenetic.

Various approaches were taken to determine whether the penetrance of the *cai* mutants with respect to virus symptoms was also incomplete. After screening on plates, "phenotypic" and "non-phenotypic" (with respect to sucrose screen phenotype) *cai 160* and *181* plants were transferred to soil. After 7 days growth, when the 2 true leaf stage was reached, 50 phenotypic and 50 non-phenotypic seedlings were infected with virus or mock inoculated. Symptoms in the *cai* phenotypic and non-phenotypic group were indistinguishable (Plate 3). In a further experiment, around 500 soil grown *cai 181* and *160* were infected with CaMV. Within each mutant group, symptoms were consistently similar. Therefore, although the *cai* mutants showed variable penetrance with respect to the sucrose phenotype, penetrance with respect to symptom phenotype was complete.

Plate 3: The effect of different *cai* phenotype penetrances on symptom development in CaMV infected plants

Approximately 1000 cai 160 and 181 seeds were sown on 100mM sucrose and 0.1 mM nitrogen (Section 2.9). After eight days growth, seedlings showing a wild type response (non-penetrant) to the media and those showing the typical *cai* phenotype (penetrant) were transferred to soil. Once plants reached the two true stage of development, they were infected with Cabb B-JI (Section 2.3.3) and the symptom phenotypes between the penetrant and non-penetrant mutants were compared. The results shown below are representative images of 50 plants.

	"Non-phenotypic" *cai* mutants	Phenotypic *cai* mutants
cai 160		
cai 181		

Virus levels in *cai* mutants (4.2.2)

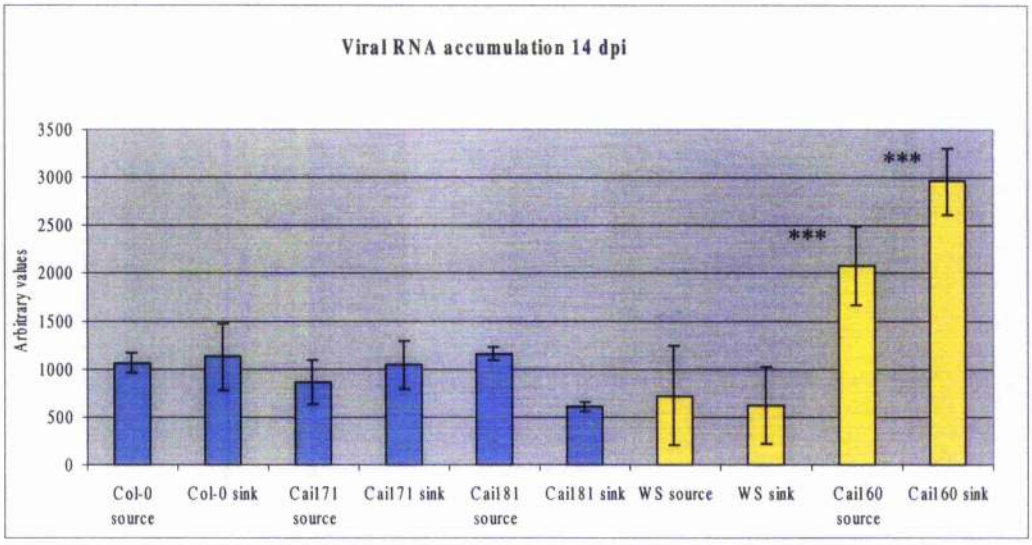
At least two of the putative sugar sensing mutants showed altered symptom expression. To test whether this was a consequence of altered virus load, virus levels were measured at 14 dpi. Triplicate samples were taken from 12 plants, each sample being derived from pooled tissues of 4 plants. Samples were taken from the source and sink leaves of infected *cai* mutants and appropriate wild type backgrounds; Col-0 for *cai 171* and *181*, WS for *cai 160*. Total RNA was extracted from the samples, and the level of replicating virus was determined by quantitating the amount of viral 35S and 19S RNA in Northern blots. This data was statistically analysed using multivariate ANOVA and Bonferroni at 95% (Section 2.12). These results are shown in Figure 9 below.

In wild type plants there was no significant difference ($P>0.05$) in levels of viral RNA between source and sink leaves; these results are in agreement with the findings of Cecchini *et al.* (2002). Levels of viral RNA accumulation in *cai 181* and *171* were not significantly different from levels in infected Col-0 ($P>0.05$), despite the fact that *cai 171* developed mild symptoms, and *cai 181* developed very severe symptoms. There was also no statistically significant difference in viral accumulation between *cai 171* and *cai 181*. In contrast to the other two mutants, *cai 160* showed highly significant ($P<0.01$), up to 4-fold elevations in viral RNA levels, although it had similar symptoms to wild type WS (Figure 9). Although one of the *cai* mutants (*cai 160*) accumulated significantly elevated levels of virus, differences in symptom phenotype in the *cai* mutants did not show any obvious correlation to the levels of virus accumulation.

Figure 9: Viral RNA concentrations in source and sink leaves of infected wild type and mutant plants at 14 dpi.

Wild type and *cai* mutant *Arabidopsis* plants were infected at the two-true leaf stage of development with Cabb B-JI (Section 2.3.3). Uninfected plants were inoculated with water only. At 14 dpi, each sample in triplicate was taken from the expanded (source) and emergent (sink) leaves of four plants. From these samples, total RNA was extracted and blotted onto filters (Section 2.4). Viral RNA levels were determined by probing Northern blots with radiolabelled viral gene VI fragments and quantifying the level of hybridisation (Section 2.6). Black stars denote significant differences between mutants and their corresponding ecotypic backgrounds (*cai 171 and181* have a Col-0 background, and *cai 160* has a WS background). Blue bars indicate a Col-0 background and yellow bars a WS background.

Black stars are used to denote significant differences between mutants and their respective ecotypic background. Levels of significance:* $P<0.05$ and *** $P<0.01$ were determined using multivariate ANOVA and Bonferroni analysis (Section 2.12).



Sugar levels in infected and uninfected wild type and *cai* mutant *Arabidopsis* (4.2.3)

Sugar signalling and/or uptake mechanisms might be important in modulating symptom development and virus accumulation in *Arabidopsis*. To further test for possible links between symptoms and sugar levels, wild type and mutant plants were infected with CaMV and sugar levels were assayed at 14 dpi. Triplicate samples were taken from 12 plants, each sample was derived from tissues pooled from 4 plants. Carbohydrate content in source and sink tissues was analysed using a linked enzyme assay (Section 2.8). Results are shown in Figures 8a-f.

As shown in Figure 10a (page 97) uninfected Col-0, *cai 181* and *cai 171* accumulated similar levels of total soluble sugars in sink leaves ($P>0.05$). However, uninfected *cai 181* and *cai 171* both showed significantly higher levels of total sugars in source leaves when compared to Col-0 ($P<0.05$). Uninfected WS and *cai 160* had similar levels of soluble sugar in source leaves and in sink leaves ($P>0.05$). In *cai 160* levels of sugar accumulation was significantly higher in source leaves when compared with sink leaves ($P<0.05$). In the other mutants and wild type plants, there was no difference in sugar levels between source and sink leaves.

As shown in Figure 10b, sugar levels in virus infected *cai 171*, *cai 181* and Col-0 were not significantly different from those measured in corresponding mock inoculated plants ($P>0.05$). When comparing sugar accumulation in infected plants (Figure 10b), the source leaves of *cai 181* and *171* had significantly higher sugar levels than Col-0 ($P<0.05$). In wild type WS the levels of sugar were not

significantly different between infected and uninfected plants ($P>0.05$). However, infected *cai 160* showed highly significant increases in sugar accumulation compared with the uninfected control; 4-fold in source leaves ($P<0.01$) and 2-fold in sink tissues ($P<0.05$). In addition, infected *cai 160* had significantly higher ($P<0.01$) sugar accumulation in source leaves when compared to infected WS. Therefore, *cai 160* hyper-accumulated soluble sugars following infection.

In contrast to turnip (which developed sugar perturbations in source and sink leaves after symptom development), virus infection had no significant impact upon sugar partitioning or accumulation in wild type *Arabidopsis*. This argues against any direct effect of soluble sugar levels in modulating symptoms. However, it is possible that virus infection might alter the normal level of sugar perception/signalling in the plant, so that induction of sugar-modulated genes occurs even in the absence of any change in the concentration of sugars.

Uninfected and infected *cai 181* and *171* also accumulated similar levels of sugar, despite differences in symptoms. Again, differences in symptom severity in these mutants do not appear to be a direct consequence of differences in the concentration of soluble sugars. However, changes in the perception of sugar levels cannot be excluded from playing a role in symptom development. *cai 160* showed highly significant increases in sugar accumulation in infected plants when compared with the mock-inoculated controls, although its symptom phenotype was similar to wild type. This mutant also showed highly significant elevations in virus accumulation. There may be a link between the elevations in levels of sugar and virus.

Levels of starch accumulation in virus and uninfected wild type and *cai* mutant *Arabidopsis* (4.2.4)

It is possible that excess sugars might be rapidly converted into starch. If this were the case, large differences in sugar levels would not be detectable. To test this hypothesis, starch levels were measured in virus and mock inoculated plants. The results are shown in Figures 8c and 8d.

There was no significant difference in starch levels in uninfected plants between mutants and their corresponding wild type backgrounds (Figure 10c). However, starch levels in source leaves were 5-fold higher ($P<0.05$) than in sink tissues, regardless of genotype (Figure 10c).

Comparing the source leaves of infected Col-0 with uninfected controls, the levels of starch were not significantly different ($P>0.05$; Figure 10d). However, virus infected Col-0 had significant 3-fold starch elevations in sink leaves ($P<0.05$) compared to uninfected plants (Figure 10d). Although there was no detectable differences in starch levels between the source leaves of virus infected Col-0, *cai 171* or *181*, both mutants had significantly less starch accumulation in sink leaves than infected Col-0 (Figure 10d). In *cai 171* and *181*, virus infection had no significant effect on starch accumulation in the source or sink leaves ($P>0.05$), when compared with the uninfected controls (Figure 10d).

In WS, although infection resulted in a 3-fold increase in starch levels in sink leaves, no obvious difference was detected in source leaves (Figure 10d). In contrast, infection in *cai 160* resulted in a significant 2-fold starch decrease in the source

leaves (P>0.05), whereas sink leaves were unaffected (Figure 10d). The starch levels in infected *cai 160* source leaves were significantly lower than those of infected WS.

Virus infected Col-0 and WS had significantly increased starch levels in sink tissues when compared with uninfected controls. Possibly stunted sink leaves are incapable of further growth, and excess resources in the form of translocated sugars that would have been used for growth might be stored as starch.

Virus infection had no statistically significant effect on starch accumulation in *cai 181* and *171*. However in infected *cai 160*, levels of starch in source leaves were significantly lower than in infected wild type WS. Interestingly, *cai 160* accumulates virus and free soluble sugars to a much greater level than infected wild type. The reduced accumulation of starch in infected *cai 160* might be a connected to the high levels of virus replication, with the latter accumulating at the expense of the former. Possibly virus infection in *cai 160* might also interfere with the ability to interconvert sugars and starches.

Sugar/starch ratios in infected and uninfected wild type and *cai* mutant *Arabidopsis* (4.2.5)

To test this, the sugar to starch ratio was analyzed (Figures 10e and f). Sugar/starch ratios were similar amongst uninfected mutant and wild type plants (P>0.05), however *cai 171* had significant 2-fold elevations in sink leaves when compared with wild type Col-0 (Figure 10e). This increase probably reflects the increased level of soluble sugars (Figure 10a), since starch levels were not significantly different from Col-0 (Figure 10c). Infection in wild type plants Col-0

and WS, led to a sugar/starch ratio reduction in sink leaves (Figure 10f). This probably reflects the increased level of starch accumulation in these tissues during infection (Figure 8d), since sugar levels were unaffected (Figure 10b). Infection had no effect on sugar/starch ratios in *cai 171* or *181* (Figure 10f). When comparing infected *cai 181* and *171* with infected Col-0, the ratios were around 3-fold higher in the mutants (Figure 10e). Again the higher levels of soluble sugars in the mutants (Figure 10b), and perhaps also their failure to accumulate starch as a result of virus infection (Figure 10d) are most likely responsible. In source leaves of *cai 160*, infection induced a significant 2-fold increase ($P<0.05$) in the sugar/starch ratio, when compared to infected WS and uninfected *cai 160* (Figure 10f). This difference probably reflects both an increase in sugar level and a decrease in the accumulation of starch in *cai 160* (Figures 10b and d).

The results indicate that virus infection has a significant effect on the levels of carbohydrate, however these changes show little obvious direct correlation with the development of symptoms. This suggests that differences in sugar or starch levels are not involved in symptom development during CaMV infection of *Arabipdopsis*. This does not exclude the possibility that altered perception of sugars may play a role in symptom development.

Figure 10: Levels and ratios of carbohydrate accumulation in infected and uninfected *cai* mutant and wildtype *Arabidopsis*

Wild type and *cai* mutant *Arabidopsis* plants were infected at the two-true leaf stage of development with Cabb B-JI (Section 2.3.3). Uninfected plants were inoculated with water only. At 14 dpi, each sample in triplicate was taken from the expanded (source) and emergent (sink) leaves of four plants. From these samples, total soluble sugar (D-glucose, D-fructose and sucrose) and starch accumulation was determined (Section 2.8). Sugar to starch ratios were calculated by dividing the appropriate sugar value by the corresponding starch value. The error bars represent the standard deviation of triplicate samples. Red stars denote significant differences between mutants and their corresponding ecotypic backgrounds (*cai 171 and181* have a Col-0 background, and *cai 160* has a WS background). Black stars are used to denote significant differences between infected and uninfected plants. Levels of significance:* $P<0.05$ and *** $P<0.01$ were determined using multivariate ANOVA and Bonferroni analysis (Section 2.12).

