

POLYSOME PROFILING OF THE PLANT PATHOGEN RESPONSE

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ABSTRACT

This study aimed to use a combination of sucrose density gradient centrifugation and RNA-Seq to investigate changes in expression at the transcriptional and translational levels and the differences therein in a susceptible cultivar of *Capsicum annuum* (pepper) during the early stages of tomato spotted wilt virus (TSWV) infection, a virus that causes significant economic loss in pepper and many other crops and to which there is little robust resistance in the pepper germplasm. Polysome profiles of pepper plants 6 hr after TSWV-inoculation showed a significant increase in polysomally associated material relative to the 80S ribosomal peak and mock- and non-inoculated controls, which is indicative of an increase in global translation upon infection. Principle component analysis (PCA) and hierarchical clustering showed that RNA-Seq results for total RNA and pooled polysomal RNA are largely similar but that significant differences were present, which indicates post-transcriptional regulation of expression at the level of translation. Sequencing results also clustered by treatment conditions, showing that expression was significantly affected by TSWV-inoculation. Gene ontology (GO) and KEGG pathway analyses revealed that upon TSWV-inoculation among the genes that reduce in abundance in total and polysomal RNA, there were many involved in promoting pathogen perception, defence signalling, autophagy, and disease resistance. Conversely many genes that were involved in suppressing the pathogen response and maintaining or promoting anabolic processes increase in abundance in total and polysomal RNA, or showed no change in abundance in such a way that may promote susceptibility to TSWV.

This study has enhanced the understanding of the compatible reaction between TSWV and pepper by highlighting multiple novel host factors that may be involved in infection. Preliminary optimisation of downstream RNA preparation and QRT-PCR demonstrated where methodological changes need to be made in order to enhance reliability of future experiments that build upon the results of this work.

1 TOMATO SPOTTED WILT VIRUS, TOSPOVIRUSES, AND TRANSLATIONAL CONTROL IN PLANTS

1.1 TOMATO SPOTTED WILT VIRUS

Tomato spotted wilt virus (TSWV) is the causative agent of spotted wilt disease, which was first identified as a disease of tomato in Australia, 1915 (Brittlebank, 1919). The disease was found to be primarily transmitted by a thrips species *Frankliniella insularis* (Franklin) (Samuel *et al.*, 1930).

This disease occurs in temperate, sub-tropical and tropical climates worldwide (Blancard, 2012). Although TSWV had been detected in various countries throughout the 20th century, spread and incidents of the disease increased during the 1980's when one of its vectors, the Western flower thrips (*Frankliniella occidentalis*) expanded its geographical range (Goldbach and Peters, 1994). Tomato spotted wilt is especially prevalent in warmer climates and glasshouse environments (Goldbach and Peters, 1994) although the vector, *F. occidentalis* is able to survive and reproduce in more temperate conditions (McDonald *et al.*, 1999). Weed plants that are able to overwinter in cold, moist climates act as reservoirs for the virus allowing the vector to complete its life-cycle (Groves *et al.*, 2001). Over 1000 plant species including economically important crops and many reservoir species are listed as hosts of TSWV comprising of 15 monocotyledonous families, 69 dicotyledonous families and one pteridophyte family (Parrella *et al.*, 2003).

Infection with TSWV leads to a reduced or total loss of yield in its host as well as a drop in the quality of the crop; this coupled with its broad host

range and the difficulty of managing thrips vectors makes it an economically important pathogen (Scholthof *et al.*, 2011). Considering these factors, it is difficult to accurately assess the total economic impact of TSWV. However, reports range in the hundreds of thousands to millions of US\$ per annum around the world; in Georgia, USA, tomato spotted wilt is estimated to have caused US\$16.9 million worth of damage to the peanut crop in 2014 (Brannen *et al.*, 2014); in Samsun, Turkey, TSWV is estimated to have caused US\$0.9 million of damage to the tomato crop in 2012 (Sevik and Arli-Sokmen, 2012).

Peppers (genus *Capsicum*) are a susceptible host and cash crop that suffers dramatic losses upon infection with TSWV and thus finding stable resistance is important (Roggero *et al.*, 2002). Little information is available on estimates of subnational, national, and international economic impact of TSWV in peppers in particular but the disease is having a marked impact on the pepper crop according to Didierlaurent, Syngenta Seeds, Sarrians (personal communications, 2013).

1.2 SYMPTOMS

Symptoms and severity of TSWV infection vary dramatically between host species and viral species, but also other factors influence infection such as; host growth conditions, host age at the point of infection, and the serotype of TSWV infecting the host (Beaudoin *et al.*, 2009; Sherwood *et al.*, 2009). Symptoms tend to include yellowing/bronzing of young leaves with subsequent formation of necrotic or chlorotic spots and wilting of leaves, as shown in figure 1.1. Other common symptoms include but are not limited to:

stunting; dieback of growing tips, as shown in figure 1.2; black streaks on stems; reduced or absent fruit set, if infected prior to fruit set; and low-yield, poor-quality, ringed/spotted, bumpy fruit with uneven ripening, if infected after fruit set, as shown in figure 1.3 (Goldberg and French, 2000).

At a cellular level in systemically infected *Datura stramonium* (a wild Solanaceous host of TSWV), 85 nm spherical particles are visible in all tissues when viewed under an electron microscope; particles are visible solely in the cytoplasm and often associated with endoplasmic reticulum (Francki and Grivell, 1970). Francki and Grivell (1970) also observed filamentous inclusions and granular viroplasms in TSWV-infected *D. stramonium*. TSWV can be differentiated from the closely related tospovirus, tomato zonate spot virus (TZSV), by comparing patterns of sub-cellular clustering and distribution of virus particles in infected plants; again, these vary between host species, host cultivar, host tissue, and virus accession (within a virus species) (Zhang *et al.*, 2016b). Zhang *et al.* (2016b) found that, in *Nicotiana tabacum*, only TSWV particles associate with the filamentous inclusions and globular viroplasms whereas TZSV only associated to the inside of endoplasmic reticulum membranes.



Figure 1.1. TSWV-infected pepper (Capsicum sp.) leaves showing chlorotic spots and rings. Picture obtained from Minuto (2018).



Figure 1.2. TSWV-infected pepper (Capsicum sp.) showing dieback of growing tips. Picture obtained from McGrath (2015)



Figure 1.3. TSWV-infected pepper (Capsicum annuum) showing necrotic lesions on fruits with uneven ripening. Picture obtained from Pappu (2018).

As a method of finding unique sources of resistance in peppers, Boiteux *et al.* (1993) used a scoring system of presence and severity of macroscopic symptoms after TSWV-infection, as well as a dot-ELISA-based system to score levels of systemic infection. Symptoms were scored from 1 to 5, depending on levels of apical leaf distortion, mature leaf mosaicism, necrosis, and general stunting. Dot-ELISA plots were scored by their relative reaction against TSWV in apical leaf extracts.

There is evidence of resistance to TSWV in pepper. Segura, a TSWV-resistant cultivar, shows localised necrotic lesions on inoculated leaves that prevented systemic movement of viral particles. Susceptible plants (Delfos) showed chlorosis and stunting upon inoculation and Segura plants co-inoculated with resistance-breaking and non-resistance-breaking TSWV accessions showed chlorosis, stunting, necrotic lesions, and occasional systemic necrosis (Aramburu *et al.*, 2015). Aramburu *et al.* (2015) also used this scoring system to corroborate earlier reports that TSWV-infected TSWV-resistant pepper plants lose resistance and exhibit systemic symptoms when grown at constant high temperature (30-35°C), in comparison to plants grown at lower temperatures (18-25°C). This phenomena may be due to the stability of the hypersensitive response, as this defence mechanism is less stable at temperatures greater than 30°C (Roggero *et al.*, 1996; Moury *et al.*, 1998).

1.3 TAXONOMY OF TOSPOVIRUSES

1.3.1 Phylogeny and evolution

TSWV is the type species of *Tospovirus*, a genus of insect-borne, plant-infecting viruses belonging to the family *Bunyaviridae*. Tospoviruses have tripartite,

single-stranded (ss)RNA genomes consisting of one negative-sense and two ambisense segments (German *et al.*, 1992). All other genera in the *Bunyaviridae* are haematophagous arthropod-borne, animal-infecting viruses and most of them are entirely negative sense. The only other exceptions to this latter rule are viruses in the genus *Phlebovirus*, which have one ambisense and two negative-sense segments (Schmaljohn and Nichol, 2007; Briesse *et al.*, 2013).

There are 11 assigned species in *Tospovirus*, according to the 2016 release by the International Committee on Taxonomy of Viruses (ICTV, 2016), as well as around 20 tentative species that have not yet been listed by the ICTV and new species are reported frequently (Kormelink, 2011; Hassani-Mehraban *et al.*, 2016; Huang *et al.*, 2017). Figure 1.4 shows the evolution of the protein sequence of the *N* gene in 29 *Tospovirus* species; species group together into clades based partly on their geographical source (Hassani-Mehraban *et al.*, 2016).

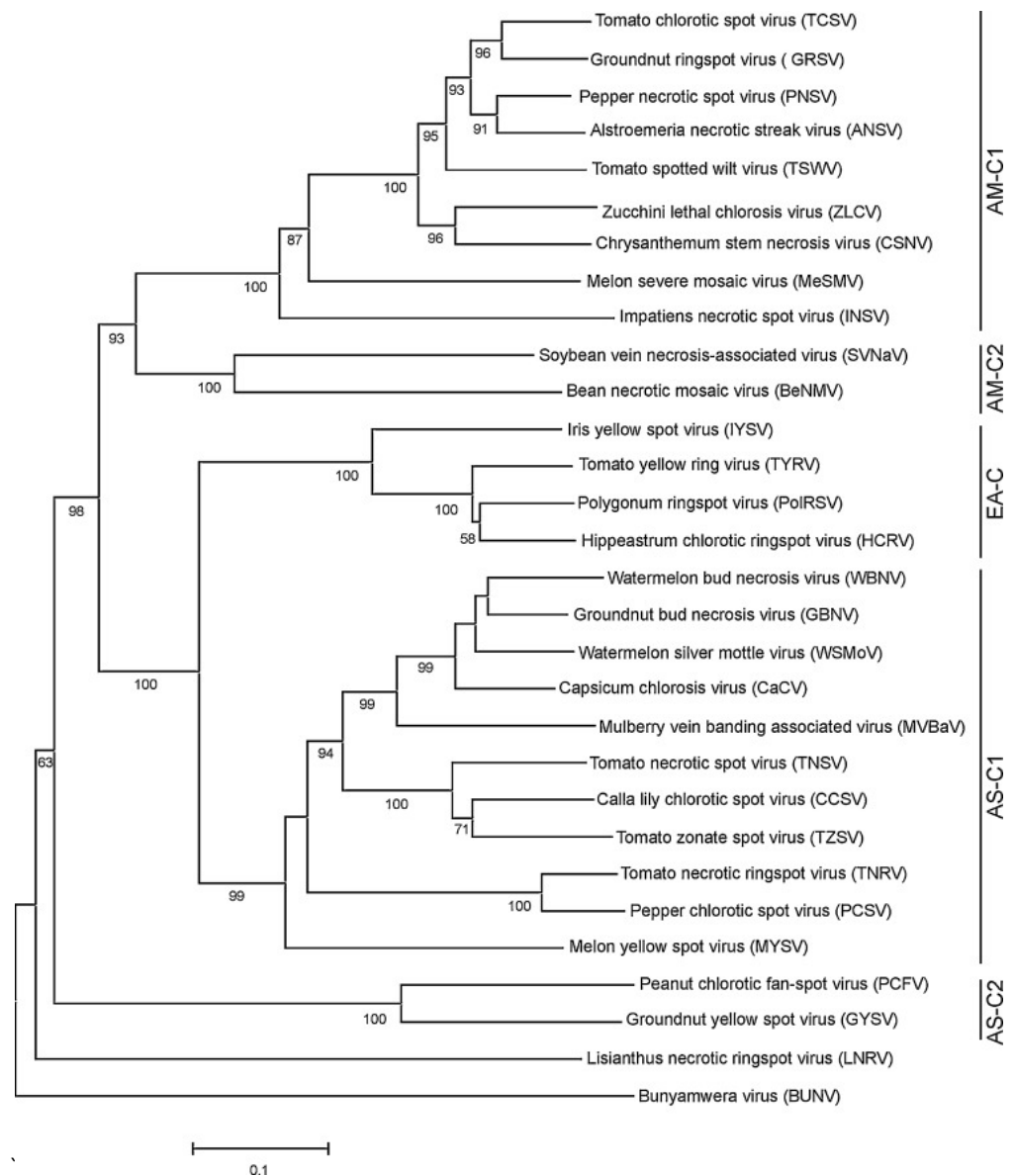


Figure 1.4. A neighbour-joining tree showing the evolution of the nucleocapsid protein sequence of 29 Tospovirus species and Bunyamwera virus (Hassani-Mehraban et al., 2016).

TSWV populations evolve rapidly as they are RNA viruses and replicate using an error-prone RNA polymerase, which allows rapid generation of highly diverse quasi-species (Andino and Domingo, 2015; Marshall *et al.*, 2017). Another layer of molecular variation in TSWV comes from recombination and reassortment (Lian *et al.*, 2013). This diversity gives TSWV the adaptability to infect new or resistant hosts (Qiu and Moyer, 1999; Margaria *et al.*, 2015a). Although heterogeneity in TSWV is limited by the fact that the viral particles must still obviously be able to interact with the hosts: both the plant host and the thrips vector, as they are not merely carried by the vector but also replicate within thrips (Tsompana *et al.*, 2005). The impact of the host on the cloud size of RNA virus quasi-species varies between hosts, as shown by the varying mutation rate and percentage of mutated viral during infection of various solanaceous and cucurbitaceous hosts; this includes whole plants and tobacco protoplasts, by Tobacco mosaic virus (TMV) and Cucumber mosaic virus (CMV) (Schneider and Roossinck, 2001).

1.3.2 Genome organisation

TSWV contains three ssRNA genomic segments consisting of five genes encoding six proteins (Cortez *et al.*, 2001). Each of the three segments has inverted complementary 5' and 3' ends so that they can circularise the linear RNA molecules; the annealed ends protrude from the pseudocircular RNA molecules in a panhandle structure (de Haan *et al.*, 1990; de Haan *et al.*, 1991; Kormelink *et al.*, 1992).

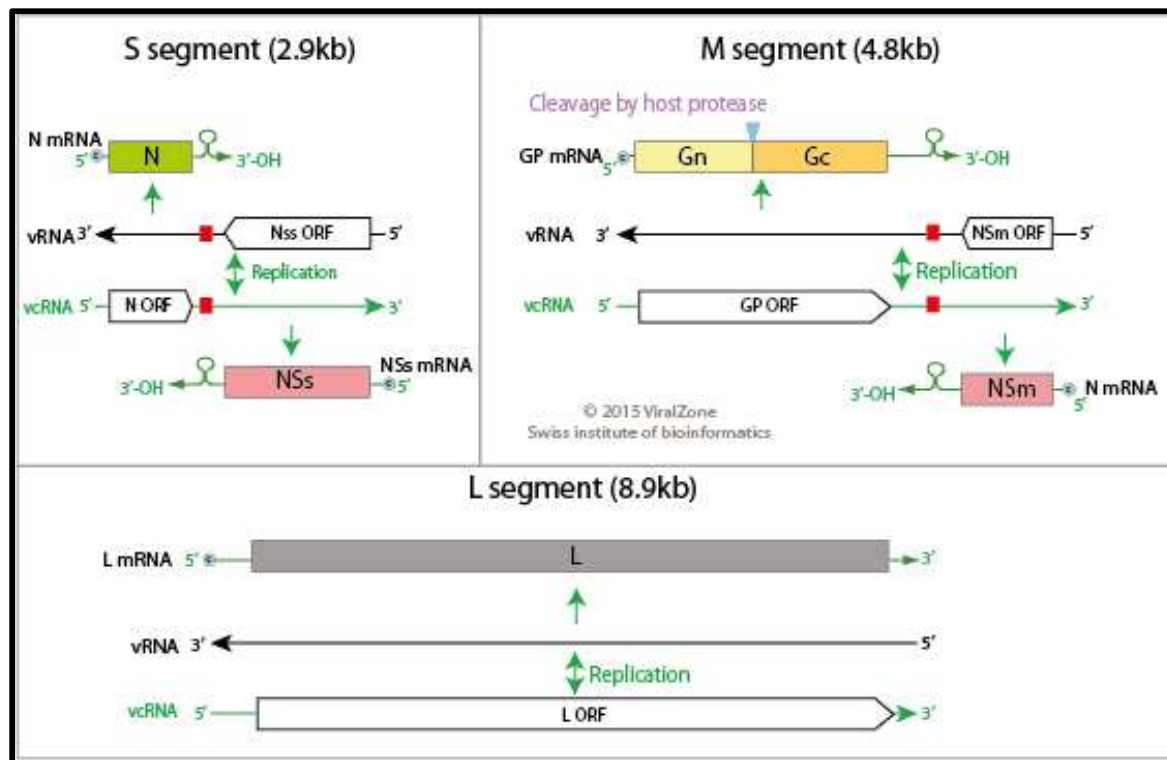


Figure 1.5. The tripartite, segmented, ambisense genome of Tomato spotted wilt virus contains five genes: on the small (S) segment are those encoding the nucleocapsid (N) in the negative sense and the RNA silencing suppressor (NSs) in the positive sense; on the medium (M) segment are a gene encoding a polyprotein, which is cleaved into two glycoproteins (Gn and Gc), in the negative sense and the movement protein (NSm) in the positive sense; and on the large (L) segment is L open reading frame, which encodes the RNA-dependent RNA polymerase. Image obtained from (ViralZone, 2012).

The S segment

The small (S) RNA segment is 2.9 kb in length and encodes two genes: one encoding the non-structural RNA silencing suppressor protein (NSs) in the viral sense and the nucleocapsid (N) gene in the viral complementary sense (de Haan *et al.*, 1990; Takeda *et al.*, 2002). The open reading frames (ORFs) of these genes do not overlap and are separated by an AU-rich intergenic region (IGR) that forms a hairpin that acts as a bidirectional transcriptional terminator and that, in the subgenomic mRNAs, may be involved in stimulating translation as a functional equivalent to a poly(A) tail (Geerts-Dimitriadou *et al.*, 2012).

The NSs protein counteracts host antiviral resistance by suppressing the RNA silencing pathway, which would otherwise block expression of viral transcripts (Takeda *et al.*, 2002).

N is the nucleocapsid protein; this protein forms oligomers, which encapsidate genomic RNAs via its positively charged RNA-binding cleft to form a ribonucleoprotein (RNP) complex with the TSWV RNA-dependent RNA polymerase (RdRp; described below) within the viral particle (de Haan *et al.*, 1990; Li *et al.*, 2015a). N also has roles in protecting genomic RNAs from host RNases (Li *et al.*, 2015a), tubule-guided intracellular and intercellular movement (Soellick *et al.*, 2000; Feng *et al.*, 2013), particle formation (Ribeiro *et al.*, 2009), and transcription and replication (Richmond *et al.*, 1998).

The M segment

Similar to the S RNA, the medium (M) RNA (4.8 kb) has two non-overlapping, ambisense ORFs: one encoding the non-structural movement protein (NSm) in the viral sense and one encoding a polyprotein precursor, which is co-

translationally cleaved and glycosylated by host enzymes in the rough endoplasmic reticulum into two glycoproteins (Gn and Gc; N-terminal and C-terminal respectively), in the viral complementary sense (Kormelink *et al.*, 1992). Again like the S RNA, both genes are separated by an IGR and analysis of the M segment IGR indicates that M-derived subgenomic mRNAs terminate with functionally similar hairpins (van Knippenberg *et al.*, 2005a; Geerts-Dimitriadou *et al.*, 2012).

NSm is involved in various aspects of movement of the infective virus particle: it associates closely with TSWV RNPs (Soellick *et al.*, 2000); it forms tubular structures, whose formation is closely linked to intercellular movement (Li *et al.*, 2009); and it associates to plasmodesmata (PD), modifying their size exclusion limit (SEL) but reports vary on whether this increases or decreases the PD SEL, making it difficult to draw conclusions on the exact reason of this association/modification (Storms *et al.*, 1998).

In plants, the two glycoproteins, Gn and Gc, are involved in particle assembly by wrapping of RNPs in Golgi cisternae to create double-enveloped virus particles (DEVs); these fuse with each other and with endoplasmic reticulum (ER)-derived membranes to produce large vesicles containing multiple single-enveloped virus particles (SEVs) that are ready for uptake by the thrips vector (Kikkert *et al.*, 1999). Movement from the ER to the Golgi and wrapping of RNPs involves the interaction between the glycoproteins and N, which cannot occur when the Gn and Gc are not glycosylated (Kikkert *et al.*, 2001; Ribeiro *et al.*, 2009). Gn and Gc are type I transmembrane proteins

whose glycosylated N-termini protrude from the surface of viral particles (German *et al.*, 1992; Kikkert *et al.*, 2001).

The L segment

In contrast to the other two segments, the large (L) RNA contains a single ORF in the negative sense. The L RNA is the largest segment, approximately 9 kb in size and encodes an RdRp in the viral complementary sense (de Haan *et al.*, 1991).

The L protein synthesises genomic, sub-genomic, and messenger RNA using viral RNPs as a template (Duijsings *et al.*, 2001b). L cleaves viral and capped non-viral RNA to prime replication and transcription respectively; when cellular mRNAs are cleaved and used to prime viral mRNA synthesis, this is called cap-snatching (Nguyen and Haenni, 2003; van Knippenberg *et al.*, 2005b). Template mRNAs are cleaved 13-18 nucleotides downstream of the cap; this leader sequence is used to as the primer for viral mRNA synthesis. Cleavage occurs preferentially at an A residue, indicating a requirement for single base complementarity with a U residue on the TSWV RNA template (Duijsings *et al.*, 2001a).

As the RdRp is needed to initiate viral gene expression, it is co-transported with viral RNPs into uninfected cells (Whitfield *et al.*, 2005).

1.4 TRANSMISSION AND EPIDEMIOLOGY

1.4.1 TSWV vectors

TSWV and the tospoviruses are transmitted between plants by thrips, which are small insects of the order Thysanoptera (Jones, 2005). At least 15 species of thrips in six genera act as vectors for tospoviruses, as listed in table 1.1

(Rotenberg *et al.*, 2015). All thrips species that act as vectors for tospoviruses are members of the suborder Terebrantia, family Thripidae and subfamily Thripinae but are not closely related; they do however share the characteristics of being able to feed and reproduce on a broad range of host plants (Mound, 2002; Buckman *et al.*, 2013; Sartiami and Mound, 2013).

The western flower thrips, *Frankliniella occidentalis* (Pergande), is the most important vector of tospoviruses, both scientifically and economically: *F. occidentalis* transmits TSWV and multiple other tospoviruses (see table 1.1) (Rotenberg *et al.*, 2015). When *F. occidentalis* expanded its geographic range from being localised to the south-west USA to being distributed globally during the 1980's, TSWV isolates became primarily transmitted by *F. occidentalis* and lost the capacity to be transmitted by a previous major vector, *Thrips tabaci* (Ullman *et al.*, 1997). *Frankliniella occidentalis* is the first member of Thysanoptera with an available genome assembly; this makes it a potential model for both vector and non-vector thrips species and facilitates its use as a model organism for the study of thrips-tospovirus interactions by allowing identification of the genetic factors in the vector that are involved in infection and transmission (Rotenberg, 2012).

Table 1.1. Verified and tentative (*) tospovirus species and the thrips species recorded as their vectors (Rotenberg et al., 2015).

Tospovirus Species	Thrips Vector
Alstroemeria necrotic streak virus, AINSV*	<i>Frankliniella occidentalis</i>
Bean necrotic mosaic virus, BeNMV*	Unknown
Calla lily chlorotic spot virus, CCSV*	<i>Thrips palmi</i>
Capsicum chlorosis virus, CaCV*	<i>Ceratothripoides claratris</i> , <i>Thrips palmi</i> , <i>Frankliniella schultzei</i>
Chrysanthemum stem necrosis virus, CSNV*	<i>Frankliniella occidentalis</i> , <i>Frankliniella intonosa</i> , <i>Frankliniella schultzei</i>
Groundnut bud necrosis virus, GBNV	<i>Frankliniella schultzei</i> , <i>Scirtothrips dorsalis</i> , <i>Thrips palmi</i>
Groundnut chlorotic fan-spot virus, GCFSV*	<i>Scirtothrips dorsalis</i>
Groundnut ringspot virus, GRSV	<i>Frankliniella occidentalis</i> , <i>Frankliniella schultzei</i> , <i>Frankliniella gemina</i>
Groundnut yellow spot virus, GYSV	<i>Scirtothrips dorsalis</i>
Hippeastrum chlorotic ringspot virus, HCRV*	Unknown
Impatiens necrotic spot virus, INSV	<i>Frankliniella occidentalis</i> , <i>Frankliniella fusca</i> , <i>Frankliniella intonosa</i>
Iris yellow spot virus, IYSV	<i>Frankliniella fusca</i> , <i>Thrips tabaci</i>
Melon severe mosaic virus, MSMV*	Unknown
Melon yellow spot virus, MYSV*	<i>Thrips palmi</i>
Pepper necrotic spot virus, PCSV*	Unknown
Polygonum ring spot virus, PoIRSV	<i>Dictyothrips betae</i>
Soybean vein necrosis virus, SVNv*	<i>Neohydatothrips variabilis</i>
Tomato chlorotic spot virus, TCSV	<i>Frankliniella occidentalis</i> , <i>Frankliniella schultzei</i> , <i>Frankliniella intonosa</i>
Tomato necrotic ringspot virus, TNRV	<i>Ceratothripoides claratris</i> , <i>Thrips palmi</i>
Tomato necrosis virus, TNeV	Unknown
Tomato spotted wilt virus TSWV	<i>Frankliniella occidentalis</i> , <i>Frankliniella fusca</i> , <i>Frankliniella schultzei</i> , <i>Frankliniella intonosa</i> , <i>Frankliniella bispinosa</i> , <i>Frankliniella cephalica</i> , <i>Frankliniella gemina</i> , <i>Thrips setosus</i> , <i>Thrips tabaci</i>
Tomato yellow (fruit) ring virus, TYRV*	<i>Thrips tabaci</i>
Tomato zonate spot virus, TZSV*	<i>Frankliniella occidentalis</i>
Watermelon bud necrosis virus, WBNV	<i>Thrips palmi</i>
Watermelon silver mottle virus, WSMoV	<i>Thrips palmi</i>
Zucchini lethal chlorosis virus, ZLCV	<i>Frankliniella zucchini</i>

1.4.2 Vector lifecycle

The thrips lifecycle consists of the following stages: eggs, first instar larvae (L1), second instar larvae (L2), pre/pro-pupae (P1), pupae (P2), and adults (Lewis, 1997). L1, L2, and adults feed on plant fluids from leaves and flowers by a “punch and suck” mechanism; P1 and P2 do not feed but reside quiescently in soil or plant growth medium (Moritz, 1997). The lifecycle of *F. occidentalis* can be seen in figure 1.6.

Generation time of thrips from eggs to ovipositing adults varies from 15 days at 30°C to 44 days at 15°C and as generation times go down population growth rate increases (Lublinkhof and Foster, 1977; Gaum *et al.*, 1994).

One population model dictates that thrips require a minimum developmental temperature of 7.9°C and in the UK using outdoor air temperatures data from 1986-95 the number of generations per year was predicted to be proportional to the accumulated number of days per year with an average air temperature greater than this threshold (degree-days) with a cofactor of roughly 268 (McDonald *et al.*, 1998). Around 3-5 generations per year were projected given no impeding factors to population growth (McDonald *et al.*, 1998). During the period of the 30th of April to the 30th of June 1989 and 1990, three distinct generations of western flower thrips were identified on field-grown tomatoes in Gadsden County, Florida; in the 1989 growing season, the temperature average was 24.3°C and range was 16.4-28.3°C and in the 1990 growing season, the temperature average was 25.0°C and ranged was 17.2-29.7°C (Puche *et al.*, 1995; Florida Climate Center, 2012). Three generations per years is fewer than expected using the model devised

by McDonald *et al.* (1998) given that the seasonal degree-day values were 1017 (1989; 3.8 predicted generations) and 1061 (1990; 4.0 predicted generations) possibly because fields used for data collection by Puche *et al.* (1995) were sprayed weekly with insecticide (esfenvalerate, methamidophos, or *Bacillus thuringiensis* or a combination thereof) impeding thrips breeding (Florida Climate Center, 2012). In the Gadsden County data, frequency of TSWV infection was measured and no association was seen between infection rates and the bursts in thrips populations but infections of tomato plants appeared at random through the field; this seems to mean that insecticide treatment was adequate to control secondary infection from thrips within the field but not primary infections from viruliferous thrips that developed on hosts outside the field then migrated in (Puche *et al.*, 1995).

Given the correct conditions with temperatures consistently over the developmental threshold for the whole year, thrips produce many overlapping, continuous generations rather than discrete generations, with reports of up to 21 generations per year in Taiwan and up to 15 generations per year in glasshouses (Reitz, 2009; Huang *et al.*, 2010; CABI, 2017). Under these constantly warmer conditions there is no inactive period for thrips allowing continuous development and breeding (Kirk, 1997). For *F. occidentalis*, temperature seems to be much more important for development and oviposition than photoperiod, as this species undergoes no reproductive diapause under short days compared with long days, whereas other thrips species' behaviour is photoperiod-dependent (Kirk, 1997; Ishida *et al.*, 2003).

Frankliniella occidentalis is more established in areas with mild winters but can overwinter in some colder regions, even in conditions which laboratory experiments have predicted to be lethal (Kirk and Terry, 2003). This thrips species overwinters as adults whereas other species can overwinter as eggs, larvae or adults but not P1 or P2; it is likely that the overwintering *F. occidentalis* adults' offspring acquire TSWV from winter weed reservoirs and infect spring planted crops (Chamberlin *et al.*, 1992; Kirk, 1997; Del Bene *et al.*, 1998; Kahn *et al.*, 2005). In colder climates, *F. occidentalis* is more associated with glasshouses, as prolonged exposure to cold reduces the ability of a proportion of the population to develop and reproduce (McDonald *et al.*, 1997).

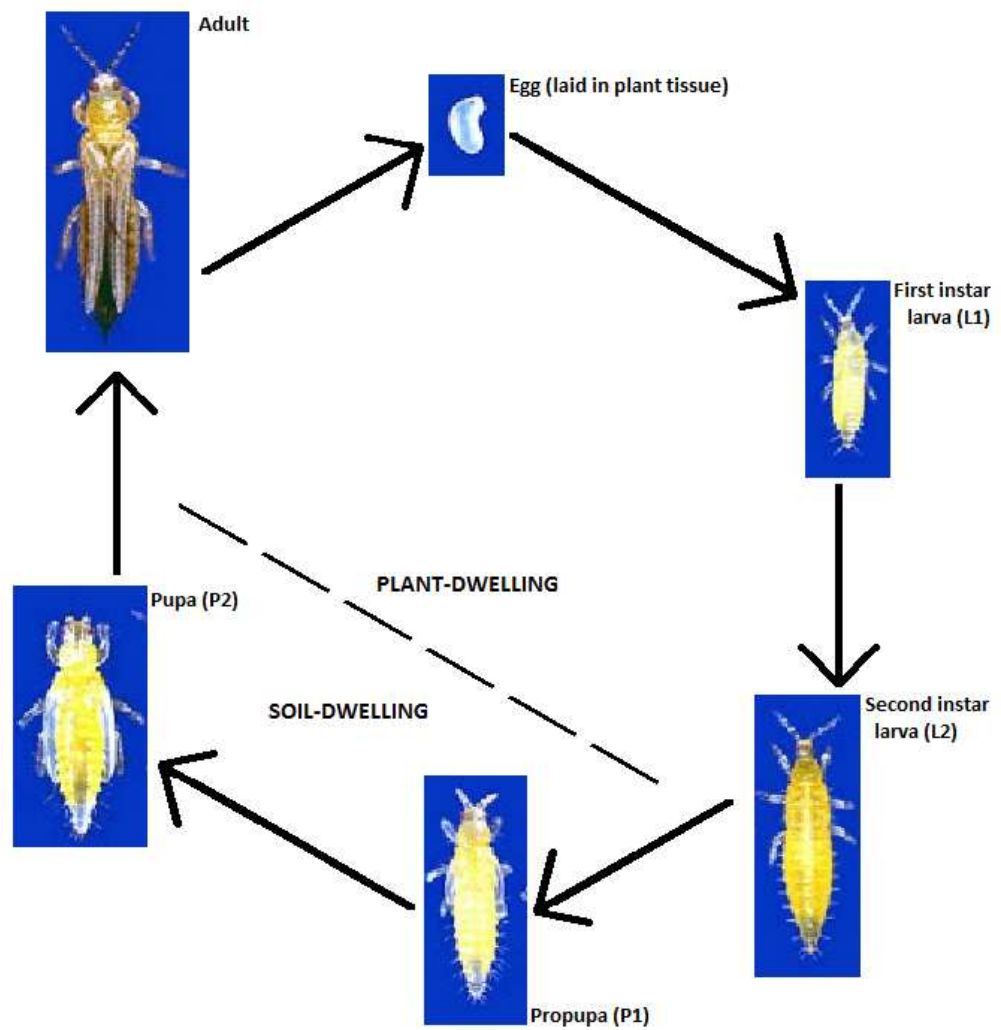


Figure 1.6. The lifecycle of western flower thrips *Frankliniella occidentalis*. Pictures of different stages obtained from Himmelein (2011).

1.4.3 Acquisition and transmission

TSWV acquisition and transmission by thrips are stage dependent; thrips can only acquire the virus if they feed on an infected plant at L1 stage and TSWV is primarily transmitted by the adults (Wijkamp *et al.*, 1993). P1 and P2 stage thrips do not feed and do not actively move unless disturbed so cannot acquire or transmit TSWV (CABI, 2017).

Thrips transmit TSWV via their saliva during feeding and TSWV replicates in the insect's salivary glands (Ullman *et al.*, 1989; Kritzman *et al.*, 2002). After an L1 larva feeds on an infected plant, the virus must migrate from the midgut and visceral muscles to the salivary glands. This can only happen in L1 larvae due to the parting of the salivary glands and first midgut loop during the L2 stage onwards. Spatial separation at later stages of development prevents further movement of viral particles to the salivary glands (Moritz *et al.*, 2004). L2 thrips that have acquired the virus at L1 are infectious but are not considered a major vector as they do not move between plants until after they have pupated into winged adults (Wijkamp and Peters, 1993).

As thrips larvae are wingless, they have a limited dispersal range and mainly feed and develop on the plant on which they hatched; this means that oviposition by the mother thrips is an important determinant of whether an L1 thrips will become viruliferous. Moreover, adult thrips actively show a preference for feeding and oviposition on TSWV-infected plants, thus increasing rates of virus acquisition by larvae (Bautista *et al.*, 1995; Maris *et al.*, 2004b). Evidence in *Arabidopsis thaliana* suggests that TSWV-infected plants are more attractive, suitable hosts for *F. occidentalis* because TSWV infection

induces salicylic acid (SA) biosynthesis; SA negatively regulates the jasmonate (JA) biosynthesis, which would otherwise reduce attractiveness of the host plant to thrips and increase the host's resistance to thrips attack (Abe *et al.*, 2012). JA and related signalling compounds play a central role in the responses to wounding, necrotrophic pathogens, and herbivory by insects (Glazebrook, 2005; Howe and Jander, 2008). JA-signalling is tightly linked to both the direct herbivory response, by which the plant endogenously deter or damage the attacking insect, and the indirect herbivory response, by which the plant attracts predators of the herbivore (Aljory and Chen, 2018).

1.4.4 Viral spread within the plant host

In order to complete the infection cycle, once acquired TSWV particles must spread systemically through the plant host to pass on the disease to the thrips vector. As is common among plant viruses, TSWV possesses a movement protein (NSm), which is required for intra cellular, intercellular, and systemic movement (von Barga *et al.*, 2001). NSm localises at the ER and plasmodesmata and is able to form tubular structures, through which infective viral RNPs move, via the interaction of NSm and the nucleocapsid N, into neighbouring cells (Kormelink *et al.*, 1994; Storms *et al.*, 1995; Feng *et al.*, 2016). TSWV NSm is able to complement movement-defective TMV mutants, allowing local and systemic symptom induction (Lewandowski and Adkins, 2005). The plant ER is one continuous network that connects cells via their plasmodesmata and along which the plant can transport macromolecules; TSWV infective RNPs are transported through the continuous plant ER (Feng *et al.*, 2016).

Numerous host factors have been identified that interact with NSm and that are vital for cell-to-cell and systemic movement and of TSWV particles. Using yeast two-hybrid traps, DnaJ-like chaperones from tobacco, *Arabidopsis*, and tomato have been identified as interacting factors of NSm (Soellick *et al.*, 2000; von Bargen *et al.*, 2001). Together with the heat shock protein Hsp70, DnaJ-like proteins are involved in protein transport into the mammalian ER, transport of proteins into the mitochondria and peroxisomes, and microtubule formation (Rassow *et al.*, 1994; Crookes and Olsen, 1998; Oka *et al.*, 1998; Zimmermann, 1998). DnaJ/Hsp40 proteins promote Hsp70 activity by recruiting Hsp70 partners and accelerating Hsp70 ATP-hydrolysis (Kelley, 1998). An Hsp70 homologue (Hsp70h) encoded by Beet yellows virus (BYV), a +ssRNA member of *Closterovirus*, acts as a chaperone to aid BYV particle assembly and transport to and through the plasmodesmata, highlighting the possibility that Hsp70 acts in a similar way during infection by other viruses (Alzhanova *et al.*, 2001). Hsp70 homologues in *Cucurbita maxima* squash have also been identified as being involved in intercellular transport of macromolecules via the plasmodesmata (Aoki *et al.*, 2002). During TSWV-infection of susceptible tomato (cv. Money Maker), *Hsp70* is upregulated transcriptionally in shoots and roots, further highlighting the possibility Hsp70 acts as a susceptibility factor therein (Catoni *et al.*, 2009).

Suppressor of the G2 allele of *skp1* (SGT1), a co-chaperone that interacts with Hsp70, has also been identified as an interacting factor of TSWV in tobacco. *SGT1* expression is upregulated by TSWV infection. Silencing of *SGT1* drastically inhibits cell-to-cell movement of TSWV NSm-GFP fusion

proteins and impedes systemic TSWV infection and *SGT1* overexpression accelerates TSWV infection (Qian *et al.*, 2018).

The *Arabidopsis* protein At-4/1 may be a key factor in the systemic movement of infective TSWV RNPs. AT-4/1 interacts with NSm and is able to perform intra- and intercellular trafficking of macromolecules via the plasmodesmata (von Bargen *et al.*, 2001; Paape *et al.*, 2006). The tobacco Nt-4/1 protein has also been shown to transport cellular and viroid RNA via the phloem (Solovyev *et al.*, 2013).

The protein ROOT HAIR DEFECTIVE 3 (RHD3) mediates the formation of the tubular ER network and *Arabidopsis rhd3* mutants produce unbranched ER (Stefano *et al.*, 2012; Zhang *et al.*, 2013). NSm physically associates to the ER in tobacco and *Arabidopsis rhd3* mutants show increased systemic resistance to TSWV due to reduced cell-to-cell spread despite viral replication continuing as normal; this demonstrates that the ER intercellular trafficking system is vital for viral movement (Feng *et al.*, 2016).

1.5 DISEASE MANAGEMENT IN PEPPER

1.5.1 Exogenous treatment in the control of TSWV

TSWV is an obligate intracellular parasite; therefore, it spends virtually all of its life cycle inside its host plant or thrips. This virus is reliant on host cell machinery for reproduction and intercellular movement via plasmodesmata and thrips for inter-host movement. Perturbing these presents numerous problems: topical pesticide treatment cannot directly attack viruses; antiviral compounds would require delivery into the plant and systemic spread in order to systemically perturb viral function and may have unpredicted systemic and

ecological effects. In addition these treatments would likely not be commercially viable for repeat or large-scale application to crops and so are not widely used.

Exogenous elicitors of plant pathogen response

However, it is possible to elicit systemic responses that prime plants against later infection by a broad range of pathogens, including viruses; these are systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Ryals *et al.*, 1996; van Loon *et al.*, 1998). Both of these signalling pathways require a functioning *NPR1* gene to trigger plant defence responses, however the processes downstream of *NPR1* are distinct between ISR and SAR (Pieterse *et al.*, 1998).

SAR is a signal transduction pathway induced after a primary infection or by application of chemicals, such as benzothiadiazole (BTH, syn. acibenzolar-S-methyl), and is associated with systemic accumulation of salicylic acid (SA) and an SAR-specific set of pathogenesis-related (PR) proteins, some of which have direct antimicrobial properties while others elicit physiological changes in the host to make it a less favourable environment for the invading pathogen (van Loon *et al.*, 1998).

Some success has been achieved using BTH to prime TSWV-resistance in Solanaceous crops. Activation of TSWV-resistance in flue-cured tobacco, based on decreased symptom severity and increased SAR marker expression, correlated with dose of BTH and was strongest from 5 days after BTH-treatment onwards (Mandal *et al.*, 2008). BTH-treatment of tomato showed some success in reducing TSWV incidence when infection rates were high but

BTH had less effect when insecticide- and mulch-based control of thrips was applied in combination (Momol *et al.*, 2002; Momol *et al.*, 2004).

ISR is distinct from SAR in that it is based on perception of jasmonic acid (JA) and ethylene (ET) instead of SA and can be elicited by the presence of plant growth promoting rhizobacteria (PGPR) (van Loon *et al.*, 1998).

In tomato, the presence of the PGPR *Pseudomonas fluorescens* in the soil increases resistance to TSWV via action of phenylalanine ammonia lyase (PAL) and peroxidase, which are involved in production of structural defences and antimicrobials as well as enhancing the ISR signal (Kandan *et al.*, 2002).

Control of thrips

Reducing populations of thrips present on host plants helps limit the spread of TSWV; this can be achieved by the use of biological controls, such as competitive non-vector thrips species and predatory pirate bugs, and UV-reflective mulch, which repels migrating adults but also deters biological controls (Funderburk *et al.*, 2011).

As mentioned in section 1.4.2, Puche *et al.* (1995) demonstrated that weekly treatment with the insecticides esfenvalerate, methamidophos, or *Bacillus thuringiensis* or a combination thereof, was sufficient for reducing thrips populations within the field to the point where secondary infections were not seen but this had no effect on primary infections from invading external thrips.

The University of California Statewide Integrated Pest Management Program, a part of their Division of Agriculture and Natural Resources, lists a number of insecticides appropriate for the control of *F. occidentalis* and

T. tabaci; in particular, Imidacloprid is recommended for the treatment of thrips on pepper seedlings and transplants before and at the time of field planting (UC IPM, 2016). It is also recommended that insecticides are rotated based on their mode of action in order to stop the development of resistance in populations of thrips; foliar spraying of the insecticides mentioned therein may reduce in-field secondary TSWV infections (UC IPM, 2016).

A number of invertebrates predatory and parasitic to thrips are commercially available, as well as pathogenic fungi and nematodes (Mouden *et al.*, 2017). Funderburk *et al.* (2000) demonstrated that the predatory bug *Orius insidiosus* is effective in reducing *F. occidentalis* populations almost to extinction in field-grown pepper when conditions are appropriate; however, during autumn, *O. insidiosus* undergoes short day-induced reproductive diapause, allowing *F. occidentalis* populations to re-establish. In glasshouse-grown pepper, the predatory mites *Amblyseius cucumeris*, *Iphiseius degenerans*, *A. andersoni* and *A. swirskii* are effective in drastically reducing thrips populations, with *A. swirskii* being the most effective and best at establishing populations (van Houten *et al.*, 2005). Little information is available on how bio-control methods of thrips affect the incidence of TSWV under experimental or commercial growth conditions.

Another approach for the control of thrips is mass-trapping in blue or yellow sticky traps and the rates of capture are enhanced by the addition of the *F. occidentalis* aggregation pheromone neryl (S)-2-methylbutanoate; this have been shown to be effective in glasshouse-grown pepper and in field-grown strawberries (Hamilton *et al.*, 2005; Sampson and Kirk, 2013).

1.5.2 Genetic control of TSWV

Genetic resistance to TSWV

The only resistance to TSWV in peppers is conferred by the dominant gene *Tsw*, which originated from the *C. chinense* accessions PI 152225 and PI 159236 (Black *et al.*, 1991). This resistance to TSWV is based on the hypersensitive response, associated with localised lesions at the inoculation site and abscission of inoculated organs, with no enduring, systemic resistance (Moury *et al.*, 1997). *Tsw* only confers resistance to TSWV (a member of *Tospovirus* serogroup I) and not to any other tospoviruses; *Tsw* has been crossed into *C. annuum*, the species of most commercially grown peppers, without affecting other important agronomic traits, such as; yield, fruit quality, fruit uniformity, tolerance to abiotic stresses etc. (Boiteux and de Ávila, 1994; van Leeuwen *et al.*, 2017). Resistance-breaking isolates of TSWV in pepper are frequently reported worldwide (Margarita *et al.*, 2004; Ferrand *et al.*, 2015; Jiang *et al.*, 2017). The lack of broad, systemic resistance to tospoviruses means finding new candidate resistance genes is imperative.

RHD3 is a gene in plants that mediates the formation of the tubular ER network (Chen *et al.*, 2011). *Arabidopsis rhd3* mutants confer some recessive resistance against TSWV because, even though viral replication is not affected, the perturbed ER stops intercellular movement (Feng *et al.*, 2016). The gene *rhd3* may be a resistance candidate for future study but *Arabidopsis* knockout mutants also possess physiological defects that would drastically affect other agronomic traits in crop plants (Chen *et al.*, 2011).

For a number of plant viruses, transgenic resistance has been achieved by expression of viral sequences; this can be protein-mediated resistance, which is generally broad, low-level resistance, or RNA-mediated, which is highly specific but gives strong resistance against large levels of inoculum (Lomonosoff, 1995). Transgenic peanuts constitutively expressing a sense copy of the TSWV *N* gene show a dramatic delay and reduction in symptom expression and total protection against systemic infection in a glasshouse environment (Li *et al.*, 1997). The resistance levels mentioned by Li *et al.* (1997) correlate to the amount of N protein present in plant tissues, indicating that resistance is due to accumulation of high levels of the N protein, which may serve to stop disassembly of otherwise infectious viral particles. Field-grown transgenic peanuts constitutively expressing an antisense copy of the *N* gene showed a strong reduction in susceptibility to TSWV compared with control plants; this is more likely to be RNA-mediated due to the orientation of the *N* gene in the construct (Magbanua *et al.*, 2000).

Transgenic tobacco expressing an intact N coding sequence showed strong and highly specific resistance to the TSWV isolate from which the sequence originated. Also, if N protein accumulation was high, some delayed symptom development after inoculation with serologically distinct TSWV isolates was shown. These data indicate that these plants exhibit a combination of protein- and RNA-mediated resistance (Pang *et al.*, 1992; Vaira *et al.*, 1995). When untranslatable sense or antisense N coding sequences are expressed, resistance is apparent against homologous and

closely related TSWV isolates but not distinct tospoviruses and appears to happen via the inhibition of viral replication (Pang *et al.*, 1994).

Genetic resistance to thrips

As an alternative to breeding for TSWV-resistance, it is possible to breed for resistance against *F. occidentalis*; resistant plants may be less likely to be infected with TSWV or to transfer TSWV to non-viruliferous larvae. Levels of resistance to *F. occidentalis* vary between cultivars of pepper (Fery and Schalk, 1991). According to the results of Fery and Schalk (1991), thrips-resistance in the pepper germplasm appears to be based more on tolerance of feeding damage, rather than non-preference of thrips or damage to the feeding thrips; therefore these plants are still likely to be infected with TSWV if fed on by viruliferous thrips. However, the thrips-resistant pepper accession CPRO-1 does exhibit reduced feeding damage, reproduction, preference, and residence time of thrips (Maris *et al.*, 2003c; Maris *et al.*, 2004a). Release of viruliferous thrips onto CPRO-1 peppers results in reduced primary infection and slowed secondary infection rates compared with the thrips-susceptible pepper variety Pikante-Reuzen in both caged and glasshouse environments as the aforementioned traits resulted in impeded population development (Maris *et al.*, 2003b). In glasshouse-grown pepper plants, reduced virus-spread occurs between thrips-resistant, TSWV-susceptible CPRO-1 plants, compared with doubly-susceptible plants, and reduced symptom expression and cosmetic damage is seen in the doubly-resistant accession PI 152225, as compared the TSWV-resistant, thrips-susceptible accession PI 159236 (Maris *et al.*, 2003a).

Transgenic potato plants expressing multi-domain protease inhibitors are unsuitable hosts for *F. occidentalis* as these proteins inhibit the cysteine proteases that predominate the thrips digestive tract; feeding damage and populations of adult and larval thrips are greatly reduced on these transgenic plants compared with controls (Outchkourov *et al.*, 2004).

1.6 BACKGROUND TO TRANSLATIONAL MACHINERY

The process of eukaryotic translation is highly conserved and consists of initiation, elongation, and termination (Sonenberg and Hinnebusch, 2009).

1.6.1 Initiation

Control of translation generally occurs at initiation by modification of eukaryotic initiation factors (eIFs) or competitive inhibition of eIFs (Haghighat *et al.*, 1995; Richter and Sonenberg, 2005). In order to be translated, an mRNA must be bound by an 80S ribosome, first by recruiting the 40S ribosomal subunit as part of the 43S preinitiation complex (PIC), scanning for the start codon, then recruiting the 60S ribosomal subunit (Jackson *et al.*, 2010). An abbreviated list of complexes and their constituent factors that are involved in eukaryotic translation initiation is given in table 1.2.

Pre-initiation complex assembly and cap-binding

Formation of the 43S PIC, whose structure can be seen in figure 1.7, requires the assembly of the 40S subunit, the ternary complex (TC), eIF1, eIF1A, eIF3, and eIF5 (Asano *et al.*, 2000). The TC consists of eIF2, GTP, and a methionyl initiator transfer RNA (Met-tRNA_i) (Jackson *et al.*, 2010).

The 5' cap (consisting of a 7-methylguanosine linked to the 5' end of the transcript by a 5'-5' triphosphate bridge) is recognised by the eIF4F

complex, which consists of eIF4G, eIF4E, and eIF4A (Merrick, 2015). 3'-polyadenylated transcripts are circularised by the association of eIF4F to poly(A) binding protein (PABP) (Jackson *et al.*, 2010).

The 43S PIC binds the 5' end of mRNA via the association between eIF3 and eIF4G (Dutt *et al.*, 2015).

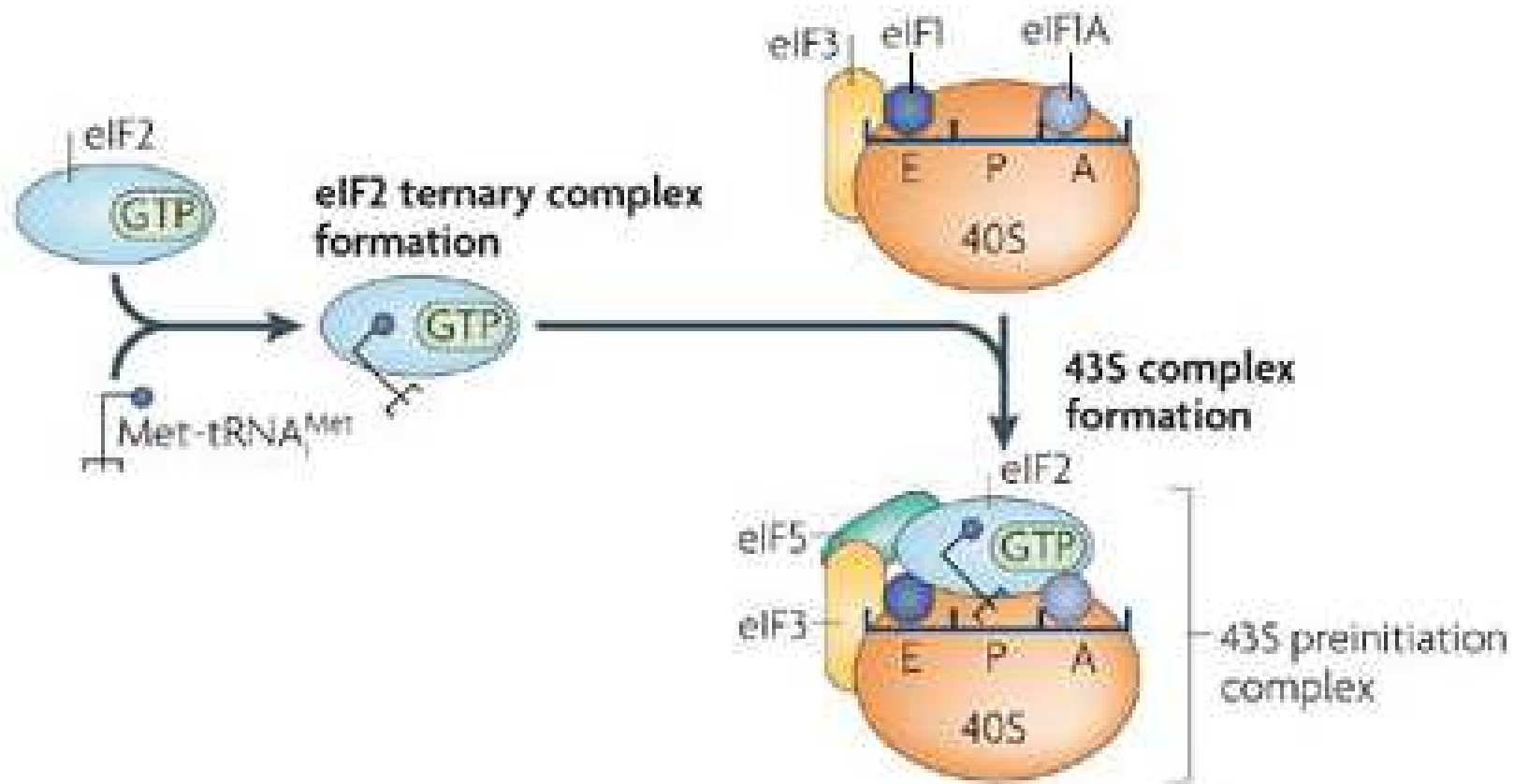


Figure 1.7. The formation of the 43S pre-initiation complex from: the eIF2 ternary complex, which consists of eIF2, Met-tRNA^{Met} and GTP; the 40S ribosomal subunit; and eIFs 1, 1A, 3 and 5.

Adapted from Jackson et al. (2010).

Scanning

Once bound, the 43S PIC scans the mRNA 5' untranslated region (UTR) in the 5' to 3' direction for an AUG start codon. Secondary structures in the 5' UTR are ATP-dependently unwound by eIF4A, allowing the 43S PIC to move along the transcript (Jackson *et al.*, 2010).

Upon recognition of the initiation codon in the 40S subunit peptidyl (P) site, eIF2 hydrolyses its GTP, which reduces the affinity of eIF2 for Met-tRNA_i^{Met} and encourages partial dissociation of eIF2-GDP from the 40S subunit (Jackson *et al.*, 2010). Start codon recognition induces the formation of the 48S initiation complex, which is able to associate with a 60S subunit to form an 80S ribosome on the transcript (Pisareva and Pisarev, 2014). When the 60S subunit binds, eIF5B, a ribosome-dependent GTPase, mediates further release of eIF2-GDP, along with eIF5, eIF3 and eIF1; eIF1A remains in the ribosome aminoacyl (A) site, blocking the recruitment of the next tRNA (Jackson *et al.*, 2010).

Table 1.2. Descriptions of complexes involved in eukaryotic translation initiation with information from Sonenberg and Hinnebusch (2009) and Jackson et al. (2010)

Complex	Role of complex	Complex constituent	Role of subunit
43S PIC	Initiation of production of the polypeptide chain	eIF2	This donates and anchors Met-tRNA _i ^{Met} to the PIC as part of the ternary complex (TC). The eIF2 γ -subunit hydrolyses eIF2-bound GTP upon recognition of an AUG initiation codon, allowing the release of eIF2 and the joining of the 60S ribosomal subunit to the 48S initiation complex.
		Met-tRNA _i ^{Met}	As part of the TC, this recognises AUG initiation codon and donates methionine at the start of translation.
		eIF1	During initiation, this promotes scanning of the 5' untranslated region, when non-AUG codons occupy the 40S ribosomal P site, and is released upon recognition of an AUG codon.
		eIF1A	This works with eIF1 to promote scanning as its C terminal region blocks the ribosomal P site until elongation and its N terminal region promotes eIF1 release upon AUG recognition.
		eIF3	This promotes 43S PIC assembly and mRNA recruitment when the TC is present and blocks 40S-mRNA binding in the absence of the TC.
		eIF5	This promotes initiation commitment by binding the eIF2 β -subunit and activating the GTPase activity of the eIF2 γ -subunit.
		40S ribosomal subunit	This is responsible for decoding mRNAs. It contains an mRNA binding cleft and three tRNA binding sites: A-site, where aminoacyl tRNAs are recruited; P-site, which holds a peptidyl tRNA attached to the nascent polypeptide; and the E-site, from which the deacylated tRNA exits after peptide bond formation.
eIF4F	Cap recognition, 43S PIC recruitment and scanning of structured 5' UTR	eIF4A	An ATP-dependent helicase that unwinds structured 5' UTR.
		eIF4E	This recognises the 5' cap.
		eIF4G	A scaffold protein that binds: eIF4A and E, promoting their respective activities; eIF3, allowing PIC recruitment; and PABP, allowing mRNA circularisation and increasing mRNA stability.

1.6.2 Elongation

GTP hydrolysis by eIF5B mediates the release of eIF5B and eIF1A from the ribosome, exposing the A site for reception of an aminoacyl-tRNA and allowing elongation (Jackson *et al.*, 2010).

Elongation is dependent on eukaryotic elongation factors (eEFs): eEF1A delivers aminoacyl-tRNAs to the ribosomal A site by GTP hydrolysis, the eEF1B complex is a GTP exchange factor for eEF1A, and eEF2 is required for ribosomal translocation (Browne and Proud, 2002; Hwang *et al.*, 2015).

1.7 HOST TRANSLATIONAL RESPONSE TO VIRAL PATHOGENS

Viruses do not possess ribosomes so require host translational machinery to produce proteins; host responses to viral infection may perturb or enhance viral translation depending on the response and the virus's method of hijacking host machinery. The degree to which cellular and viral mRNAs are translated affects viral pathogenesis (Gale *et al.*, 2000).

1.7.1 Global translation repression

Translation is often suppressed in virus-infected cells; this can be due to viral manipulation of host translational machinery to enhance production of viral proteins while disabling the host antiviral response in a process called "host shut-off". Alternatively, this can be a result of the host cell inhibiting both cellular and viral protein production for the duration of infection, curtailing viral replication and possibly inducing apoptosis to stop viral spread (Walsh and Mohr, 2011; Walsh *et al.*, 2013).

Viruses that induce host shut-off are able to utilise various means of non-canonical cellular translation to produce their proteins. Different viruses will induce host shut-off by targeting different components of the 43S and 48S complexes,

depending on the structure of viral transcripts and their means of translation (Gradi *et al.*, 1998; Piron *et al.*, 1998; Cuesta *et al.*, 2004; Blakqori *et al.*, 2009; Garrey *et al.*, 2010; Lokugamage *et al.*, 2012).

Translation inhibition as a part of the innate antiviral defence response is well characterised in mammals but less so in plants. In mammals, this inhibition is mediated by protein kinase R or interferon-induced eukaryotic initiation factor 2-alpha kinase (PKR), which phosphorylates the eIF2 α subunit upon detection of dsRNA and RNA molecules with secondary structures; PKR-directed phosphorylation of eIF2 α prevents the formation of the ternary complex (Walsh *et al.*, 2013). Plants may or may not possess a functional homologue of PKR (pPKR) but do possess a putative cellular inhibitor of pPKR (P58^{IPK}), which is a key susceptibility factor in viral pathogenesis (Bilgin *et al.*, 2003; Chan, 2009).

1.7.2 Mutations identify key genes in susceptibility and resistance

As viruses exploit key host cellular factors to reproduce, absence of the appropriate host factors, with which viral factors can interact, gives the host a passive resistance to that virus. Thus, the mutations of susceptibility factors to a given virus confer a recessive resistance to said virus (Truniger and Aranda, 2009).

In crop plants, almost all known recessive resistance genes to viruses encode eIFs 4E and 4G and their isoforms; however, not all viruses interact with these factors, meaning there are many more susceptibility genes, whose mutation could be exploited for resistance against viruses (Hashimoto *et al.*, 2016). A short list of recessive resistance genes can be seen in table 1.3.

Table 1.3. A short list of selected plant virus recessive resistance genes, including translation initiation factors and other proteins.

Host plant	Recessive resistance gene	Pathogen	Gene product	Reference
<i>Arabidopsis</i>	<i>rhd3</i>	TSWV	RHD3 (a GTP-binding membrane protein involved ER formation and vesicle trafficking)	Chen <i>et al.</i> (2011), Bin <i>et al.</i> (2012), Ueda <i>et al.</i> (2015) and Feng <i>et al.</i> (2016)
Barley	<i>rym4</i>	Barley yellow mosaic virus (BaYMV), a Bymovirus	eIF4E	Kanyuka <i>et al.</i> (2005) and Stein <i>et al.</i> (2005)
	<i>rym5</i>	BaYMV	eIF4E	
	<i>rym6</i>	BaYMV	eIF4E	Kanyuka <i>et al.</i> (2005)
<i>Brassica rapa</i>	<i>retr01</i>	Turnip mosaic virus (TuMV), a potyvirus	eIF(iso)4E	Rusholme <i>et al.</i> (2007) and Nellist <i>et al.</i> (2014)
Pepper	<i>pvr1</i>	Potato virus Y (PVY)	eIF4E	Kang <i>et al.</i> (2005)
	<i>pvr2</i>	PVY	eIF4E	Ruffel <i>et al.</i> (2002)
	<i>pvr6</i> (complementary to some <i>pvr2</i> alleles)	PVY	eIF(iso)4E	Rubio <i>et al.</i> (2009)
Rice	<i>rymv1</i>	Rice yellow mottle virus (RYMV), a sobemovirus	eIF(iso)4G	Albar <i>et al.</i> (2006)
Tomato	<i>pot1</i>	PVY	eIF4E	Ruffel <i>et al.</i> (2005)
	<i>ty-5</i>	Tomato yellow leaf curl virus (TYCLV), a begomovirus	mRNA surveillance factor Pelota (PELO)	Lapidot <i>et al.</i> (2015)

1.7.3 Known polysome profiling experiments to abiotic and biotic stresses

Many gene expression studies tend to focus exclusively on the transcriptome when identifying genes involved in resistance and susceptibility (Huang *et al.*, 2012; Cai *et al.*, 2013; Lopez-Kleine *et al.*, 2013). This has the disadvantage that mRNA levels do not necessarily correlate to the levels of their respective proteins (Gygi *et al.*, 1999; Vogel and Marcotte, 2012). During the stress response, global translation is drastically reduced and the translation of certain mRNAs, set apart by elements in their 5' and 3' un-translated regions, is selectively increased, which allows the cell to rapidly respond to an immediate stress using existing transcripts; thus translation is a critical step in gene expression and investigation thereof can give data that relates more to the proteins being produced (Holcik and Sonenberg, 2005; Spriggs *et al.*, 2010).

Polysome profiling is one technique that can be used to investigate translation-level expression and changes or defects therein: during development; caused by mutations; or in response to a given stimulus or stress (Coudert *et al.*, 2014). Polysome profiling has been used to investigate a multitude of stresses, including: heat-shock in *Drosophila melanogaster* (Glover, 1982); hypoxia in human cultured cells and in plants (Koritzinsky and Wouters, 2007; Thomas and Johannes, 2007; Mustroph *et al.*, 2009b); radiation in both human cancer cell lines and normal cell lines (Kumaraswamy *et al.*, 2008); and heat-shock, fungal infection and herbicide-treatment of maize leave (Wu *et al.*, 1988).

In animal cells, as a response to both biotic and abiotic stress, translation is generally repressed by eIF2 α phosphorylation. This is performed

by the following protein kinases under distinct stress conditions: PKR, upon viral dsRNA detection; PKR-like endoplasmic reticulum kinase (PERK), upon detection of unfolded proteins in the ER; haem-regulated eIF2 α kinase (HRI), upon haem deprivation; and general control nonderepressible 2 (GCN2), upon amino acid starvation (Sudhakar *et al.*, 2000; Sonenberg and Hinnebusch, 2009).

Plant homologues of PKR, PERK and HRI have not been identified in the *Arabidopsis* genome but plants do possess a GCN2 homologue, which is activated upon amino acid starvation by herbicide treatment (Lageix *et al.*, 2008). However, PKR-like activity has been observed in response to TMV (Crum *et al.*, 1988) and Potato spindle tuber viroid (Hiddinga *et al.*, 1988; Zhang *et al.*, 2008). A dsRNA-dependent PKR-like kinase that cross reacts with antibodies against human PKR has been reported (Langland *et al.*, 1995). A homologue of P58^{IPK}, a PKR inhibitor, is expressed in plant cells, is required for TMV and Tobacco etch virus (TEV) infection, and, interacts with mouse PKR (Bilgin *et al.*, 2003). Also upon viral infection of P58^{IPK}-silenced plants, eIF2 α is phosphorylated substantially more, resulting in a lack of protein synthesis and host death; expression of a non-phosphorylatable mutant eIF2 α rescues P58^{IPK}-silenced plants (Bilgin *et al.*, 2003). These results indicate but do not prove the presence of a putative, functional plant homologue to PKR.

Contrary to the results mentioned above, another study showed no eIF2 α phosphorylation in *Arabidopsis* in response to RNA viruses and no eIF2 α phosphorylation at all in *gcn2* null mutants under all conditions (Zhang *et al.*, 2008). Polysome profiling was used to track translation in response to amino

acid starvation by Chlorsulfuron in wild-type (WT) and *gcn2* mutant *Arabidopsis*; these results showed translation suppression in WT plants in order to conserve amino acids during starvation and an inability of *gcn2* mutants to perform the same suppression (Lageix *et al.*, 2008).

Another mechanism of translational suppression in begomovirus (ssDNA) infected plants is mediated by nuclear shuttle protein (NSP)-interacting kinase 1 (NIK1), a transmembrane, leucine-rich repeat receptor-like kinase (LRR-RLK), whose action triggers shuttling of ribosomal protein L10 (RPL10) from the cytoplasm to the nucleus (Machado *et al.*, 2015). Once in the nucleus, RPL10 interacts with L10-interacting MYB domain-containing protein (LIMYB), repressing the transcription of ribosomal protein (RP) genes; this in turn results in repressed global translation, presumably via reduced ribosome assembly, and subsequent tolerance to infection (Zorzatto *et al.*, 2015).

1.8 PATHOGEN STRATEGY TO DEFEAT HOST TRANSLATIONAL RESISTANCE

As mentioned previously, viruses lack translational machinery so must perturb canonical host translation, either by host shut-off or avoidance of host translational inhibition, in order to translate viral mRNAs for replication, spread and infection (Aranda and Maule, 1998). Considering all viruses, host shut-off targets virtually all stages of cellular gene expression, not necessarily at the point of translation, to ensure production of viral proteins (Rivas *et al.*, 2016).

1.8.1 Viral strategies to escape general host-derived inhibition of translation

Viruses have evolved a number of strategies to escape global repression of translation by the host that would otherwise cripple protein production and viral gene expression for the duration of infection (Walsh and Mohr, 2011).

In animals and fungi, viruses often subvert or escape PKR-based translation initiation repression by inhibiting its activation, inhibiting or reversing eIF2 α phosphorylation, PKR degradation, or independence of viral mRNA from eIF2 Ternary Complex (TC) for initiation (Mathews and Shenk, 1991; Schneider and Mohr, 2003; Garcia-Moreno *et al.*, 2013).

In plants, begomoviruses escape NIK1-RPL10-LIMYB antiviral translation suppression by the action of their nuclear shuttle protein (NSP), which binds NIK1 and blocks its kinase signalling activity; in turn this stops the nuclear localisation of RPL10 and LIMYB-directed suppression of RP expression (Fontes *et al.*, 2004).

1.8.2 Viral hijacking of host translational machinery

Owing to the diversity of virus genome structures and mechanisms of mRNA synthesis, viral strategies to hijack host machinery to preferentially translate viral mRNAs are diverse. These strategies include inactivating at least one of the factors involved in cap-dependent initiation so that the virus can utilise non-canonical translation rarely used for cellular mRNAs (Schneider and Mohr, 2003). For example, many members of the single stranded, positive sense RNA virus family *Picornaviridae*, such as poliovirus, rhinovirus, and coxsackievirus, possess proteases that cleave eIF4G into two fragments; the C-terminal eIF4G

fragment preferentially binds the uncapped viral mRNAs with internal ribosome entry sites (IRES; secondary structures in the 5' UTR of an mRNA) and, without the eIF4G N-terminus, does not associate with eIF4E and PABP, thus inhibiting cap-binding and circularisation of cellular mRNAs (Ali *et al.*, 2001).

Hepatitis C virus (HCV) of the *Flaviviridae* has a single stranded, positive sense RNA genome containing a single ORF that encodes a polyprotein, which is processed by viral and host proteases into smaller functional structural and non-structural proteins, flanked by conserved, structured 5' and 3' UTRs (Han *et al.*, 1991; Kato, 2000). The HCV polyprotein is translated cap-independently by direct recruitment of the 40S ribosomal subunit to an IRES in the 5' UTR and this is enhanced by a structured *cis*-element in the 3' UTR, which aids in delivering the 40S subunit to the IRES and attenuates host translation inhibition of the HCV RNA by binding the polypyrimidine tract binding protein (PTB) that would normally block 40S-IRES binding (Ito and Lai, 1999; Bai *et al.*, 2013). Many translation factors are not required for HCV IRES-mediated translation except for the eIF2 TC and eIF2B; RNA aptamers directed against the gamma subunits of these eIFs block HCV translation (Krüger *et al.*, 2000).

Bunyaviridae mRNAs are not polyadenylated; Bunyamwera virus (BUNV; genus *Orthobunyavirus*), a member of *Bunyaviridae* that infects humans and *Aedes aegypti* (yellow fever mosquito), force host shut-off of poly(A) tail-dependent translation and encourage PABP-independent translation by nuclear retention of PABP via interaction of cytoplasmic PABP and the BUNV N protein (Blakqori *et al.*, 2009). In another member of this

family, the rift valley fever virus (RVFV; genus *Phlebovirus*) that infects humans, numerous livestock animals, and the vector mosquito species *Aedes vexans* and *Culex tritaeniorhynchus*, PABP nuclear retention is mediated by the NSs protein rather than N (Copeland *et al.*, 2013).

1.9 AIMS AND OBJECTIVES

The main aim of this study was to identify defence response genes that are differentially translated in *C. annuum* upon infection with TSWV using a combination of polysome profiling and RNA-Seq. This study aims to provide information on how these genes are regulated at the level of translation and find genes that may provide novel, durable and broad spectrum resistance to TSWV in cultivated peppers and other crops.

In order to meet these aims, the levels of global translation were investigated between TSWV-inoculated pepper plants and control plants by sucrose density gradient polysome profiling. RNA samples from these extractions were sequenced and RNA-Seq results were compared to identify genes that were differentially expressed upon inoculation and between pool-types (i.e. total RNA, sub-polysomal, and polysomal pools), in order to identify whether any genes were differentially translated. Sets of differentially expressed genes were interpreted using gene ontology and pathway enrichment analyses to investigate whether any biological processes are over-represented in these sets and how these results might underlie the susceptible phenotype observed.

In order to validate the results of RNA-Seq analysis, it was planned to use QRT-PCR on the sequenced samples. This would demonstrate whether fold-

changes in expression correlated strongly between RNA-Seq and QRTPCR results (Fang and Cui, 2011).

2 POLYSOME PROFILING OF THE EARLY TRANSLATIONAL RESPONSE OF PEPPER TO VIRULENT TOMATO SPOTTED WILT VIRUS

2.1 INTRODUCTION

2.1.1 The plant response to RNA viruses

Very little is known about the response to tospoviruses at the level of translation so researching this could be used as an approach to find new resistance to these pathogens.

Plant cells utilise RNA silencing, which is triggered by dsRNA and hairpin RNA (hpRNA), as an innate response to viruses, as well as other exogenous and endogenous nucleic acid elements that could perturb cellular process, such as transposons and transgenes; RNA silencing also acts as an evolutionarily conserved mechanism of regulating gene expression and changing chromatin states (Baulcombe, 2004).