(a) Total soluble sugar levels in uninfected plants
(b) Total soluble sugar levels in infected and uninfectedplants
(c) Starch accumulation in uninfected plants
(d) Starch accumulation in infected and uninfected plants
(e) Sugar/starch ratios in uninfected plants
(f) Sugar/Starch ratios in infected and uninfected plants

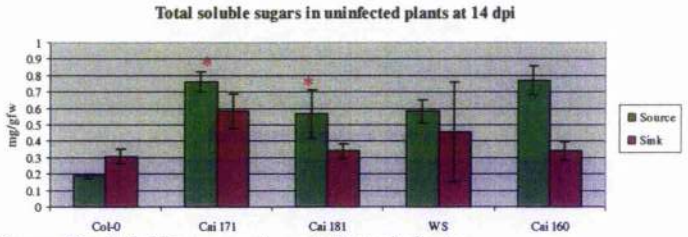


Figure 8a: Soluble sugars in uninfected plants

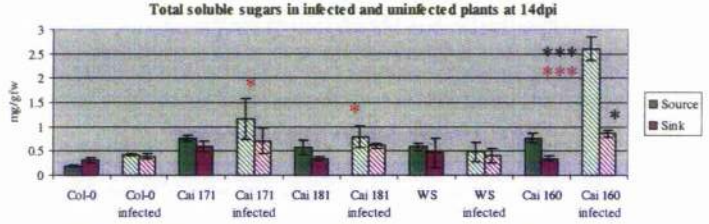


Figure 8b: Soluble sugars in uninfected and infected plants

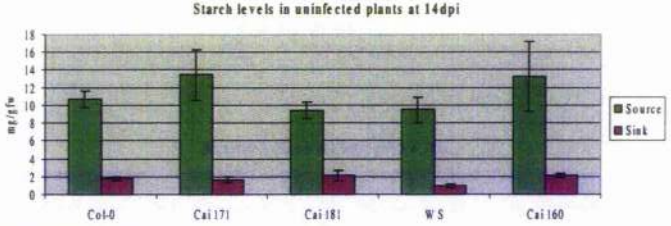


Figure 8c: Starch in uninfected plants

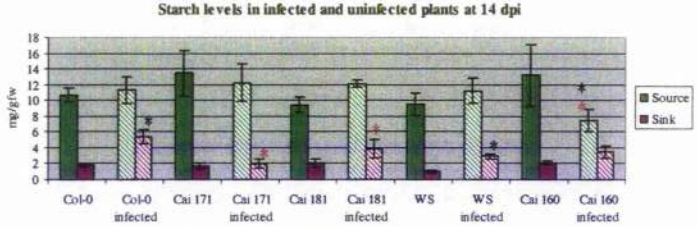


Figure 8d: Starch in uninfected and infected plants

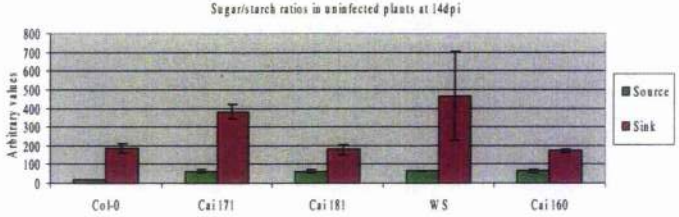


Figure 8e: Sugar/starch ratios in uninfected plants

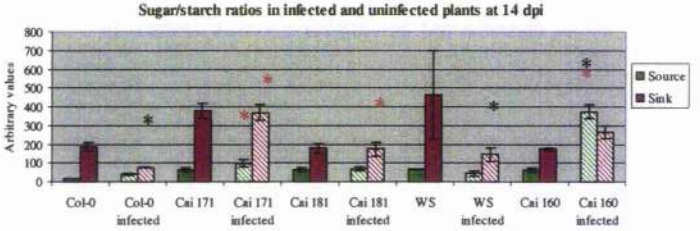


Figure 8f: Sugar/starch ratios in uninfected and infected plants

Sugar related defence pathways and analysis of PR defence gene expression during CaMV infection of *Arabidopsis* (4.2.6)

It has been reported that plants with perturbations in sugar sensing or signalling can also be altered in their defence responses (Herbers *et al.*, 1997). Perhaps in the *cai* mutants, altered defence responses may account for differences in symptom severity and level of virus accumulation.

Preliminary results (Barrett, S. J., Love, A. J., Milner, J. J. unpublished) showed that CaMV infection could strongly up-regulate levels of PR-1 mRNA. The following experiments reported in this section expands upon these preliminary observations and investigates the expression of additional PR defence genes and their possible interactions with sugar signalling and sugar accumulation.

PR-1 (specific marker of SA pathway activation), PR-2 and PR-5 (expression can be activated by SA and other pathways) have been used to monitor the extent of SA related defence responses during infection in *cai* mutant and wild type plants. The expression of these markers was investigated in infected plants at 14 dpi using quantitative Northern blotting (Sections 2.4 and 2.6). RNA samples were obtained from source and sink leaves of infected and uninfected plants. Three samples were taken from a total of 12 plants, with each sample being derived from 4 plants. The results were analysed statistically using a multivariate Analysis of Variance (ANOVA) and Bonferroni test, as described in Section 2.12.

Data from uninfected plants are not shown since PR-1, PR-2 and PR-5 levels were undetectable, even after over exposure of the autoradiographs. PR-1 transcript

levels were greatly elevated in infected wild type and mutant plants at 14 dpi (Figure 11a). The levels observed in *cai 181* and *171* were not significantly different (P>0.05) from their wild type background (Col-0) in either source or sink leaves (Figure 11a). *cai 181* had significantly higher (P<0.05) expression in source leaves than *cai 171*, although sink leaves between the two genotypes were not significantly different (P>0.05; Figure 9a). The PR-1 transcript accumulation in *cai 160* was not significantly different (P>0.05) from its wild type background WS (Figure 11a).

Infected wild type and sugar signalling mutants all had strongly elevated PR-2 transcript levels at 14dpi. PR-2 transcript levels in *cai 171* were not significantly different (P>0.05) from those seen in Col-0 (Figure 11b). In contrast, *cai 181* had significantly higher (P<0.05) PR-2 mRNA levels than both Col-0 and *cai 171* (Figure 11b). No significant differences (P>0.05)were observed between WS and *cai 160* (Figure 11b).

PR-5 transcript levels were highly elevated in all infected plants at 14 dpi. There was no significant difference in levels of PR-5 transcripts between *cai 171* and Col-0 (P>0.05; Figure 11c). However, *cai 181* had significantly higher levels of expression in sink leaves than Col-0 and *cai 171* (P<0.05), although the source leaves between these plants were similar (Figure 11c). When comparing *cai 160* with its background WS, there were no detectable differences (P>0.05) between them with regard to PR-5 transcript levels detected (Figure 11c).

These results indicate that infection can induce a large array of defence responses. Since the previous chapter has shown that sugar levels are not significantly affected by virus infection in wildtype plants, this suggests that sugars

may not be involved in the elicitation of these defences. Differential expression of these defence genes was observed on several occasions in *cai* mutants. It is possible that these differences are due to the pleiotropic effect of the mutations, rather than being directly linked with sugars.

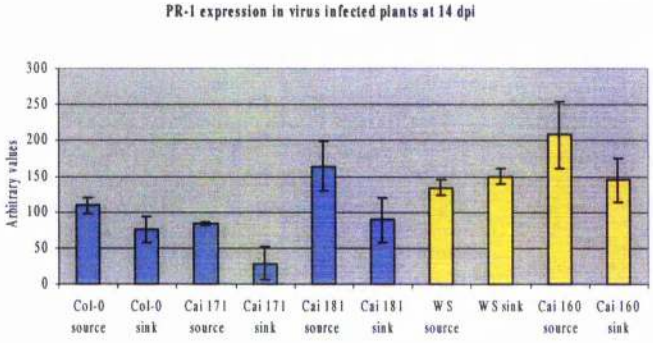
Figure 11: PR defence gene expression in wildtype and *cai* mutant *Arabidopsis* at 14 dpi

Defence gene expression was quantified arbitrarily by probing Northern blots with radiolabelled PR-1, PR-2 and PR-5 DNA fragments, and then measuring the level of hybridisation (Sections 2.5 and 2.6). These Northern blots were the same ones that were previously used to determine the level of viral RNA accumulation in infected *cai* mutant and wild type *Arabidopsis*. The error bars represent the standard deviation of triplicate samples. Black stars indicate significant difference between the *cai* mutants and their respective wild type backgrounds: * $P<0.05$. Statistical significance was determined by using multivariate ANOVA and Bonferroni analysis (Section 2.12). Blue bars indicate a Col-0 background and yellow bars a WS background.

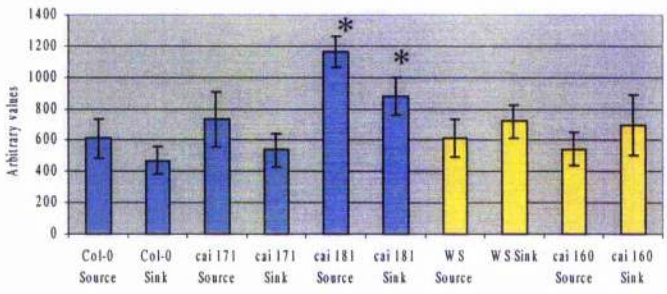
(a) PR-1 gene expression in *cai* mutant and wild type *Arabidopsis* at 14 dpi

(b) PR-2 gene expression in *cai* mutant and wild type *Arabidopsis* at 14 dpi

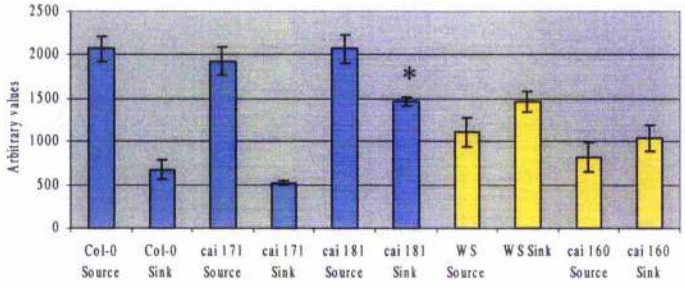
(c) PR-5 gene expression in *cai* mutant and wild type *Arabidopsis* at 14 dpi



a



b



c

Summary (4.3)

cai 171, 160 and *181* are regarded as sugar signalling/uptake mutants. However, it is unknown what these mutations are since they have not been mapped. It has to be considered that the mutations may have no direct involvement in sugar related pathways, with reduced sensitivity to screening conditions being a result of pleiotropic effects. Examining carbohydrate partitioning in these mutants enabled us to determine in what ways the mutations could affect the responsiveness of sugar transport and allocation. *cai 171* and *181* showed elevated sugar levels in source leaves compared to wildtype Col-0, but starch levels were similar. It is possible that *cai 171* and *181* may accumulate sugars until they can be sensed by the plant. Alternatively, these mutants may be less able to convert soluble sugars into starch. It is unlikely that the increase in sugar levels is due to a lesion in sugar transport, since there is little difference in accumulation between source and sink leaves.

There was no significant difference in either sugar or starch levels between *cai 160* and wildtype WS. In wildtype plants there was no difference between levels of soluble sugar in source or sink leaves. However, in *cai 160* the level of sugars was significantly higher in source than in sink leaves. This could suggest that *cai 160* has a lesion in the translocation of sugars, which could impinge on phloem long-distance transport. If indeed *cai 160* is affected in transport of metabolites, this could account for the phenotype seen in the *cai* screen. A mutant that was unable to transport sugars from the plate would not show the repression of gene expression resulting from high levels of sucrose in leaf tissue. Although *cai 160* has altered partitioning of sugars between source and sink leaves, the distribution of virus is unaffected. It is

possible that the lesions in sugar partitioning may manifest themselves at later stages of plant development, which are less critical for virus movement.

The *cai* mutants which clearly have lesions in carbohydrate partitioning, also have abnormal symptom responses to infection when compared with wild type plants. For example both *cai 171* and *181* showed significantly altered symptom phenotypes when compared to wild type plants. However, infection caused no significant changes in sugar levels in wild type *Arabidopsis* or in *cai 171* or *181*. Thus, symptoms cannot be attributable to changes in sugar levels, but an indirect role involving alteration in some aspect of sugar signalling cannot be excluded.

It is possible that there may be some correlation between levels of carbohydrate and virus accumulation in some circumstances. Following infection, *cai 160* had significantly elevated levels of both sugar and virus compared to wildtype. One explanation might be that increased sugar levels are necessary to support the extra drainage of resources required for increased virus replication. The reduced starch levels in source leaves of infected *cai 160* is consistent with this hypothesis. It is interesting to note that neither do extensive changes in carbohydrate partitioning or level of virus accumulation have any effect on symptom severity in this mutant.

Since symptoms and virus levels were affected in the *cai* mutants, it was a possibility that altered sensitivity to sugars could affect the expression of genes involved in limiting pathogen spread and replication. This was examined by assessing the expression of several PR defence genes during infection. Herbers *et al.*, (1996a, 1996b) demonstrated that sugars can increase the expression of PR

proteins in both an SA dependent and independent fashion. In *cai* mutants and wildtype plants at 14 dpi, expression of the PR proteins (which are SA/SAR markers) was detectable only in virus infected plants. Since sugar levels were unaffected in wildtype plants as a result of infection but expression levels of PR-1, PR-2 and PR-5 were greatly elevated, this suggests that signals other than sugars are responsible for elicitation of these defence mechanisms. Despite the differences in symptoms, sugar levels and virus accumulation between *cai 160*, *cai 171* and their respective wildtype backgrounds, the levels of PR-1 expression was similar. Thus sugar signalling probably does not play a direct role in the elicitation of these genes during CaMV infection.

Chapter 5

Analysis of defence pathway activation during CaMV infection

Introduction (5.1)

It was unexpected that such an extensive array of defence genes could be elicited during infection with a compatible pathogen. Since the results suggested that the SA pathway is strongly invoked after infection with CaMV, it was decided that other major defence reactions, such as the oxidative burst and JA/ethylene pathway should be examined.