In plants, dsRNA and hpRNA are processed by Dicer-like proteins (DCLs) into small interfering RNA (siRNA) and micro RNA (miRNA), depending on the DCL protein and the structure of the precursor RNA (Baulcombe, 2004). These small RNA molecules are amplified by cellular RNA-dependent RNA polymerases (RdRP) then used by the RNA-induced silencing complex (RISC) to direct degradation of and block translation of transcripts with sequence homology to the small RNA (Baulcombe, 2004).

Virus-derived siRNAs (vsRNA) that target viral RNAs accumulate in virus-infected plants because of the presence of RNA precursors, such as

genomic replicative intermediates or secondary structures in viral genomes and transcripts (Molnár *et al.*, 2005). These vRNAs can provide virus species-specific resistance in organs distal to the point of infection and resistance to secondary infection (Ratcliff *et al.*, 1997).

Evidence in plants of the response to viruses in general, let alone to tospoviruses, at the point of translation is minimal. Profiling of the transcriptional response to TSWV infection of tomato does not highlight any differential expression of genes or pathways involved in translation or suppression/activation thereof (Catoni *et al.*, 2009).

2.1.2 Tospovirus strategy to defeat host translational resistance mechanisms

Tospoviruses have multiple putative mechanisms for ensuring the expression of their own genes while reducing host translation, both in order to down-regulate the defence response and divert cellular resources to the production of viral proteins.

eEF1A has been proposed as an important factor during TSWV reproduction, as it seems to facilitate TSWV transcription *in vitro*; this is, however, independent of translation and no changes in translation as a result of this interaction have been noted or proposed (Komoda *et al.*, 2014).

Translation of viral transcripts

TSWV mRNAs are produced by the viral RNA-dependent RNA polymerase (RdRp) by one round of transcription of its negative-sense genes or by two rounds of transcription for positive-sense genes on the genomic segments that possess genes in both orientations; this consists of primary production of a

complementary, sub-genomic RNA and secondary production of the sense mRNA (Duijsings *et al.*, 2001b; Geerts-Dimitriadou *et al.*, 2012). In order to prime transcription, the RdRp must first cleave either a cellular 5'-capped transcript, using the capped fragment to prime production of viral mRNA containing a capped host-derived leader sequencing (cap-snatching), or cleave a copy of the viral genomic RNA to prime replication of full-length genomic segments; RNA in ribonucleoprotein complexes (RNPs) with the TSWV nucleocapsid (N) protein act as templates in these processes (Richmond *et al.*, 1998; Nguyen and Haenni, 2003; van Knippenberg *et al.*, 2005b). Transcription of mRNAs from the ambisense segments terminates in the intergenic hairpin region (IR), preventing the production of a full-length dsRNA (van Knippenberg *et al.*, 2005a). These processes produce 5'-capped, non-polyadenylated transcripts; mRNAs from ambisense segments have predicted stable A-rich hairpin structures at their 3' ends, which may act in the place of poly(A) tails in concert with poly(A) binding proteins (PABP) or functional PABP substitutes; these structures allow ribosomal recruitment and translation of viral mRNAs by the mimicry of host mRNA structure (Geerts-Dimitriadou *et al.*, 2012; Clabbers *et al.*, 2014). Less information is available on the structure of the L segment-derived mRNA but it is assumed that the mechanism of transcription initiation and thus the cap structure for this transcript is the same as for those derived from the S and M segments (van Knippenberg *et al.*, 2005b); transcription of L is assumed to terminate in the ~62 nucleotides at the 5' end of the segment or by the RdRp running to the 5' end and falling off

but little information is present on this subject so 3' UTR structure and function for this transcript are not clear.

The TSWV N protein and the S segment-encoded, non-structural protein (NSs) stabilise transcripts and enhance translation. N may be required to prevent de-capping of viral mRNAs. N and NSs may act as a functional analogue to PABP or in concert with PABP (Geerts-Dimitriadou *et al.*, 2012).

RNA silencing suppression

TSWV defeats part of the host innate defence using NSs, which acts as an RNA silencing suppressor, thus inhibiting viral mRNA degradation and translational blockage (Takeda *et al.*, 2002; Bucher *et al.*, 2003). TSWV NSs blocks both the siRNA and miRNA pathways by binding small and long dsRNA, blocking cleavage of long dsRNA by DCL proteins and blocking loading of small dsRNA into active RISC (Schnettler *et al.*, 2010). Infection of tobacco by TSWV RSS-defective NSs mutants leads to greater accumulation of vsRNAs, particularly those targeting the *NSm* gene and the 3' terminal region of the L segment, where little change was observed in endogenous sRNA abundance (Margaria *et al.*, 2015b). Results from the same study show that S and M segment IGR hairpin structures are poor inducers of RNA silencing even in NSs-defective mutants, either meaning that the IGRs are protected from the RNA silencing machinery independently of NSs or, in the NSs mutants used in the study, global RSS ability and IGR-/hairpin-masking have been separated (Margaria *et al.*, 2015b).

Virus-derived small RNAs that target host transcripts

As well as their role in inhibiting viral replication and gene expression, vsRNAs can also target host transcripts and contribute to disease development (Shimura *et al.*, 2011; Smith *et al.*, 2011; Wang *et al.*, 2016). Using a TSWV vsRNA profile from tomato developed by Mitter *et al.* (2013), vsRNA-targets were predicted and the expression and degradation states of a subset of the predicted targets in susceptible tomato cv. Marglobe (-Sw-5) and resistant tomato cv. Red Defender (+Sw-5) were confirmed by Q-RTPCR and 5' Rapid amplification of cDNA ends (5'-RACE). These results show that targets in various metabolic pathways are down-regulated by TSWV vsRNAs in cv. Marglobe; the lack of vsRNA-directed down-regulation/degradation of various transcripts in cv. Red Defender may be an important part of the resistance mechanism (Ramesh *et al.*, 2017). Results from the same study also corroborate the down-regulation of genes associated with secondary metabolite biosynthesis upon TSWV infection presented by Catoni *et al.* (2009) (Ramesh *et al.*, 2017).

2.2 MATERIALS AND METHODS

2.2.1 Plant material and inoculation

Plant growth

Plant growth and inoculation was performed at a Syngenta research facility in Sarrians, France. *Capsicum annuum* pepper plants were grown in trays containing compressed compost. One hundred and sixty seeds were sown per tray in eight lines of 20 seeds; two cultivars (Syngenta 14PEF990007 and 14PEF990010) were sown with four lines each per tray. In prior experiments,

susceptibility of these varieties to Syngenta TSWV1 (previously named TSWV-RZ) was verified by symptom expression of six week old plants three weeks after inoculation using the same method described below.

Trays were placed in a climatic chamber with a 15 hour day length. During the day the light intensity was 10,000 lux and the temperature was $25\pm 2^{\circ}\text{C}$ and during the night the temperature was $20\pm 2^{\circ}\text{C}$.

As germination rates were higher than expected, two weeks after sowing plants were suppressed to keep only 10 seedlings per line.

Inoculation

The TSWV1 inoculum consisted of young leaves from a systemically infected susceptible tomato plant. Inoculum leaves were harvested 8-12 days post-inoculation and stored at -80°C .

TSWV1 is a Syngenta internal virus *Tsw*-resistance breaking isolate that was previously named TSWV-RZ. The geographical origin of TSWV1 is not known.

Presence of TSWV in the inoculum was verified by RT-PCR using standard TSWV-diagnosis primers JLS90-46 (AGCTAACCATGGTTAAGCTCACTAAGGAAAGC) and JLS90-47 (AGCATTCATGGTTAACACACTAAGCAAGCAC), which are designed against a conserved region of the *N* gene on the S genomic segment (Pang *et al.*, 1992) and Qiagen One-Step RT-PCR according to manufacturer conditions. The results of this can be seen in figure 2.1.

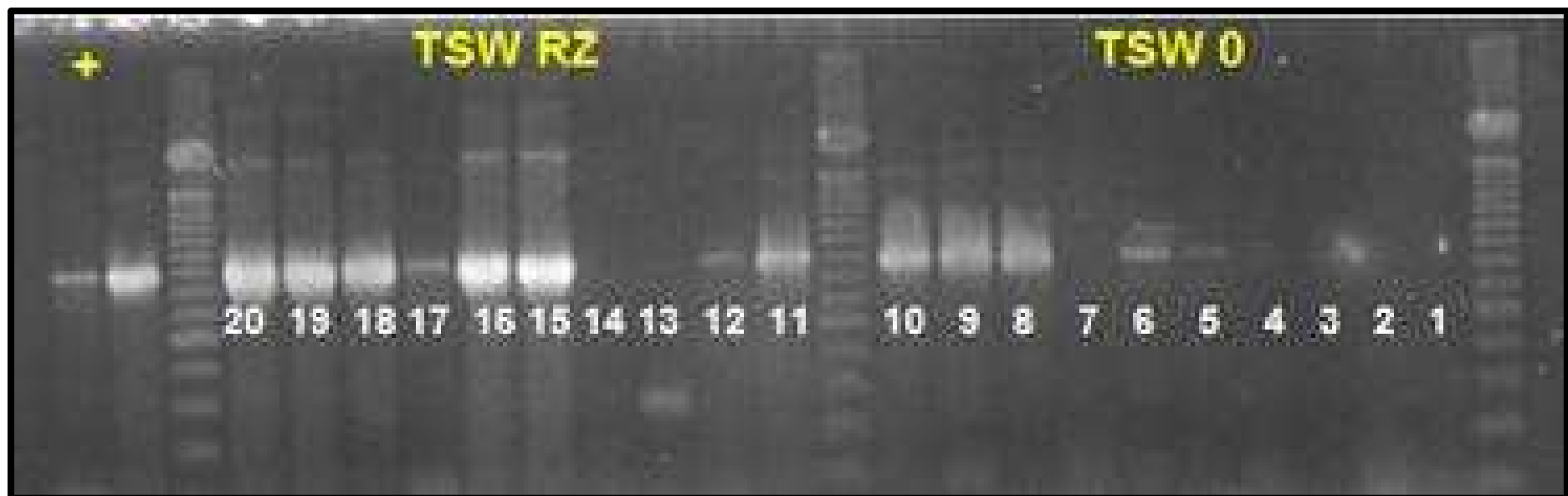


Figure 2.1. Gel electrophoresis results showing the presence of TSWV in tomato leaves used as inocula in this experiment. Only samples that produced the expected 760bp band after Qiagen One-Step RT-PCR with the primers JLS90-46 and JLS90-47 were used as inocula. “+” denotes positive controls, “TSW RZ” is TSWV1, “TSW 0” is a TSWV isolate (elsewhere named TSWV0) used to inoculate tissue for primer optimisation.

One litre of inoculation buffer was made up containing 15.08 mM di-sodium hydrogen phosphate (Na_2HPO_4), 22.19 mM sodium diethyldithiocarbamate ($\text{C}_5\text{H}_{10}\text{NNaS}_2$), and 1.9-2.0% (w/v) sodium bisulphite in de-ionised water.

To prepare the TSWV1 inoculum, 1 g of frozen leaves of tomato plants systemically infected with TSWV1 were crushed in a pre-cooled mortar and mixed with 5 ml of inoculation buffer, 0.1 g of powdered charcoal, and 0.1 g of powdered carborundum.

Mock inoculum was prepared with the same procedure, omitting the TSWV1-infected tomato leaves.

Twenty-five day old plants were inoculated by rubbing 100 μl of inoculum into both cotyledons.

Sampling

Six hours post-inoculation first leaves were harvested from four plants per sample. Leaves were immediately halved; separated into two clean 2 ml polypropylene Eppendorf tubes, so that duplicates existed for each sample; then frozen in liquid nitrogen. Leaves were stored at -86°C , shipped from Syngenta, Sarrians to the University of Nottingham on dry ice then stored at -80°C . Table 2.1 gives a summary of plant first leaf samples harvested for this experiment. Other samples were retained for future experiments.

Table 2.1. Summary of plant material used for sucrose density centrifugation.

Sample number	Condition	Repeat number	Syngenta cultivar ID
29	TSWV1-inoculated	1	14PEF990007
30		2	14PEF990007
31		3	14PEF990007
53	Mock-inoculated	1	14PEF990007
54		2	14PEF990007
55		3	14PEF990007
77	Non-inoculated	1	14PEF990007
79		3	14PEF990007
80		4	14PEF990007

2.2.2 Sucrose density centrifugation

Buffers and sucrose density gradient preparation

Ten times lysis buffer and gradients was made with 5 ml of cycloheximide (10 mg/ml) stock, 500 mg of sodium heparin salt, 7.5 ml 1 M Tris-HCl pH 7.5, 3.75 ml 2 M MgCl_2 and brought up to 50 ml with 4 M NaCl. Cycloheximide was kept in a 10mg/ml (aq.) stock stored at -20°C .

Lysis buffer was prepared on the day the gradients were run using 1 ml 10x buffer, 1 ml 10x Triton X100 and 8 ml RNase-free water.

Sucrose density gradients were prepared in advance of running samples and stored at -80°C .

Sucrose solutions were prepared the day before the sucrose density gradients. Solutions of 10, 18, 26, 34, 42, 50 and 60% sucrose (w/v) were prepared with 5 ml each of 10x buffer and brought up to 50 ml with RNase-free water. These were left overnight on a rocker at 4°C to allow the heavier sucrose solutions to dissolve fully.

Into one 13.2 ml Polyallomer 14×89 mm centrifuge tube, 0.5 ml of 60% sucrose solution was pipetted and frozen in a covered container at -80°C . Next a 1.6 ml layer of 50% sucrose was added and frozen again. Then 1.6 ml of each sucrose solution was added in order of density. The final volume is thus 10.1 ml.

The sucrose gradients were stored at -80°C . These were taken out the night before the gradients were due to be run, placed into the ultracentrifuge buckets and left in the 4°C cold room.

Lysis

Roughly 100 mg of leaf tissue of each sample was ground under liquid nitrogen inside 2 ml polypropylene Eppendorf tubes using tube pestles.

One ml of lysis buffer was added and the tube was immediately vortexed. This was repeated for all samples to be run on that day; up to six samples can be run at once. The samples were centrifuged at 18000 g for 1 minute.

A 500 µl aliquot of each leaf sample lysate was then transferred onto the corresponding pre-prepared, numbered sucrose gradient. Each sucrose gradient in its bucket was balanced using lysate to the tube and bucket opposite it to within 10 mg. If there was not sufficient lysate, lysis buffer was used to balance tubes. From the lysate, 250 µl was retained for extraction of total RNA.

Ultracentrifugation

Using a Beckman-Coulter Optima XL-70 Ultracentrifuge and SW41Ti rotor, samples were centrifuged at 38000 rpm (178305 g) for 2 hr at 4°C. The acceleration profile was set to profile 9 (500 rpm in 6:00 minutes) and the deceleration profile was set to profile 6 (500 rpm in 4:30 minutes).

Once out of the ultracentrifuge, samples were kept refrigerated at 4°C and agitation was avoided where possible. All gradients were separated on the same day they were centrifuged.

Separation

Separation of centrifuged sucrose gradients was performed using a Cole-Palmer Syringe Pump (SP) and a Teledyne-ISCO Foxy R1 Fraction Collector

(FR1). Polysome profiles were constructed by pumping the gradient through a Teledyne-ISCO Type 11 Optical Unit (OU) connected to a Teledyne-ISCO UA6 UV/VIS Detector (UA6). UA6 settings were: noise filter “1.5”, sensitivity “0.5”, peak separator “off”, and chart speed while running gradients was 60. FR1 settings were: dispensing speed “3”, dispensing pattern “Serpentine”, and rack type was “12 mm x 144”. Interchangeable parts for OU were: metal washer C and rubber gasket collar B. On SP: syringe-type was “Air-tite 20 ml,” force level was 50%, baud rate was 115200, pump rate while running gradients was 1 ml/min.

Before separation all tubing was cleaned by pumping through sterile, distilled water, 70% ethanol and sterile, distilled water again, then retracting all remaining fluid. Between samples all tubing was washed through with sterile, distilled water.

The OU measured the relative absorbance at 254 nm (A_{254}) of fluids passing through its flow cell, which is coupled to a spectrophotometer; the A_{254} was continuously plotted onto graph paper with the UA6. The baseline was drawn using 30% sucrose (w/v). Blank sample and gradients were chased using 70% sucrose (w/v) to push them to the OU and FR1. After separation all fractions were kept on ice for clean-up.

Total RNA extraction from lysate

In the 6 hr inoculation experiment, total cellular RNA was extracted from the cell lysate using Trizol LS Reagent (Invitrogen).

In a 2 ml polypropylene Eppendorf tube, 750 μ l of Trizol LS reagent was added to 250 μ l of the remaining lysate and incubated on ice for 5 minutes.

To this, 200 μ l of chloroform was added and the sample was mixed vigorously. The sample was incubated from 2-15 minutes on ice then centrifuged at 12000 g at 4°C for 15 minutes.

The aqueous phase was transferred into a new 2 ml tube and 500 μ l of isopropanol was added. This was incubated on ice for 10 minutes then centrifuged at 12000 g at 4°C for 10 minutes.

The supernatant was discarded and RNA pellets were washed with 1 ml 75% ethanol then centrifuged at 7500 g at 4°C. Ethanol was removed and the pellets were air-dried and re-suspended in 50 μ l of RNase-free water and stored at -80°C prior to removal of heparin with lithium chloride.

Clean-up

Once separated into 1 ml fractions, 1 ml of 100% isopropanol was added to each fraction. All fractions with isopropanol were kept at -20°C overnight. The following day, fractions were centrifuged at 18000 g at 4°C for 30 minutes. Pellets were washed with 70% ethanol to remove residual isopropanol. Pellets were re-dissolved in 100 μ l of RNase-free water and kept at -80°C until needed.

Fractions were pooled according to whether they were sub-polysomal or polysomal according to their polysome profile. Fifty μ l of each fraction was taken and put into the corresponding pool in a 2 ml tube. Sub-polysomal fractions were defined as those containing material that sedimented above or at the 80S ribosomal peak, which is material that is dispensed first; polysomal fractions were those containing material that sedimented below the 80S ribosomal peak, which is material that is dispensed last.

Each pool, including total RNA samples from Trizol LS extraction, was brought up to 500 µl with RNase-free water. To remove heparin, 500 µl of 5 M LiCl were added to each pool, bringing the solution to a final concentration of 2.5 M LiCl, and pools were chilled at -20°C overnight then centrifuged 18000 g at 4°C for 30 minutes. Samples were washed with 70% ethanol twice to remove residual LiCl.

Pool pellets were dissolved in RNase-free water and run on an Agilent 2100 Bioanalyzer with the assay Eukaryote Total RNA Nano Series II (v.2.5) to check quantity and quality of RNA for sequencing. Pools were precipitated in 75% ethanol 5% 3 M sodium acetate pH 5.2 and the supernatant was removed. One ml of 70% ethanol was added onto each of the dried pellets and tubes were kept at -80°C until they were shipped on dry ice for sequencing.

2.3 RESULTS

2.3.1 Gradient Profiles

Twenty-five day old pepper plants underwent three treatment conditions (TSWV-inoculation, mock-inoculation and no treatment) and, six hours after treatment, their first leaves were harvested. In order to measure relative levels of translation and to separate actively translated, polysome-associated transcripts from free ribosomal subunits and transcripts not associated with the translational machinery, polysome profiling was undertaken on these leaf samples using sucrose density gradient centrifugation coupled with UV detection at 254 nm. This separation allows both a global view of translation, as an increase in relative abundance of polysome-associated material is indicative of a global increase of translation, and the measurement of

translational activity of specific sequences, based on where they sediment in the sucrose gradient (Chassé *et al.*, 2017). Polysome profiles for first leaf samples harvested from these plants can be seen in figures 2.2-2.10.

As peak heights and underlying area vary with the amount of material present and with small adjustments to the measuring equipment, these measurements were internally normalised against the 80S peak and an average was taken for each peak and for the collective area underneath all polysome peaks. Normalised peak heights and collective polysome peak underlying area values can be seen in table 2.2.

These data show a statistically significant (ANOVA, $p < 0.05$) increase: in the average normalised height of polysome peak 3 in polysome profiles of TSWV-inoculated pepper plants compared with non-inoculated plants and in the average normalised heights of polysome peaks 4-8 in TSWV-inoculated plants compared with both mock- and non-inoculated pepper plants. Also a statistically significant (ANOVA, $p < 0.05$) increase in the normalised area underneath the polysome peaks is seen in polysome profiles of TSWV-inoculated plants compared with both mock- and non-inoculated pepper plants. No statistically significant effect was seen in normalised peak heights or polysome area in polysome profiles of mock-inoculated plants compared with non-inoculated plants. The increase of polysome-associated RNA in TSWV-inoculated plants shown in these data indicates an increase in global translation six hours after inoculation.

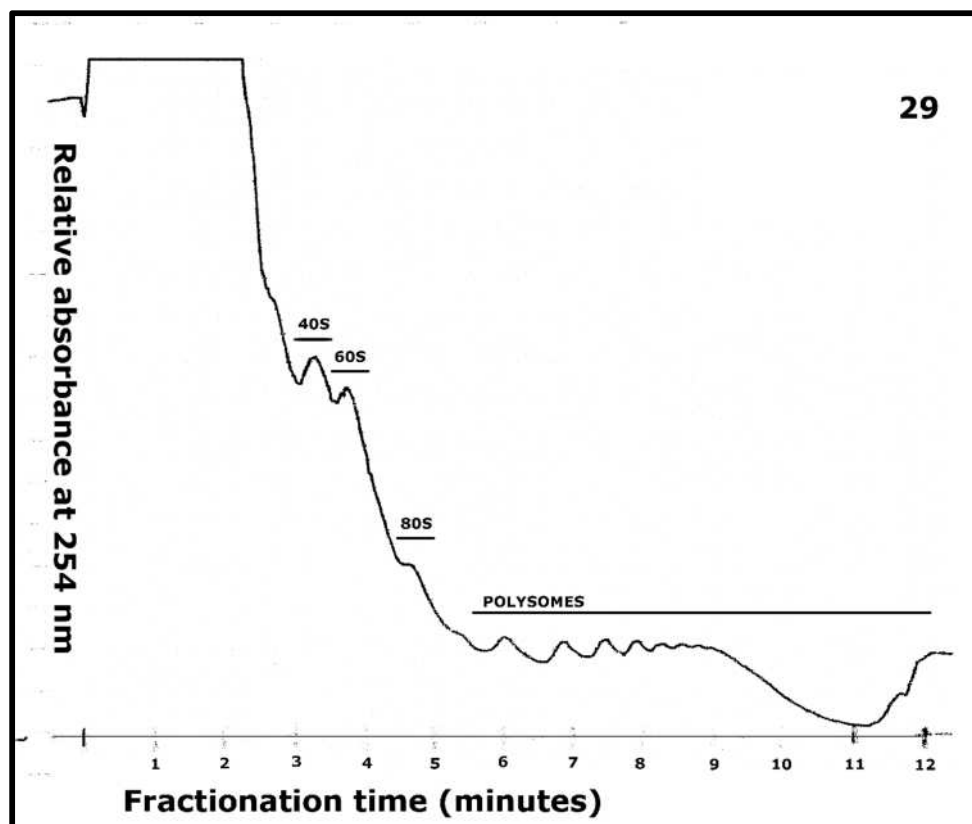


Figure 2.2. Polysome profile of 6hr TSWV1-inoculated pepper plant leaf sample 29. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

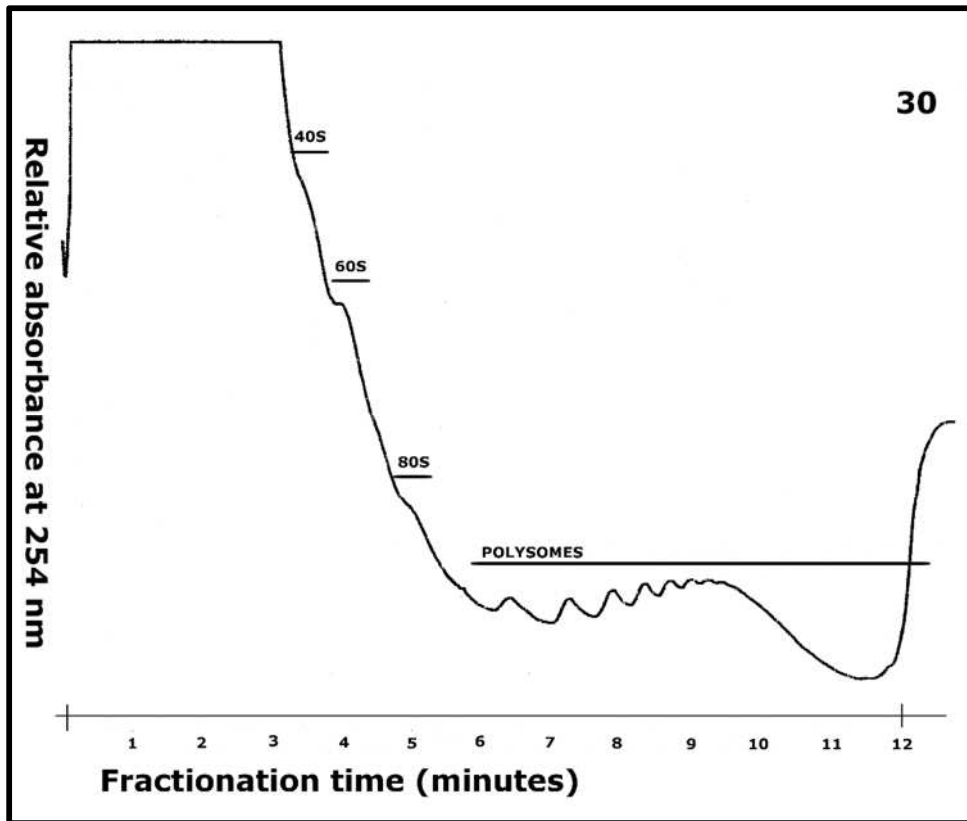


Figure 2.3. Polysome profile of 6hr TSWV1-inoculated pepper plant leaf sample 30. Ribosomal subunit and polysome peaks are marked. A₂₅₄ (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

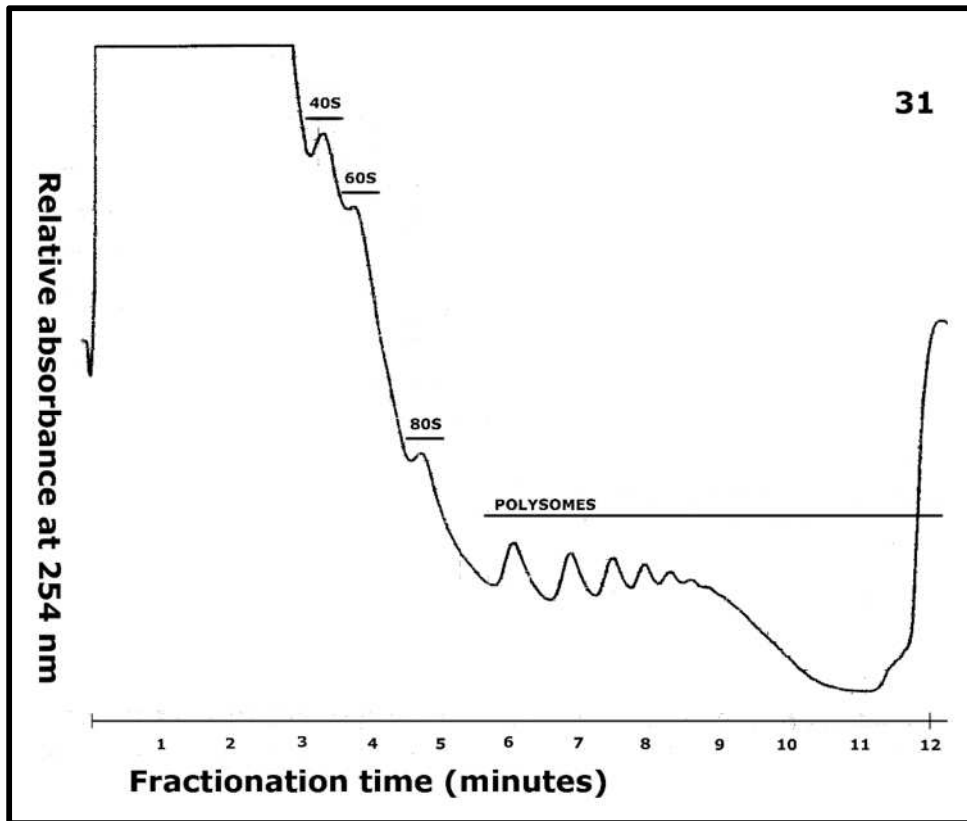


Figure 2.4. Polysome profile of 6hr TSWV1-inoculated pepper plant leaf sample 31. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

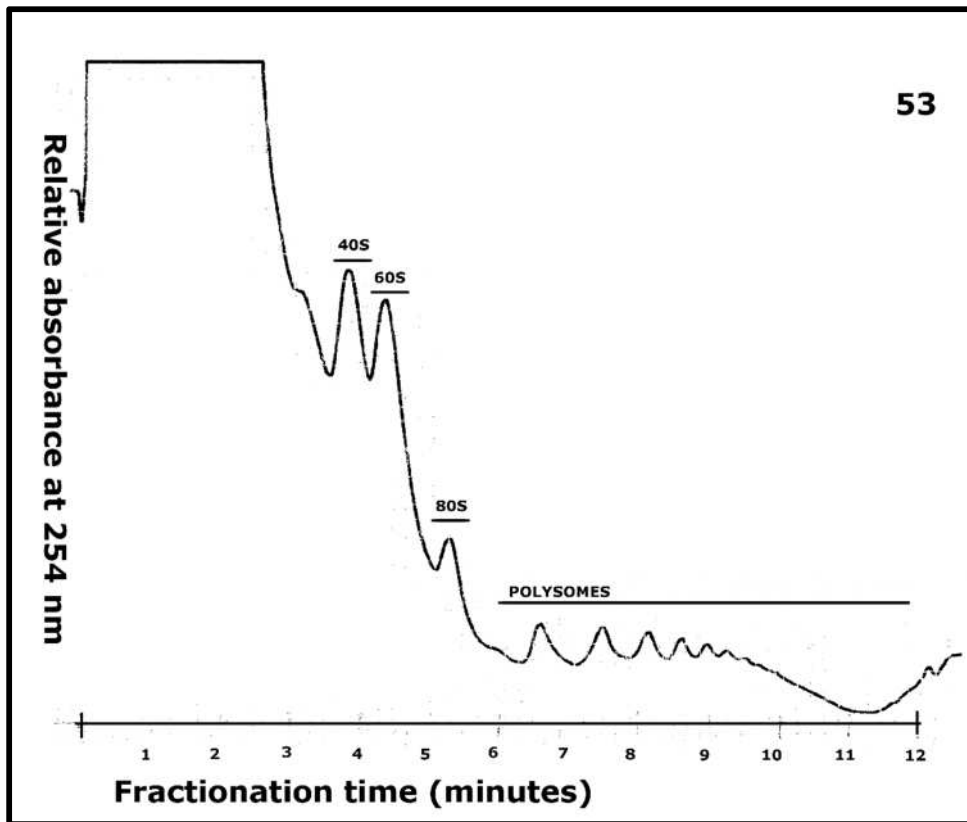


Figure 2.5. Polysome profile of 6hr mock-inoculated pepper plant leaf sample 53. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

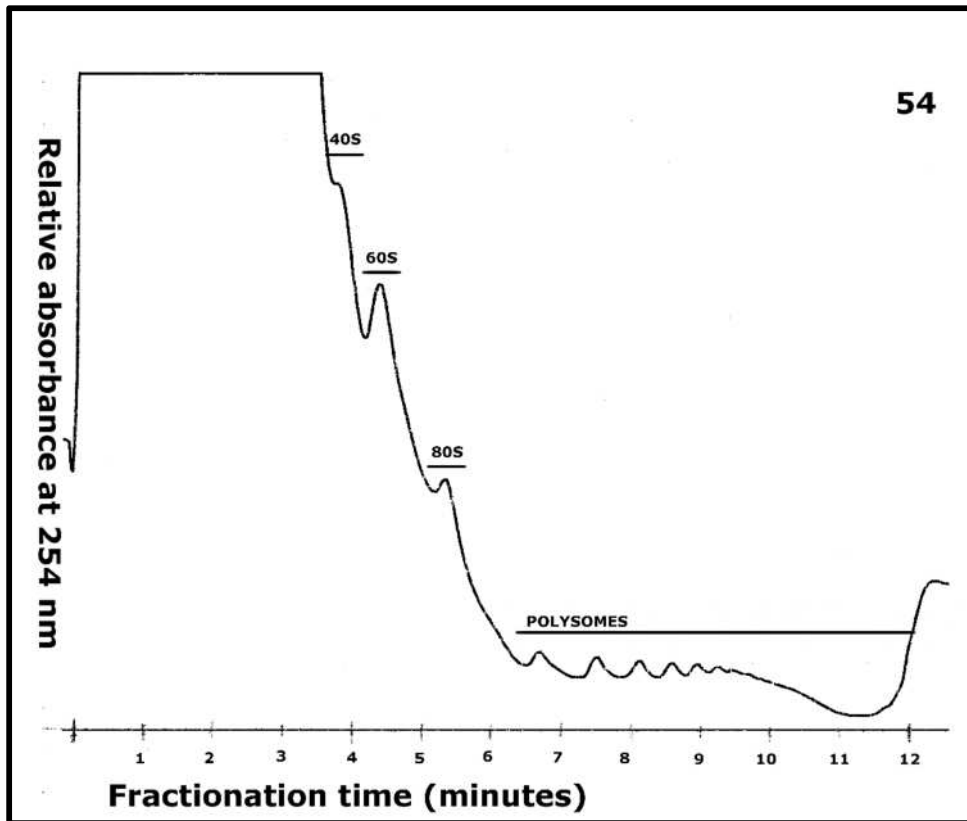


Figure 2.6. Polysome profile of 6hr mock-inoculated pepper plant leaf sample 54. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

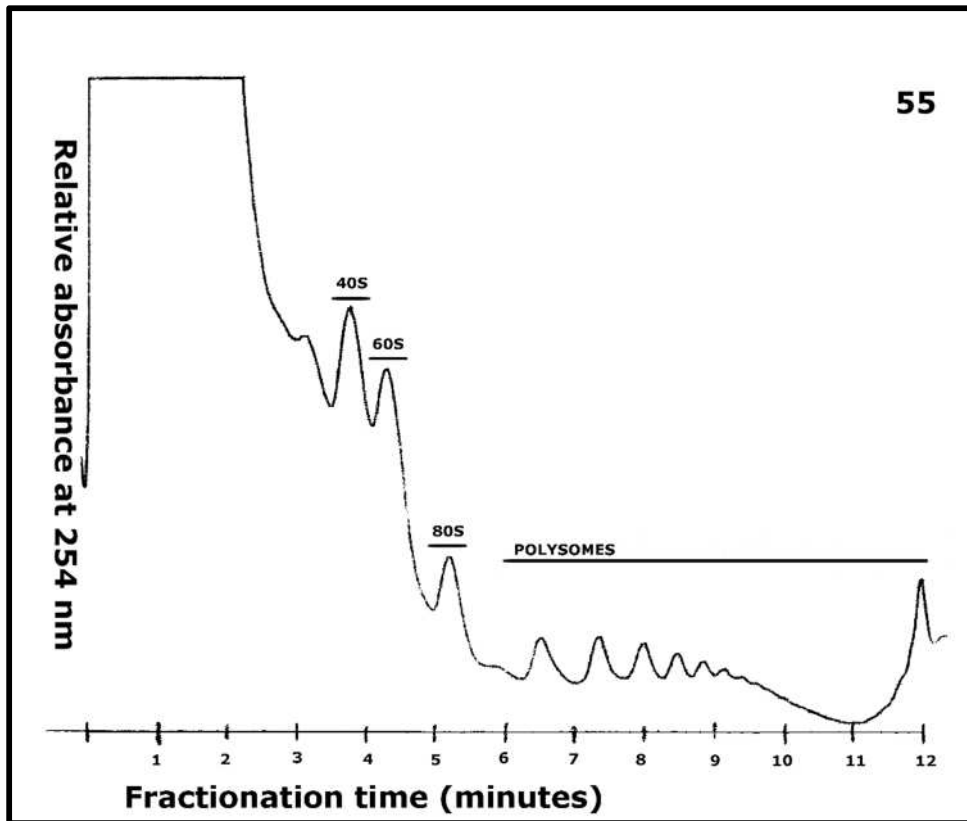


Figure 2.7. Polysome profile of 6hr mock-inoculated pepper plant leaf sample 55. Ribosomal subunit and polysome peaks are marked. A₂₅₄ (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