The behaviour of the oxidative burst, SA and JA/ethylene pathways during CaMV infection was monitored using *Arabidopsis* defence gene promoter:: luciferase reporter lines. The defence gene promoters in these lines are from classical marker genes, which are known to be up-regulated by specific defence pathways (Loake, G. and Yun, B. W., unpublished). The SA and JA/ethylene pathways were scrutinized using transgenic reporter lines of *Arabidopsis* in which corresponding tobacco *PR-1*, and *Arabidopsis PDF1.2* promoters drove luciferase expression.

The oxidative burst can be toxic to plant cells, therefore during ROI formation potent anti-oxidants such as GST1 are expressed in order to limit damage. Since GST1 is a useful marker for the oxidative burst, Grant et al. (2000) designed *Arabidopsis thaliana GST1* promoter::Luciferase reporter plants, which have also been used in experiments in this chapter. Previous work has shown that luciferase activity in all of these luciferase lines reflect the accumulation of defence gene transcripts (Loake, G. and Yun, B. W., unpublished). In addition the

AtGST1::Luciferase lines have been shown to faithfully report H_2O_2 accumulation in a number of different conditions, and during infection by incompatible pathogens (Grant et al, 2000).

Experimental Approach (5.2)

In order to analyse defence gene expression from the very early stages of virus infection right up to and beyond symptom development, luciferase activity was measured at appropriate time intervals after groups of plants were inoculated with CaMV isolate Cabb B-JI (Sections 2.3.3 and 2.10). Controls were mock inoculated with H_2O or received no treatment at all. In the *PR-1*::*Luc* lines, luciferase activity was assessed at 2 hours, 2, 5, 8, 12, 15 and 19 days after inoculation. *PDF1.2*::*Luc* activity was examined at 2 hours, 1, 2, 3, 5, 6, 8, 15 and 19 dpi. Also, *GST1*::*Luc* activity was observed at 2 hours, 1, 2, 3, 4, 5, 6, 8, 12, 15 and 19 dpi. At each time point in the experiment, batches of 2-3 plants from each treatment group were viewed under a photon counting camera, 5 minutes after D-luciferin application. Plants were treated with D-luciferin once only, and viewed immediately because Jorda *et al.* (1999) have reported that luciferin application can give rise to increased *PR-1* expression 48 hours after application. Presymptomatic plants were retained after viewing to check for the subsequent development of symptoms; this ensures that any possible changes in induction would be indicative of successful virus infection. In order to ascertain that the position of the reporter transgene did not affect defence gene expression, for each promoter::Luc construct two independently transformed *Arabidopsis* lines containing the same construct were used in the

experiments. Both of these lines produced similarly consistent and reproducible results, thus the positional effect of the transgene can be discounted.

In addition to using the *AtGST1*::*Luc* lines to assess the oxidative burst during infection, an *in planta* stain was used to visualise the accumulation of H_2O_2. The 3,3' diaminobenzidine (DAB) stain infiltrates the plant and precipitates in contact with H_2O_2 – peroxidase complexes. Regions of elevated H_2O_2 accumulation can be seen as dark brown in the chlorophyll cleared plants (Section 2.11). The staining procedure involved killing the plants, therefore fresh batches of 5 plants were used for each time point and treatment. Three independent replications of each DAB experiment were carried out.

Results (5.3)

Analysis of SA signalling using *PR-1*::Luciferase promoter-reporter plants (5.3.1)

Plants from all three treatment groups (mock-, virus-inoculated and uninoculated plants) showed little luciferase activity up to and including 5 dpi (Plate 4a). However at 8 dpi, in virus inoculated plants high levels of activity in the inoculated leaf, and slightly increased levels throughout the rest of the plant were apparent (Plate 4a). Such increases in expression were not observed in the mock-inoculated and untreated controls (Plate 4a). At 12 dpi, the virus-inoculated plants showed further elevated luciferase activity in the inoculated leaves and some peripheral leaves; however the level of activity in other parts of the plant had fallen compared to 8 dpi (Plate 4a). In contrast, luciferase levels were consistently low to

undetectable in the mock-inoculated and uninoculated controls (Plate 4a). At 15 dpi when systemic symptoms became visible, the level of luciferase activity increased in localised regions of older peripheral leaves, whereas a reduction was detected in other areas of the plant (Plate 4a). Luciferase activity was consistently undetectable in the mock inoculated and uninoculated plants (Plate 4a).

When CaMV infections in *Arabidopsis thaliana* are carried out, approximately 85% of plants displayed similar symptoms, however some plants develop unusually mild symptoms. At 19 dpi, plants that had developed the atypical mild symptoms (with virtually no crinkling or distortion of emergent tissues) had high levels of luciferase activity in the peripheral leaves, and virtually none in the emergent tissues (Plate 4b). Plants that developed typical severe symptoms, had low levels of luciferase activity in the peripheral leaves, but high levels in the emergent tissues (Plate 4b). At 19 dpi both the mock-inoculated and uninoculated plants showed low but detectable luciferase activity in some older leaves. However these levels were consistently very much lower than the corresponding virus infected plants (Plate 4b). The location of specific symptoms on the leaf (vein clearing, chlorsis and stunting) did not correlate with the regions of elevated luciferase activity.

Since PR-1::Luc activity was observed in infected plants from 8 dpi to 19 dpi, this strongly implies that the SA pathway is activated before and after the development of systemic symptoms. By 19 dpi, some of the CaMV-infected PR-1::Luc plants were showing only mild symptoms. Such occasional variation does occur routinely, and possibly as a result of natural plant-to-plant variation or slight

differences in the inoculation process. Plants with typical symptoms had high levels of luciferase activity in the emergent tissues, whereas in plants with mild symptoms the activity was predominantly observed in expanded leaves. Possibly the plants with atypical mild symptoms may have unusual accumulation or partitioning of virus between emergent and expanded leaves. The spatial pattern of virus location and luciferase activity may be related. In addition higher levels of luciferase activity in the inoculated leaves at 8-12 dpi may reflect the early accumulation of virus in the inoculated zone. Indeed previous work (Barrett, S. unpublished) has shown that the amount of virus accumulation correlates with the level of PR-1 expression. Since induction of *PR-1* seems to coincide with the accumulation and spread of virus, the trigger for *PR-1* expression may be the local presence of the virus.

Although differences in the pattern of luciferase activity were observed between plants with mild and normal symptoms, luciferase activity showed no obvious fine scale spatial correlation with specific symptoms (vein clearing, stunting and chlorosis) or their development. This implies that PR-1 expression is probably not directly associated with the development of these symptoms, although a correlation with the level of virus replication is possible.

Plates 4a and 4b: Luciferase activity in CaMV infected PR-1::Luc transgenic plants

Two *Arabidopsis* PR-1::Luc lines were inoculated with either CaMV isolate Cabb B-JI or water at the two-true leaf stage of development (Section 2.3.3). As additional controls for the wound response, a proportion of plants were untreated. Levels of luminescence were determined as described previously (Section 2.10), with the images shown being representative of 3 or more plants. Activities in uninoculated plants (not shown) were similar to those in the mock-inoculated plants. The pictures are composed of brightfield images overlaid with false colour images of luciferase acitivity. An indicator bar on the right shows a colour coding of luciferase activity.

(a) shows the luciferase activity at various timepoints from 2 hours pi to 15 dpi.
(b) shows luciferase activity at 19 dpi in plants showing normal and atypical mild symptoms.

Plate 4a

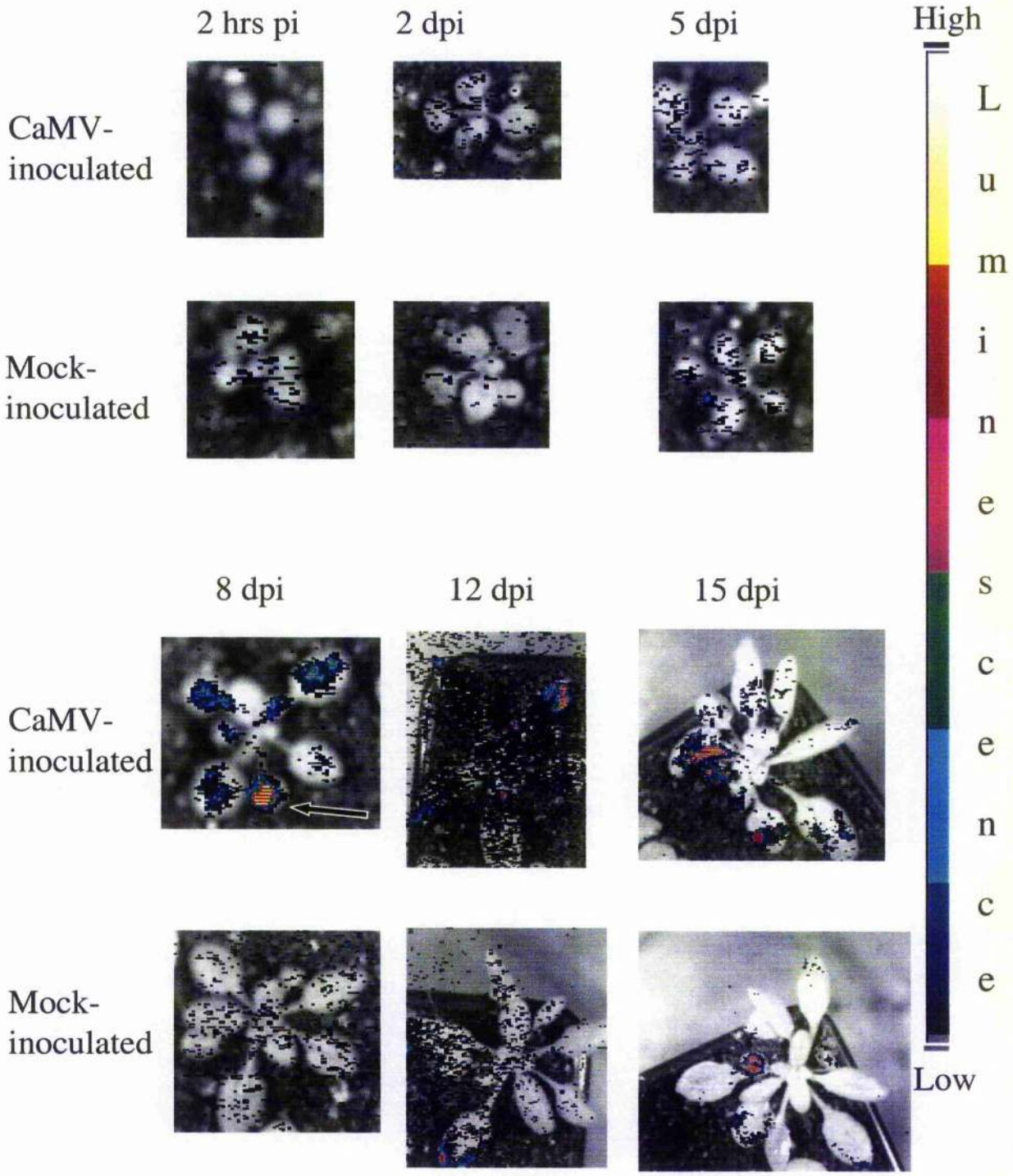
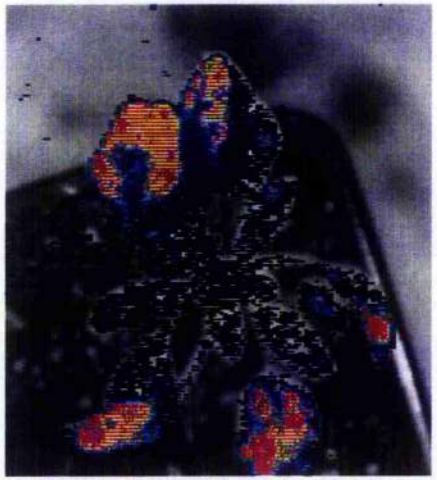

2 hrs pi
2 dpi
5 dpi
CaMV-inoculated
Mock-inoculated
8 dpi
12 dpi
15 dpi
CaMV-inoculated
Mock-inoculated
High
Luminescence
Low

Plate 4b

CaMV-inoculated



Mild symptoms

Normal symptoms

Mock-inoculated

Analysis of JA/ethylene signalling using *PDF1.2*::Luciferase promoter-reporter plants (5.3.2)

At 2 hours pi, both the virus and mock-inoculated plants showed moderate but consistent systemic elevations in luciferase expression, compared to uninoculated controls. However luciferase activity was always higher in the virus-inoculated when compared with the mock-inoculated leaves (Plate 5a). Luciferase expression was undetectable in the uninoculated controls. Luciferase expression patterns at 1 dpi were similar to 2 hours pi except that further increases in activity were apparent in the virus-inoculated leaf (Plate 5a). At 2 dpi both virus and mock-inoculated leaves showed increased luciferase activity when compared with uninoculated controls (Plate 5a). However, luciferase levels were again consistently and clearly higher in the virus-inoculated plant compared with the mock-inoculated plants (Plate 5a). Uninoculated controls showed no detectable luciferase activity (Plate 5a). From 3-5 dpi, elevated luciferase activity was observed systemically in virus-inoculated plants; the levels were substantially greater than those seen in the mock and uninoculated controls (Plate 5b). Although luciferase activity in the virus inoculated plants fell at 6 (Plate 5b) and 8 dpi (Plate 5b), levels were still higher than in the mock and the untreated controls (Plate 5b). By 15 and 19 dpi, although patches of high expression could still be detected on some of the older peripheral leaves in the virus inoculated plants, systemic activity was very much reduced (Plate 5c). In comparison mock-inoculated and uninoculated controls had very low to undetectable levels of luciferase activity (Plate 5c). The location of specific

symptoms on the leaf (vein clearing, chlorsis and stunting) did not show any obvious correlation with the regions of elevated luciferase activity (Plate 5c).

A rapid systemic increase in the level of luciferase expression in *PDF1.2::Luc* plants was observed 2 hours after inoculation with CaMV. This response, which is distinct from the wound-inducible response seen in the mock-inoculated leaf, indicates that the virus must be recognised very rapidly and the ethylene/JA-mediated defence pathway invoked (Thomma *et al.*, 1998; Penninckx *et al.*, 1996). For systemic induction to occur a mobile signal must be required. At 2 hours pi this cannot be virus particles, since systemic movement of CaMV is not detectable until at least 2-3 dpi (Laval, V., unpublished data).

Virus induced systemic luciferase activity in *PDF1.2*::Luc transgenics lasted from 2 hours to 5 dpi. Thus the JA/ethylene signalling pathway must be activated in response to CaMV infection, but only in the early pre-symptomatic stages of infection. It is possible that the rising luciferase activity may reflect the sensitivity of the JA/ethylene pathway to early virus replication and movement. However, although virus titre progressively increases over time, the absence of luciferase activity after 5 dpi suggests that the level of virus replication and movement is not tightly correlated to JA/ethylene pathway activation.

Although the JA/ethylene defence pathway is activated in the early responses to compatible CaMV infections, this is insufficient to prevent viral spread and concomitant symptom development.

Plates 5a, 5b and 5c: Luciferase activity in CaMV infected PDF1.2::Luc transgenic plants

Two *Arabidopsis* PDF1.2::Luc lines were inoculated with either CaMV isolate Cabb B-JI or water at the two-true leaf stage of development (Section 2.3.3). As additional controls for the wound response, a proportion of plants were untreated. Levels of luminescence were determined as described previously (Section 2.10), with the images shown being representative of 3 or more plants. Activities in uninoculated plants are shown only when they have a different response to the mock inoculated plants. The pictures are composed of brightfield images overlaid with false colour images of luciferase acitivity. An indicator bar on the right shows a colour coding of luciferase activity. Where appropriate, black arrows highlight the inoculated leaf.

(a) shows the luciferase activity at various timepoints from 2 hours pi to 2 dpi.
(b) shows luciferase activity at several timepoints from 3 dpi to 8 dpi.
(c) shows luciferase activity at 15 and 19 dpi. The normal colour pictures next to infected plants show symptoms not visible on the brightfield images.

Plate 5a

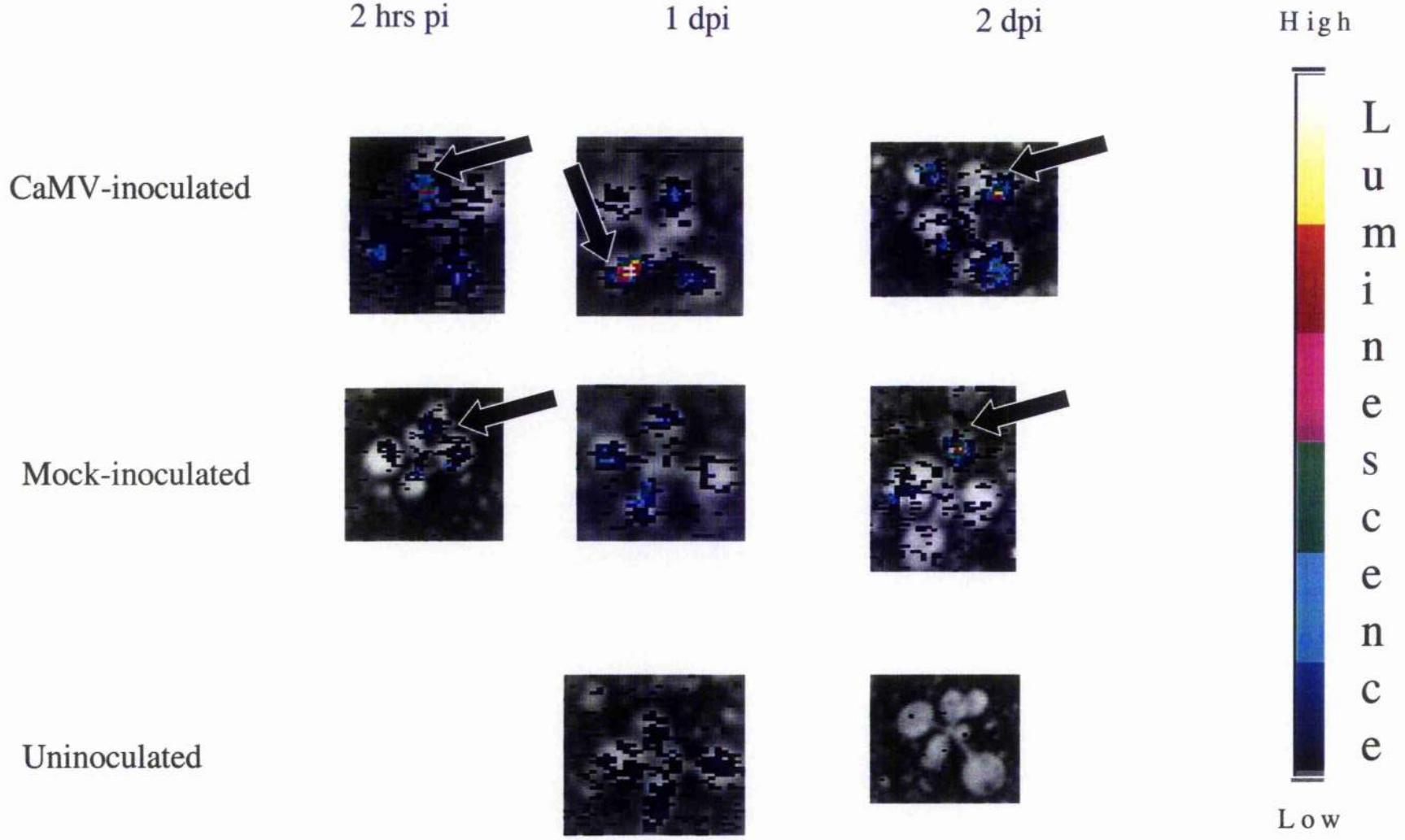

2 hrs pi
1 dpi
2 dpi
CaMV-inoculated
Mock-inoculated
Uninoculated
High
Luminescence
Low

Plate 5b

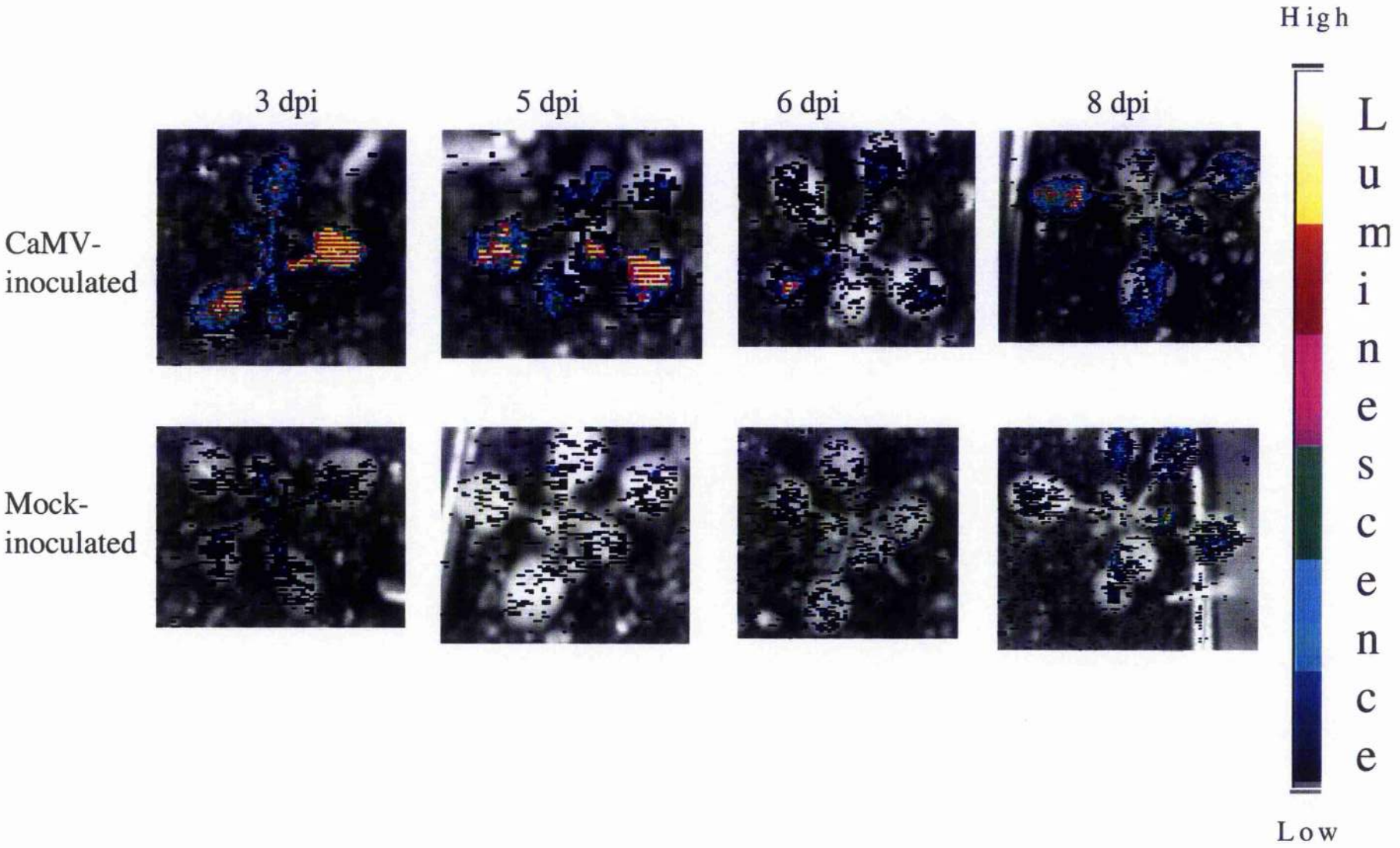
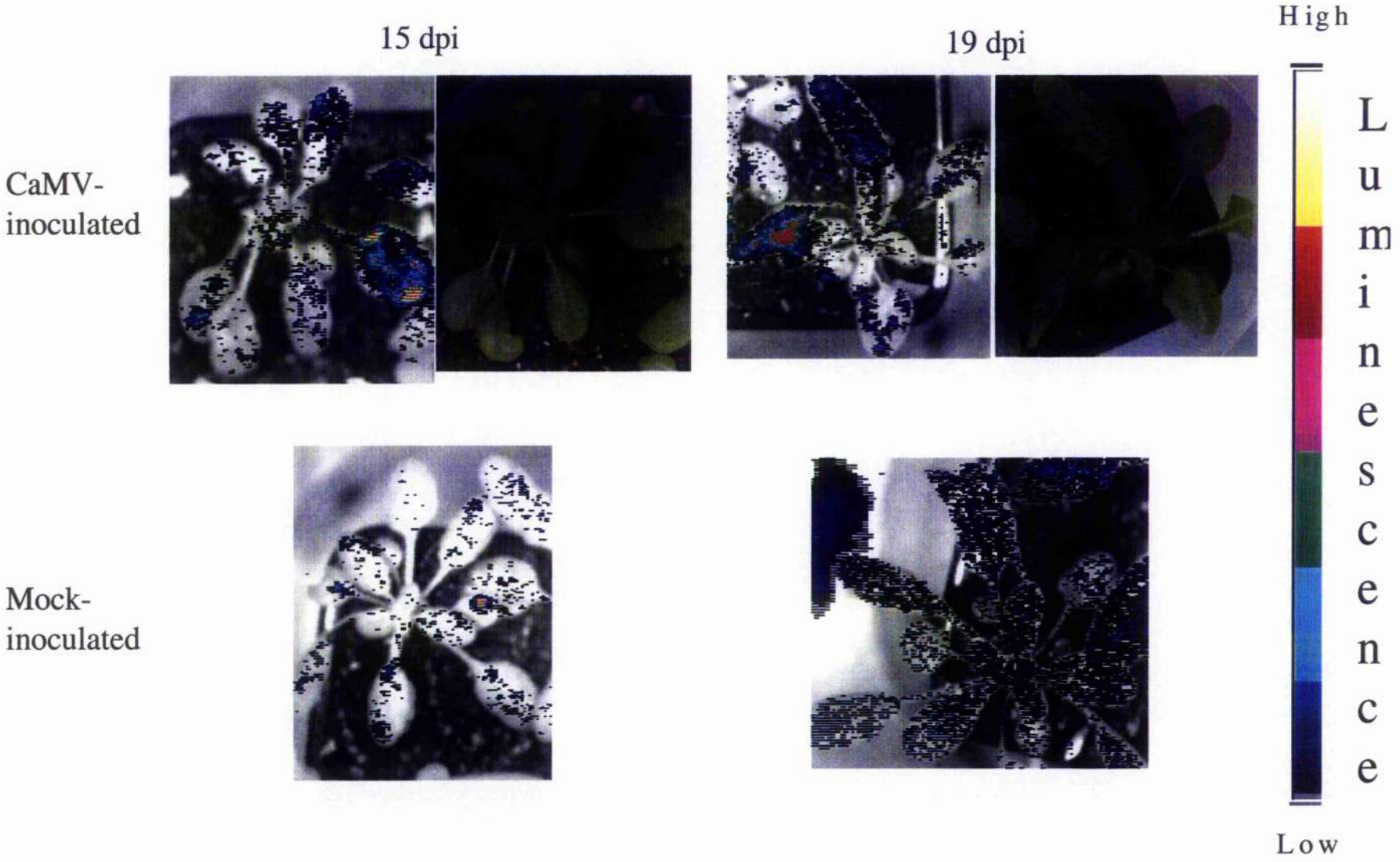

3 dpi
5 dpi
6 dpi
8 dpi
CaMV-inoculated
Mock-inoculated
High
Luminescence
Low

Plate 5c


15 dpi
19 dpi
CaMV-inoculated
Mock-inoculated
High
Luminescence
Low

Analysis of the oxidative burst during infection using *GST1*::Luciferase promoter-reporter plants and *in planta* H_2O_2 staining (5.3.3)

At 2hrs pi both mock and virus inoculated leaves showed elevated levels of luciferase activity compared to non-inoculated controls. However, as with *PDF1.2*::Luc plants, the mock-inoculated *AtGST1*::Luc plants consistently lacked the strong systemic expression seen in the virus-inoculated plants (Plate 6a). Luciferase activity was not observed in the uninoculated controls (Plate 6a). At 1 dpi, the virus-inoculated plants had increased luciferase activity throughout the whole plant, but especially on the inoculated leaf (Plate 6a). In contrast, luciferase activity in mock-inoculated plants was almost undetectable and was similar to uninoculated controls (Plate 6a). This trend of increased systemic luciferase activity in virus infected plants continued at 2 dpi (Plate 6a) and 3 dpi (Plate 6b). From 3-6 dpi the systemic levels of luciferase activity fell in the virus-inoculated plants (Plate 6b), but they remained consistently higher than in the mock and untreated controls (Plate 6b).