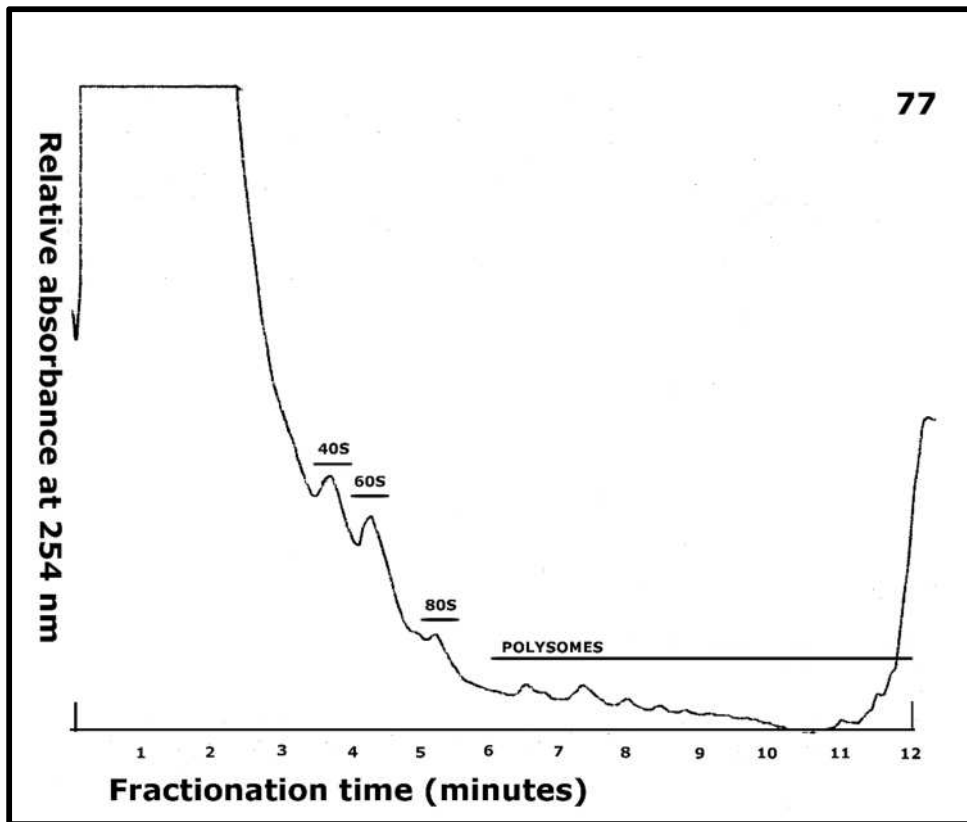


Figure 2.8. Polysome profile of 6hr non-inoculated pepper plant leaf sample 77. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

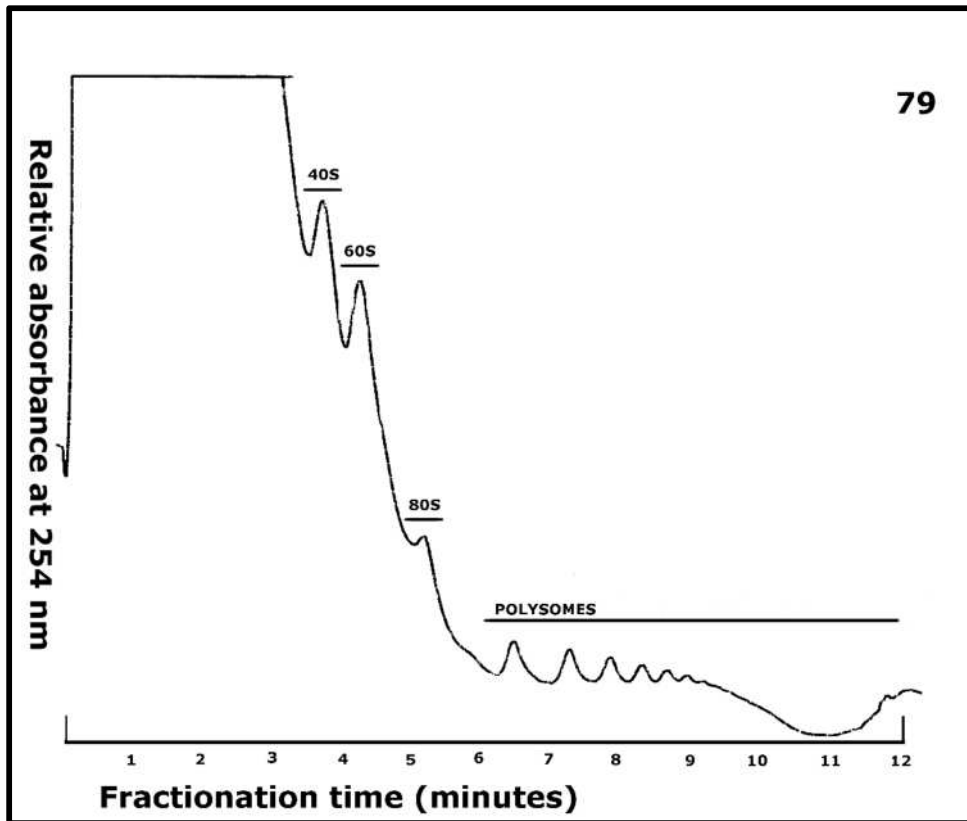


Figure 2.9. Polysome profile of 6hr non-inoculated pepper plant leaf sample 79. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

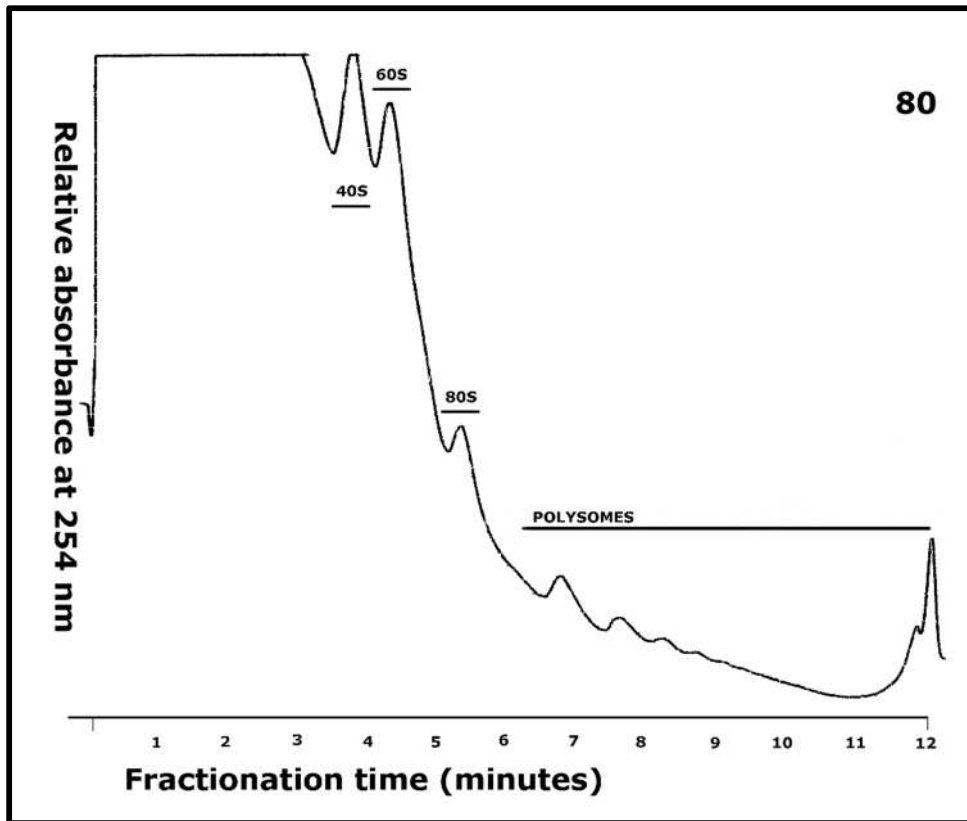


Figure 2.10. Polysome profile of 6hr non-inoculated pepper plant leaf sample 80. Ribosomal subunit and polysome peaks are marked. A_{254} (arbitrary units) of the sample passing through the flow-cell was plotted against fractionation time (minutes), which relates directly to the density of the sample being pumped through the flow cell as denser fractions are separated later than lighter fractions.

Table 2.2. Average heights of ribosomal subunit (40S, 60S, and 80S) and polysome peaks (P1-P8) and the average polysome areas from polysome profiles of TSWV-, mock-, and non-inoculated pepper plants (shown in figures 2.2-2.10); all heights and areas were normalised against the 80S ribosomal peak.¹

	TSWV-inoculated plants	Mock- inoculated plants	Non-inoculated plants
40S	2.31±0.11	2.36±0.09	2.54±0.12
60S	1.97±0.03	2.06±0.15	2.21±0.05
80S	1.00±0.00	1.00±0.00	1.00±0.00
P1	0.60±0.03	0.46±0.08	0.49±0.00
P2	0.58±0.02	0.45±0.08	0.42±0.04
P3	0.59±0.01*	0.42±0.08	0.33±0.04*
P4	0.59±0.02* **	0.39±0.06*	0.28±0.05**
P5	0.58±0.03* **	0.36±0.05*	0.25±0.05**
P6	0.57±0.04* **	0.33±0.04*	0.22±0.05**
P7	0.56±0.05* **	0.30±0.03*	0.20±0.05**
P8	0.58±0.06* **	0.25±0.03*	0.18±0.05**
polysome area	48.64±2.12* **	28.55±4.10*	25.21±1.34**

¹ Within rows, "*" and "***" indicate statistically significant differences between marked values (ANOVA, p<0.05). Values are shown graphically in figures 2.11 and 2.12. Standard error values are also shown.

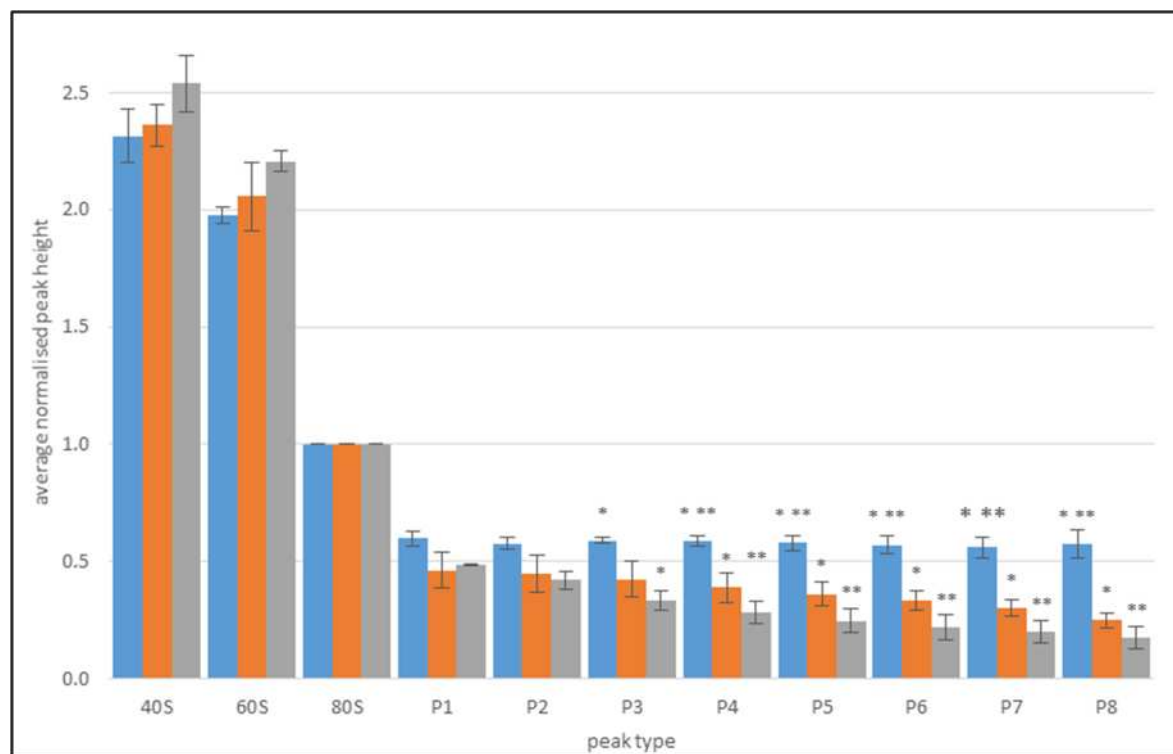


Figure 2.11. A bar chart showing average peak heights of ribosomal subunits and polysomes (P) from polysome profiles of TSWV- (blue), mock- (orange), and non-inoculated (grey) pepper plants, normalised against the 80S ribosomal peak with error bars.²

² Within each set of peaks, “*” and “***” indicate a statistically significant difference between marked peaks (ANOVA, $p < 0.05$). Values can be seen numerically in table 2.2 .

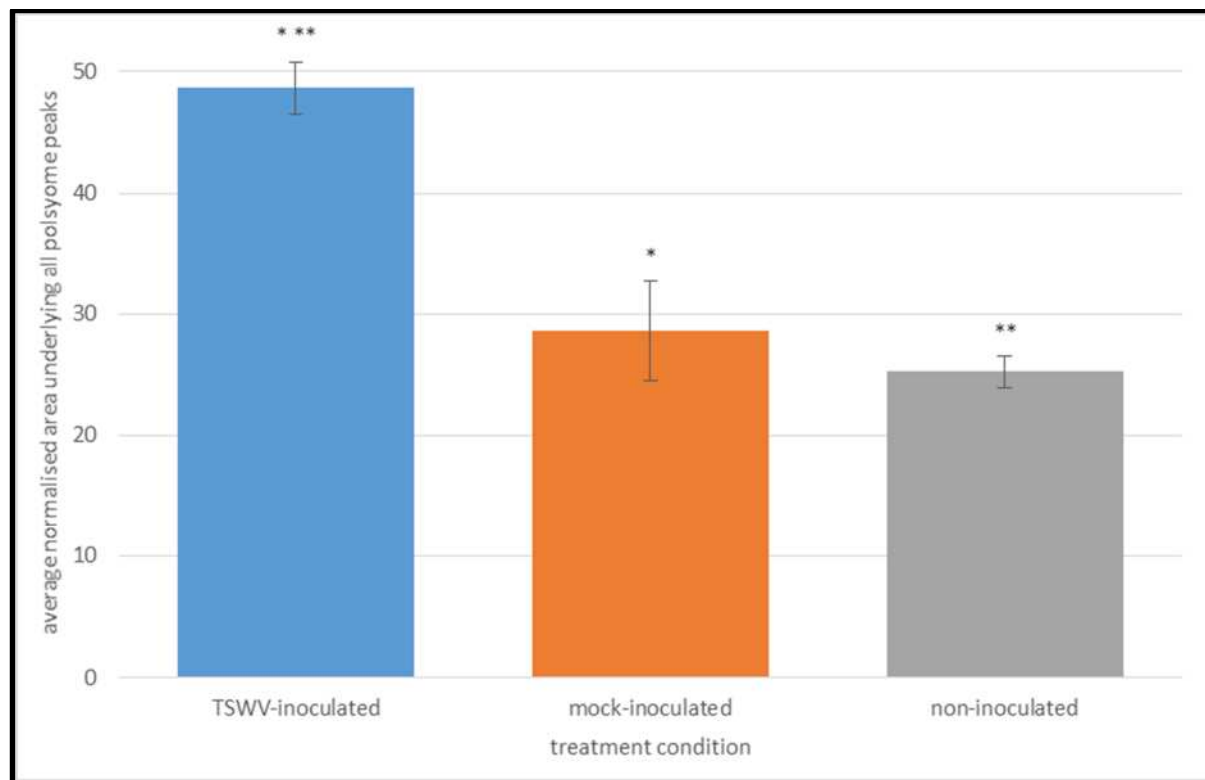


Figure 2.12. A bar chart showing the average areas underlying all polysome peaks from polysome profiles of TSWV- (blue), mock- (orange), and non-inoculated (grey) pepper plants, normalised against the 80S ribosomal peak, with error bars. ³

³ “*” and “***” indicate a statistically significant difference between marked peaks (ANOVA, $p < 0.05$). Values can be seen numerically in table 2.2 .

2.4 DISCUSSION

2.4.1 Gradient profiles

The polysome profiles shown in figure 2.2 in section 2.3 show a statistically significant increase in the relative abundance of polysome-associated material in TSWV-inoculated plants compared with those from mock-inoculated plants 6 hr after inoculation. This indicates an increase in global translation, thus showing no effective translational repression during the compatible interaction between the strain of virus (Syngenta TSWV1; *Tsw*-gene resistance-breaking) and the susceptible pepper line (Syngenta 14PEF990007; *Tsw*⁻) used in this study. Information on the source and RSS ability of Syngenta TSWV1 in relation to other strains was not available.

Drawing conclusions on the molecular causes and relevance of these results is difficult due to the lack of corresponding data from a non-resistance-breaking TSWV isolate, to which Syngenta 14PEF990007 is still susceptible, and from a *Tsw*⁺ pepper line. In addition to these factors, the functional RSS activity of the Syngenta TSWV1 NSs is not known. This is relevant as a loss of RSS activity of NSs is somewhat functionally linked to *Tsw*-gene resistance-breaking and the lack of RSS activity may reduce global translation by degradation of target transcripts of host- and viral-derived siRNAs (Molnár *et al.*, 2005; de Ronde *et al.*, 2014).

A lack of effective translational repression in response to TSWV1 infection may contribute to the susceptibility of Syngenta 14PEF990007 as this could mean translation of viral transcripts is unimpeded, where a global repression of translation in response to TSWV infection may inhibit viral

reproduction and systemic spreading, as is the case in animals (McCormick and Khaperskyy, 2017). However a global increase in translation does not necessarily mean that TSWV transcripts are being translated at the same rate as cellular transcripts as these viral transcripts have a different structure and are non-canonically translated (Geerts-Dimitriadou *et al.*, 2012). A pepper line which exhibits inhibition of translation in TSWV-infected cells, thus reducing viral effector synthesis, reproduction, and spread, may represent a possible means of resistance and provide more insight into how tospoviruses hijack cellular translation machinery.

Although a significant difference is seen in heavy polysome peaks and polysome-associated material relative to the monosomal peak between TSWV1- and mock-inoculated plants in polysome profiles, the protocol used was not optimised for plant tissue extracts, which may reduce the reliability of these data or other effects may be present during the TSWV response that are not shown therein (Mustroph *et al.*, 2009a). Using the animal-optimised protocol may have caused a number of problems. A general drop in RNA yield may have occurred as the buffers used did not possess EGTA, a Ca^{++} chelator that inhibits Ca^{++} -dependent RNases, or KCl, which inhibits ribosome-associated RNases; extraction and separation buffers in this study were pH 7.5, as animal-optimised protocols claim buffers greater than pH 8.8 reduce polysome stability, where plant-specific protocols call for a buffer at pH 9.0, which is also recommended to reduce RNase activity (Bonanou-Tzedaki and Arnstein, 1972). The protocol described by Mustroph *et al.* (2009a) also recommends not using heparin in extraction or separation buffers

unless tissues have a high RNase content or where RNA degradation is evident as heparin interferes with downstream analysis. Organellar translation was not included in profiles as the buffers used did not contain chloramphenicol, which inhibits organellar elongation as cycloheximide does for cytosolic elongation. A reduced yield and a poorer view of translation by endoplasmic reticulum- and cytoskeleton-associated ribosomes may have occurred as plant-specific protocols call for additional ionic and non-ionic surfactants in the extraction buffer to disrupt these organelles. The large pre-ribosomal subunit peak, which includes cellular debris such as soluble protein and often made ribosomal subunit peaks to visualise, could have been removed by a brief centrifugation of the cellular extract through a 2 M sucrose cushion, thus obtaining pellets enriched in ribosomal subunits, ribosomes, and polysomes; this however may have also removed free mRNA from the analysis as mRNA sediments in the same range of sucrose concentration as other components that comprise this cellular debris (Birnie *et al.*, 1972; Davies and Larkins, 1980; Mustroph *et al.*, 2009a). As yields were lower this may have increased the signal-to-noise in the polysome profiles, making the test less sensitive to changes in peak height and potentially causing false positive increases in the area underlying peaks. Low yield and heparin contamination also caused downstream difficulties in data validation.

3 IDENTIFYING GENES INVOLVED IN EARLY MECHANISMS OF RESISTANCE AGAINST TOSPOVIRUSES IN PEPPER

3.1 INTRODUCTION

3.1.1 Mechanisms of resistance against tospoviruses in Solanaceae

Pepper resistance gene *Tsw*

In pepper, the only known resistance to TSWV is conferred by the dominant gene *Tsw*, which originated from three *C. chinense* accessions (PI152225, PI159236, and CNPH 275 syn. “Panca”) (Black *et al.*, 1991; Boiteux *et al.*, 1993). This resistance to TSWV is based on the hypersensitive response (HR), associated with localised lesions at the inoculation site and abscission of inoculated organs 3-4 days post-inoculation (Moury *et al.*, 1997; de Ronde *et al.*, 2013). *Tsw* only confers resistance to TSWV (a member of *Tospovirus* serogroup I), but yields no resistance to tospoviruses of other serogroups. Resistance gene *Tsw* has been crossed into *C. annuum*, the species of most commercially grown peppers (Boiteux and de Ávila, 1994). Resistance-breaking isolates of TSWV in pepper are frequently reported worldwide (Margaria *et al.*, 2004; Ferrand *et al.*, 2015; Jiang *et al.*, 2017). The lack of broad, systemic resistance to tospoviruses means finding new candidate resistance genes is imperative.

Tsw was mapped to a locus on chromosome 10 containing multiple tightly linked dominant pathogen resistance genes but has only recently been cloned to a particular nucleotide-binding and leucine-rich domain protein (NLR) coding gene; this cluster was identified in *C. chinense* PI159236

(Grube *et al.*, 2000; Kim *et al.*, 2017). NLR-coding genes are expanded significantly in the pepper genome compared with other plants (Kim *et al.*, 2014). In the *C. chinense* progenitor, the resistance gene *Tsw* is physically located among a cluster of multiple related genes and pseudogenes encoding NLRs. *Pvr4*, which is associated with complete resistance to three pathotypes of potato virus Y (PVY) and to pepper mottle virus (PepMoV; a potyvirus), is a gene that originated from the corresponding region to the *C. chinense Tsw* locus in *C. annuum* var. CM334 (Caranta *et al.*, 1999; Grube *et al.*, 2000; Kim *et al.*, 2017). The exact nature of other genes at this locus remains unclear; these include *Pvr7*, which confers dominant resistance to PepMoV, and the *Lt10.1* QTL, which is associated with resistance to powdery mildew caused by *Leveillula taurica* and came from the powdery mildew resistant *C. annuum* var. H3 (Lefebvre *et al.*, 2003).

The avirulence factor (*Avr*) for *R* gene *Tsw* was determined by transiently expressing candidate *Avr* genes from resistance-inducing (RI) and resistance-breaking (RB) TSWV isolates in peppers possessing *Tsw* (de Ronde *et al.*, 2013). The *Avr* factor was identified as NSs, which also acts as the suppressor of the host's antiviral RNA silencing response, by sequestering various dsRNA molecules away from host RNA silencing machinery, and enhances translation of viral transcripts, possibly by acting as a functional equivalent PABP (Schnettler *et al.*, 2010; Geerts-Dimitriadou *et al.*, 2012; de Ronde *et al.*, 2013). Expression of NSs^{RI} and NSs^{RB} in tobacco expressing a sense GFP construct indicates that NSs^{RI} demonstrated RNA silencing suppression (RSS) but NSs^{RB} has lost this ability, although the RB virus isolates, from which

the NSs^{RB} came, are still able to suppress RNA silencing just as well as the RI virus isolates (de Ronde *et al.*, 2013). A mutant screen of NSs^{RI} indicated that the N-terminal domain is important for both RSS and Avr activity as mutations in the first 133 amino acids of NSs^{RI} normally caused a loss of both functions; this was corroborated by domain swaps between NSs^{RI} and NSs^{RB}, in which the RSS/Avr activities of the chimeric proteins were determined by the source of the N-terminal domain (de Ronde *et al.*, 2014). Single mutations in the C-terminal region had little effect on either RSS or Avr activity but deletion of the final 97 amino acids caused a total loss of both functions (de Ronde *et al.*, 2014). The N-terminal NSs mutants that cause loss of only RSS or *Tsw* Avr function show that these two activities are not tightly functionally linked (de Ronde *et al.*, 2014).

Tomato resistance gene *Sw-5*

In tomato, a gene called *Sw-5b* confers broad-spectrum, dominant, HR-based resistance against TSWV (Jahn *et al.*, 2000). *Sw-5b* is part of a the *Sw-5* gene cluster, which contains at least five paralogues (*Sw-5a* through *Sw-5e*), on chromosome 9; *Sw-5b* is the only gene at this locus that confers TSWV-resistance and, like *Tsw*, encodes an coiled-coil NLR (Spassova *et al.*, 2001; Kim *et al.*, 2017). *Sw-5* originated from *Solanum peruvianum*; this was shown after TSWV resistance was observed in the tomato cultivar “Stevens,” which was developed from a cross between *S. lycopersicum* and *S. peruvianum* (Stevens *et al.*, 1991).

The *Avr* for tomato *Sw-5b* was mapped to the M segment by coinfection of tobacco with TSWV-A, an *Sw-5b* RB strain, and TSWV-D, an

Sw-5b RI strain, in order to produce viruses with reassorted genomes; only TSWV-A and reassortant isolates possessing a TSWV-A-derived M segment were able to induce symptoms and spread systemically in TSWV-D resistant plants (Hoffmann *et al.*, 2001).

The Avr determinant for *Sw-5b* is NSm; agroinfiltration of tobacco plants transgenically expressing *Sw-5b* and *Sw-5*-containing tomato plants with NSm^{RI} induces HR-development, which does not occur upon agroinfiltration with NSm^{RB} or with any other candidate TSWV proteins (Hallwass *et al.*, 2014).

Even though the tomato and pepper genomes exhibit synteny, *Sw-5* and *Tsw* do not appear to share a recent common evolutionary ancestor as they are found at non-corresponding loci (Jahn *et al.*, 2000; Wu *et al.*, 2009).

Expression level studies

Identifying differentially expressed (DE) genes at the transcriptional level upon viral infection of a given host provides a means of highlighting cellular, viral genes and processes involved in the disease development and host defence responses; this has been done for various model and crop plants (Ramonell and Somerville, 2002; Marathe *et al.*, 2004; Espinoza *et al.*, 2007; Fernández-Calvino *et al.*, 2016; Anjanappa *et al.*, 2018). Data on transcription level responses of pepper to TSWV is lacking but published studies exist for other TSWV-susceptible plants including many solanaceous hosts (Senthil *et al.*, 2005; Catoni *et al.*, 2009; Abe *et al.*, 2012; Nachappa *et al.*, 2013; Choi *et al.*, 2015).

In tobacco, oxylipin biosynthesis genes are up-regulated during infection by TSWV or by a synergistic combination of potato virus X and potato virus Y (PVX-PVY) where programmed cell death (PCD) is associated with infection, both when PCD is part of necrotic lesions caused by a systemic compatible interaction and when it is part of an HR-based incompatible interaction (García-Marcos *et al.*, 2013). Oxylipin biosynthesis genes are also induced in pepper showing HR to the incompatible (+)ssRNA virus *Obuda pepper virus* (ObPV), a tobamovirus, but this induction is much less pronounced during the compatible interaction with the tobamovirus pepper mild mottle virus (PMMoV; not to be confused with PepMoV), where inoculation only causes mild chlorotic symptoms but not systemic necrosis (Gullner *et al.*, 2010). There are two groups of LOX enzymes: 9-LOXs and 13-LOXs, which produce 9- and 13-fatty acid hydroperoxides respectively; *13-LOX* genes are induced in pepper leaves inoculated with either ObPV or PMMoV but *9-LOX* genes are much more strongly induced in those inoculated with ObPV than with PMMoV, suggesting a link between *9-LOX* induction with HR symptom expression and with suppression of viral replication, whereas *13-LOX* induction is more likely linked to general virus sensing during both compatible and incompatible reactions (Juhász *et al.*, 2015).

Transcriptomic analysis of the response of *C. annuum* pepper showing HR-based resistance to capsicum chlorosis virus (CaCV), a related tospovirus, has been performed; this CaCV resistance (identified in the *C. chinense* accession PI290972) segregates independently from *Tsw* and does not confer resistance to TSWV (Widana Gamage *et al.*, 2016). Differential expression

results of Widana Gamage *et al.* (2016) showed up-regulation of genes involved in defence and stress responses, PCD, signal transduction, and antimicrobial compound synthesis, as well as identifying two potential CaCV resistance genes, which are also annotated as NLRs.

The systemic transcriptional response during a compatible interaction between tomato and TSWV has been investigated using microarrays to profile expression in roots and shoots; many more genes were regulated in shoots than in roots with only a small overlap between them and many pathways showed organ-specific transcriptional regulation (Catoni *et al.*, 2009). In shoots, genes involved in defence and signal transduction were induced and genes involved in primary, secondary and amino acid metabolism were repressed (Catoni *et al.*, 2009). In roots, transcription of genes involved in signal transduction and primary metabolism was unchanged, as was the case with amino acid metabolism apart from genes involved in secondary compound synthesis, which showed marked induction; biotic stress response genes were also induced in roots but expression of abiotic stress response genes was either repressed or unchanged (Catoni *et al.*, 2009).

3.2 MATERIALS AND METHODS

3.2.1 RNA sequencing

Library preparation

Library preparation was performed externally. NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) was used to isolate mRNA from the total, sub-polysomal, and polysomal RNA samples described in Chapter 2. Libraries were prepared and enriched with NEBNext Ultra RNA

Library Prep Kit for Illumina (New England Biolabs), a fragmentation and random primer-based kit. These were prepared according to the manufacturer's instructions. One µg of RNA was used for library preparation. The RNA integrity number (RIN) score for this had to be greater than 7 and the RNA had to be free of organic and inorganic contaminants and DNA.

NEBNext Poly(A) mRNA Magnetic Isolation Module

Extraction of poly(A)+ RNA for RNA library preparation and sequencing was done by pulling down all RNA that anneals to oligo-d(T)₂₅, which had been coupled to magnetic beads, via various wash and binding steps. All annealed RNA was eluted with First Strand Reaction Buffer and Random Primer Mix for the next step.

NEBNext Ultra RNA Library Prep Kit for Illumina

Samples were fragmented to 200 bp by incubation at 94°C for 15 minutes. First and second strand syntheses were performed, followed by end repair, adapter ligation with P5 and P7 adaptor oligos, and PCR library enrichment.

Sequencing

Samples were sequenced using a HiSeq2000 Illumina platform with 100 bp PE reads at a depth of around 50 million reads per sample.

3.2.2 RNA sequencing analysis

All R scripts for read alignment, differential expression analysis, and gene set enrichment analysis (GSEA) are available in the "Supplementary files" folder under "Scripts." GSEA demonstrates whether any sets of genes are significantly enriched in a list of DE genes.

Reads as FastQ files were aligned to the reference genome with HiSAT2, version hisat2-2.0.1-beta (Kim *et al.*, 2015). The reference genome (DAN2305_REF.gff) consisted of: the public version of the CM334 pepper genome version 1.55, named PEPPER_CM334_REF9_GENOME (Kim *et al.*, 2014); an addendum list of potential reference and DE genes, /project/DID714/DID714_genelist, which can be seen in table 3.1; and a copy of the pepper chloroplast genome, Genbank: NC_018552.1 (Jo *et al.*, 2011).

Differential expression data was generated with the R package “edgeR” (Robinson *et al.*, 2010). Significant differential expression was demonstrated using a minimum counts per million (CPM) of 2, a maximum false discovery rate (FDR) of 0.05, and a maximum P-value of 0.05. Direction of regulation for a given gene was determined using log2-fold change (LogFC); if LogFC was greater than 0 in a given comparison the gene was considered to be up-regulated and if LogFC was less than 0 the gene was considered to be down-regulated.

Table 3.1. A list of potential reference and pathogen-induced genes from literature for use in RNA-Seq.

Predicted expression	Gene name	Accession number	Citation
Reference	<i>ACT</i>	AY572427.1	(Wan <i>et al.</i> , 2011)
	<i>UBI-1</i>	DQ924970.1	
	<i>UBI-3</i>	AY486137.1	
	<i>GAPDH</i>	AJ246013.1	
	<i>EIF5A2</i>	AY484392.1	
	α - <i>TUB</i>	EF495257.1	
	β - <i>TUB</i>	EF495259.1	
	<i>EF1α</i>	DQ975458	(Bin <i>et al.</i> , 2012)
	<i>UEP</i>	AY496125	
Pathogen-induced	<i>CaBPR1</i>	AF053343	(Üstün <i>et al.</i> , 2013)
	<i>CaPR-Q</i>	XM016704913	
	<i>CaSAR82A</i>	AF112868	
	<i>CaGLU</i>	AY497910	(Tuyong <i>et al.</i> , 2005)
	<i>CaPR10</i>	AF244121	(Shin <i>et al.</i> , 2001)
	<i>CaLTP</i>	AF118131	
	<i>CaPAL</i>	KF279696	(Kim and Hwang, 2014)
	<i>CaPR3</i>	FJ596176	Unpublished; later published by Mishra <i>et al.</i> (2017)
	<i>CaBGL2/PR2</i>	AF294849	(Pflieger <i>et al.</i> , 2001)
	<i>CaIBI1</i>	XM016716647	In <i>Arabidopsis thaliana</i> (Luna <i>et al.</i> , 2014)
	<i>CaDEF1</i>	AF442388	(Mee Do <i>et al.</i> , 2004)
	<i>CaCAT1</i>	AF227952	(Lee and An, 2005)

Gene ontology enrichment

Gene ontology (GO) enrichment was performed for each pool-type on sets of genes DE between conditions using the R package “mroast,” which is a version of “ROAST” that allows multiple comparisons during GSEA (Wu *et al.*, 2010; Young *et al.*, 2010). A maximum FDR of 0.05 and a maximum P-value of 0.05 were used to demonstrate significant change in enrichment and the direction of the change.

KEGG pathway enrichment

Pathway enrichment using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database was performed on sets of genes DE in each pool-type upon inoculation with TSWV1. This was performed using the “GOstats” R package (Falcon and Gentleman, 2011). Pathway enrichment was performed on the “both” set of DE genes for each pool-type, i.e. the set that includes both up- and down-regulated genes. In this way, pathways that are affected in any way by TSWV-inoculation.

Results of KEGG pathway enrichment and the relative expression levels of pathway members were painted onto KEGG pathway maps.

3.3 RESULTS

3.3.1 Global overview of translome change

Having ascertained that there is a greater quantity of RNA associated with polysomes in TSWV-inoculated pepper plants than in mock-inoculated and non-inoculated plants, RNA sequencing was used to investigate which transcripts were differentially represented in total RNA, sub-polysomal, and polysomal pools. This aimed to show whether any transcripts are preferentially

translated or sequestered away from the translational machinery and whether there is a difference between the transcriptome and the translome.

RNA sequencing of the 27 samples submitted found an average of 52.0 million reads per sample. Early in analysis, total RNA from non-inoculated plant replicate 3 (Nt3) was flagged for per-base sequence content, per sequence GC content, sequence duplication levels, over-represented sequences (including polyA, 6 unknown sequences, and Illumina primer sequences), and K-mer content.

Later analyses found that Nt3 was an outlier, with the fewest unique concordant pairs, the highest number of concordant pairs that aligned to the genome multiple times, and the highest number of orphan sequences that aligned to the genome multiple times. The polysomal pool from TSWV-inoculated plants replicate 1 (Ip1) had the highest number of discordant pairs and the sub-polysomal pool from the same replicate (Is1) had the lowest total read count. The sub-polysomal pool from non-inoculated replicate 3 (Ns3) had the lowest total alignment rate. Nt3, however, was the only sample shown to drastically skew principle component analysis (PCA) plots and clustering and so was omitted from downstream analyses. Figures mentioned here are shown in table 3.2.

In this experiment, 16280 genes were expressed above a counts per million (CPM) cut-off of 2, to remove low read count genes, and a false discovery rate (FDR) less than 0.05, in at least three samples across sub-polysomal, polysomal and total RNA pools extracted from first leaves of 25 day old pepper plants. No GC content bias and little length bias were found by

analysis using NOISeq. Figure 3.1 shows a sensitivity plot with potential CPM cut-offs. Figure 3.2, a plot of expression against GC content, shows that no normalisation was needed to correct GC bias. Figure 3.3, a plot of expression against gene length, shows a possible relationship between length and expression, although this is likely due to outlying data points caused by a few large genes, so no normalisation to correct for length bias was performed. Figures 3.1-3.3 were generated using NOISeq (Tarazona *et al.*, 2011; Tarazona *et al.*, 2015).

PCA and hierarchical clustering were performed on CPM- and FDR-normalised read counts to generate PCA plots (figures 3.4-3.6), a scree plot of standard deviation against principal component (PC) number (figure 3.7), and a clustering dendrogram of samples (figure 3.8). These show that the greatest source of variation in the experiment is the pool-type, with all sub-polysomal pools clustering away from polysomal and total RNA pools.

The standard deviations and proportional contributions to variance of each principle component are given in the “Supplementary files” folder under “summaryPCA_norm.xlsx”.

Table 3.2. Summary of Alignments results from HiSAT for raw reads from all libraries. Outlying values ($-2.9 < z < 2.9$) are highlighted blue if low or red if high. ⁴

Leaf sample	Sample	Total reads	Total alignment rate	Unique concordant pairs	Multi-concordant pairs	Discordant pairs	Unaligned reads	Unique orphans	Multi orphans
29	lp1	55250374	80.45%	33490945	8515955	1349017	21605385	1472505	711024
30	lp2	45931383	80.82%	27851775	7476033	781874	17621255	1411159	610988
31	lp3	62668276	84.57%	38612584	12557626	523006	19339778	1815046	795296
29	ls1	38285596	83.89%	22182344	8753812	470688	12338405	884360	534739
30	ls2	46568484	83.78%	26176372	11442035	458685	15111417	1142991	728376
31	ls3	53520754	80.03%	27110080	14047369	534793	21371102	1320785	965137
29	lt1	49808111	84.77%	31143636	9630399	373988	15167908	1526356	625912
30	lt2	47386053	85.71%	30768232	8512338	406859	13547079	1314567	535602
31	lt3	53110050	84.92%	34063915	9363379	574636	16021469	1545387	649384
53	Mp1	53700366	85.59%	34831032	9686707	331152	15472192	1580367	650391
54	Mp2	55924114	84.15%	35465385	9812956	473584	17725056	1864265	755057
55	Mp3	50187007	85.02%	31672931	9650297	258258	15038475	1533056	639511
53	Ms1	56248566	86.58%	32415300	14671648	342715	15101533	1527273	1009000
54	Ms2	52689064	82.38%	28996759	12580739	603344	18572097	1477152	967195
55	Ms3	52811167	82.87%	29205073	13188643	223738	18088584	1407988	890854
53	Mt1	50635693	85.58%	30899406	10887865	402844	14599475	1513192	778489
54	Mt2	60299027	85.99%	38164691	11859875	301924	16890631	2140619	913824
55	Mt3	56928136	83.78%	35356790	10458196	560363	18472699	1828687	804188
77	Np1	49684384	85.31%	29691695	11294590	234909	14594711	1517779	813890
79	Np2	59102888	82.70%	36457799	10524882	640292	20447620	1750322	761888
80	Np3	55746489	84.52%	35078951	10462146	396217	17254466	1666984	696900
77	Ns1	53989166	84.96%	29781059	14377859	495011	16234768	1391150	1044556
79	Ns2	55715806	85.73%	33536780	12655052	231881	15898964	1734557	950665
80	Ns3	49926055	77.56%	25532696	10923386	1141961	22404303	1324559	927162
79	Nt1	50555508	86.30%	30876933	11262621	275264	13849049	1654726	777605
80	Nt2	58711656	85.58%	35907759	12587548	249799	16930132	2074932	928036
77	Nt3	52439166	86.46%	17356920	26399228	387900	14205424	907148	1477664

⁴ Total RNA from non-inoculated leaf samples 79 and 80 were renamed Nt1 and Nt2 respectively when sequencing results of total RNA from leaf sample 77 (henceforth Nt3) were shown to skew PCA and hierarchical clustering analysis. Samples are labelled by condition and pool-type and numbered by repeat number: lp is Tomato Spotted Wilt Virus-inoculated polysomal RNA, ls is TSWV-inoculated sub-polysomal RNA, lt is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA.

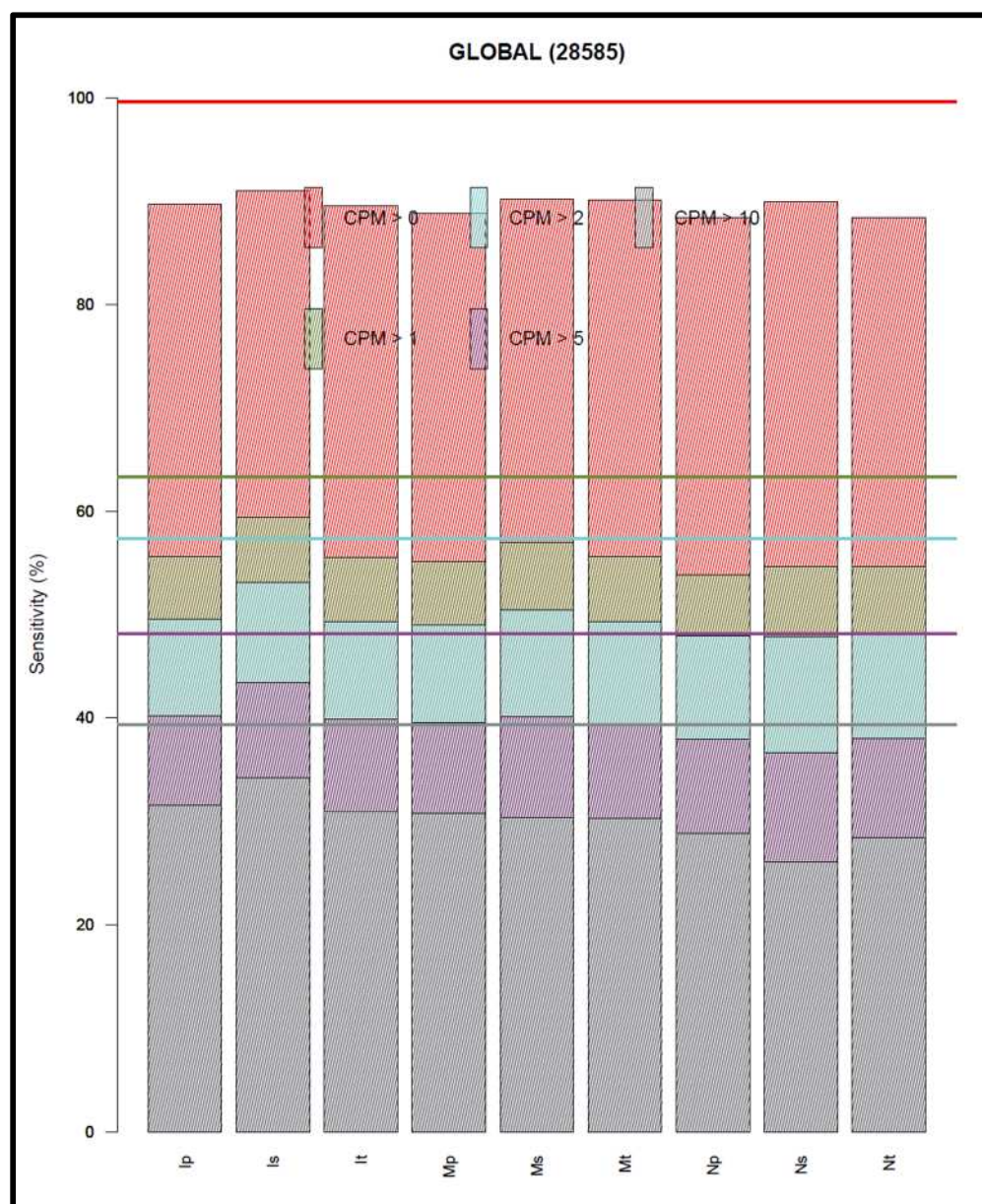


Figure 3.1. A graph summarising the proportion of genes expressed above 0 (red), 1 (green), 2 (blue), 5 (purple) and 10 (grey) CPM in at least three samples against all genes in the pepper genome in each condition (bars) and all samples (horizontal lines).⁵

⁵ Samples are labelled by condition and pool-type: Ip is Tomato Spotted Wilt Virus-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA.

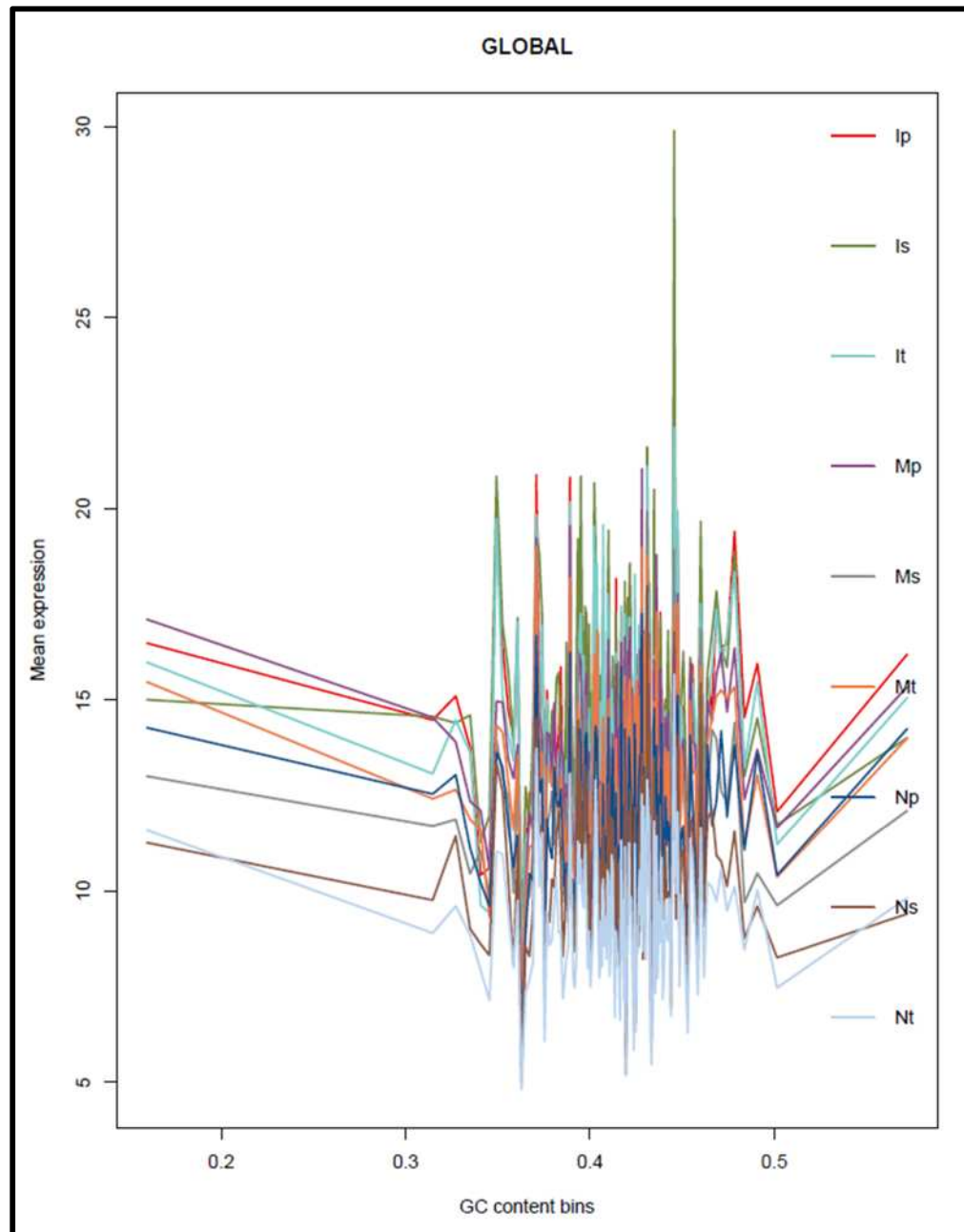


Figure 3.2. A plot of read count against GC content bins showing no dependence of read count on GC content ($P < 0.05$, $R^2 < 70\%$).⁶

⁶ Samples are labelled by condition and pool-type: Ip is Tomato Spotted Wilt Virus-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA.

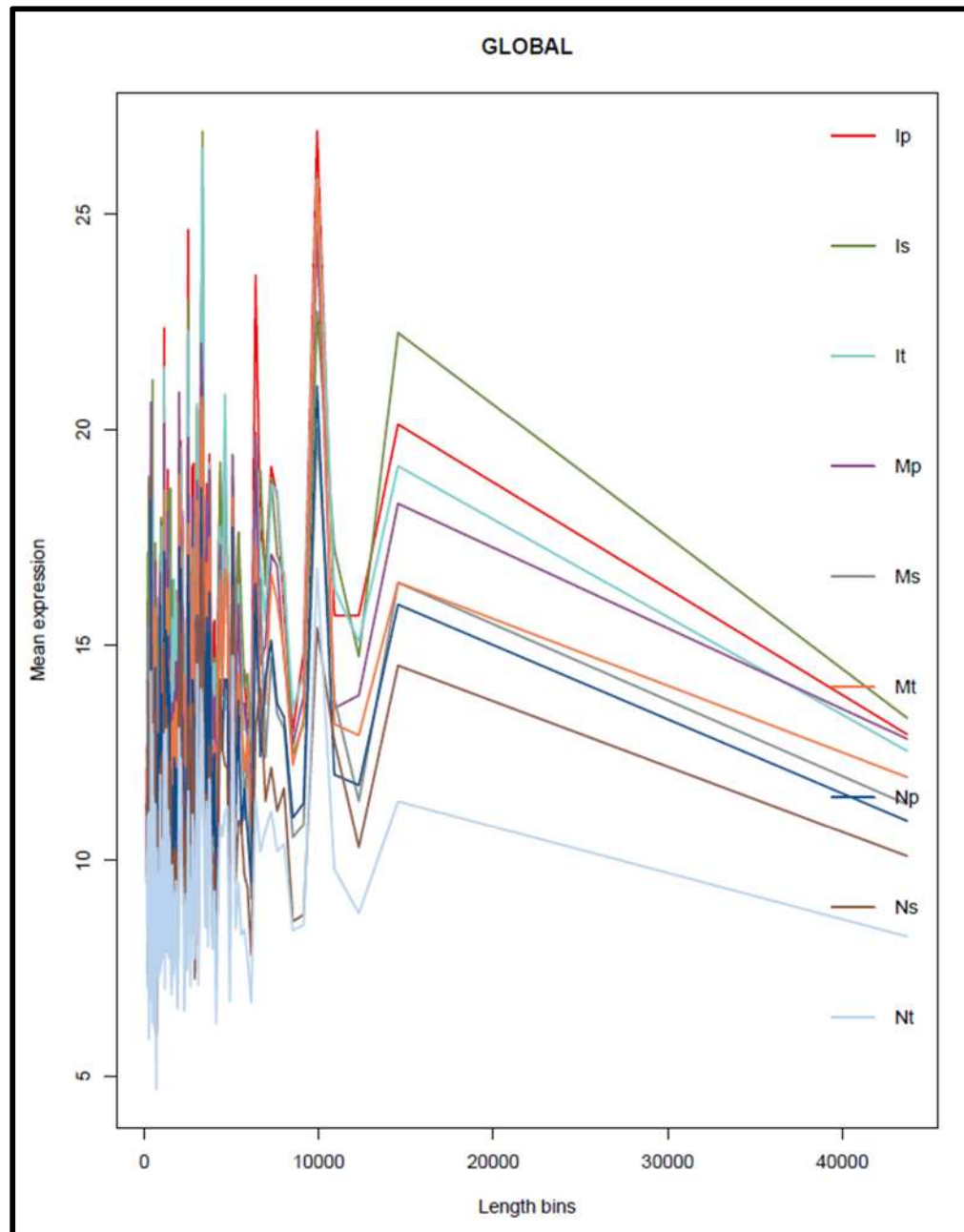


Figure 3.3. A plot of read count against gene length bins in order to find any transcript length bias. At least one model P value was lower than 0.05, indicating that normalisation for correcting length bias could be advisable, but R^2 was lower than 70% in all conditions. This apparent correlation was likely due to one gene with a high read count so no normalisation was undertaken.⁷

⁷ Samples are labelled by condition and pool-type: Ip is Tomato Spotted Wilt Virus-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA.

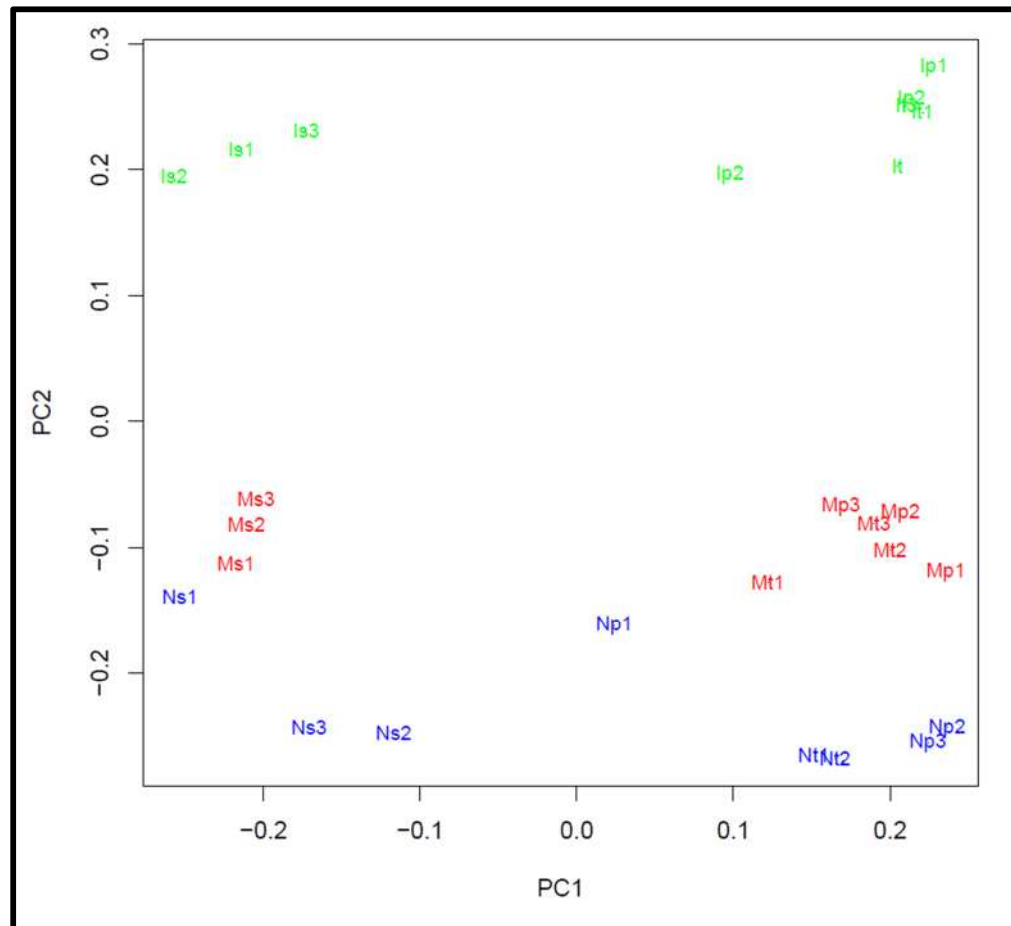


Figure 3.4. Principle component analysis (PCA) plot of principle component (PC) 1 vs PC2. Analysis was performed on CPM-normalised count data (CPM>2, FDR<0.05). Sample Nt3 was omitted from these and future analyses as it was flagged as a statistically significant outlier and skewed PCA plots. Samples segregate on PC1 according to their pool-type, wherein sub-polysomal samples cluster together and polysomal and total RNA samples cluster together. Samples segregate on PC2 according to condition, wherein Tomato spotted wilt virus-inoculated samples cluster together and mock- and non-inoculated samples cluster together.⁸

⁸ Samples are labelled by condition and pool-type: Ip is Tomato spotted wilt virus (TSWV)-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA. Samples are also coloured by condition type: TSWV-inoculated samples are green, mock-inoculated samples are red, and non-inoculated samples are blue.

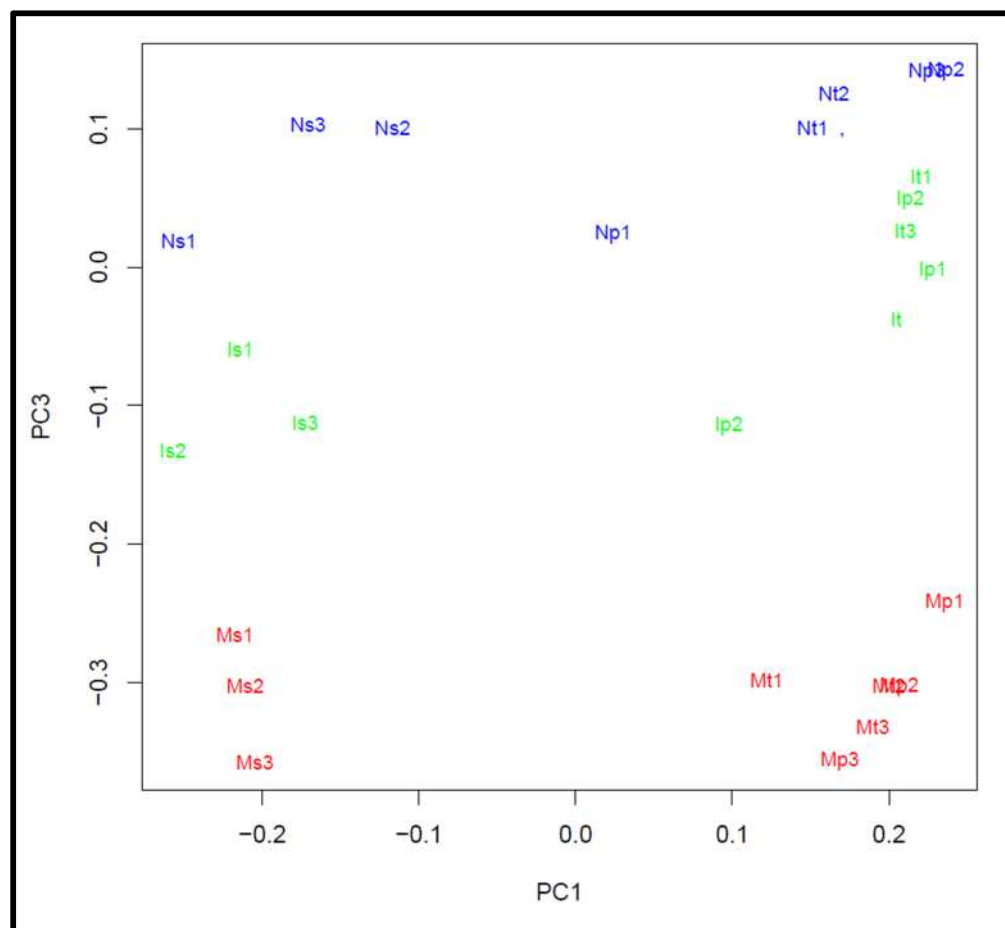


Figure 3.5. Principle component analysis (PCA) plot of principle component (PC) 1 vs PC3. Analysis was performed on CPM-normalised count data (CPM>2, FDR<0.05). Sample Nt3 was omitted from these and future analyses as it was flagged as a statistically significant outlier and skewed PCA plots. Samples shown some clustering on PC3 according to condition, where mock-inoculated samples cluster together and Tomato spotted wilt virus- and non-inoculated samples cluster with some cross-over.⁹

⁹ Samples are labelled by condition and pool-type: Ip is Tomato spotted wilt virus (TSWV)-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA. Samples are also coloured by condition type: TSWV-inoculated samples are green, mock-inoculated samples are red, and non-inoculated samples are blue.

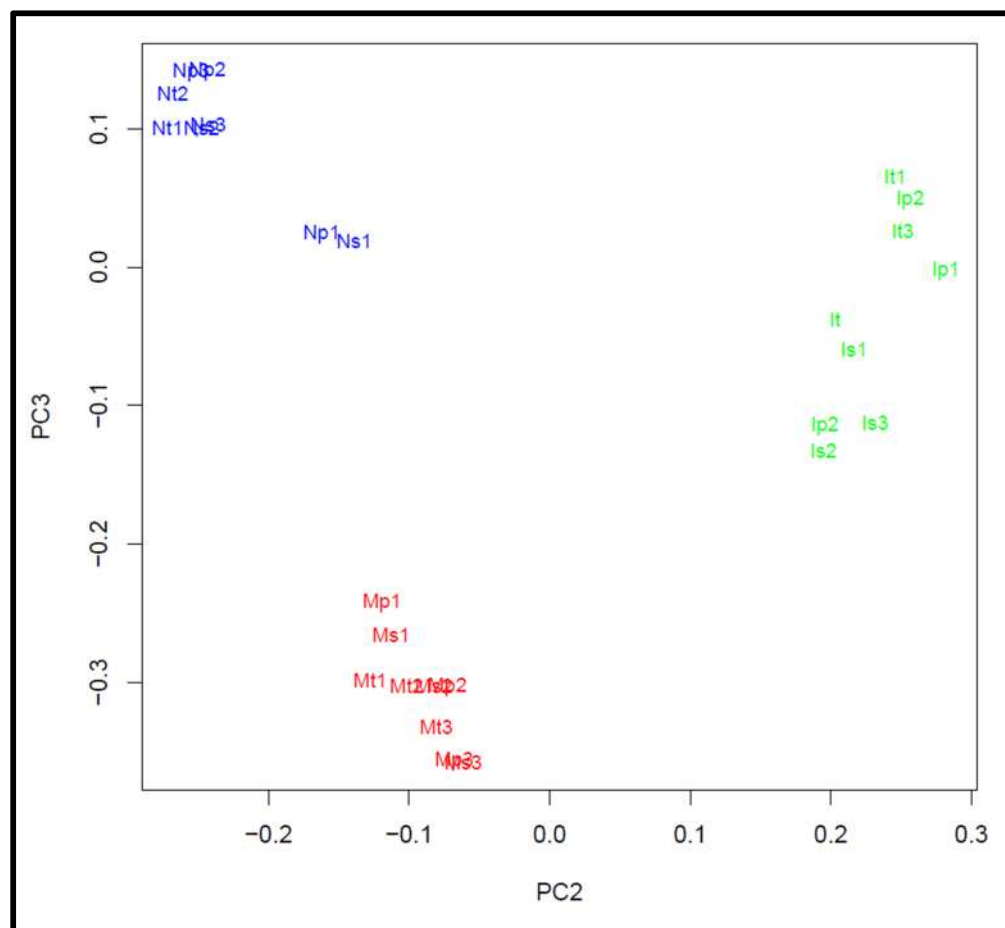


Figure 3.6. Principle component analysis (PCA) plot of principle component (PC) 2 vs PC3. Analysis was performed on CPM-normalised count data (CPM>2, FDR<0.05). Sample Nt3 was omitted from these and future analyses as it was flagged as a statistically significant outlier and skewed PCA plots.¹⁰

¹⁰ Samples are labelled by condition and pool-type: Ip is Tomato spotted wilt virus (TSWV)-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA. Samples are also coloured by condition type: TSWV-inoculated samples are green, mock-inoculated samples are red, and non-inoculated samples are blue.

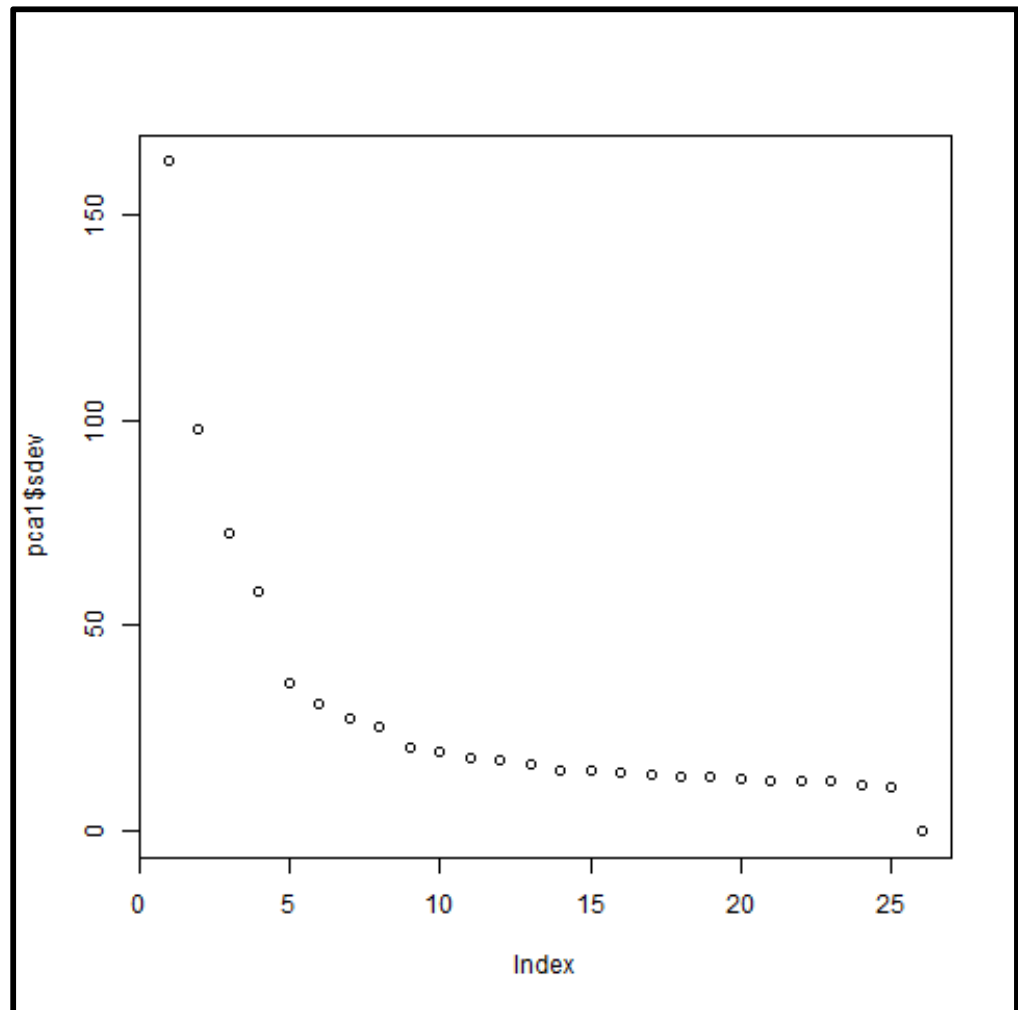


Figure 3.7. A scree plot of the proportion of standard deviation in the entire data set explained by each principle component (PC) against PC number ordered by decreasing standard deviation; standard deviations and proportion of variance for each PC can be found in the “Supplementary files” folder under “summaryPCA_norm.xlsx”.

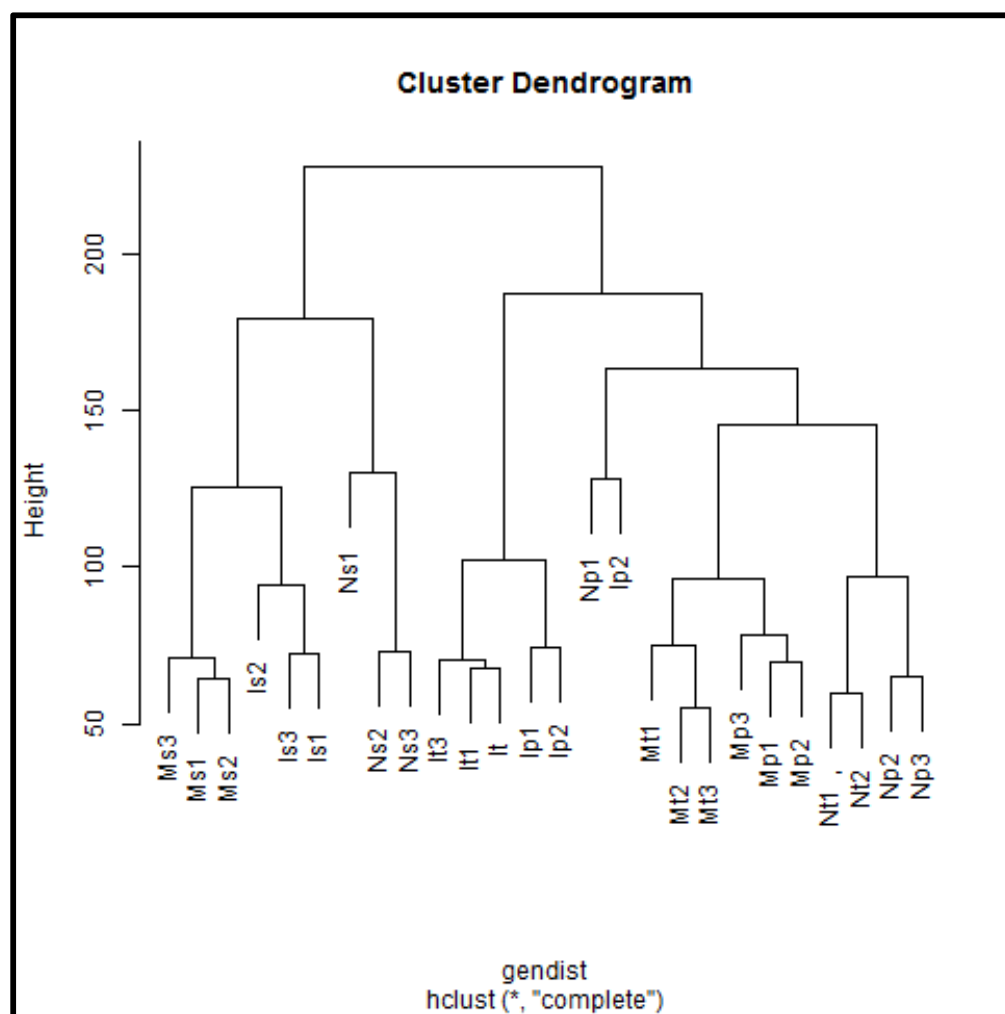


Figure 3.8. A hierarchical clustering of CPM-normalised count data ($CPM > 2$, $FDR < 0.05$); samples are labelled as in Figure 3.4. Sample Nt3 was shown to be a statistically significant outlier and was omitted from clustering.¹¹

¹¹ Samples are labelled by condition and pool-type: Ip is Tomato spotted wilt virus (TSWV)-inoculated polysomal RNA, Is is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA.

Sets of genes over- and under-represented were uniquely in each pool-type in a comparison between TSWV-inoculated and mock-inoculated plants, as well as gene sets that were DE in two out of three pools, and gene sets that were DE in all pools.

These results are summarised in table 3.3 and figures 3.9 and 3.10. Table 3.3 shows differential expression results that show there are genes that increase or decrease in abundance between conditions within pool-types; lists of up- and down-regulated genes with their annotations according to the SolGenomics CM334 reference genome can be found in the supplementary folder “Chapter 3 Genes up and down”. Abbreviated lists of annotated DE genes between TSWV- and mock-inoculated plants ($\text{LogFC} > 3$ or < -3 ; $P < 0.05$; $\text{FDR} < 0.05$) for each pool-type can be seen in tables 7.1-7.6 in the appendices.

Figures 3.9 and 3.10 show two Venn diagrams of differential expression results for six hr TSWV-inoculated plants versus mock-inoculated plants; lists from these contrasts are available in the “Supplementary files” folder under “lvM.sig DE Venn contrasts.xlsx”. Venn diagrams and gene lists were generated using the University of Ghent Venn Diagram Tool (Sterck, 2010).

In these comparisons, genes that are induced upon TSWV inoculation only in polysomal pools and not in sub-polysomal pools or in total RNA are likely to be actively translated but not differentially transcribed in response to TSWV. Conversely genes induced only in sub-polysomal pools are likely to be sequestered in response to TSWV. The results shown in figure 3.9 and table 3.3 indicate that genes are differentially transcribed and translated in every condition in this study.

Table 3.3. Differential expression results from nine contrasts between sub-polysomal, polysomal, and total RNA pools of 6hr TSWV1-, mock-, and non-inoculated plants. ¹²

	Contrast	Genes Up	Genes Down
Polysome	TSWV v mock	2062	2179
	TSWV v non	2756	2674
	Mock v non	1564	1326
Sub-polysome	TSWV v mock	2214	2283
	TSWV v non	3698	3653
	Mock v non	1899	1762
RNA	TSWV v mock	2141	2351
	TSWV v non	2421	2157
	Mock v non	1150	832

¹² For “Genes Up”, LogFC>0, p-value<0.05; for “Genes Down”, LogFC<0, p-value<0.05. FDR<0.05, CPM>2. Lists of up- and down-regulated genes can be found in the supplementary folder “Chapter 3 Genes up and down”. Excel files are named after the comparison of conditions and pool-type therein; inoculated (I), mock-inoculated (M), non-inoculated (N), polysomes (p), sub-polysomes (s), and total RNA (t).