From 8 dpi onwards virus-inoculated plants showed a second phase of increased luciferase activity; these levels were significantly higher than those observed in the mock and un-inoculated controls, and those detected at 6 dpi (Plate 6b). From 8 dpi, luciferase activity progressively increased in the virus inoculated plants, whereas mock and uninoculated plants consistently showed low to undetectable levels (Plate 6b and Plate 6c). The spatial localisation of luciferase

activity in virus-inoculated plants did not correlate well with the location of specific symptoms such as vein clearing, chlorosis or stunting (Plate 6c).

A proportion of plants do not develop symptoms after infection, this could possibly reflect variation in the inoculation process or natural plant variability. Some GST1::Luciferase plants that were tested for luciferase activity at 2 hours pi later failed to develop symptoms. Interestingly, the level and location of luciferase activity in these plants were similar to those that successfully developed symptoms.

To test whether increased *GST1::Luc* activity was associated with increased H_2O_2, DAB staining was performed on 5 Col-0 plants. Plants were analysed at 3.5 hours pi, 1, 7, 11, 15 and 19 dpi. At 3.5 hours pi, compared with uninoculated controls, both virus and the mock-inoculated plants showed light but increased staining in the inoculated leaves (Plate 7). However, the virus inoculated plants also showed a light but consistent staining in the cotyledons that was never observed in the mock-inoculated plants (Plate 7). This elevated staining was maintained at 1 dpi (Plate 7). At 7 and 11 dpi, levels of DAB staining in both the mock and virus-inoculated plants were reduced compared with earlier time points (Plate 7). Similar low or undetectable levels of staining were also observed in virus and mock-inoculated plants at 15 and 19 dpi (not shown). At no time was any significant DAB staining observed in uninoculated plants.

Both the *AtGST1*::Luciferase and DAB stain data show that wounding can induce an early (2hrs to 1dpi) increase in *GST1* expression and H_2O_2 accumulation. However, over and above this wound response, in the virus-inoculated plant there is also a consistent up-regulation of *GST1* expression and an increased accumulation of

H_2O_2 in cotyledons and the inoculated leaf. This was never observed in mock-inoculated plants and must therefore be a specific response to the presence of the virus. The plants must sense the presence of the virus very rapidly since this effect was detectable as little as 2 hours after inoculation. At 2 hours pi all virus-inoculated plants had high levels of systemic *GST1::Luc* expression, although not all these plants subsequently developed symptoms. This suggests that in some plants, although virus is present and has been recognized, unknown factors prevent virus replication and spread. Since the early burst of luciferase activity was the same in plants that did or did not develop symptoms, it is possible that the oxidative burst probably reflects a recognition event, unknown factors subsequently preventing full-blown systemic infection developing in a proportion of plants.

AtGST1::Luc expression showed a biphasic response to infection. However, the DAB staining data suggests that the second burst of *AtGST1* expression is not associated with H_2O_2 accumulation. Cues other than the oxidative burst are most probably involved. The later increase in *AtGST1::Luc* expression (8-19 dpi) seems to correlate with both increased PR-1 expression, and systemic movement of the virus and the associated rise in virus titre (Cecchini *et al.*, 1998; Cecchini *et al.*, 2002; Laval and Barrett., unpublished observations). Although symptoms such as vein clearing, mosaics and stunting start to appear during this period, their location does not correlate with the spatial regulation of *AtGST1*.

Plates 6a, 6b and 6c: Luciferase activity in CaMV infected GST1::Luc transgenic plants

Two *Arabidopsis* GST1::Luc lines were inoculated with either CaMV isolate Cabb B-JI or water at the two-true leaf stage of development (Section 2.3.3). As additional controls for the wound response, a proportion of plants were untreated. Levels of luminescence were determined as described previously (Section 2.10), with the images shown being representative of 3 or more plants. Activities in uninoculated plants are shown only when they have a different response to the mock inoculated plants. The pictures are composed of brightfield images overlaid with false colour images of luciferase acitivity. An indicator bar on the right shows a colour coding of luciferase activity. Where appropriate, black arrows highlight the inoculated leaf.

(a) shows the luciferase activity at various timepoints from 2 hours pi to 2 dpi.
(b) shows luciferase activity at several timepoints from 3 dpi to12 dpi.
(c) shows luciferase activity at 15 and 19 dpi. The normal colour pictures next to infected plants show symptoms not visible on the brightfield images.

Plate 6a

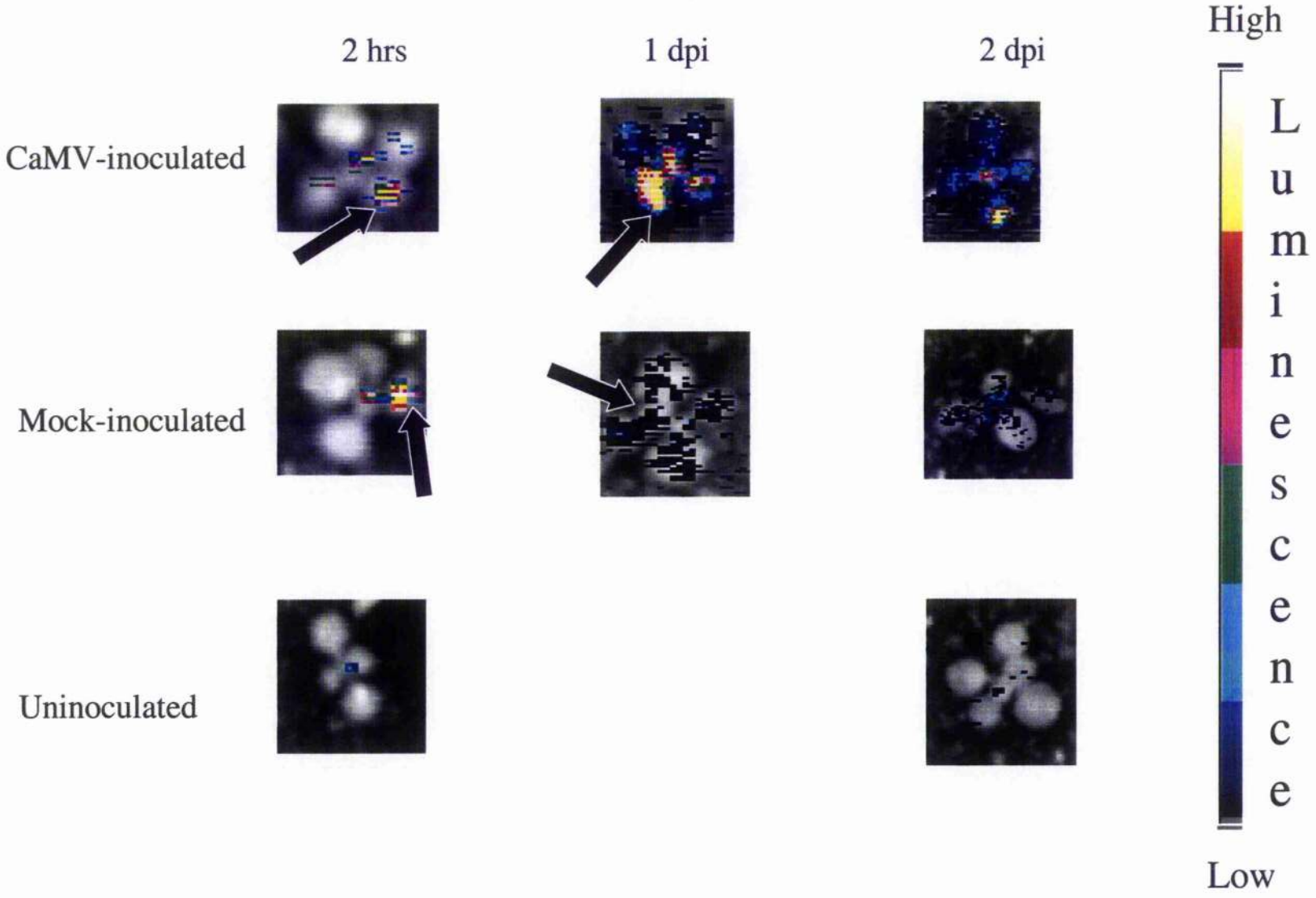

2 hrs
1 dpi
2 dpi
CaMV-inoculated
Mock-inoculated
Uninoculated
High
Luminescence
Low

Plate 6b

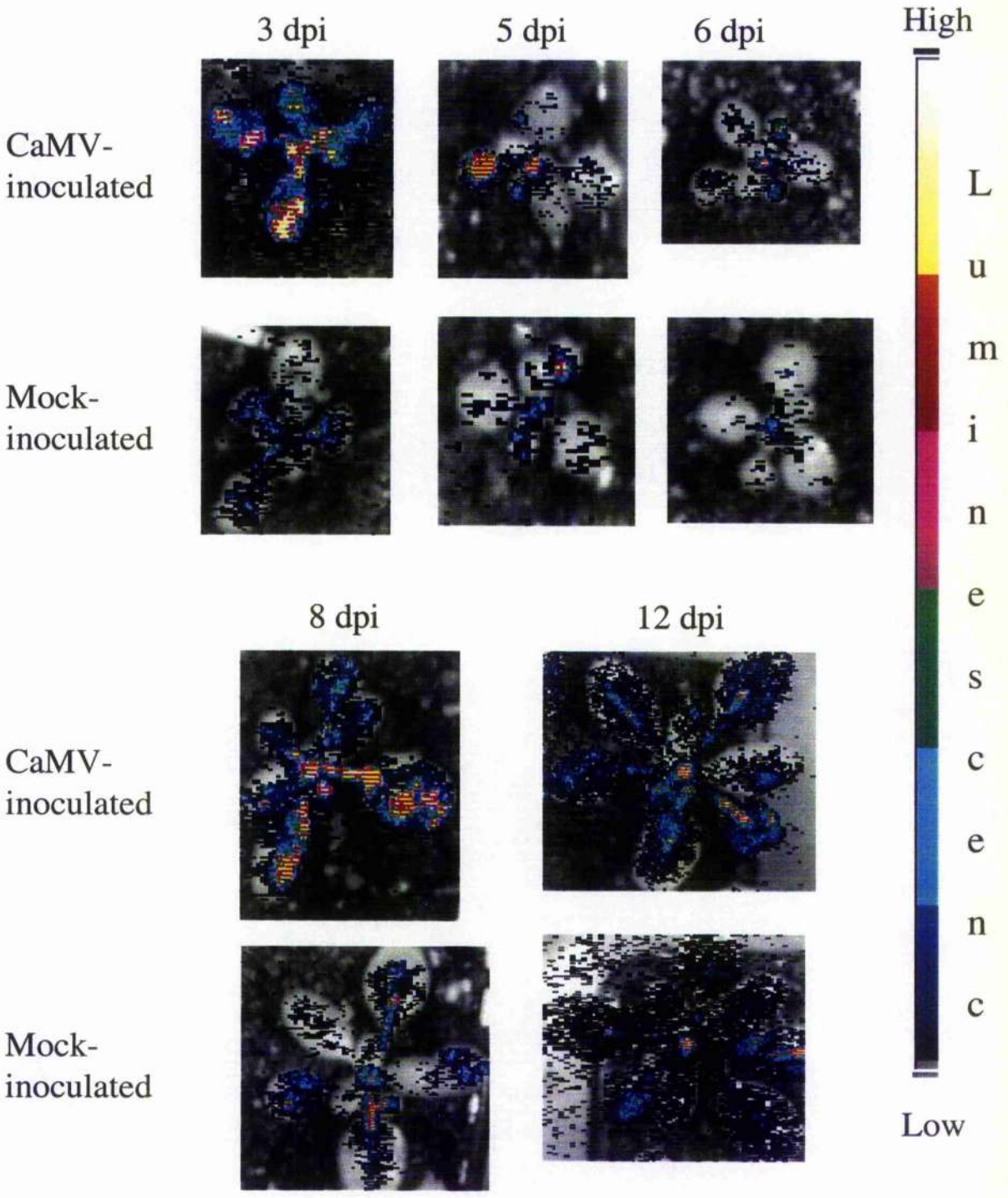

3 dpi
5 dpi
6 dpi
CaMV-inoculated
Mock-inoculated
8 dpi
12 dpi
CaMV-inoculated
Mock-inoculated
High
Luminescence
Low