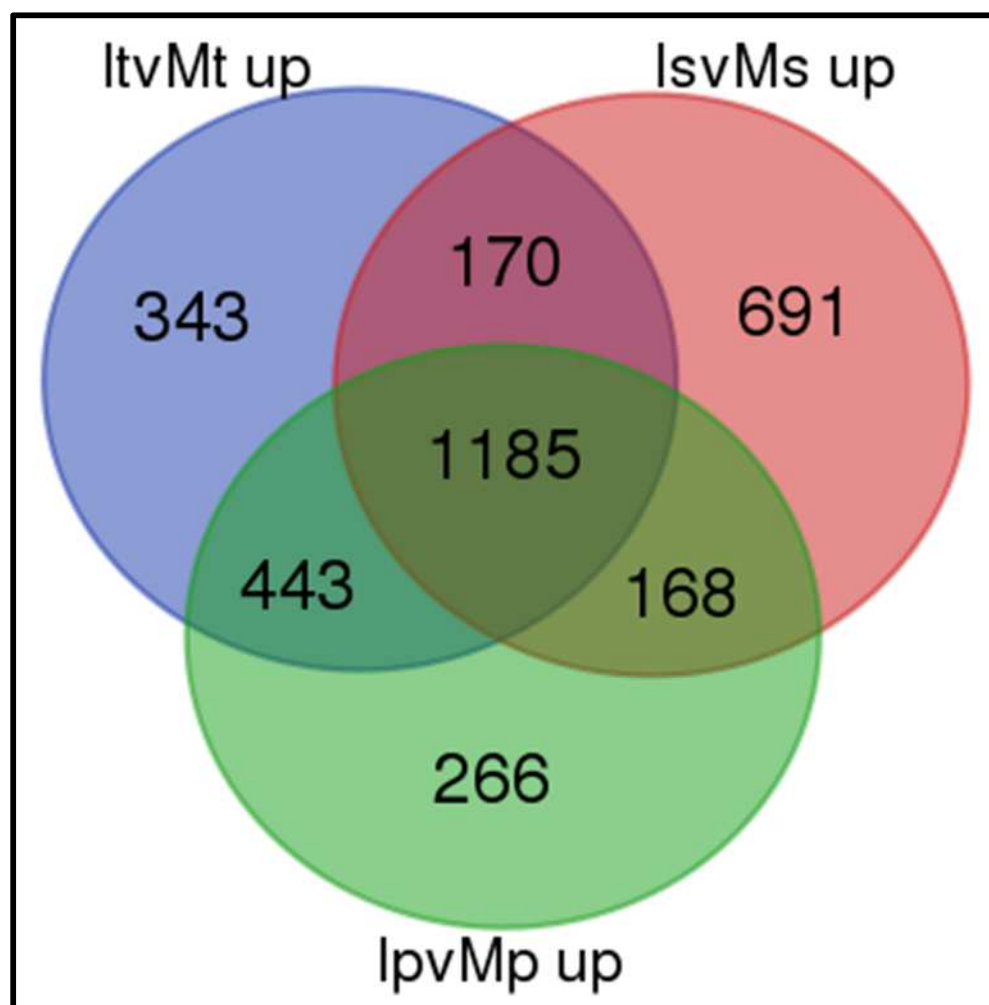


Figure 3.9. A Venn diagram showing numbers of DE genes that show an increase in abundance ($\text{LogFC} > 0$, $p\text{-value} < 0.05$, $\text{FDR} < 0.05$, $\text{CPM} > 2$) in one, two, or all pool-types in the comparison between TSWV- and mock-inoculated plants.¹³

¹³ Samples are labelled by condition and pool-type: lp is Tomato spotted wilt virus (TSWV)-inoculated polysomal RNA, ls is TSWV-inoculated sub-polysomal RNA, It is TSWV-inoculated total RNA, Mp is mock-inoculated polysomal RNA, Ms is mock-inoculated sub-polysomal RNA, Mt is mock-inoculated total RNA, Np is non-inoculated polysomal RNA, Ns is non-inoculated sub-polysomal RNA, and Nt is non-inoculated total RNA. Lists from these contrasts are available in the supplementary file “lvM.sig DE Venn contrasts.xlsx”.

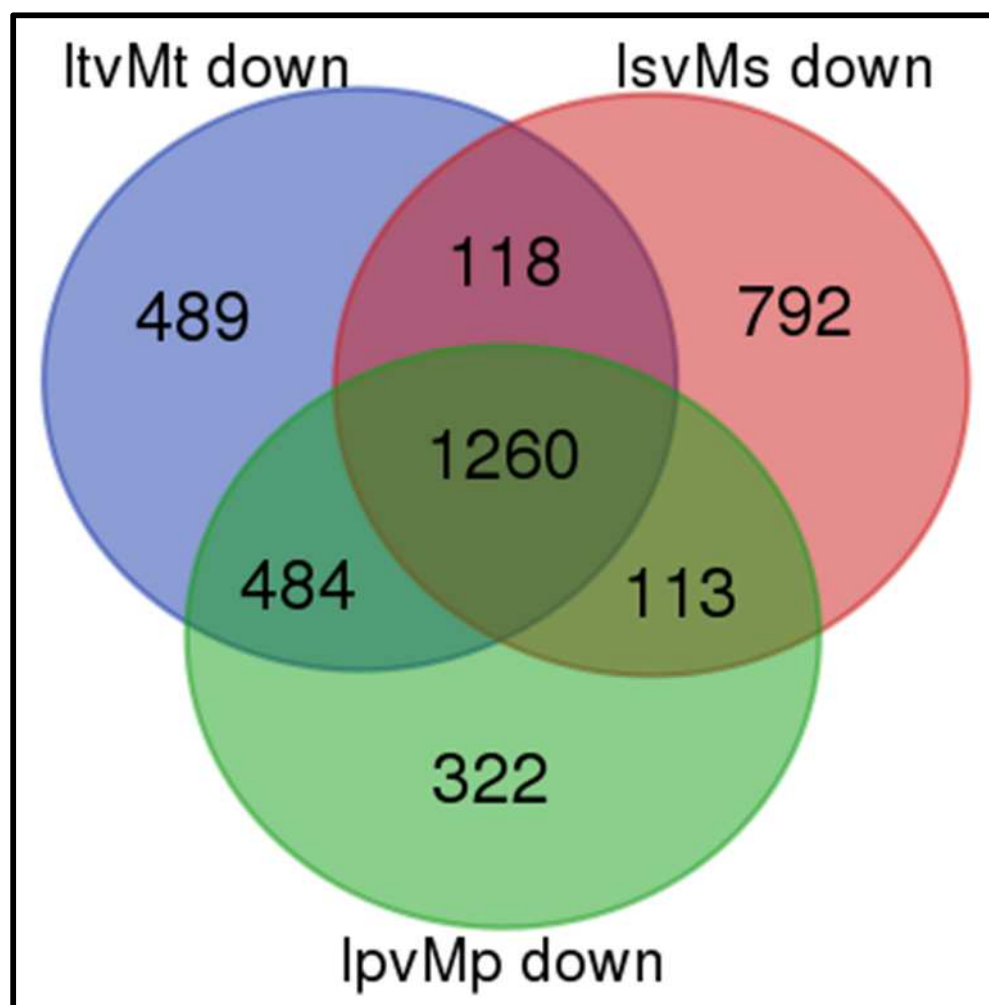


Figure 3.10. A Venn diagram showing numbers of DE genes that show a decrease in abundance ($\text{LogFC} < 0$, $p\text{-value} < 0.05$, $\text{FDR} < 0.05$, $\text{CPM} > 2$) in one, two, or all pool-types in the comparison between TSWV- and mock-inoculated plants.¹⁴

¹⁴ Samples are labelled by condition and pool-type: lp is Tomato spotted wilt virus (TSWV)-inoculated polysomal RNA, ls is TSWV-inoculated sub-polysomal RNA, lt is TSWV-inoculated total RNA, mp is mock-inoculated polysomal RNA, ms is mock-inoculated sub-polysomal RNA, mt is mock-inoculated total RNA, np is non-inoculated polysomal RNA, ns is non-inoculated sub-polysomal RNA, and nt is non-inoculated total RNA. Lists from these contrasts are available in the supplementary file "lvM.sig DE Venn contrasts.xlsx".

3.3.2 Gene ontology enrichment

In order to interpret the large gene sets found using RNA Seq and understand how the plant is affected by TSWV-infection in comparison with carborundum treatment, DE genes were analysed against the background of genes expressed in the experiment in order to see which GO terms were differentially enriched in each pool-type during the TSWV response. GO analysis is commonly used to find categories of genes, grouped by a common biological category or purpose, that are over-represented in a set of DE genes (Young *et al.*, 2010); GO terms for molecular function (MF), cellular component (CC) and biological processes (BP) were generated by running InterProScan on the CM334 pepper genome version 9 (Jones *et al.*, 2014; Kim *et al.*, 2014).

In total RNA during the response to TSWV, 127 GO terms (54 BP, 11 CC, and 62 MF) proportionally increased in enrichment and 69 GO terms (25 BP, 8 CC, 34 MF, and 2 terms with no annotation) proportionally decreased. In polysomal pools, 92 GO terms (41 BP, 11 CC, and 40 MF) proportionally increased and 87 GO terms (33 BP, 7 CC, 44 MF, and 3 terms with no annotation) proportionally decreased. In sub-polysomal pools, 83 GO terms (32 BP, 6 CC, and 45 MF) proportionally increased and 72 GO terms (31 BP, 8 CC, 32 MF, and 1 term with no annotation) proportionally decreased.

Tables 3.4, 3.5, and 3.6 show the five most up-regulated and down-regulated BP, CC, and MF GO terms in the compatible response to TSWV in total RNA, polysomal pools, and sub-polysomal pools respectively. Full GO term lists from these analyses can be found in the supplementary files “ItvMt.roast.xlsx,” “IpvMp.roast.xlsx,” and “IsvMs.roast.xlsx” respectively.

Table 3.4. The results of GO term enrichment of RNA Seq data from total RNA extracts of first leaves of TSWV-inoculated pepper plants compared with those of mock-inoculated plants.¹⁵

Direction	Domain	GO ID	Term	N	Down	Up	P	FDR
Up	BP	GO:0006526	arginine biosynthetic process	4	0	1	0.006	0.024
		GO:0006559	L-phenylalanine catabolic process	4	0	1	0.001	0.004
		GO:0006414	translational elongation	18	0.06	0.89	0.001	0.004
		GO:0006796	phosphate-containing compound metabolic process	6	0	0.83	0.001	0.004
		GO:0000162	tryptophan biosynthetic process	4	0	0.75	0.001	0.004
	CC	GO:0042555	MCM complex	6	0	1	0.001	0.004
		GO:0005741	mitochondrial outer membrane	11	0	0.91	0.001	0.004
		GO:0005852	eukaryotic translation initiation factor 3 complex	11	0	0.73	0.001	0.004
		GO:0019867	outer membrane	5	0	0.6	0.005	0.021
		GO:0005759	mitochondrial matrix	4	0	0.5	0.001	0.004
	MF	GO:0016841	ammonia-lyase activity	6	0	0.83	0.001	0.004
		GO:0030060	L-malate dehydrogenase activity	6	0	0.83	0.001	0.004
		GO:0004427	inorganic diphosphatase activity	10	0	0.8	0.001	0.004
		GO:0004664	prephenate dehydratase activity	4	0	0.75	0.001	0.004
		GO:0008235	metalloexopeptidase activity	4	0	0.75	0.001	0.004
Down	BP	GO:0009966	regulation of signal transduction	5	1	0	0.001	0.004
		GO:0019318	hexose metabolic process	6	0.83	0	0.001	0.004
		GO:0000398	mRNA splicing, via spliceosome	5	0.6	0	0.002	0.01
		GO:0006813	potassium ion transport	12	0.5	0	0.001	0.004
		GO:0015937	coenzyme A biosynthetic process	4	0.5	0	0.003	0.014
	CC	GO:0009538	photosystem I reaction center	6	1	0	0.004	0.017
		GO:0005669	transcription factor TFIID complex	4	0.75	0	0.008	0.03
		GO:0000775	chromosome, centromeric region	5	0.4	0	0.002	0.01
		GO:0030131	clathrin adaptor complex	10	0.5	0.1	0.004	0.017
		GO:0005875	microtubule associated complex	7	0.57	0.29	0.012	0.042
	MF	GO:0003857	3-hydroxyacyl-CoA dehydrogenase activity	4	0.75	0	0.001	0.004
		GO:0005249	voltage-gated potassium channel activity	8	0.75	0	0.001	0.004
		GO:0003712	transcription cofactor activity	9	0.67	0	0.001	0.004
		GO:0016811	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	6	0.67	0	0.002	0.01
		GO:0033926	glycopeptide alpha-N-acetylgalactosaminidase activity	6	0.83	0.17	0.001	0.004

¹⁵ The five most over-represented and under-represented terms for BP, CC, and MF are listed. ($P < 0.05$, $FDR < 0.05$). “N” is the number of genes within the term expressed in the experiment; “Down” and “Up” represent the proportions of N that are down-regulated or up-regulated respectively.

Table 3.5. The results of GO term enrichment of RNA Seq data from polysomal pools of first leaves of TSWV-inoculated pepper plants compared with those of mock-inoculated plants. ¹⁶

Direction	Domain	GO ID	Term	N	Down	UP	P	FDR
Up	BP	GO:0006559	L-phenylalanine catabolic process	4	0	1	0.001	0.005
		GO:0006796	phosphate-containing compound metabolic process	6	0	0.83	0.001	0.005
		GO:0006546	glycine catabolic process	5	0	0.8	0.006	0.028
		GO:0006414	translational elongation	18	0	0.78	0.001	0.005
		GO:0000162	tryptophan biosynthetic process	4	0	0.75	0.001	0.005
	CC	GO:0042555	MCM complex	6	0	1	0.002	0.011
		GO:0005730	nucleolus	5	0	0.8	0.008	0.035
		GO:0005741	mitochondrial outer membrane	11	0.09	0.82	0.001	0.005
		GO:0005852	eukaryotic translation initiation factor 3 complex	11	0	0.73	0.001	0.005
		GO:0032040	small-subunit processome	7	0	0.57	0.001	0.005
	MF	GO:0016841	ammonia-lyase activity	6	0	0.83	0.001	0.005
		GO:0000774	adenyl-nucleotide exchange factor activity	4	0	0.75	0.001	0.005
		GO:0004664	prephenate dehydratase activity	4	0	0.75	0.001	0.005
		GO:0030060	L-malate dehydrogenase activity	6	0.17	0.83	0.001	0.005
		GO:0003746	translation elongation factor activity	13	0.08	0.69	0.002	0.011
Down	BP	GO:0019318	hexose metabolic process	6	1	0	0.001	0.005
		GO:0006885	regulation of pH	4	0.75	0	0.002	0.011
		GO:0006816	calcium ion transport	5	0.6	0	0.008	0.035
		GO:0009966	regulation of signal transduction	5	0.6	0	0.001	0.005
		GO:0009873	ethylene-activated signaling pathway	4	0.5	0	0.005	0.024
	CC	GO:0005777	peroxisome	4	0.75	0	0.001	0.005
		GO:0030131	clathrin adaptor complex	10	0.6	0	0.001	0.005
		GO:0005829	cytosol	4	0.5	0	0.001	0.005
		GO:0048046	apoplast	20	0.5	0.15	0.013	0.048
		GO:0005856	cytoskeleton	7	0.29	0	0.006	0.028
	MF	GO:0005249	voltage-gated potassium channel activity	8	0.88	0	0.001	0.005
		GO:0016811	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	6	0.83	0	0.001	0.005
		GO:0003857	3-hydroxyacyl-CoA dehydrogenase activity	4	0.75	0	0.001	0.005
		GO:0015385	sodium:proton antiporter activity	4	0.75	0	0.001	0.005
		GO:0033926	glycopeptide alpha-N-acetylgalactosaminidase activity	6	0.83	0.17	0.001	0.005

¹⁶ The five most over-represented and under-represented terms for BP, CC, and MF are listed. ($P < 0.05$, $FDR < 0.05$). “N” is the number of genes within the term expressed in the experiment; “Down” and “Up” represent the proportions of “N” that are down-regulated or up-regulated respectively.

Table 3.6. The results of GO term enrichment of RNA Seq data from sub-polysomal pools of first leaves of TSWV-inoculated pepper plants compared with those of mock-inoculated plants. ¹⁷

Direction	Domain	GO ID	Term	N	Down	Up	P	FDR
Up	BP	GO:0006559	L-phenylalanine catabolic process	4	0	1	0.001	0.004
		GO:0009094	L-phenylalanine biosynthetic process	4	0	1	0.001	0.004
		GO:0008272	sulfate transport	9	0	0.78	0.001	0.004
		GO:0006207	'de novo' pyrimidine nucleobase biosynthetic process	4	0	0.75	0.002	0.011
		GO:0006270	DNA replication initiation	9	0.11	0.78	0.001	0.004
	CC	GO:0042555	MCM complex	6	0	1	0.001	0.004
		GO:0016602	CCAAT-binding factor complex	4	0	0.75	0.001	0.004
		GO:0032040	small-subunit processome	7	0	0.43	0.001	0.004
		GO:0005694	chromosome	6	0.17	0.5	0.002	0.011
		GO:0000015	phosphopyruvate hydratase complex	6	0	0.17	0.001	0.004
	MF	GO:0004664	prephenate dehydratase activity	4	0	1	0.001	0.004
		GO:0016841	ammonia-lyase activity	6	0	0.83	0.001	0.004
		GO:0016671	oxidoreductase activity, acting on a sulfur group of donors, disulfide as acceptor	5	0	0.8	0.001	0.004
		GO:0016891	endoribonuclease activity, producing 5'-phosphomonoesters	5	0	0.8	0.003	0.015
		GO:0015116	sulfate transmembrane transporter activity	9	0	0.78	0.001	0.004
Down	BP	GO:0019318	hexose metabolic process	6	1	0	0.001	0.004
		GO:0006885	regulation of pH	4	0.75	0	0.001	0.004
		GO:0008612	peptidyl-lysine modification to peptidyl-hypusine	4	0.75	0	0.003	0.015
		GO:0033014	tetrapyrrole biosynthetic process	6	0.67	0	0.001	0.004
		GO:0006014	D-ribose metabolic process	7	0.57	0	0.003	0.015
	CC	GO:0009538	photosystem I reaction center	6	1	0	0.001	0.004
		GO:0005956	protein kinase CK2 complex	5	0.8	0	0.003	0.015
		GO:0000775	chromosome, centromeric region	5	0.6	0	0.001	0.004
		GO:0005743	mitochondrial inner membrane	13	0.46	0.08	0.002	0.011
		GO:0005681	spliceosomal complex	6	0.33	0	0.009	0.04
	MF	GO:0016811	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides	6	0.83	0	0.001	0.004
		GO:0019887	protein kinase regulator activity	5	0.8	0	0.002	0.011
		GO:0043022	ribosome binding	5	0.8	0	0.003	0.015
		GO:0003849	3-deoxy-7-phosphoheptulonate synthase activity	4	0.75	0	0.001	0.004
		GO:0003857	3-hydroxyacyl-CoA dehydrogenase activity	4	0.75	0	0.001	0.004

¹⁷ The five most over-represented and under-represented terms for BP, CC, and MF are listed. ($P < 0.05$, $FDR < 0.05$). “N” is the number of genes within the term expressed in the experiment; “Down” and “Up” represent the proportions of “N” that are down-regulated or up-regulated respectively.

3.3.3 KEGG pathway enrichment

In order to further investigate the early response of pepper to virulent TSWV, metabolic pathway analysis was performed on the sets of DE genes using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Kanehisa *et al.*, 2017). This provides information about pathways and individual members of pathways that are differentially represented in the dataset, which may show which of these are being targeted during the compatible interaction with TSWV and how this contributes to susceptibility. The top five most enriched KEGG pathways among DE genes can be seen in table 3.7. Full lists are shown in the “Supplementary files” folder in the folder “KEGG GOstats.”

KEGG pathway maps for a selection of enriched pathways can be seen in figures 3.11-3.16.

Among the KEGG pathways showing significant enrichment in the results of this study was the term “Ribosome” (map3010). A number of RP genes show no significant differential expression in total RNA, up-regulation in polysomal pools, and down-regulation in sub-polysomal pools. These are listed table 3.8.

Table 3.7. The results of KEGG pathway enrichment on RNA extracted by sucrose density centrifugation of first leaves of TSWV-inoculated pepper plants compared with those of mock-inoculated plants.¹⁸

Pool	KEGGID	P	OR	EC	Count	Size	Term
T	4075	4.72E-08	6.538781	14.14856	31	40	Plant hormone signal transduction
	1110	2.05E-06	1.755272	122.7388	161	347	Biosynthesis of secondary metabolites
	3010	1.40E-05	2.231778	43.50682	66	123	Ribosome
	940	2.41E-05	13.03061	5.659424	14	16	Phenylpropanoid biosynthesis
	500	0.000106	4.364261	10.61142	21	30	Starch and sucrose metabolism
P	3010	9.03E-12	3.631848	43.07175	79	123	Ribosome
	4075	3.60E-08	6.644344	14.00707	31	40	Plant hormone signal transduction
	940	1.43E-06	28.4292	5.60283	15	16	Phenylpropanoid biosynthesis
	4016	4.74E-05	3.683398	14.35725	27	41	MAPK signalling pathway – plant
	941	0.00022	Inf	2.801415	8	8	Flavonoid biosynthesis
S	4016	2.33E-06	4.622378	13.34209	28	41	MAPK signalling pathway – plant
	940	8.00E-06	14.81111	5.20667	14	16	Phenylpropanoid biosynthesis
	1110	4.31E-05	1.629844	112.9197	145	347	Biosynthesis of secondary metabolites
	4075	8.23E-05	3.55412	13.01668	25	40	Plant hormone signal transduction
	196	9.88E-05	21.04101	3.579586	10	11	Photosynthesis - antenna proteins

¹⁸ The five KEGG terms most significantly enriched among DE genes in each pool-type are listed here. “OR” is “odds ratio,” “EC” is “expected counts,” “T” is “total RNA,” “P” is “polysomal RNA,” and “S” is “sub-polysomal RNA”. P<0.05.

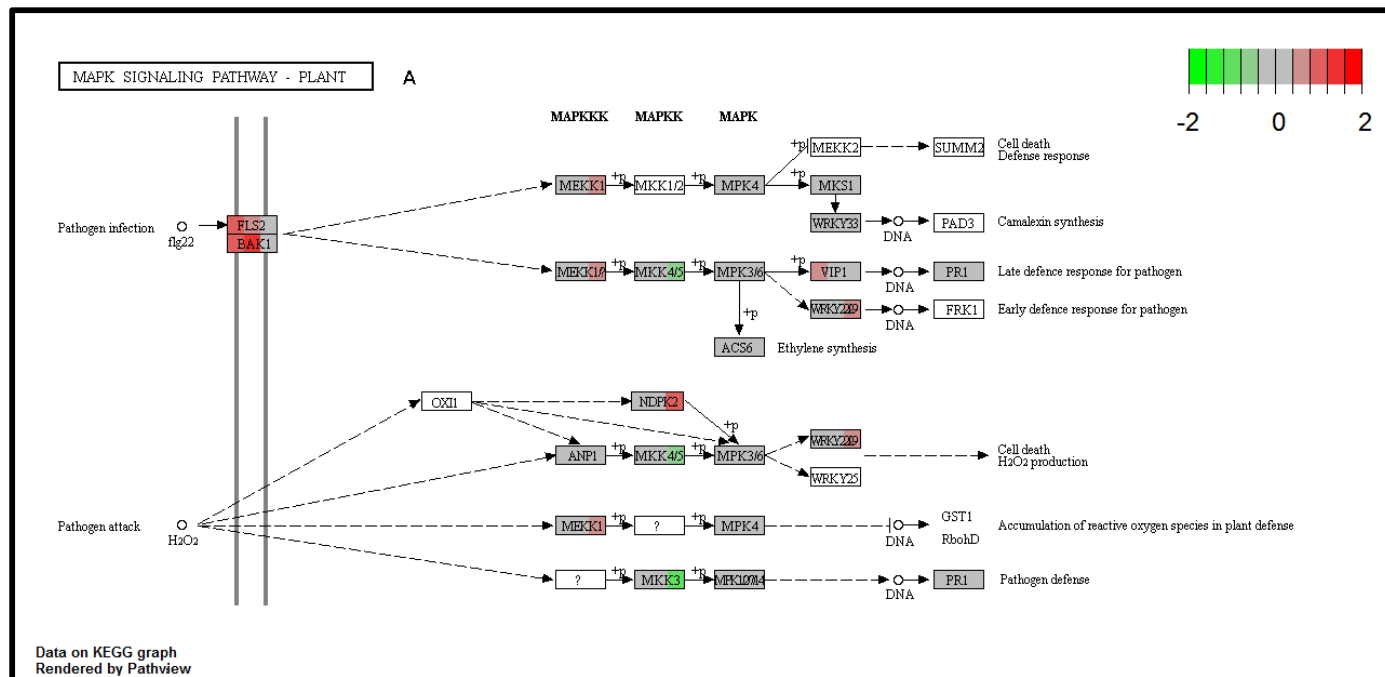


Figure 3.11. The first half of the KEGG pathway map for "MAPK signalling pathway – plant" (map04016) with enzymes coloured by the fold-change of their orthologue in pepper 6hr after inoculation with TSWV1.¹⁹

¹⁹ Cells are coloured by their fold change: if LogFC>0, the cell is red; if LogFC<0, the cell is green; if LogFC=0, the cell is grey. The cell for each enzyme is coloured in three sections: the left section represents the fold change in total RNA, the central section represents the fold change in polysomal RNA, and the right section represents the fold change in sub-polysomal RNA. Where multiple enzymes carry out the same function, the mean fold-change of their respective KEGG orthologue IDs is used to colour the cell.

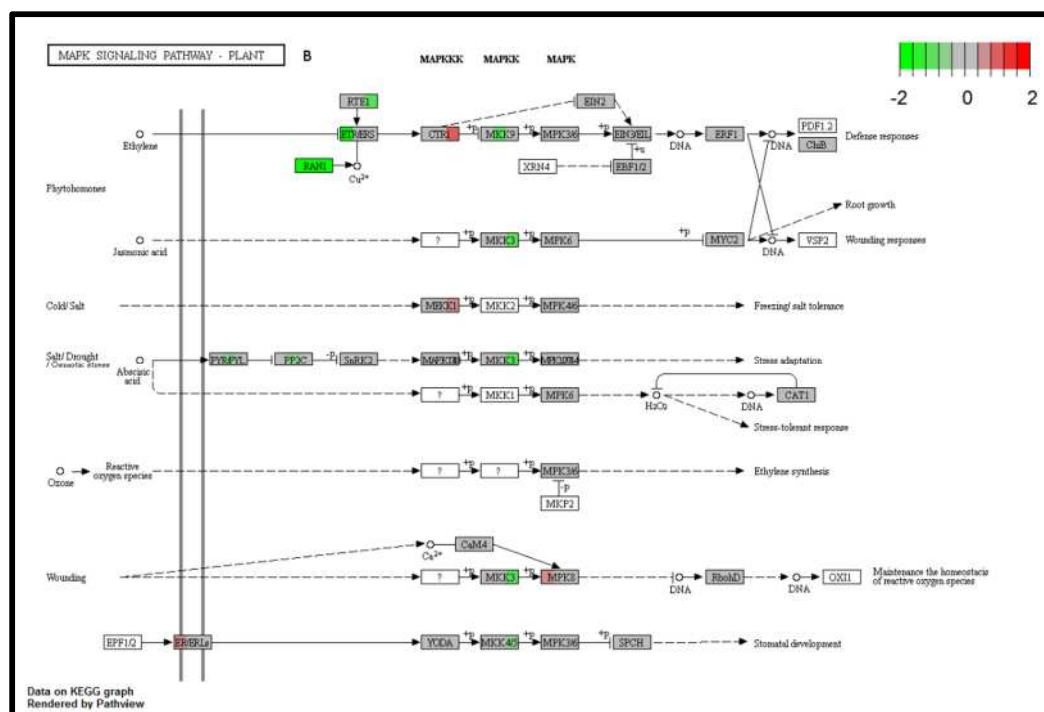


Figure 3.12. The second half of the KEGG pathway map for “MAPK signalling pathway – plant” (map04016) with enzymes coloured by the fold-change of their orthologue in pepper 6hr after inoculation with TSWV1.²⁰

²⁰ Cells are coloured by their fold change: if LogFC>0, the cell is red; if LogFC<0, the cell is green; if LogFC=0, the cell is grey. The cell for each enzyme is coloured in three sections: the left section represents the fold change in total RNA, the central section represents the fold change in polysomal RNA, and the right section represents the fold change in sub-polysomal RNA. Where multiple enzymes carry out the same function, the mean fold-change of their respective KEGG orthologue IDs is used to colour the cell.

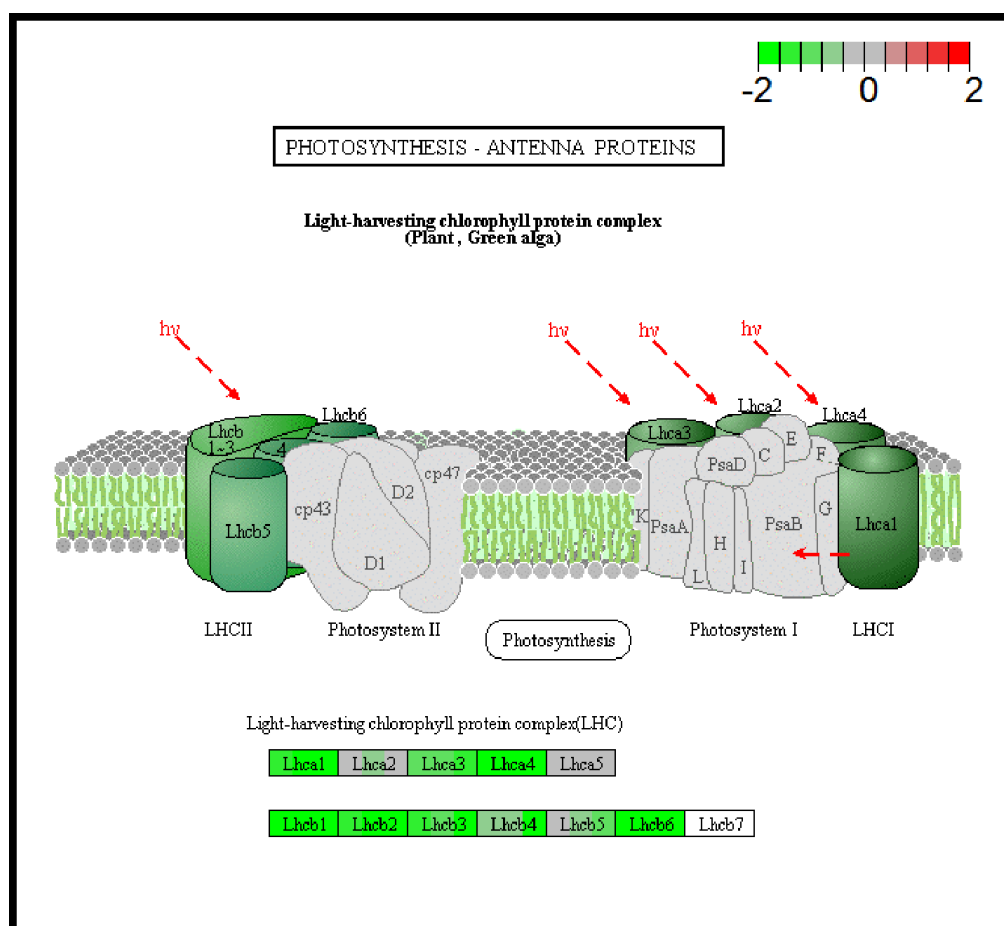


Figure 3.13. The KEGG pathway map for "Photosynthesis – antenna proteins" (map00196) with antenna subunits coloured by the fold-change of their orthologue in pepper 6hr after inoculation with TSWV1.²¹

²¹ Cells are coloured by their fold change: if LogFC>0, the cell is red; if LogFC<0, the cell is green; if LogFC=0, the cell is grey. The cell for each subunit is coloured in three sections: the left section represents the fold change in total RNA, the central section represents the fold change in polysomal RNA, and the right section represents the fold change in sub-polysomal RNA. Where multiple enzymes carry out the same function, the mean fold-change of their respective KEGG orthologue IDs is used to colour the cell.

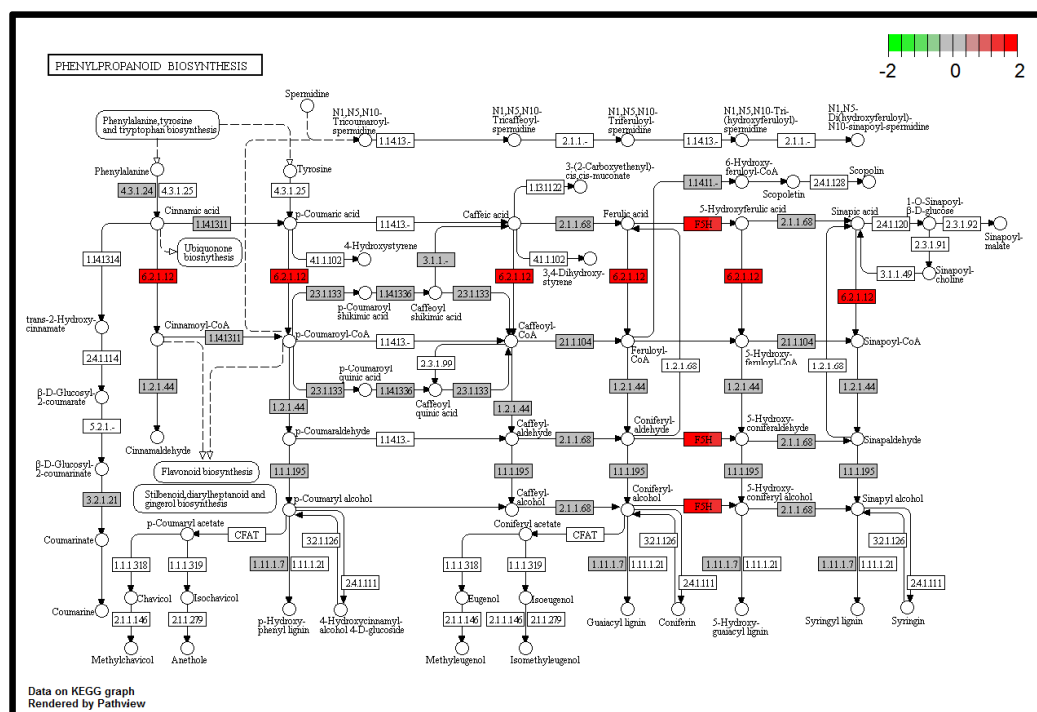


Figure 3.14. The KEGG pathway map for “Phenylpropanoid biosynthesis” (map00940) and with enzymes coloured by the fold-change of their orthologue in pepper 6hr after inoculation with TSWV1²²

²² Cells are coloured by their fold change: if LogFC>0, the cell is red; if LogFC<0, the cell is green; if LogFC=0, the cell is grey. Each cell is coloured in three sections: the left section represents the fold change in total RNA, the central section represents the fold change in polysomal RNA, and the right section represents the fold change in sub-polysomal RNA. Where multiple enzymes carry out the same function, the mean fold-change of their respective KEGG orthologue IDs is used to colour the cell

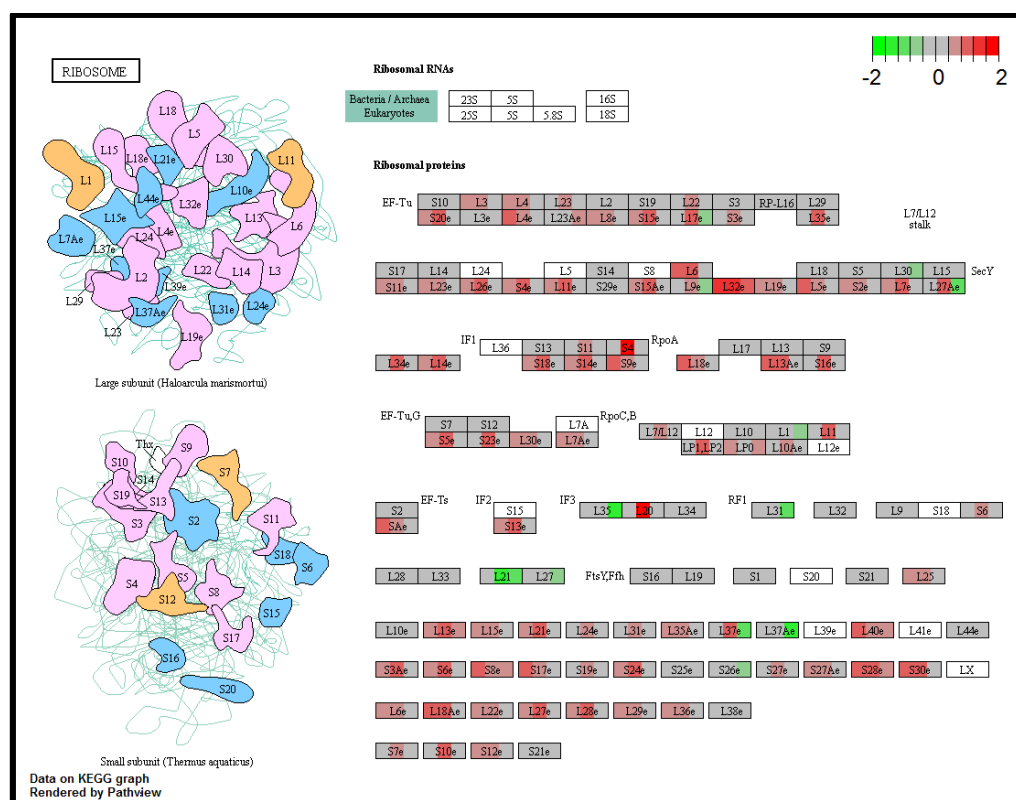


Figure 3.16. The KEGG pathway map for “Ribosome” (map03010) with ribosomal protein genes coloured by the fold-change of their orthologue in pepper 6hr after inoculation with TSWV1.²⁴

²⁴ Cells are coloured by their fold change: if $\text{LogFC} > 0$, the cell is red; if $\text{LogFC} < 0$, the cell is green; if $\text{LogFC} = 0$, the cell is grey. The cell for each ribosomal protein gene is coloured in three sections: the left section represents the fold change in total RNA, the central section represents the fold change in polysomal RNA, and the right section represents the fold change in sub-polysomal RNA. Where multiple ribosomal protein gene homologues are present in the pepper genome the mean fold-change of their respective KEGG orthologue IDs is used to colour the cell

Table 3.8. A subset of ribosomal protein genes, all of which are members of the KEGG pathway “Ribosome” (map03010) that, upon inoculation of pepper plants with TSWV1, show significant up-regulation (LogFC>0; P<0.05; FDR<0.05) only in polysomal pools and down-regulation (LogFC<0; P<0.05; FDR<0.05) only in sub-polysomal pools, indicating selective translation of these genes.²⁵

KEGG ID	Accession number	T FC	P FC	S FC	Gene product
K02880	CA03g24510	#N/A	0.438833	-0.56963	RPL17e
K02888	CA06g14020	#N/A	0.556811	-0.82723	RPL21c
K02900	CA06g19960	#N/A	0.865027	-0.81004	RPL27Ae
K02940	CA06g21890	#N/A	0.539299	-0.68114	RPL9e
K02932	CA06g26200	#N/A	0.445602	-0.62796	RPL5e
K02868	CA09g13560	#N/A	0.609758	-0.74614	RPL11e

²⁵ “#N/A” indicates no significant change in expression.