Plate 6c

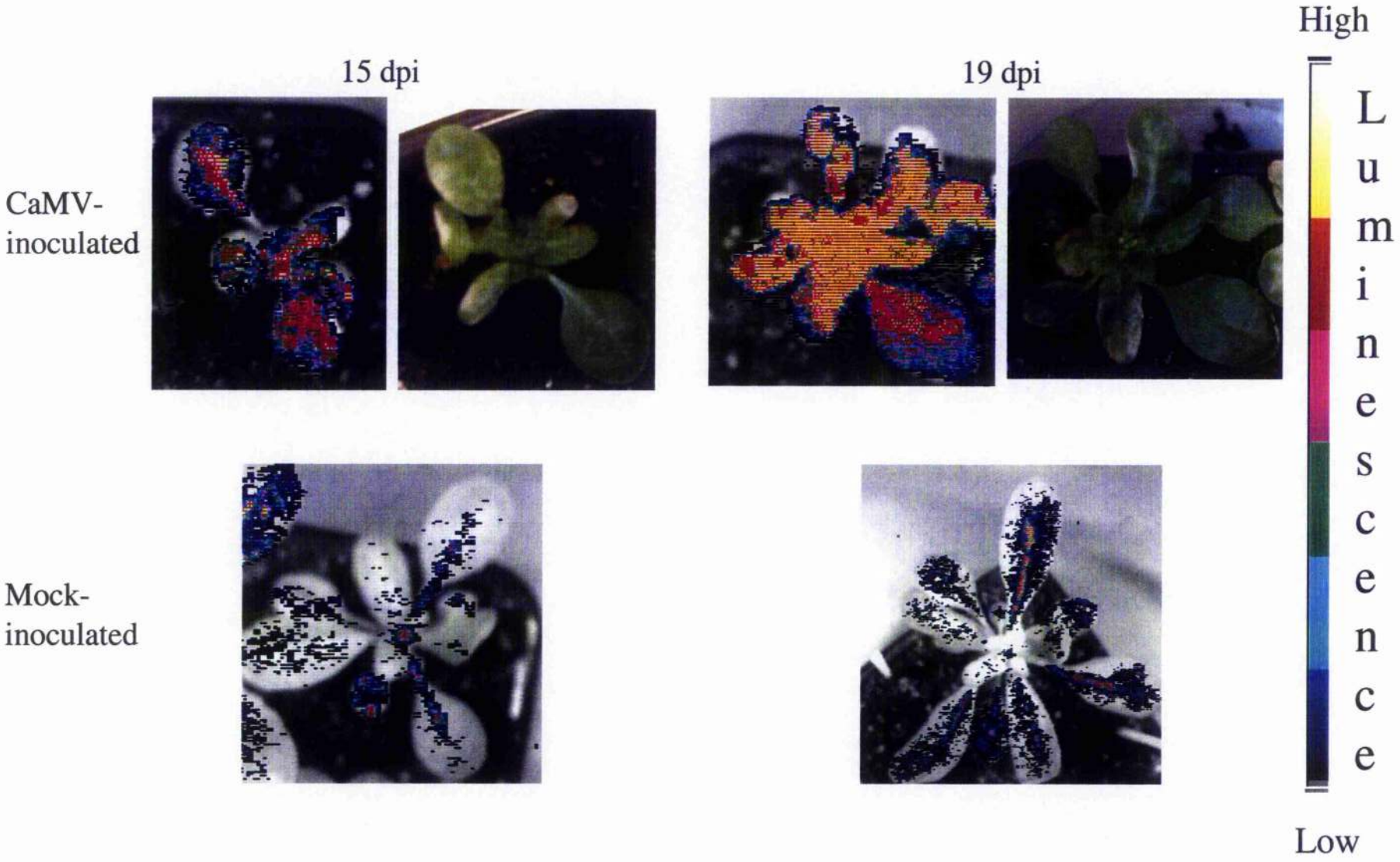
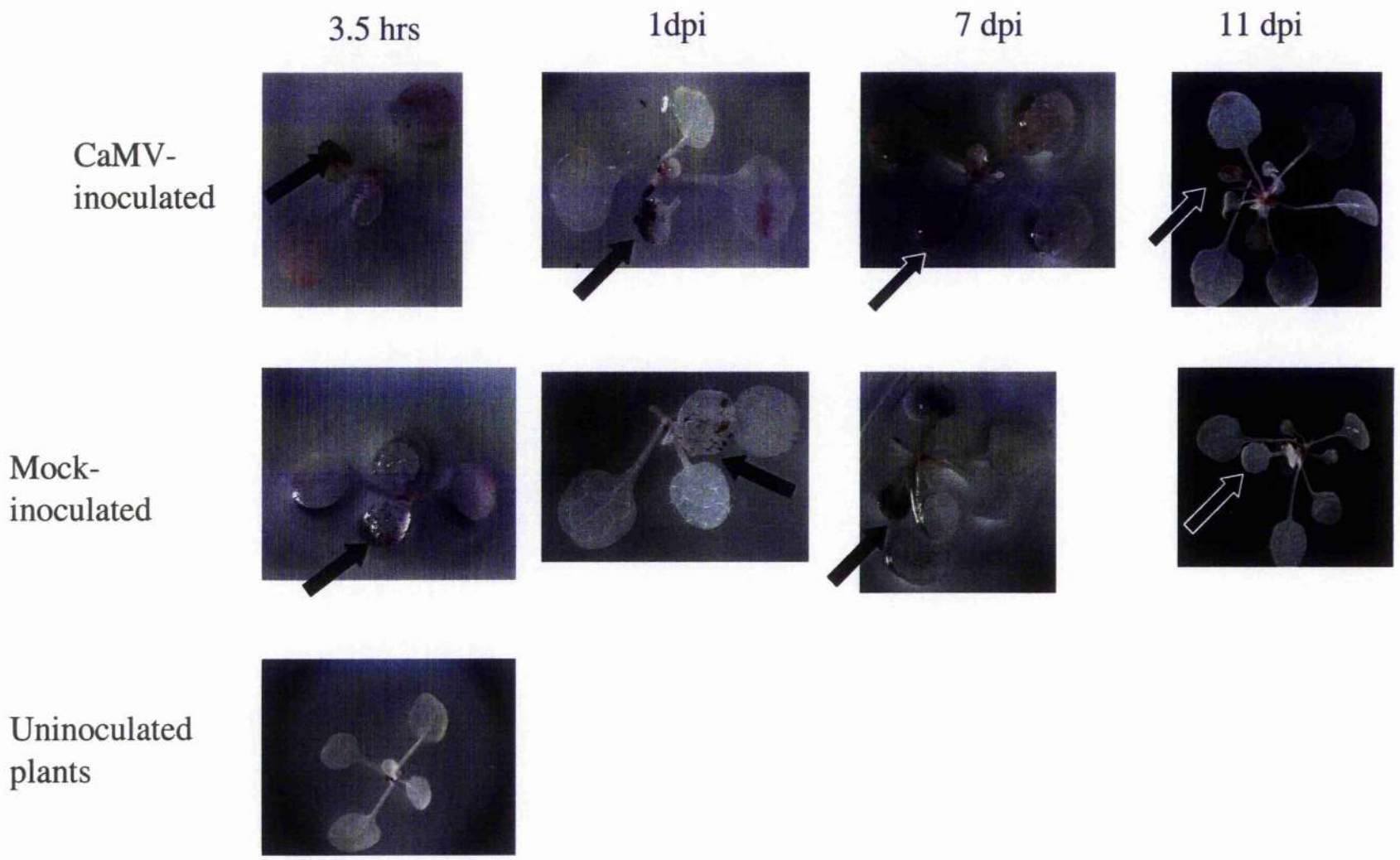

15 dpi
19 dpi
CaMV-inoculated
Mock-inoculated
High
Luminescence
Low

Plate 7: Visualisation of H_2O_2 accumulation by DAB staining

Spatial H_2O_2 accumulation in CaMV-, mock-inoculated and uninoculated plants. Brown areas represent H_2O_2 accumulation, as detected by DAB staining (Section 2.11). Arrows highlight the inoculated leaves. Uninoculated plants at other timepoints were free of staining (not shown).


3.5 hrs
1dpi
7 dpi
11 dpi
CaMV-inoculated
Mock-inoculated
Uninoculated plants

Effect of H_2O_2 on symptoms and virus level (5.3.4)

Inoculation with CaMV is associated with an early oxidative burst. However the impact of this defence response on virus spread and concomitant symptom development is unknown. To test whether there might be any correlation between the oxidative burst, symptom severity and viral titre, H_2O_2 accumulation was assayed in *Arabidopsis* mutants/transgenic lines with altered symptom responses and/or virus levels. The *Arabidopsis* lines used were *ein2, etr1-1, cai 171, cai 181, cai 160* and *NahG. ein2* and *etr1-1* (Guzman *et al.,* 1990; Bleecker *et al.,* 1988) are two ethylene response mutants that exhibit milder symptoms and accumulate reduced levels of virus when compared with wildtype *Arabidopsis* (Cecchini, E. and Barrett, S. J., unpublished). *cai 171*, *cai 160* and *cai 181* have been described earlier in this thesis (Chapter 4). *NahG* transgenic plants have been engineered to express a bacterial SA hydroxylase which degrades SA (Lawton *et al.,* 1995). *NahG* plants cannot accumulate SA and are deficient in all aspects of SA-mediated defence. These plants exhibit very severe symptoms and accumulate 4-fold more virus than wildtype controls (Laird, J., Geri, C., Giuntini, P. unpublished). A summary of symptom severity and virus accumulation is shown in table 4. DAB staining was used to visualize the H_2O_2 accumulation in mock inoculated, virus inoculated and uninoculated plants at 1 dpi (the optimal time for H_2O_2 accumulation). Each timepoint consisted of 5 plants, and each experiment was repeated 3 times.

Virus and mock-inoculated *ein2-1* and *etr1-1* showed similar levels of staining in the inoculated leaves when compared with wildtype plants. However the staining in the cotyledons, a characteristic of virus-inoculated wildtype *Arabidopsis*,

was consistently absent in both mutants (Plate 8a). No staining was detected in uninoculated plants of all genotypes (Plate 8a). In contrast virus-, mock-inoculated and uninoculated *NahG* plants all showed high levels of DAB staining in cotyledons when compared to the wildtype plants (Plate 8a).

Virus infected *cai 181* and *cai 171* also showed very high levels of staining in the cotyledons when compared to wildtype (Plate 8b). In contrast in virus inoculated *cai 160* plants, staining in cotyledons was lower than in wildtype (Plate 8b). Mock-inoculated and uninoculated *cai* mutant plants showed similar staining to wildtype plants (Plate 8b).

The two ethylene response mutants which showed milder symptoms and lower virus levels also lacked the systemic accumulation of H_2O_2 in response to virus infection than wildtype. In contrast *NahG,* which accumulates more virus and develops more severe symptoms than wildtype, had highly elevated levels of systemic H_2O_2 accumulation compared with wildtype plants. This implies a possible association between levels of H_2O_2 accumulation, symptom severity, and virus accumulation. However, *cai 171* and *181* accumulated similar levels of H_2O_2 in response to virus infection, despite the difference in symptom severity. This argues against a direct correlation between symptom severity and the level of H_2O_2 accumulation. Also *cai 160,* which hyper-accumulates virus but developed a similar symptom response to wildtype, showed similar oxidative burst responses to mutants which have lower virus levels. Again, this argues against any direct correlation between virus titre and the magnitude of the first phase oxidative burst response.

Interestingly it is possible that the reduced amount of H_2O_2 accumulation in both ethylene mutants could suggest that perhaps the JA/ethylene pathway may influence the magnitude of the oxidative burst.

Table 4: Mutants and transgenics with differences in symptoms and virus levels

Mutant	Description	Phenotype (Symptom severity)	Virus load
etr1-1	Ethylene insensitive (receptor)	Milder	Reduced
ein2	Ethylene insensitive (signaling intermediate)	Milder	Reduced
NahG	Salicylic acid hydroxylase transgenic	More severe	4-fold higher
cai 160	Putative sugar signaling/uptake mutant	Normal	4-fold higher
cai 171	Putative sugar signaling/uptake mutant	Milder	Normal
cai 181	Putative sugar signaling/uptake mutant	More severe	Normal

Plates 8a and 8b: Visualisation of H_2O_2 accumulation in mutants at 1 dpi

Spatial H_2O_2 accumulation in CaMV-, mock-inoculated and uninoculated wild type and mutant plants at 1dpi. Brown areas represent H_2O_2 accumulation, as detected by DAB staining (Section 2.11). Arrows highlight the inoculated leaves. Images represent replicates of 5 plants.

(a) shows H_2O_2 accumulation in Col-0, *ein 2-1*, *etr1* and *NahG*.

(b) shows H_2O_2 accumulation in Col-0, *cai 171*, *160* and *181*. Mutant uninoculated plants (not shown) were similar to wildtype.