3.4 DISCUSSION

3.4.1 Global overview of translome change

The results of RNA sequencing of total RNA, polysomal RNA pools, and sub-polysomal RNA pools from TSWV-infected susceptible pepper plants show differential expression of genes between treatments and differential representation of these genes between pool-types. This demonstrates both a transcriptomic and a translomic change during this compatible reaction.

Figure 3.9 shows genes that are commonly up- or down-regulated in both polysomal and sub-polysomal pools but do not change in total RNA pools may indicate some bias in the extraction procedures or statistical testing. Lists of DE genes and how they segregate by pool-type can be seen in the supplementary file “lvM.sig DE Venn contrasts.xlsx”.

Among the 691 genes significantly over-represented solely in sub-polysomal pools, CA10g21190/*CaNBARC309*, which is syntenic and homologous to *CcNBARC309* the *C. chinense* paralogue of *Tsw*, is present but is not DE in either direction in any other pool-types, indicating a possible lack of increased translation despite some increase in transcription. *CaNBARC309* transcripts are found associated to polysomes at steady levels in both treatments. The identity of *CaNBARC309* was inferred by strong alignment of translated nucleotide sequence to *C. chinense* Tsw protein sequence, UniProt accession A0A1C9TCM9, and by the position of *CaNBARC309* relative to *Pvr4*/CA10g21170 according to results of Kim *et al.* (2016). *CaNBARC309* encodes an intact coiled-coil NBS-LRR, whereas other members of this cluster are pseudogenes (Kim *et al.*, 2016). Repression of NBS-LRR, such as the

product of *Tsw*, expression at multiple levels is thought to repress aberrant plant immune response activation; thus, stopping the TSWV-responsive translational repression of *CaNBARC309* may provide an effective means of TSWV-resistance (Staiger *et al.*, 2013; Gloggnitzer *et al.*, 2014).

It is not surprising that *CaNBARC309* is not translated during the response to TSWV-inoculation as it is not a known TSWV resistance gene. It may, however, be an interesting candidate for TSWV-resistance as it shows some TSWV-responsiveness and is paralogous to *Tsw*. Overexpression analysis may demonstrate whether *CaNBARC309* is able to induce a pathogen response on its own or in the presence of TSWV RNPs.

The *CaNBARC309* UTRs may also be targeted to enhance transcript stability and translation. Mutation of miRNA responsive elements (MRE) in the 5' or 3' UTRs of plant genes can enhance translation of a transcript and precociously induce processes controlled by that gene's product. The *Arabidopsis SPL3* transcript, whose product is involved in floral transition, 3' UTR contains an MRE; removal of this MRE causes a dramatic increase in *SPL3* translation and premature flowering (Gandikota *et al.*, 2007). Similar results have been observed for transcripts containing 5' UTR MREs. The transcript encoding ubiquitin-conjugating E2 enzyme contained an MRE in its 5' UTR, which is targeted by the phosphate stress-responsive miR399; miR399-overexpression suppresses E2 enzyme production and the transgenic plants accumulate five to six times the normal level of phosphate, showing a similar phenotype to E2 loss-of-function mutants (Chiou *et al.*, 2006). *CaNBARC309*

may contain an MRE targeted by a miRNA that suppresses the pathogen response.

Also of interest among the genes that are overrepresented in sub-polysomal pools in response to TSWV at this time point but not DE in any other pools, is *LOX3.1* (Ca03g35180; a 13-lipoxygenase, which produces a JA precursor). In potato *LOX3.1* shows constitutive, low-level expression as well as an early peak in expression after wounding; thus *LOX3.1* appears to contribute to normal physiological processes regulated by JA, as well as acting early in JA-regulated stress pathways (Royo *et al.*, 1996). The observed *LOX3.1* transcript sequestration may be a host-directed down-regulation of the JA pathway at the level of translation as part of canonical SA-JA antagonism in order to induce SA production and subsequent downstream HR or SAR (Preston *et al.*, 1999). However, it is worth noting that no differential expression is seen in any *9-LOX* genes, whose products are involved in HR-based viral resistance partly by the diversion of substrates away from 13-LOX enzymes and by the production of polyunsaturated fatty acid (PUFA) hydroperoxide (Royo *et al.*, 1996; Juhász *et al.*, 2015). Also Royo *et al.* (1996) demonstrated that, 6 hr after wounding, *LOX3.1* expression was already receding and *LOX2*, which encodes a 13-LOX enzyme linked to sustained induction of the JA stress pathway at later time points, had not yet shown a strong up-regulation. This would be in accordance with the results from this study, whereby it appears that the JA stress signalling pathway is being induced due to an inability to sense TSWV and induce the SA pathogen response,

although without a full time course of LOX expression or activity this cannot be verified.

Among the genes significantly up-regulated uniquely in polysomal pools upon TSWV inoculation is *eEF1A* (AY496125), which was initially included in the reference genome as a potential control gene. As mentioned earlier in the chapter, *eEF1A* is an important host factor in tospoviral mRNA transcription and genomic replication; this function is not paired with protein synthesis (Komoda *et al.*, 2014). These results indicate an increase in *eEF1A* production; this may be either a host-driven translational increase to replace depleted *eEF1A* or a virus-driven increase for use of this factor in constructing viroplasm. A gene encoding *eEF1B2* (CA11g14580) is also over-represented in polysomal pools and under-represented in sub-polysomal pools of TSWV-inoculated plants, indicating a strong up-regulation of *eEF1B* translation. During canonical translation, *eEF1B* acts as a GTP exchange factor, which allows *eEF1A* to transfer aminoacylated tRNAs to ribosomes during elongation of the nascent polypeptide (Hwang *et al.*, 2015). There is no putative role of *eEF1B* in TSWV replication but the *eEF1By* subunit has been shown to act synergistically with *eEF1A* to support Tomato bushy stunt virus (TBSV; (+)ssRNA) RdRp activity in a yeast model (Sasvari *et al.*, 2011); *eEF1B* may perform a similar function here and thus show similar regulatory patterns *eEF1A* or may merely just be commonly regulated with *eEF1A*. Sasvari *et al.* (2011) also showed that symptom development is attenuated in knockdowns of *eEF1By*. Similar roles have been observed for *eEF1A* and *eEF1B* as host factors that interact with viral RdRps, with some evidence that they support

infection and RdRp activity during interactions with potexviruses, potyviruses, tobamoviruses, and tymoviruses, all of which possess (+)ssRNA genomes (Yamaji *et al.*, 2006; Thivierge *et al.*, 2008; Hwang *et al.*, 2013; Hwang *et al.*, 2015). Owing to their putative roles in multiple RNA virus-plant interactions and the translational up-regulation observed in this study, eEF1A and B may be key susceptibility factors during the compatible reaction between TSWV and pepper.

Of the genes that are up-regulated in polysomal pools but show no significant differential expression in total and sub-polysomal samples, the most extreme is CA01g20700, which encodes a putative polygalacturonase (PG); PG is an enzyme that breaks down pectin, an important constituent of the plant cell wall that is involved in cell-to-cell adhesion (Wang *et al.*, 2018). Many fungal and bacterial pathogens secrete PGs in order to promote colonisation and spread within the host plant and PG-inhibiting proteins (PGIP) have been shown to enhance basal resistance and defence marker expression in response to virulent fungal pathogens infection of cotton (Liu *et al.*, 2017). The translational increase in host-derived PG expression seen herein may promote viral spread and host susceptibility by cell wall remodelling.

Commonly used markers for SA-dependent SAR are the pathogenesis-related genes *PR-1*, *PR-2*, and *PR-5* (Molinari *et al.*, 2014). Of these, *PR1* (CA01g31060) and *PR2* (CA01g15970) were down-regulated in all pool-types in response to TSWV infection; the pepper *PR5* gene is not assigned to a chromosome in the CM334 genome (v. 1.55) so was not seen in sequencing data.

3.4.2 Gene ontology enrichment

GO term enrichment using mroast shows significant differential enrichment between pool-types upon TSWV-inoculation of pepper plants. This demonstrates regulation of functional categories of genes at both transcription and translation.

Glycerol-3-phosphate metabolism

Among the GO terms that are under-represented in all pool-types upon TSWV inoculation are multiple terms relating to glycerol-3-phosphate (G3P) metabolism and NAD⁺-dependent G3P catabolism (GO:0004367, GO:0006072, GO:0009331, and GO:0046168). One member of all these terms (*GLY1/SFD1*, CA06g07170), which is down-regulated and encodes a chloroplastic G3P-dehydrogenase (G3Pdh), is involved in reduction of dihydroxyacetone phosphate (DHAP) resulting in G3P accumulation (Chanda *et al.*, 2008; Mandal *et al.*, 2011). G3P is an important signalling molecule in the SA-/NPR1-independent branch of SAR; impaired G3P synthesis in *Arabidopsis gly1* mutants compromises SAR against the hemi-biotrophic pathogens *Pseudomonas syringae* pv. *maculicola* and *Colletotrichum higginsianum* and *GLY1* over-expression enhances basal resistance against *C. higginsianum* (Nandi *et al.*, 2004; Chanda *et al.*, 2008). G3P can also be produced via glycerol phosphorylation by cytosolic glycerol kinase *GLI1/NHO1* and, similarly to *GLY1*, *gli1* mutants show increased susceptibility to *C. higginsianum* (Chanda *et al.*, 2008). The pepper *GLI1* gene (CA03g32600) is annotated in GO:0006072 and is significantly down-regulated in all pool-types. The gene *DEFECTIVE IN INDUCED RESISTANCE 1* (*DIR1*) encodes a lipid transfer protein that

chaperones G3P or a G3P-derived signal symplastically to tissues distal to the point of infection during SAR; *DIR1* is not annotated in the GO terms mentioned above (Champigny *et al.*, 2013; Carella *et al.*, 2015). In *Arabidopsis* G3P and DIR1 positively regulate each other as part of a feedback loop, whereby G3P induces DIR1 accumulation and DIR1, as part of a complex with AZELAIC ACID INDUCED 1 (AZI1), is required for pathogen-induced G3P production (Yu *et al.*, 2013).

Arabidopsis also possesses a *DIR1* paralogue putatively from a duplication event after the divergence of *Arabidopsis* and *Brassica* called *DIR1-like*; thus it is unlikely that the pepper genome contains a true *DIR1-like* orthologue (Champigny *et al.*, 2013). DIR1-like shows less capacity for systemic movement than DIR1 and possibly provides a smaller contribution to SAR with some redundancy to *DIR1*, as *dir1* mutants occasionally regain some SAR competency (Champigny *et al.*, 2013). DIR1-like function also seems to be important in seed development as reducing *DIR1-like* expression by RNAi stops the production of viable seeds (Champigny *et al.*, 2013).

The pepper genome possesses four genes showing homology to *DIR1* (CA01g26160, CA03g35530, CA07g20040, and CA08g18360) using Sol Genomics and NCBI BLAST platforms (Altschul *et al.*, 1990; Fernandez-Pozo *et al.*, 2014). The gene showing strongest homology to *Arabidopsis DIR1* CA03g35530 was down-regulated in polysomal and sub-polysomal pools, indicating reduced translation upon infection. CA01g26160, which shows similarly strong homology, was up-regulated in polysomal and total pools, indicating increased translation. CA07g20040 and CA08g18360 show much

weaker homology to *AtDIR1* than CA03g35530 and CA01g26160 and did not meet the CPM cut-off in any of the samples sequenced.

One member gene of the GO terms mentioned above (CA01g21440) encodes a cytosolic G3Pdh isoform (GPDHC1) and is up-regulated in all pool-types. GPDHC1 is a cytosolic component of the mitochondrial G3P shuttle, a mechanism by which NADH is oxidised to regenerate NAD⁺ while DHAP is reduced to generate G3P; G3P is then shuttled to the mitochondria to supply reducing power for respiration, at which point it is converted back to DHAP by the mitochondrial FAD-GPDH (Shen *et al.*, 2006). GPDHC1 is involved in reducing cellular ROS levels; *gpdhc1 Arabidopsis* mutants exhibit hypersensitivity to abiotic stresses owing to poor redox homeostasis and increased cellular ROS (Shen *et al.*, 2006).

A lack of *GLY1* and *GLI1* expression upon TSWV-inoculation may contribute to the inability of peppers to induce SAR to this pathogen; the action of GPDHC1 may also stop the threshold ROS level being reached for the plant to perceive the attack pathogen and mount a response (Shen *et al.*, 2006; Yu *et al.*, 2013). The different expression patterns of the pepper *DIR1* homologues may indicate functional diversification of these genes. Yu *et al.* (2013) suggest that in *Arabidopsis* G3P-induced DIR1 accumulation at 24 hr after exogenous G3P application involves post-transcriptional regulation as no difference is seen in *DIR1* transcription. The apparent translational down-regulation of *GLY1*, *GLI1*, and the *DIR1* homologue CA03g35530 in pepper at 6hr may show post-transcriptional down-regulation of this branch of the plant SAR pathway during the compatible interaction with TSWV. This cannot be

assumed, however, as the other *DIR1* homologue CA01g26160 shows the opposite pattern regulation pattern and the functions of all of the products of these genes are inferred by homology.

Phenylalanine metabolism and ammonia lyase activity

The GO terms “L-phenylalanine catabolic process,” “L-phenylalanine biosynthetic process,” and “ammonia-lyase activity” (GO:0006559, GO:0009094, and GO:0016841 respectively) are over-represented in all pool-types in response to TSWV inoculation. Together GO:0006559 and GO:0016841 contain five loci annotated as phenylalanine ammonia lyase (PAL) enzymes (CA05g20790, CA09g02410, CA09g02420, CA09g02430, and CA10g12380) and GO:0009094 contains two genes annotated as arogenate dehydratase 3 (ADT3; CA06g23200 and CA11g17670), all of which are up-regulated in all pool-types. An arogenate/prephenate dehydratase gene (CA02g18350) was up-regulated only in sub-polysomal pools. An arogenate dehydratase 2 gene (CA11g14180) in GO:0009094 and a putative histidyl-tRNA synthetase (CA08g13690) in GO:0016841 were expressed in the leaves but not significantly DE; two *PAL* genes (CA00g95510 and CA12g15510) and a homogentisate 1,2-dioxygenase (CA00g19890) were either not expressed above the CPM threshold or were not included in analyses as they were not assigned to a chromosome in the reference genome.

Phenylalanine (Phe) is the precursor to many phenolic secondary metabolites that play active roles in biotic and abiotic stress responses in plants (Dal Cin *et al.*, 2011). ADT3 is an enzyme that catalyses the final stage in Phe production and is required for ROS homeostasis via the production of Phe-

derived antioxidants (Para *et al.*, 2016). PAL is the first enzyme in production of Phe-derived secondary metabolites, such as phenylpropanoids (Tohge *et al.*, 2013). In peppers, PAL activity is required to accumulate SA and ROS, to induce *PR1* expression, and to mount an immune response; silencing of *PAL1* (CA09g02410) compromises the response to virulent and avirulent microbial pathogens whereas *PAL1* over-expression increases resistance (Kim and Hwang, 2014). In tobacco, suppression of *PAL* expression leads to an inability to develop SAR in response to TMV due to reduced SA/phenylpropanoid production and a lack of distal PR protein production; *PAL*-suppressed plants do not show reduced susceptibility to TMV upon secondary infection (Pallas *et al.*, 1996).

In this study, multiple *PAL* genes are shown to be over-expressed in susceptible pepper plants in response to TSWV. This *PAL*-induction must therefore be insufficient to induce systemic immunity. The increase in expression seen may not be enough to meet the threshold *PAL* activity to mount the pathogen response or the *PAL* expression seen under these conditions may have no correlation with *PAL* activity. Additionally, the cause of susceptibility may be due to the down-regulation of an enzyme up- or downstream of *PAL* in the phenylpropanoid or shikimate pathways or diversion of substrates away from the SAR-related branch of the phenylpropanoid pathway (Tohge *et al.*, 2013).

RNA pseudouridylation

The GO terms “pseudouridine synthesis,” “RNA modification,” and “pseudouridine synthase activity” (GO:0001522, GO:0009451, and

GO:0009982 respectively) appear to be up-regulated in all pool-types. The gene sets contained within these terms are very similar; only a relatively small proportion of each set is up regulated in each pool-type and the expression patterns of the genes within the terms varies with pool-type. A gene belonging to all three terms encoding a chloroplastic RNA pseudourine synthase (CA04g22190) is up-regulated in total and polysomal pools, indicating a strong push from transcription to translation. A putative tRNA-pseudouridine synthase gene (CA02g30120), which also belongs to all three terms, is up-regulated only in sub-polysomal fractions, indicating sequestration in response to TSWV. CA06g27540, which encodes a putative ribosomal pseudouridine synthase and is included in all three terms, and CA10g21580, which encodes a putative H/ACA ribonucleoprotein complex subunit 1-like protein 1 similar to the *Arabidopsis* GAR1 protein and is exclusively annotated as part of GO:0009982, are up-regulated only in polysomal pools, indicating an increase in translation of these genes.

Pseudouridylation is a process in which uridine in RNA is post-transcriptionally converted to its isomer pseudouridine; pseudouridine is the most abundant modified nucleoside found in cellular RNAs (Hamma and Ferré-D'Amaré, 2006). Pseudouridine is primarily found in non-coding RNAs, such as tRNA, rRNA, small nuclear RNA (snRNA), or small nucleolar RNA (snoRNA), where it enhances function and stability (Charette and Gray, 2000). More recently, pseudouridylation has been observed in mRNA of yeast and mammals and pseudouridylation sites are dynamically and differentially modified tissue-specifically and during stress; however, their effect on mRNA

function is not clear (Carlile *et al.*, 2014; Lovejoy *et al.*, 2014; Schwartz *et al.*, 2014; Li *et al.*, 2015b). Transcriptome-wide mapping of pseudouridylation sites has not been performed in plants although, as the machinery that performs mRNA pseudouridylation is conserved across eukaryotes, pseudouridine may occur in plant mRNAs (Burgess *et al.*, 2016). Pseudouridylation of the GUA anticodon of tRNA^{Tyr} is required for infection by TMV as this allows non-canonical base-pairing and suppresses translational termination at an in-frame UAG stop codon between ORF1 and ORF2 of the TMV genome; this in turn allows read-through and production of RdRp, whose coding sequence spans both reading frames (Zerfass and Beier, 1992).

Single peptide pseudouridine synthases catalyse this reaction on substrate RNAs with high site-specificity; H/ACA RNPs are complexes that perform pseudouridylation via a guide RNA (Hamma and Ferré-D'Amaré, 2010). The guide RNA is characterised by two hairpin structures separated by a 'box H' sequence motif and followed by an ACA trinucleotide; these hairpins also contain internal bulges complementary to the sequences flanking the pseudouridylation site (Reichow *et al.*, 2007). The protein components of H/ACA RNPs are: CBF5, which catalyses RNA pseudouridylation; NOP10, which stabilises the active site of CBF5; GAR1, which positions the substrate uridine into the active site of CBF5 and promotes product release; and NHP2, which, in concert with CBF5, holds the guide RNA in place for recognition of the substrate RNA (Hamma and Ferré-D'Amaré, 2010; Wang *et al.*, 2015).

The role pseudouridylation plays during TSWV infection of pepper is not clear; it may serve to stabilise viral RNA structures, as in cellular non-coding

RNAs, or modify viral/cellular RNA elements in such a way that enhances non-canonical translation of viral transcripts (Charette and Gray, 2000).

Translation

A number of GO terms associated with translation were differentially enriched between TSWV-inoculated and mock-inoculated plants and between pool-types. “Translation initiation factor activity,” “translation elongation factor activity,” “eukaryotic translation initiation factor 3 complex,” and “translational elongation” (GO:0003743, GO:0003746, GO:0005852, and GO:0006414 respectively) were significantly enriched in total and polysomal pools. Members of “translational termination” (GO:0006415) were enriched among down-regulated genes in sub-polysomal pools. “SRP-dependent cotranslational protein targeting to membrane” (GO:0006614) were down-regulated in polysomal pools. “tRNA aminoacylation for protein translation” (GO:0006418) were up-regulated in total pools. The two very similar and large terms “structural constituent of ribosome” and “ribosome” (GO:0003735 and GO:0005840) and the terms “translation,” “translational initiation,” and “tRNA processing” (GO:0006412, GO:0006413, and GO:0008033) were all specifically enriched in polysomal pools.

Of the genes within GO:0003735 and GO:0005840: in total pools, 111 are up-regulated and 20 are down-regulated; in sub-polysomal pools, 29 are up-regulated and 34 are down-regulated; and in polysomal pools, 147 are up-regulated and 10 are down-regulated. This is indicative that there is a strong increase in translation of proteins related to ribosomal structure.

Ribosomal proteins (RP) interact with viruses during infection. Hepatitis C virus (HCV) requires ribosomal protein eS25 (RPS25) for IRES-mediated translation and this is also enhanced by the presence of RPL22, which interacts with the HCV 3'UTR (Wood *et al.*, 2001; Fuchs *et al.*, 2015). RPS5 also interacts with the HCV IRES and is required for 40S recruitment (Filbin *et al.*, 2012). Of these, an RPS5-encoding gene (CA10g21500) is up-regulated in total and polysomal pools and down-regulated in sub-polysomal pools. An increase in RPS5 production may facilitate 40S recruitment to TSWV transcripts.

RPL10 is involved in resistance to the begomovirus cabbage leaf curl virus (CaLCuV) in *Arabidopsis* as NIK1-mediated RPL10 nuclear shuttling represses transcription of translation-related genes; in turn this represses viral translation (Carvalho *et al.*, 2008; Zorzatto *et al.*, 2015). *RPL10*-overexpression also down-regulates RP expression (Carvalho *et al.*, 2008). Three *RPL10* genes are up-regulated in total RNA and polysomal pools (CA02g20330, CA06g03060, and CA11g17050), of which CA02g20330 and CA11g17050 are also down-regulated in sub-polysomal pools; this may be an early step in RPL10-associated translational repression or be part of an inadequate response to TSWV infection.

DNA damage response

The GO terms “damaged DNA binding” and “base-excision repair” (GO:0003684 and GO:0006284 respectively) are enriched against the background only in polysomal pools upon TSWV inoculation. Within GO:0003684: no genes are DE in total RNA; two genes are down-regulated in sub-polysomal pools (CA05g09620, encoding a predicted ubiquitin receptor

RAD23c-like protein, and CA02g23340, encoding RAD23) and two genes are up-regulated (CA08g16740, encoding a DNA polymerase IV/Kappa, and CA01g11100, encoding a putative Ku P80 DNA helicase); and in polysomal pools CA01g11100 is up-regulated. Within GO:0006284: in total RNA, CA04g20160, which encodes a detected protein of unknown function containing a domain conserved among DNA-3-methyladenine glycosylase I enzymes, is up-regulated (Marchler-Bauer *et al.*, 2017); in sub-polysomal pools, two genes are down-regulated (CA02g05320 and CA02g22510, which both encode DNA glycosylase superfamily proteins showing homology to DNA-3-methyladenine glycosylase 2) and two genes are up-regulated (CA10g05220, encoding a GMP-synthase/DNA-3-methyladenine glycosylase 1-like protein, and CA02g07210, encoding a uracil-DNA glycosylase); and in polysomal pools, three genes are up-regulated (CA04g20160, CA10g05220, and CA12g05790, encoding a DNA glycosylase with regions associated with base-excision and endonuclease activity during DNA repair and CpG demethylation (Marchler-Bauer *et al.*, 2017). Other DNA damage markers that are not included in the above GO terms but show differential expression are: CA03g06920 is up-regulated in all pools and encodes the double strand break (DSB) associated histone variant H2AX homologue H2AXb; CA04g00220 is down-regulated in total RNA pools and encodes the H2AX homologue H2AXa; CA06g19680, a gene encoding a homologue of the DSB-responsive phosphatidylinositol 3-kinase-related kinase (PIKK) ataxia telangiectasia mutated (ATM), is down-regulated in total and polysomal pools; and two genes encoding homologues of the DSB- and stalled replication fork-responsive PIKK ataxia telangiectasia-

and RAD3-related (ATR; CA08g13620 and CA12g02990) are down-regulated in total and polysomal pools respectively (Maréchal and Zou, 2013). In eukaryotes, ATM and ATR are also involved in promoting programmed cell death (PCD) in response to DNA damage and, in the case of ATM, directly to oxidative stress (Fulcher and Sablowski, 2009; Alexander *et al.*, 2010; Ditch and Paull, 2012).

RAD23 is involved in nucleotide excision repair (NER), whereby a wide range bulky DNA lesions that distort DNA structure are detected and excised (Schärer, 2013). DNA glycosylases are involved in the repair of DNA lesions by base excision repair (BER) whereby specific cytotoxic bases are excised from DNA, particularly in the reversal of oxidative DNA damage (Metz *et al.*, 2007). DNA polymerase IV/Kappa (POLK) repairs DNA lesions by reading through at points where normal high-fidelity DNA polymerases have stalled (Prakash and Prakash, 2002).

Activation of the DNA damage response (DDR) during viral infection is a known phenomenon, even among RNA viruses that reproduce entirely in the cytoplasm; a large part of DDR activation is due to infection-induced oxidative stress although the DDR can also be directly caused by the virus and contribute to viral pathogenesis (Ryan *et al.*, 2016). Human-infecting members of the order *Bunyavirales* induce DDR independently of the presence of DNA damage by the action of their NSs proteins. RVFV causes cell cycle arrest to enhance viral replication by the activation of the ATM pathway (Baer *et al.*, 2012). LACV suppresses the host's interferon-based immune response by inducing DDR-associated degradation of RNA polymerase II (Verbruggen *et al.*, 2011). It is

possible that the observed DDR activation is due to a non-canonical function of a TSWV protein, such as NSs, or this may exclusively be due to DNA damage caused by infection-induced oxidative stress (Barzilai and Yamamoto, 2004). It is also possible that the up-regulated DNA damage-responsive genes are rescuing the host from DNA damage while the lack of ATM-/ATR-signalling inhibits autophagy, in accordance with their canonical functions as PCD inducers (Alexander *et al.*, 2010), and allows the obligatorily biotrophic TSWV to reproduce and spread, thus contributing to disease progression.

3.4.3 KEGG pathway enrichment

Below is a discussion of a selection of KEGG pathways that are significantly enriched among DE genes in this study.

MAPK signalling pathway – plant

Six hours after inoculation with TSWV1, the KEGG term “MAPK signalling pathway – plant” (map04016) is enriched in the “both” (26 genes in total RNA, 27 genes in sub-polysomal RNA, and 27 genes in polysomal RNA) and down-regulated (15 genes in total RNA, 14 genes in sub-polysomal RNA, and 16 genes in polysomal RNA) gene sets for all pool-types and in the up-regulated gene set in the sub-polysomal pool (13 genes). Eleven members of map04016 are up-regulated in total and polysomal pools even though the KEGG pathway term is not enriched in up-regulated gene sets in these pools. Figures 3.11 and 3.12 show the plant MAPK kinase signalling pathway with the average LogFC of each member in each pool-type painted onto the member’s cell.

Mitogen-activated protein kinases (MAPK) are a class of serine/threonine protein kinase ubiquitous to eukaryotes that are involved in

signalling cascades during various fundamental cell processes and responses; these are generally activated by posttranslational phosphorylation by MAPK kinases (Cargnello and Roux, 2011). In plants, MAPK signalling pathway component families have expanded over the course of evolution; these are activated by abiotic and biotic stimuli and their functions are interconnected with those of plant signalling molecules (Rodriguez *et al.*, 2010).

MAPK cascades play a central role in the pathogen response in plants (Cardinale *et al.*, 2002; Del Pozo *et al.*, 2004). During the response to TMV by tobacco carrying the *N* resistance gene, wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK) are activated; in the absence of *N*, WIPK is not activated by inoculation with TMV. Unlike most mammalian MAPKs and SIPK, WIPK requires both transcriptional up-regulation and posttranslational phosphorylation in order to be activated (Zhang and Klessig, 1998). SIPK and WIPK and two of their upstream MAPKKs MEK1 and MKK1 are required for the HR-like cell death response in tobacco systemically co-infected with PVX and PVY; the 9-lipoxygenase LOX5, which is associated with HR-like cell death, is epistatic to SIPK (Aguilar *et al.*, 2017).

The most extremely up-regulated gene in map04016 in all pool-types is CA07g11530 (SolGenomics annotation: PREDICTED: mitogen-activated protein kinase kinase kinase A-like [*Solanum tuberosum*], NCBI BLAST annotation: PREDICTED: mitogen-activated protein kinase kinase kinase ANP1-like) and the most extremely down-regulated member in all pool-types is Ca03g05640 (SolGenomics annotation: S25-XP1 DNA binding protein, NCBI BLAST annotation: PREDICTED: ethylene-responsive transcription factor 1B-like).

ANP1 is one of three *Arabidopsis* orthologues of the tobacco MAPK kinase kinase (MAPKKK) NPK1 (Nishihama *et al.*, 1997). NPK1 and ANP1, 2, and 3 positively regulate cell division (Nishihama *et al.*, 2001; Krysan *et al.*, 2002). ANP/NPK1 MAPKKKs are activated by oxidative stress, at which point they initiate stress signalling cascades and suppress an auxin-responsive promoter, and expression of constitutively active NPK1 enhances resistance to various abiotic stresses (Kovtun *et al.*, 2000). In tobacco, NPK1 functions upstream of some TIR-NBS-LRR resistance genes but not cytosolic NBS-LRR resistance genes (Jin *et al.*, 2002). In *Arabidopsis*, ANP1 functions in a MAPK cascade upstream of AtMPK3 and AtMPK6, orthologues of SIPK and WIPK, which leads to the downstream activation of the WRKY transcription factors 22/29 and 25 causing subsequent H₂O₂ production and cell death (Kovtun *et al.*, 2000). It is not clear that this is true in tobacco as SIPK and WIPK are activated in a separate MAPK signalling cascade, which starts with MAPKKK α for SIPK and involves the MAPKK MEK2 for both SIPK and WIPK (Del Pozo *et al.*, 2004).

Among the genes contained in map04016, three genes annotated as WRKY transcription factors in the reference genome were expressed in RNA samples sequenced in this experiment: CA01g01920, which encodes WRKY22 according to SolGenomics but encodes WRKY29 according to NCBI BLAST, is down-regulated in all pool-types; CA06g13580, which encodes a double WRKY type transcription factor according SolGenomics and a probable WRKY25 homologue according to NCBI BLAST, is not differentially expressed in any pool-type; and CA08g07730, which is annotated as a putative WRKY transcription factor in the SolGenomics database and is annotated as WRKY22

according to NCBI BLAST, is up-regulated in all pool-types.

WRKY22 and *WRKY29* expression is an early marker of defence in *Arabidopsis* upon detection of bacterial flagellin through the activation of the MAPK cascade involving MPK3 and MPK6; activation of this pathway and ectopic expression of *WRKY29* both enhance innate resistance against bacterial and fungal pathogens (Asai *et al.*, 2002). The down-regulation of CA01g01920 upon TSWV1 infection may thus be an important contributory factor to susceptibility. *WRKY22* and *WRKY29* have been suggested to be functionally redundant in *Arabidopsis*; the results described herein indicate that this may not be true in pepper (Asai *et al.*, 2002). Resistance to TSWV1 may require synergistic activity of both *WRKY22* and *WRKY29* or be independent of *WRKY22* activity.

The pepper homologue of SIPK (CA03g14570) is down-regulated in all pool-types and the homologue of WIPK (CA06g06270) is up-regulated only in sub-polysomal pools. MAPKKK α (CA12g08170) acts upstream of SIPK via MEK2 (CA03g36820), which also activates WIPK, and MAPKKK α activity is associated with both HR-like and disease-associated cell death (Del Pozo *et al.*, 2004). Neither CA12g08170 nor CA03g36820 are differentially expressed in this experiment; the failure to induce activity of this pathway during the early stages of infection may be a large part of why the pepper line is unable to mount HR against TSWV1.

Ethylene-responsive transcription factor 1 (ERF1) expression is activated by ethylene, JA, and synergistically by a combination of both; ERF1 is a key element in the response to necrotrophic pathogens, such as *B. cinerea*

and *Plectosphaerella cucumerina* (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003). It is possible that the down-regulation of Ca03g05640 (*ERF1B*) is caused by general antagonism between the SA and JA/ethylene defence pathways although SA-treatment has been shown to induce *ERF1*-expression in wheat and *ERF1*-expression is unchanged by phloem-feeding silverleaf whitefly-induced SA-signalling (Xu *et al.*, 2007; Zarate *et al.*, 2007). In *Arabidopsis*, SA has been shown to suppress ERF1-mediated expression of *PLANT DEFENSIN 1.2* (*PDF1.2*) in plants overexpressing *ERF1* in a JA-insensitive *coi1-1* background (van der Does *et al.*, 2013). It is not clear how these mechanisms are conserved in solanaceous plants and how the down-regulation of *ERF1B* affects susceptibility of this host to TSWV1. In tomato, a related but not orthologous AP2-like ethylene-responsive transcription factor (Soly11g008560.1.1; a predicted target of TSWV vsRNAs) shows drastic down-regulation 17 days after TSWV inoculation of the susceptible cultivar Marglobe and marginal up-regulation after inoculation of the resistant cultivar Red Defender (Ramesh *et al.*, 2017). It is thus possible that ERFs are more closely linked to TSWV resistance in Solanaceae than one would expect from canonical descriptions of biotrophic pathogen responses in plants.