Plate 8a

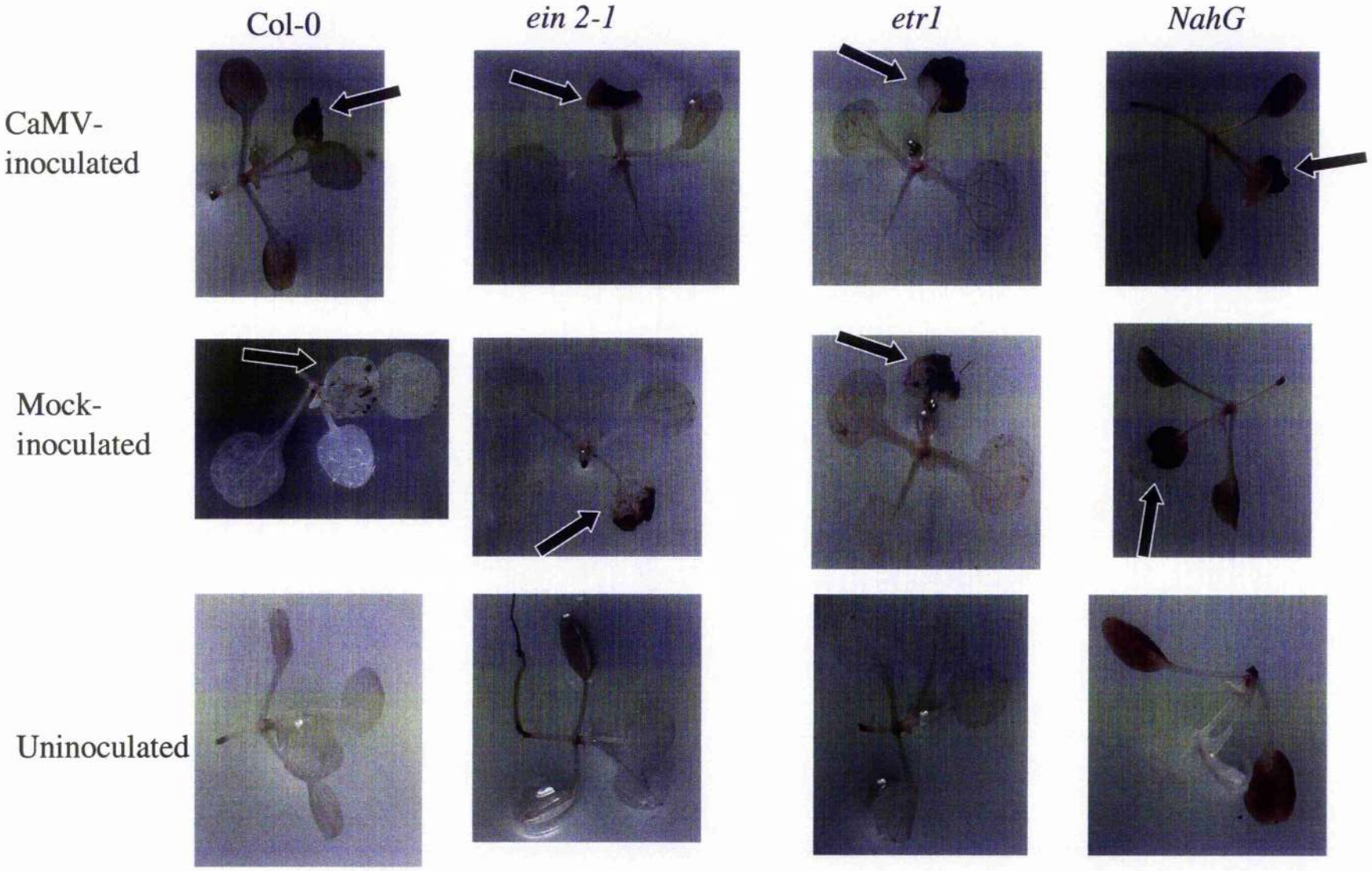
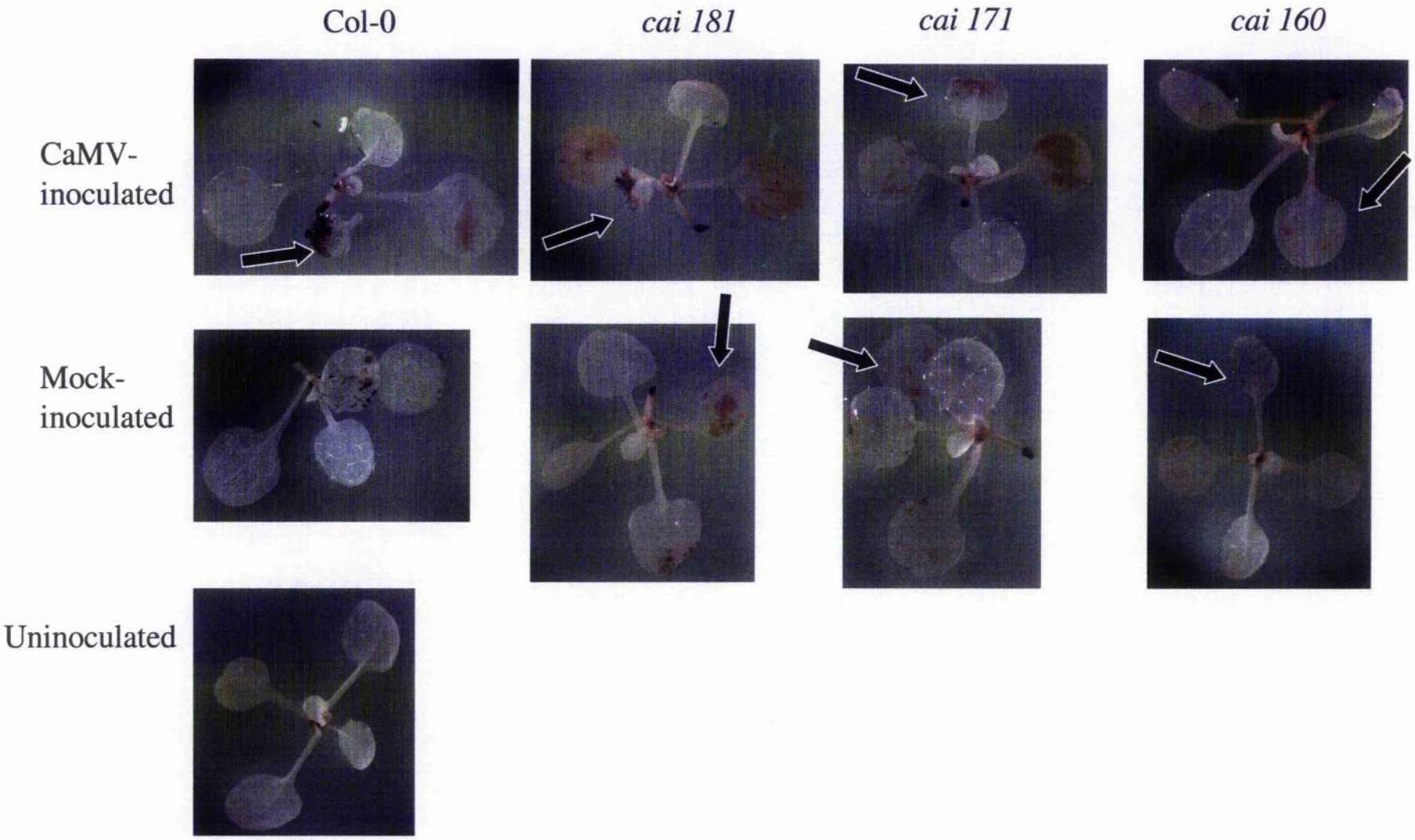

Col-0
ein 2-1
etr1
NahG
CaMV-inoculated
Mock-inoculated
Uninoculated

Plate 8b


Col-0
cai 181
cai 171
cai 160
CaMV-inoculated
Mock-inoculated
Uninoculated

Summary (5.4)

Compatible CaMV infection of *Arabidopsis* can result in early induction of the oxidative burst and JA/ethylene pathway, followed by a later activation of the SA pathway. Despite the extensive induction of these pathways, they are unable to prevent virus replication, movement and the concomitant development of symptoms. Although the pattern of increased gene expressions did not correlate well with symptom development, it is possible that the location and level of virus accumulation may mirror the activity of the SA pathway.

It is possible that the decline in activity of some pathways, while others increase (JA/ethylene pathway is suppressed as SA pathway is induced) may represent a complex cross-talk which may in part be regulated developmentally and/or by the virus.

Chapter 6

General Discussion (6.1)

Introduction (6.1.1)

The symptoms produced by compatible plant-virus interactions are a major source of crop loss around the world. By developing an understanding of the interactions between virus and host, it could enable us to design strategies which either prevent infection or lead to the amelioration of symptoms. The aim of this project was to identify metabolic and host gene expression changes, which participate in symptom development and pathogenesis during compatible virus infection. Of primary interest were soluble sugars, since they have been implicated in the development of symptom-like phenotypes in the absence of compatible infection, and have also been closely associated with the regulation of plant defence genes (Herbers *et al.*, 1996a, 1996b), which are crucial components that can limit plant disease. The studies reported in this thesis strongly argue against the role of soluble sugars as elicitors of symptoms or regulators of the defence response during compatible virus infections. However, the results show for the first time that a compatible virus infection can activate a large array of defence responses, although these are unable to prevent the subsequent development of disease.

Role of soluble sugars in compatible virus disease (6.1.2)

One of the aims of the work presented in this thesis was to assess the role of soluble sugars in the development of symptoms. The first approach involved infecting turnip with CaMV isolates that induced symptoms of different severity, and examining sugar, starch, levels of virus accumulation, and symptoms at weekly intervals. The second approach involved infecting wildtype and mutant *Arabidopsis*

lines with a severe isolate, Cabb B-JI, and analysing symptoms, virus accumulation and carbohydrate levels.

In turnip, there was no relationship between virus levels and symptom severity. This implies that there cannot be a simple causal correlation between the metabolic drain incurred in supporting virus replication and symptom development. The genetic background of the virus rather than the rate of replication may be the most important factor in determining symptom character, again confirming observations made by Cecchini *et al.*, (1998). Similar findings have been reported for some other host-virus combinations; for example in TMV infected tomato plants no correlation was found between levels of virus accumulation and symptom severity (Fraser *et al.*, 1986). In other host-virus combinations, for example TMV infection of tobacco, Fraser *et al.*, (1986) reported a correlation between the level of symptoms and the level of virus accumulation. This suggests that several factors may be important in symptom development and that these may vary, depending on the host and virus.

Although levels of CaMV accumulation do not show a direct correlation with symptom severity, other factors, in particular sugar signalling may play a role. It was found that although CaMV infection produced changes carbohydrate accumulation in turnip, they did not correlate with symptoms or virus levels; thus ruling out sugar accumulation as being a direct potentiator of symptoms. However, CaMV infection of the *cai* mutants resulted in unusual symptoms and virus loading when compared to wild type plants. This could suggest that the altered ability to sense sugars has an impact on symptom development. This supports the suggestion that sugar signalling

is to some extent involved in potentiating symptoms. In contrast, analysis of carbohydrate levels in infected *Arabidopsis* revealed that sugar and starch levels do not correlate well with either virus accumulation or symptoms. It is therefore possible that symptoms may arise due to an altered perception of sugars rather than direct changes in the level of soluble sugars. In turn, it is possible that changes in carbohydrate levels may be a consequence of the symptoms induced during infection.

When comparing the carbohydrate levels in *Arabidopsis* with turnip during infection, the patterns of sugar and starch accumulation in source leaves at 14 dpi were similar. However, in sink leaves at the same time point during infection, *Arabidopsis* had elevated levels of starch, whereas turnip had reductions in starch. It is unknown why carbohydrate accumulation changes during CaMV infection and why both infected hosts show marked differences in carbohydrate levels. In other virus-host interactions a diverse range of effects on carbohydrate partitioning has been reported, including decreases in soluble sugars and increases in starch accumulation (Lindner *et al.*, 1959; Israel *et al.*, 1967; Cohen *et al.*, 1975; Roberts *et al.*, 1982; Tomenius *et al.*, 1982). These differences could be ascribed to a number of possibilities, such as the variable effect of virus infection on photosynthesis (Rahoutei *et al.*, 2000; Mayarosy *et al.*, 1973; Bedbrook and Matthews, 1973), plasmodesmatal structure (Wolf *et al.*, 1989; Herbers *et al.*, 1997), and carbohydrate metabolism (Tecsi *et al.*, 1994). The effect of infection on metabolic pathways both up-stream and down-stream of carbohydrate biosynthesis clearly have an effect on sugar and starch levels. Variable effects of virus infection on these processes may be

due to the intrinsic physiological differences between each of the host plants used in these studies.

Although physiological differences between turnip and *Arabidopsis* (in particular those impinging on sugar partitioning) have not been extensively catalogued, there are several lines of evidence that suggest they do exist. Turnip has tubers that act as both a source and a sink for carbohydrates, whereas *Arabidopsis* has roots only. A further contributor to the differences between turnip and *Arabidopsis* is the speed of scenescence. *Arabidopsis* grows much more rapidly than turnip, and it was found that *Arabidopsis* plants have a higher ratio of source-to-sink leaves than turnip at 14 dpi. Possibly *Arabidopsis* source leaves may start to accumulate starch earlier than turnip. Thus even in the absence of virus infection, the two plants probably differ in the partitioning of carbohydrates. Such physiological differences could be partly responsible for the differences in the effect of infection on carbohydrate levels. A further factor may be the differences in levels of virus in the two hosts, with turnip accumulating about ten times as much virus as *Arabidopsis* (Cecchini *et al.*, 1998).

These data provide convincing evidence to indicate that changes in sugar level are not a direct trigger for the development of symptoms in either turnip or *Arabidopsis*. Since the major alterations in sugar levels are first detected after the appearance of symptoms, these changes may be a **consequence** of symptoms and the associated up- and down-regulation of genes typically observed in compatible virus interactions. Therefore the disturbance in carbohydrates during virus infection could be regarded as part of the symptom response, albeit a non-visible one. In

contradiction, infection studies on the putative sugar mutants do not rule out the possibility that the ability to sense sugars could interfere with the development of symptoms and the level of virus replication.

Since close relationships have been identified between sugar signalling and plant defence activation (Herbers et al., 1997), it was thought that different defence responses in the *cai* mutants may account for altered symptoms and virus loading. Subsequent analysis of sugar and salicylic acid inducible PR-1, PR-2 and PR-5 defence gene expression showed that these defence responses were unaltered in the mutants. Therefore, altered defence responses are not a plausible explanation for the difference in pathologies between wild type and the *cai* mutants.

Defence pathways are activated by a compatible virus interaction (6.1.3)

SA pathway (6.1.3.1)

The activation of PR-1, PR-2 and PR-5 demonstrated for the first time that an extensive array of defence genes can be induced during a compatible virus interaction. Therefore the SA and additional defence pathways were scrutinised using defence gene promoter::Luciferase reporter lines and H_2O_2 staining. As previously demonstrated, PR-1, PR-2 and PR-5 expression is strongly up-regulated at 14 dpi, but the PR-1::Luciferase lines have shown that induction first occurs at around 8 dpi and lasts probably beyond 19 dpi. Although this indicates involvement of the SA defence pathway, this is not sufficient to prevent virus spread or the development of symptoms.

Different classes of PR proteins possess a variety of enzymatic activities (Jach *et al.*, 1995; Stintzi *et al.*, 1993), which may be effective in defence against fungal and bacterial pathogens (Roberts *et al.*, 1990). However, no direct anti-viral activity has been attributed to any of these classes and it is unlikely that PR proteins provide an effective defence against CaMV infection. In addition, it has been reported that TMV virus spread is unaffected in tobacco plants which over-express PR genes (Cutt *et al.*, 1989; Linthorst *et al.*, 1989). The expression of PR proteins indicates that there is increased activity of the SA-mediated defence pathway. It has been reported that the branch of the SA pathway leading to PR protein production is ineffective against viruses (Chivasa *et al.*, 1997; Murphy *et al.*, 1999).

In SA-deficient (*NahG*) *Arabidopsis*, CaMV infection resulted in more severe symptoms and a 4-fold increase in virus levels (Geri, C., Guintini, P., Laird, J. unpublished). This suggests that some aspect of the SA-signalling pathway is partially effective in limiting CaMV infection. Murphy *et al.*, (1999) reported that the SA pathway branches into SHAM sensitive and insensitive routes, the SHAM sensitive branch serving to limit virus spread. It is possible the SHAM sensitive branch of the SA pathway might be involved moderating CaMV accumulation and symptom severity. Possibly, the late activation of these defense reactions is a factor mitigating against their effectiveness.

JA/ethylene pathway (6.1.3.2)

The pattern of *PDF1.2* expression implicates activation of the JA/ethylene defence pathway in the early responses to compatible CaMV infections (2hours pi to 5 dpi). For systemic induction of *PDF1.2* to occur a mobile signal must be required.

At 2 hours pi this cannot be virus particles, since systemic movement of CaMV is not detectable until at least 2-3 dpi (Laval, V., unpublished data).

Since symptoms later develop after induction of the JA/ethylene pathway, this suggests that viral replication and spread is not halted by this defence. This is perhaps not surprising since the JA/ethylene defence pathway has not been implicated in defence against viruses (Broekaert *et al.*, 1995; Penninckx *et al.*, 1996; Penninckx *et al.*, 1998). In addition, the induction of *PDF1.2* expression by CaMV is the first reported case of viral activation of the JA/ethylene pathway.

Possible interactions between the JA/ethylene and SA defence pathways during infection (6.1.3.3)

The fall in *PDF1.2* expression at 8 dpi coincides with an increase in SA associated *PR-1* expression. Cross-regulation, whereby induction of the SA pathway can inhibit the JA/ethylene pathway, has been reported by Doherty *et al.*, (1988) and Doares *et al.*, (1995). These authors demonstrated that JA/ethylene dependent *PDF1.2* expression can be reduced by increased levels of SA. It is thought that SA can achieve this by inhibiting the expression of enzymes that are critical to the biosynthesis of JA (Pena-Cortes *et al.*, 1993). In addition there is genetic evidence to support antagonism between the two pathways. When *Arabidopsis* mutants which constitutively express PR proteins and have elevated SA, were crossed to SA deficient *NahG* lines, SAR related gene expression fell. In contrast *PDF1.2* expression increased. This suggested that the elevated levels of SA in the mutants may suppress *PDF1.2* expression (Clarke *et al.*, 1998). Thus, cross-talk between the

SA pathway and the JA/ethylene pathway may contribute to suppressing *PDF1.2* expression during the later stages of CaMV infection. It is possible that the co-ordinated up and down regulation of PDF1.2 and PR-1 may not be due to direct interaction of both pathways, since developmental regulation of these genes cannot be ruled out.

Developmental regulation of PR-1 and some other aspects of the SA pathway have recently been described- with older tissues showing higher expression than younger leaves Herbers *et al.*, (1998). It is commonly accepted that *Arabidopsis*, a short-lived plant (an annual), senesces faster than turnip (a perennial). Interestingly, unlike in *Arabidopsis*, CaMV-infected turnip shows little PR-1 expression at 14 dpi (Love, A. J. unpublished). Since turnip eventually develops strong PR-1 expression, this suggests that the presence of the pathogen and host developmental stage may influence the level of expression. It is possible that during CaMV infection of *Arabidopsis*, increases in PR-1 expression can only take place when plants reach a particular developmental stage, in this case 8 dpi. Such developmental cues may exist for *PDF1.2* expression since the SA pathway can be developmentally regulated and interact with the JA/ethylene pathway.

The oxidative burst (6.1.3.4)

In addition to activating the SA and JA/ethylene mediated defence pathways, virus inoculation induced a systemic oxidative burst, as evidenced by increased *GST1::Luc* expression and DAB staining, as rapidly as 2 hours after inoculation. This observation reinforces the idea that CaMV must be recognised very rapidly and that some mobile signal is produced, which can elicit a systemic oxidative burst. The

signal has still to be identified, and might or might not be the same as the one involved in the activation of the JA/ethylene defence responses. However, as demonstrated by DAB staining, it is possible that an ethylene component may be involved in potentiating the systemic oxidative burst.

Early oxidative bursts have been reported for other compatible virus-host interactions. For example, compatible cucumber mosaic virus (CMV) infection of tobacco resulted in a single early oxidative burst after pathogen challenge (Allan *et al.*, 2000). In contrast, TMV-tobacco infections did not induce any oxidative burst (Allan *et al.*, 2000). From these data, Allan *et al.*, (2000) suggested that a very early oxidative burst is not enough to limit virus replication or spread. This must be true for CaMV infected *Arabidopsis*, since virus spread usually follows the oxidative burst.

It has been suggested that a single oxidative burst is merely a recognition event. In contrast, a biphasic oxidative burst occurs with true resistance, associated with the successful activation of resistance pathways, defence genes and limitation of pathogen spread (Baker and Orlandi, 1995; Allan *et al.*, 2000). For example, a biphasic oxidative burst was detected during the incompatible infection of N gene tobacco with TMV (Allan *et al.*, 2000). This could imply that the oxidative burst observed during CaMV infection is merely a recognition event.

Interactions between the oxidative burst, JA/ethylene and SA pathways (6.1.3.5)

The oxidative burst is often regarded as a pleiotropic modulator of defence mechanisms such as SA and JA/ethylene pathways. It has been reported that the

build up of ROIs can induce the expression of *PDF1.2* in a SA independent manner (Mitter *et al.,* 1998). It is therefore likely that there is cross-talk between the oxidative burst and the JA/ethylene pathway. Interestingly, in CaMV-infected *Arabidopsis*, *PDF1.2::Luc* expression coincided with the oxidative burst during the early stages of infection. Possibly increased accumulation of ROIs might be involved in the activation of the JA/ethylene mediated defence response.

The oxidative burst can also work upstream of SA, and in some cases can induce SA accumulation (Bi et al 1995; Neuenschwander et al 1995; Chamnongpol et al 1998). Conversly SA can potentiate the increase of ROIs in the cell (Chen *et al.*, 1995; Conrath *et al.*, 1995; Wendehenne *et al.*, 1998). However, ROIs were not detected by DAB staining at 8- 19 dpi in CaMV infected *Arabidopsis*, when *PR-1::Luc* was expressed. Conversely *PR-1::Luc* activity was undetectable at 2 hours pi- 5 dpi, when the oxidative burst was occurring. Thus the oxidative burst is not responsible for the activation of the SA pathway in this case. During CaMV infection of *Arabidopsis* there may be little direct cross-talk between ROIs and the SA pathway.

Temporal and spacial correlation between defence gene induction and symptoms (6.1.3.6)

As well having role in defence, ROIs might participate in CaMV induced stunting and chlorosis, since they have been shown to affect pigmentation (Elstner, 1990; Rice-Evans et al,. 1991) and lead to the production of growth inhibitors (Elstner, 1990). The timing of the systemic oxidative burst does not support this hypothesis, since the first appearance of symptoms and the accumulation of H_2O_2

were separated by about 11 days. In addition the DAB staining results (Section 6.3.4) suggests that the oxidative burst is not directly connected to the altered symptom severity observed amongst the different *Arabidopsis* mutants.

Also, no correlation between the location of symptoms with the expression of either *GST1*, *PDF1.2* or *PR-1* was detected. This suggests that the activation of these defence pathways is not involved in symptom development, however it is still possible that defence gene expression may correlate with the location of the virus.

Conclusions (6.2)

It has been suggested that virus-induced changes in the levels of soluble sugar might be a possible candidate for potentiating symptoms during infection. Analysis of sugar and starch accumulation in turnip and *Arabidopsis* infected with CaMV has revealed that although variations in carbohydrate level occurred, there was no obvious direct link between carbohydrate levels, development of symptoms or virus load. Most probably changes in the level of free soluble sugars do not have an influence on symptom development. Rather the converse is likely to be true, and such changes may be brought about by the development of symptoms and the associated up- and down-regulation of host genes. Alterations in sugar levels can therefore be regarded as part of the host response to virus infection, albeit an invisible symptom of virus infection.

Compatible infection of *Arabidopsis* with CaMV results in the activation of three defence pathways. Two of these are activated very rapidly after inoculation, the third is activated later (just prior to and after development of symptoms). SA-mediated defence pathways are central to the production of necrotic lesions and also

limitation of virus spread, responses normally associated with incompatible interactions. However, neither of these responses were observed during CaMV infection, which suggests that only a subset of possible defence responses may be activated, and that they are insufficient to prevent virus spread. The results reported in this thesis provide the first evidence for such a co-ordinated and extensive induction of defence responses during infection by a compatible virus. The invocation of defence responses during compatible host-virus interactions has often been ignored since the pathogen appears to replicate and move freely in the plant. However, the simplistic division of host-pathogen interactions into discrete classes, incompatible (defence response initiated) and compatible (no defence response) is clearly no longer tenable.

Future Work (6.3)

In order to further analyse the role of sugar signalling in the potentiation of symptom development, several approaches could be taken. The first approach would involve screening mapped *Arabidopsis* sugar mutant lines such as *sun6* (sucrose uncoupled; Pego, 2000), or transgenic lines that over-express genes involved in sugar signalling (such as HXK over-expressing lines; Jang *et al.*, 1994) for altered responses to infection. Since characterised sugar mutants which represent multiple alleles for different genes are available, these could be used to test the importance of specific sugar signalling pathways in symptom development. These studies could be combined with analysis of gene expression in order to find out how the behaviour of specific sugar signalling genes may be affected by infection. It may also be

possible to create sugar signalling gene promoter::Luciferase reporter plants, enabling temporal and spacial correlations between the development of specific symptoms and the behaviour of sugar pathways to be investigated. Furthermore, the exogenous application of inhibitors of specific sugar signalling pathways (non metabolisable sugar analogues; Pego, 2000) to the sugar gene luciferase lines would not only confirm repression of specific sugar pathways, but would also help us understand the effect of inhibition on symptom development.

Of possible benefit to future investigations, would be a sampling method of much finer resolution than the ones used in this thesis. The development of single cell sampling techniques would enable investigators to pinpoint cells that are either dark green, chlorotic or stunted, and extract the cytoplasm for further analysis (Laval *et al.*, 2001). Carbohydrates (D-glucose, D-fructose, sucrose, starch and conjugates) and levels of virus could therefore be measured in regions of the plant that display specific symptoms. It is possible that correlations between symptoms, virus levels and sugars may become more apparent by using this approach.

The early induction of the defence responses upon inoculation with CaMV implies that the plant can sense the presence of the virus. The induction of these early events may be triggered either by viral proteins and/or events involved in virus replication. The identification of specific viral proteins as elicitors could be tested by inoculating defence promoter::Luciferase reporter lines with purified virus proteins, and observing luminescence. In order to rule out virus replication as an eliciting event, these luciferase lines could be inoculated with replication deficient virus.

It is also important to consider the types of signaling pathways and interactions that take place during these responses. This could be examined by infecting defence promoter::Luciferase lines with CaMV, then applying inhibitors that are specific to early signalling pathways (for example catalase/super oxide dismutase may be used to prevent the oxidative burst), and then monitoring the level of luminescence. This method could be used to assess the involvement of a vast array of pathways, such as calcium/calmodulin and nitric oxide signaling during the early responses to infection. However, a more genetic approach could be carried out, whereby characterized transgenic/mutant *Arabidopsis* could be crossed with defence promoter::Luciferase reporter lines. For example, infecting GST1::Luciferase/NahG crosses with CaMV and measuring luminescence, would enable us to determine how crucial SA is for ROI formation and GST1 induction. Since characterized mutant/transgenic and defence promoter::Luciferase reporter lines are readily available, very extensive studies could be carried out.

Luciferase promoter reporter plants are useful tools for analyzing the temporal and spacial induction of genes. However, other approaches could be taken in viewing the defence responses to infection *in planta*. For example aequorin experiments could be used to quantify calcium involvement in signal transduction during infection, and/or specific staining could be employed to temporally and spatially detect plant products closely associated with defence (i.e. DAF staining may be used to detect the accumulation of nitric oxide). Therefore, a broad array of techniques should be employed to find out the extent of defence activation during infection, thereby building a coherent map of pathological events. These approaches

should also exploit the large array of different viruses and CaMV isolates that are available, helping us to understand why some viruses can elicit but evade the defence response.

It is also important that we scrutinize the ability of each defence pathway to limit virus accumulation or symptom development. This would involve classic infection studies, whereby virus titre and symptom severity would be measured in plants that have one or more lesions in defence pathway(s).

Thus an integrated approach should be taken to identify the responses elicited by the virus and the impact that it has on pathogenesis.

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WS sugar (7.1.1.4)

WS starch (7.1.1.5)

WS ratio (7.1.1.6)

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WS PR genes (7.1.2.2)

Virus levels (7.1.3)

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Disk 2: Turnip statisitics (8.1)

Carbohydrates (8.1.1)

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