Photosynthesis - antenna proteins

The KEGG term “Photosynthesis - antenna proteins” (map00196) is enriched in down-regulated gene sets and in the “both” gene sets for all pool-types. Nine genes in map00196 show significant down-regulation in total and polysomal pools and ten show significant down-regulation in sub-polysomal pools. Figure 3.13 shows the “Photosynthesis - antenna proteins” pathway

with the average LogFC of each member in each pool-type painted onto the cells.

Antenna proteins are the pigment-binding protein constituents of the light-harvesting complexes (LHC), whose major function is to transfer excitation energy from absorbed light to the reaction centres of photosystems (PS) I and II, and are thus a major component in the photosynthetic machinery (Govindjee, 2002).

Photosynthetic capacity is suppressed during viral infection, owing to drastically reduced sucrose transport out of infected tissues and conversion of sucrose into hexose by cell-wall invertases (Herbers *et al.*, 2000). Ectopic expression of yeast-derived invertases in the vacuole and cell wall induces HR-like necrotic lesions and enhanced resistance to PVY; this effect is not seen for ectopic expression of cytosolic invertases. Vacuolar and apoplastic invertase expression and subsequent necrosis is paired with localised accumulation of hexose sugars, SA, and PR transcripts, and with reduced expression of the antenna protein chlorophyll a/b binding protein (CAB) (Herbers *et al.*, 1996).

Down-regulation of nuclear-encoded, photosynthesis-related genes is a common feature of biotic stress and this repression promotes accumulation of various unrelated viruses; the mechanisms by which plastid-associated repression promotes replication vary between viruses (Abbink *et al.*, 2002; Jiménez *et al.*, 2006; Dardick, 2007). As this repression appears to contribute to disease progression it is unlikely that it is merely coincidental. It has been proposed that viruses circumvent or inhibit the pathogen response by perturbing normal functions of chloroplast, which are the source of multiple

stress signals or of upstream components in the formation of signals, such as SA and JA (Dardick, 2007). Transcriptional repression of photosynthesis-related genes has also been observed systemically in TSWV-infected tomato plants (Catoni *et al.*, 2009); thus it is reasonable to conclude that repression of photosynthesis antenna proteins may be a major contributory factor to TSWV-susceptibility in solanaceous hosts.

Phenylpropanoid biosynthesis

Complementing the results of GO enrichment, which showed that genes involved in Phe metabolism and ammonia lyase activity are enriched among up-regulated genes, the KEGG term “Phenylpropanoid biosynthesis” (map00940) is enriched among differentially expressed genes in all pool-types. Figure 3.14 shows the phenylpropanoid biosynthesis pathway with the average LogFC of each member in each pool-type painted onto the member’s cell.

Among the members of the phenylpropanoid biosynthesis pathway are enzymes needed to produce SA (Chen *et al.*, 2009; Kanehisa *et al.*, 2017). In plants, SA is produced through three pathways: the cytoplasmic, non- β -oxidative pathway and the peroxisomal, β -oxidative pathway that both involve trans-cinnamic acid and benzoic acid; and the chloroplastic pathway occurs via prephenate, chorismate, and isochorismate by the action of chorismate mutase (CM; CA02g27850, up-regulated in all pool-types) and isochorismate synthase (ICS; CA06g19210, down-regulated in total RNA only) respectively (Widhalm and Dudareva, 2015). The conversion of benzoic acid to salicylic acid is performed by benzoic acid 2-hydroxylase (BA2H), a soluble cytochrome P450 monooxygenase, whose amino acid and nucleotide sequences are as yet

unidentified (Leon *et al.*, 1995). The final conversion of isochorismate to SA is performed by an as yet unidentified enzyme with isochorismate pyruvate lyase (IPL) activity; in *Arabidopsis*, neither of the two ICS enzymes possesses any IPL activity (Macaulay *et al.*, 2017). The chloroplastic isochorismate route of SA production is not shown in map00940 and is the major source of SA during the pathogen response (Wildermuth *et al.*, 2001; Kanehisa *et al.*, 2017). The down-regulation of CA06g19210 in total RNA may cause reduced ICS activity after the post-inoculation time-point used in this experiment, which in turn would reduce SA production and contribute heavily to TSWV1 susceptibility.

In this experiment, as well as PAL, later stages of the phenylpropanoid pathway show significant differential expression. Phenylpropanoids comprise a diverse class of metabolites with a wide range of functions, including plant defence (Naoumkina Marina *et al.*, 2010). Accumulation of phenylpropanoids and associated transcripts has been observed during various plant-pathogen interactions, including during viral infection of tomato, cucumber, tobacco (Choi *et al.*, 2006; Bellés *et al.*, 2008; López-Gresa *et al.*, 2012). Induction of the phenylpropanoid pathway in tomato by treatment with *Pseudomonas fluorescens* reduces TSWV disease incidence (Kandan *et al.*, 2002).

In this experiment a number of genes encoding downstream enzymes in the phenylpropanoid pathway show significant down-regulation in polysomal pools, as is shown in figure 3.14, even when there is no change or up-regulation observed in total RNA. This is indicative that these nodes of the phenylpropanoid pathway are being blocked at the translational level and this

may contribute to susceptibility. The polysomally down-regulated pathway nodes encode enzymes associated with monolignol biosynthesis and lignification (Kanehisa *et al.*, 2017). Lignification and phenolic compound accumulation appears to impede TSWV movement in petunia (Quecini *et al.*, 2007); thus it is possible that a lack of lignin production is an important contributory factor to host susceptibility in this experiment.

Flavonoid biosynthesis

The KEGG term “Flavonoid biosynthesis” (map00941), which branches off from the phenylpropanoid biosynthesis pathway, is enriched in up-regulated genes and in the “both” gene set for all pool-types. One KEGG orthologue ID (K00588) has a pepper orthologue that is down-regulated in all pool-types (CA02g14470) and one that is down-regulated (CA02g14450) that is down-regulated only in polysomal pools. The enzyme encoded by these genes is caffeoyl-CoA O-methyltransferase, which catalyses the conversion of caffeoyl-CoA to feruloyl-CoA and the conversion of 5-hydroxy-feruloyl-CoA to sinapoyl-CoA; caffeoyl-CoA O-methyltransferase is also included in the phenylpropanoid pathway and is involved in lignin production, as mentioned above (Li *et al.*, 2013; Kanehisa *et al.*, 2017). Figure 3.15 shows the flavonoid biosynthesis pathway with the average LogFC of each member in each pool-type painted onto the member’s cell.

Flavonoids are a diverse class of secondary metabolite that are involved in protection of plants against various abiotic and biotic stresses as well as having roles in development (Pourcel *et al.*, 2007). The flavonoid biosynthetic

pathway begins with the addition of malonyl-CoA to the phenylpropanoid coumaroyl-CoA (Winkel-Shirley, 2002).

Flavonoids as phytoalexins (molecules produced upon infection that inhibit pathogen development) or phytoanticipins (molecules produced constitutively prior to infection that inhibit pathogen development) are not well studied in Solanaceae; there is strong evidence that flavonoids act as phytoalexins in rice, legumes and citrus, and this is primarily in response to fungal pathogens (Cho and Lee, 2015; Desta *et al.*, 2016).

Flavonoid biosynthesis has also been linked to oxidative stress and flavonoids are involved in scavenging ROS, as well as having roles in stabilising cellular and organellar membranes during cellular dehydration and cold stress (Agati *et al.*, 2012).

High-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) profiling of the response to the fungal pathogen *Colletotrichum gloeosporioides* in post-harvest pepper fruits revealed a number of polyphenols as phytoalexins and phytoanticipins including two flavonoid phytoanticipins: kaempferol O-pentosyldihexoside and dihydroxyflavone O-hexoside (Park *et al.*, 2012).

As shown in figure 3.15, a number of KEGG orthologues involved in flavonoid biosynthesis are up-regulated: CA03g02050 (EC2.3.1.74 and EC2.3.1.170, K00660), which encodes a chalcone synthase (CHS) and is up-regulated in all pool-types; CA05g15880 (EC5.5.1.6, K01859), which encodes a chalcone isomerase and is up-regulated in all pool-types; CA03g29350 (EC1.14.13.21, K05280), which encodes a flavonoid 3'-hydroxylase and is up-

regulated in all pool-types; CA09g02570 (EC1.14.11.23, K05278), which encodes a flavonol synthase (FLS) and is significantly up-regulated in polysomal and sub-polysomal pools. These are involved in the production of various biologically active flavonoids, including dihydroxyflavone and kaempferol, as well as butin, quercetin, luteolin, and myrecitin, all of which have been reported to act protectively against primarily fungal plant pathogens or as ROS quenchers (Mierziak *et al.*, 2014; Kanehisa *et al.*, 2017).

Ribosome

As was also shown in the results of GO enrichment, the KEGG term “Ribosome” (map03010), which contains the ribosomal protein (RP) genes, is enriched in the both and up-regulated gene sets of total RNA (66 and 65 KEGG orthologues respectively) and polysomal pools (79 and 78 KEGG orthologues respectively). Of these, among the 66 orthologues enriched in the both set of total RNA, 52 are in the KEGG eukaryotic ribosome module and 14 are in the prokaryotic ribosome module, and among the 79 orthologues enriched in polysomal pools, 65 orthologues are eukaryotic and 14 are prokaryotic (Kanehisa *et al.*, 2017). The large number of RP genes showing differential expression herein may highlight a number of possible host factors involved in TSWV infection and susceptibility. Figure 3.16 shows the KEGG “ribosome” pathway with the average LogFC of each member in each pool-type painted onto the member’s cell.

One orthologue (K02888; CA02g16160, which encodes mitochondrial 50S RPL21 [RPL21m]) is down-regulated in all pool-types; the chloroplastic 50S RPL21 orthologue (RPL21c; CA06g14020) is significantly down-regulated in

polysomal and sub-polysomal pools but not total RNA. RPL21 is required for formation of the prokaryotic 50S ribosomal subunit; mutation of the *Arabidopsis* gene encoding RPL21m causes failed nuclear fusion of polar nuclei during female gametophyte development, possibly due to impaired phosphatidylcholine (PC) synthesis as PC is a major constituent of the plant nuclear membrane (Portereiko *et al.*, 2006). PC accumulation during positive-sense RNA virus infection is observed at viral replication complexes (VRC) in a process that is conserved across eukaryotes; enhanced PC production is believed to promote cellular membrane re-organisation and VRC formation (Zhang *et al.*, 2016a). The down-regulation of CA02g16160 may thus be an inadequate host response to ambisense TSWV. RPL21c is required for embryogenesis and plastid-development; *Arabidopsis rpl21c* mutants are unable to produce well-organised thylakoid membranes and arrest between the globular and heart stage of development (Yin *et al.*, 2012). It is also possible that down-regulation of organellar *RPL21* genes will, at later time-points, perturb normal organellar function in such a way that impairs the host response to TSWV1; both chloroplasts and mitochondria are important players in pathogen response signal pathway, redox-homeostasis, and programmed cell death (Colombatti *et al.*, 2014; Serrano *et al.*, 2016).

Differential expression is seen among some of the genes cytoplasmic ribosomal stalk proteins P0 (CA02g04760, up-regulated in total RNA and sub-polysomal pools), P2 (CA06g23130, up-regulated in polysomal pools, and CA11g17410, up-regulated in total RNA and polysomal pools), and the plant-specific P3 protein (CA07g12300, up-regulated in total RNA); no differential

expression is seen in CA01g73410, which encodes P1. P0 functions as an anchor for the ribosomal stalk by binding the 28S rRNA and the N-terminal domains of heterodimerised P1 and P2 proteins, which recruit translational GTPases, such as eIF2 and eEF1, via their C-terminal domains in order to promote elongation (Gonzalo and Reboud, 2003). P3 contains the conserved C-terminal domain of P1 and P2 but a distinct N-terminus (Bailey-Serres *et al.*, 1997). The exact role of P3 during translation is unclear.

All four plant ribosomal stalk proteins have been implicated in infection by the (+)ssRNA virus *Potato Virus A* (PVA) but P0 has a distinct role in protecting viral RNA and enhancing viral translation as a component of the PVA membrane-associated RNP, which also contains the PVA genome-linked protein (VPg) and eIF(iso)4E (Hafrén *et al.*, 2013). RNAi depletion of P1 and P2 in human cells enhances translation of transcripts containing a (+)ssRNA foot-and-mouth disease virus (FMDV; picornavirus) IRES but not that of transcripts with an HCV IRES (Martínez-Azorín *et al.*, 2008).

The *Arabidopsis* genome contains two loci encoding P3 proteins (P3A and P3B); AtP3B has been shown to act as a chaperone for both RNA and proteins during cold and heat stress respectively (Kang Chang *et al.*, 2016). Increased expression of *AtP3B* enhances tolerance to both these abiotic stresses, both in *Arabidopsis* and sweet potato (*Ipomoea batatas* [L.] Lam); tubers of sweet potato plants over-expressing *AtP3B* also exhibit improved post-harvest storage at low temperatures (Kang Chang *et al.*, 2016; Ji *et al.*, 2017).

Due to their involvement in non-canonical translation and stress tolerance and their differential expression seen herein, ribosomal stalk P-protein genes may represent novel candidates for viral resistance; however, as P0, P1, and P3 appear to be encoded at single loci in pepper and as the ribosomal stalk is important for normal translation, recessive resistance may result in dramatic loss of yield (Tchórzewski, 2002; Kim *et al.*, 2014).

Six RP genes, listed in table 3.8, show no significant abundance change in total RNA, significantly increased abundance in polysomal pools, and significantly decreased abundance in sub-polysomal pools; this indicates preferential translation of these genes, as there is no transcriptional change but increased presence in pools of ribosome-associated RNA. Due to their apparent translational up-regulation, the RP genes listed could potentially be key susceptibility factors or part of an inadequate pathogen response during the compatible interaction between virulent TSWV and susceptible varieties of pepper. In *Drosophila*, RPL5 and RPL11, which are mentioned in table 3.8, are involved in the translational inhibition of specific mRNAs in an RNP with FMR1, the 5S rRNA, and members of the RNA-induced silencing complex (RISC) such as AGO2 (Ishizuka *et al.*, 2002); plants do not possess an orthologue to FMR1 but RPL5 and RPL11 may be involved in translational regulation in virus-infected plants by an analogous pathway.

4 METHOD DEVELOPMENT FOR VALIDATION OF CANDIDATE GENES AND REGULATION OF EXPRESSION AT THE POINT OF TRANSLATION

4.1 INTRODUCTION

In order to validate relative expression levels of DE and reference genes in RNA samples extracted by sucrose density gradient centrifugation, as determined by RNA-Seq, it was planned to use quantitative reverse transcription PCR (QRT-PCR), which is an established and robust method to quantify levels of specific mRNA sequences (Bustin, 2002). Thus it was necessary to optimise the protocols for downstream RNA processing, cDNA synthesis, and QRT-PCR.

4.2 MATERIALS AND METHODS

4.2.1 Heparin removal

During polysome profiling, heparin was added as a general inhibitor of RNase-activity; heparin is also an effective inhibitor of reverse transcription, PCR, and QPCR (Izraeli *et al.*, 1991; Del Prete *et al.*, 2007). Because of this, heparin removal was an important processing step during the clean-up of pooled RNA extracted by sucrose density gradient centrifugation prior to transcript quantification by QRT-PCR.

Due to tissue constraints, for the experiments described below, a separate TSWV accession (Syngenta TSWV0) was used to the one used in previous chapters (Syngenta TSWV1).

Lithium chloride precipitation

RNA samples were precipitated in 2.5 M LiCl to remove heparin, as described in Chapter 2.

Heparinase I digestion

As an alternative to LiCl precipitation, heparin was removed from RNA samples by heparinase I digestion in the presence of the RNasin RNase Inhibitor. *Bacteroides* Heparinase I enzyme and 10× *Bacteroides* Heparinase Reaction Buffer were obtained from New England Biolabs (P0735S) and RNasin was obtained from Promega (N211A). Heparinase I was supplied at 12 U/μl in 100 mM NaCl, 20 mM Tris-HCl (pH 7.5 at 25°C), 1 mM Na₂EDTA, and 5 mM CaCl₂. Ten times *Bacteroides* Heparinase Reaction Buffer contained 200 mM Tris-HCl (pH 7.5 at 25°C), 1 M NaCl, and 15 mM CaCl₂. RNasin Ribonuclease Inhibitor was supplied at 40 U/μl in 20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 8 mM DTT, and 50% (v/v) glycerol. The reaction mixture for heparinase I digestion is described in table 4.1.

Reaction vessels were incubated at room temperature for 1-2 hr. RNA was then purified by ethanol precipitation into 50 μl of RNase-free water and concentration was measured using a NanoDrop spectrophotometer.

Heparin digestion yields oligosaccharide products containing unsaturated uronic acids that absorb UV at 232 nm (Mourier *et al.*, 2015). Samples that have undergone heparinase I digestion may thus have a low 260/230 ratio, which would normally be indicative of poor quality, contaminated RNA, but the breakdown products of heparin are not inhibitory of any stage of QRT-PCR (Izraeli *et al.*, 1991; Desjardins and Conklin, 2010).

Table 4.1. The reaction mixture required for removal of heparin by heparinase I digestion.

Reagent	Volume (μl)
RNA sample	85
10× Heparinase Buffer	10
RNAsin	4
Heparinase I	1
Total	100

4.2.2 Reverse transcription

Complementary DNA (cDNA) libraries were generated from RNA by reverse transcription primed with random hexamers. Random hexamers were supplied at 200 ng/μl from ThermoFisher Scientific (SO142). SuperScript II reverse transcriptase, 5× First-Strand Buffer, and 0.1 M DTT were supplied from ThermoFisher Scientific (18064014). SuperScript II was supplied in 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% (v/v) NP-40, 50% (v/v) glycerol and 5× First-Strand Buffer contained 250 mM Tris-HCl (pH 8.3 at 25°C), 375 mM KCl, and 15 mM MgCl₂. The SuperScript II mixture is described in table 4.3.

The annealing stage of the reaction was carried out with the reaction mixture described in table 4.2. The mixture was heated at 65°C for 5 min then immediately chilled on ice. Ten μl of SuperScript II mixture was added to the annealing mixture for each RNA sample, the tube was mixed gently, and then incubated at room temperature for 10 min. The reaction mixture was incubated at 42°C for 50 min and then inactivated by incubation at 70°C for 15 min. A reverse transcriptase-free control was made to control for possible contamination of DNA in reagents and template RNA by using 1 μl of water in the place of the SuperScript II. Seventy-five μl of nuclease-free water was added to each reaction mixture to bring each cDNA sample to its working concentration. During method development, cDNA synthesis was performed on polysomal and sub-polysomal RNA samples from pepper first leaves that were harvested 24 hr after inoculation using the same procedures and virus samples described in Chapter 2.

Table 4.2. The reaction mixture required for primer-annealing stage of cDNA synthesis.

Reagent	Volume (μl)
RNA sample	x (for 50 ng)
Random hexamers	1
dNTP mix (10mM each)	1.25
RNase-free water	Bring up to 15
Total	15

Table 4.3. The SuperScript II reaction mixture used to construct cDNA libraries for each RNA sample.

Reagent	Volume (μl)
5× First-Strand Buffer	5
RNasin	0.5
SuperScript II	1
DTT	2.5
RNase-free water	Bring up to 10
Total	10

4.2.3 Quantitative reverse transcription PCR

QRT-PCR was carried out using fluorescent FAM-TAMRA probes in order to improve specificity of product detection. Roche LightCycler 480 Probes Master (04707494001) reaction mixture was used. For QRT-PCR, cDNA was diluted in a 1:1 ratio with nuclease-free water prior to being added to the reaction mixture. The reaction mixture used for QRT-PCR is described in table 4.4. Each primer/template combination was performed in quadruplicate.

During method development, primers and probes (Eurofins Genomics) were designed against the pepper alpha-tubulin gene (α -TUB; GenBank accession: EF495257.1) as a positive control using Primer3Plus with settings optimised for QPCR (Untergasser *et al.*, 2007). To test for presence of TSWV in samples, primers and probes designed against a conserved region of the TSWV nucleocapsid (N) gene were used (Roberts *et al.*, 2000). Primer and probe sequences are shown in table 4.5.

Conditions for QRT-PCR are described in table 4.6. The excitation and emission wavelengths of the fluorescent dye used (FAM) are 495 and 520 nm respectively according to manufacturer specifications. QRT-PCR was run using LightCycler480 System (Roche Applied Science).

Total RNA from 3 wk TSWV0-inoculated and mock-inoculated pepper leaves (samples 91 and 88) to act as positive controls for α -TUB primers and respectively as positive and negative controls for TSWV N primers.

Table 4.4. The reaction mixture used for QRT-PCR on cDNA made from RNA samples extracted from pepper leaves by sucrose density gradient centrifugation.

Reagent	Volume (μ l)
Nuclease-free water	1.7
2 $\times$ LC480 Probes Master	6
Forward primer (10 μ M)	0.6
Reverse primer (10 μ M)	0.6
FAM-TAMRA probe (10 μ M)	0.6
cDNA	2.5
Total	12

Table 4.5. Primers and probes used to develop QRT-PCR methods for validation of RNA-Seq data generated from RNA extracted pepper leaves using sucrose density gradient centrifugation.

	TSWV N	α -TUB
Forward primer	GCTTGTTGAGGAACTGGGAATT	GAACCGCCTTATTTACAGG
Reverse primer	AGCCTCACAGACTTTGCATCATC	TGAATCCTGGGATAGGGAAC
FAM-TAMRA probe	AAATCTAAGATTGCTTCCCACCCTTTGATTCAA	TGATGGAGCCCTGAATGTTGATGTG

Table 4.6. Thermal cycle conditions for QRT-PCR detection of α -TUB and TSWV N in pepper cDNA.

Step	Temperature (°C)	Duration (m:s)	Cycles
Initial denaturation	95	10:00	1
Denaturation	95	0:10	>45
Annealing	60	0:50	
Extension	72	0:01	
Cooling	40	10:00	1

4.3 RESULTS

Herein the efficacies of both heparin removal strategies are discussed.

4.3.1 Lithium chloride precipitation

When cDNA libraries were made using LiCl-precipitated RNA from polysome profiling, no fluorescence was observed during QRT-PCR; agarose gel electrophoresis of QRT-PCR products showed that no extension had occurred.

4.3.2 Heparinase I digestion

Heparinase I treatment of pooled RNA extracts from sucrose density gradient centrifugation yielded the results seen in table 4.7.

A two-tailed t-test shows no statistically significant difference in RNA quality between pre- and post-digest samples judging by the 260/280 and 260/230 ratios ($P_{260/280}=0.70$ and $P_{260/230}=0.89$).

Considering 85 μ l of pre-digest RNA were put into each reaction and post-digest RNA was re-suspended in 50 μ l of RNase-free water, the average amount of RNA in each reaction dropped from 9702.75 ng to 485.75 ng (a drop of 95.0%). A one-tailed t-test showed this reduction in yield to be statistically significant ($P=0.03$).

Table 4.7. The concentrations, absorptions, and absorption ratios of pooled polysomal (p) and sub-polysomal (s) RNA samples from 24h TSWV1 inoculated (38) and mock-inoculated (5) pepper plants before and after heparinase I digestion.

Pre-digest					
	ng/μl	A260	260/280	260/230	Constant
5s	158.36	3.959	1.91	1.96	40
5p	39.32	0.983	1.49	1.14	40
38s	192.76	4.819	1.78	1.5	40
38p	66.16	1.654	1.5	1.28	40
Average	114.15	2.85375	1.67	1.47	
Post-digest					
	ng/μl	A260	260/280	260/230	Constant
5s	21.41	0.535	2.01	2	40
5p	5.52	0.138	1.16	1.07	40
38s	7.82	0.195	1.74	1.88	40
38p	4.11	0.103	1.6	1.01	40
Average	9.715	0.24275	1.6275	1.49	

QRTPCR

QRTPCR performed on total RNA from systemically infected 3 wk TSWV0-inoculated (91) and mock-inoculated (88) samples yielded the results seen in table 4.8, which shows the number of PCR cycles (C_P) required to meet a threshold fluorescence value for each reaction. Reactions containing RT-free controls in the place of cDNA and reactions containing cDNA generated from leaf sample 88 and TSWV N primers and probes never met the threshold fluorescence value.

Having ascertained that the primers and probes used above are effective in the detection of *C. annuum* α -TUB and TSWV N sequences, QRTPCR was performed on cDNA generated from sub-polysomal (s) and polysomal (p) RNA from first leaves of 24 hr mock-inoculated (5) and TSWV1-inoculated (38) pepper plants. Results of this can be seen in

table 4.9. One replicate of the 38s RT-free control (reaction A12) met the threshold fluorescence value but this is likely due to contamination. None of the reactions containing primers and probes for TSWV N met the threshold fluorescence.

Table 4.8. The results of QRT-PCR to demonstrate function of primers and probes designed against the *C. annuum* α -TUB gene and the TSWV N gene on total RNA of 3 wk mock-inoculated (88) and TSWV0-inoculated (91) pepper plants.²⁶

	α -TUB				TSWV N				α -TUB				TSWV N			
	88+RT		88-RT		88+RT		88-RT		91+RT		91-RT		91+RT		91-RT	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	25	25							24	24			21	21		
B	26	25							24	24			21	22		

²⁶ "+RT" indicates reactions performed on cDNA and "-RT" indicates reactions on reverse transcriptase-free controls. Numbers indicate C_p values and empty cells are reactions where fluorescence never met the detection threshold.

Table 4.9. The results of QRTPCR to test for efficacy of heparinase I treatment in the preparation of RNA extracted by sucrose density gradient centrifugation for cDNA synthesis. QRTPCR was performed on sub-polysomal (s) and polysomal (p) pools extracted from 24 hr mock-inoculated (5) and TSWV1-inoculated (38) pepper plants. ²⁷

		5s				5p				38s				38p			
		+RT		-RT		+RT		-RT		+RT		-RT		+RT		-RT	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
α -TUB	A	28	28			28	28			29	29		34	28	28		
	B	28	27			28	28			29	29			28	28		
TSWV N	C																
	D																

²⁷ “+RT” indicates reactions performed on cDNA and “-RT” indicates reactions on reverse transcriptase-free controls. Numbers indicate C_p values and empty cells are reactions where fluorescence never met the detection threshold.

4.4 DISCUSSION

The results described in

table 4.9 demonstrated that heparinase I treatment of RNA samples extracted from first leaves of 5 wk old pepper plants by sucrose density gradient centrifugation is adequate for removal of 1 mg/ml heparin to the point that there is no inhibitory effect on cDNA synthesis and QRT-PCR. LiCl precipitation removed heparin sufficiently for cDNA synthesis, PCR, and RNA-Seq but not for QRT-PCR.

The heparinase I treatment protocol used herein did however lead to a 95.0% reduction in RNA yield, as shown in table 4.7, likely due to the long room temperature incubation step. No figures for direct comparison of RNA yield by LiCl precipitation with that of heparinase I are available for the current experiment as LiCl precipitation was paired to pooling of polysomal and sub-polysomal fractions. Loss of RNA during LiCl precipitation was quantified while optimising the extraction of total RNA from heparinised plant lysates using Trizol LS reagent on tomato leaves lysed using lysis buffer; the Trizol LS protocol can be seen in Chapter 2. Data from this optimisation can be seen in the “Supplementary files” folder under “Trizol LS heparin removal.xlsx” and represent concentrations of RNA in ng/μl after a paired Trizol LS-LiCl procedure compared with Trizol LS extraction alone. These data show a 43.6% reduction (two-tailed t-test, $P=0.02$) in the average yield when RNA samples are LiCl precipitated. Recovery of RNA after LiCl precipitation was much higher than recovery after heparinase I treatment.

RNA quality after heparinase I treatment was not assessed herein. Using an Agilent Bioanalyzer 2100, Johnson *et al.* (2003) reported similar RNA integrity between heparinase I-treated RNA from sheep granulosa cells

containing heparin and untreated RNA extracted from sheep thecal cells that did not contain heparin. As mentioned in the materials and methods section of Chapter 3, for sequencing the RIN score of RNA samples had to be at least 7 and this was verified using an Agilent Bioanalyzer 2100 (data not shown). This is in agreement with other experiments that have shown LiCL precipitation of RNA to remove heparin yields high quality RNA (Hebels *et al.*, 2014). Future experiments that require further optimisation of the procedures described in this chapter should monitor RNA quality using an Agilent Bioanalyzer 2100 or by the visual assessment of 18S and 28S ribosomal RNA on an agarose gel (Masek *et al.*, 2005).

As explained in the closing paragraphs of Chapter 2, the presence of heparin in plant RNA extracted using sucrose density gradient centrifugation was a major drawback when validating RNA-Seq in the current experiment. Successful polysome profiling has been achieved using lower concentrations of heparin (0.53-0.65 mg/ml), only using heparin (0.5 mg/ml) in the lysis buffer and not in cushion or gradient buffers, or totally removing heparin from all buffers for isolation of RNA from tissues with low RNase activity (Del Prete *et al.*, 2007; Melamed and Arava, 2007; Mustroph *et al.*, 2009a).

No conclusions on the presence of TSWV N sequences in sub-polysomal and polysomal cDNA libraries of TSWV1-inoculated plants can be drawn from the above data. It is likely that template quality in heparinase I treated RNA samples was low prior to cDNA synthesis due to degradation during room temperature incubation; RNA quality can cause false-positives and false-negatives in the results of QRT-PCR (Bustin *et al.*, 2009; Die and Román, 2012).

Due to tissue restraints, the TSWV N primers and probes used were only shown to amplify and fluoresce in the presence of TSWV0 N sequences. TSWV1 was not tested. While these were designed against a purportedly conserved region, sequence variation between the strains may stop amplification when using TSWV1 as a template (Lemmon and Gardner, 2008). TSWV N primers and probes were checked against cDNA constructed from a total RNA template; the results shown in

table 4.9 are for cDNA from polysomal and sub-polysomal RNA samples.

As mentioned in the closing paragraphs of Chapter 2, the reagents used during sucrose density gradient centrifugation in this experiment did not include sufficient ionic and non-ionic surfactants to disrupt the cytoskeleton and endoplasmic reticulum, as described by Mustroph *et al.* (2009a). Bunyaviral transcripts are purportedly translated solely on the rough endoplasmic reticulum, intercellular transport of TSWV RNPs is carried out via the endoplasmic reticulum desmotubule, and intracellular transport of the TSWV viral replication complex (VRC) happens along actin microfilaments; thus it is possible that insufficient TSWV N template RNAs were released during tissue lysis for detection by QRT-PCR (Feng *et al.*, 2013; Feng *et al.*, 2016; Sun *et al.*, 2018).

As well as the particular potential problem with detection of TSWV RNA, the omission of sufficient surfactants may have also led to a larger scale representational bias in RNA-Seq and QRT-PCR data towards soluble RNA and against RNA that was part of an insoluble, membrane- or cytoskeleton-associated complex, such as ribosomes performing ER-associated translation (Mustroph *et al.*, 2009a).

5 GENERAL DISCUSSION

5.1 TSWV1 INFECTION OF SUSCEPTIBLE PEPPER INCREASES GENERAL TRANSLATION

The polysome profiling results in Chapter 2 show an increase in polysomally associated material in TSWV1-inoculated susceptible pepper plants in comparison with mock-inoculated plants of the same variety. This is indicative of a global increase in translation in virally infected plants (Coudert *et al.*, 2014).

These results differ from other experiments investigating early induced nucleotide-binding site leucine-rich repeat protein (NBS-LRR)-mediated pathogen responses in *Arabidopsis* and tobacco, which showed no significant increase in global translation (Meteignier *et al.*, 2016; Meteignier *et al.*, 2017). During *Pseudomonas syringae* infection of *Arabidopsis*, the type III effector AvrRpm1 is recognised by the NBS-LRR RPM1; once RPM1 is activated, the hypersensitive response (HR) is induced and pathogen growth is inhibited (Mackey *et al.*, 2002); Meteignier *et al.* (2017) used dexamethasone (DEX)-inducible AvrRpm1 expression in *RPM1⁺ Arabidopsis* to profile the translational response 2 hr post-induction. Meteignier *et al.* (2016) used a similar method to profile the tobacco N-dependent response to the presence of the tobacco mosaic virus (TMV) p50 replicase fragment in potato virus X (PVX)-infected tobacco. During this DEX-induced response, eIF2 α -phosphorylation was not observed 8 hr post-induction, which would otherwise cause translational repression, and PVX transcripts are selectively segregated into processing

bodies (PBs) and not translated, despite continued viral RNA accumulation and no immediate viral RNA degradation (Meteignier *et al.*, 2016).

As a lack of translational activation appears to be a hallmark of an effective defence response against obligate biotrophs (Meteignier *et al.*, 2016; Meteignier *et al.*, 2017), the increased polysomal load seen in the results of this study indicates an inability to defend against TSWV attack by repressing global translation. Thus it may be inferred that selective translation of defence-related genes and control of global translation are important determining factors for the efficacy of the defence response in plants against TSWV.

The conserved translational regulator Target of Rapamycin (TOR) is a phosphatidylinositol 3-kinase-related kinase (PIKK) that promotes growth by activating anabolic processes and negatively regulating autophagy in eukaryotes; TOR is also involved in antagonising the actions of the plant hormones salicylic acid (SA) and jasmonic acid (JA) during the defence response (Deprost *et al.*, 2007; Liu and Bassham, 2010; De Vleeschauwer *et al.*, 2017). The results of Meteignier *et al.* (2017) show that during the DEX-induced NBS-LRR defence response, TOR is translationally down-regulated. Using TOR over-expression and knockdown *Arabidopsis* lines, it was also shown that TOR expression is inversely correlated with resistance to biotrophic bacterial pathogens (Meteignier *et al.*, 2017). Moreover, TOR is a target for viral attack in plants. During Cauliflower mosaic virus (CaMV) infection of *Arabidopsis*, the viral reinitiation factor transactivator–viroplasm (TAV) binds TOR; this causes TOR hyperactivation and activation of the 40S ribosomal protein S6 kinase 1 (S6K1), which enhances reinitiation on CaMV

polycistronic transcripts via retention of host translational machinery (Schepetilnikov *et al.*, 2011).

In the current experiments, the pepper *TOR* homologue CA08g16500 shows no differential expression in any pool-type. Additionally the pepper homologue *S6K1* CA10g10590, a conserved downstream effector of TOR signalling (Fingar *et al.*, 2004; Schepetilnikov and Ryabova, 2018), shows significant up-regulation in all pool-types. In accordance with the explanation above, the failure to transcriptionally or translationally down-regulate *TOR*-expression and the subsequent failure to suppress TOR-derived signalling may explain the increase in global translation observed upon TSWV inoculation, as well as underpinning susceptibility of the pepper cultivar used to TSWV infection.

5.2 A SUBSET OF GENES SHOW DIFFERENTIAL TRANSLATION DURING TSWV INFECTION

In Chapter 1, the potential problem of using transcriptomic data to represent expression was discussed because post-transcriptional regulation, such as translational regulation and protein stability, can dramatically affect protein levels and functionality (Gutierrez *et al.*, 1999; Hellmann and Estelle, 2002; Kawaguchi and Bailey-Serres, 2002). In Chapter 3, principle component analysis (PCA) and hierarchical clustering of normalised RNA sequencing (RNA-Seq) results revealed that expression data derived from polysomal and total RNA were largely similar but that significant differences were present.

The signalling pathways that caused an increase in global translation upon TSWV-inoculation may also be involved in causing the differential

translation seen herein. As explained above, TOR activity promotes anabolic processes and antagonises defence pathways (De Vleeschauwer *et al.*, 2017). The data presented in Chapter 3 shows that upon TSWV1 infection, members of multiple canonical defence pathways, such as glycerol-3-phosphate (G3P) metabolism (Chanda *et al.*, 2008) and the SAR markers *PR1* and *PR2* (Molinari *et al.*, 2014), as well as the canonical upstream TOR antagonist *ATM* (Alexander *et al.*, 2010) are down-regulated. Various anabolic pathways are enriched among genes up-regulated in total and polysomal RNA. These up-regulated members of anabolic processes include: those involved in ribosome biogenesis, such as ribosomal proteins (RP) and pseudouridine synthases (Henras *et al.*, 2015; Weis *et al.*, 2015); those involved in DNA replication and mitosis, such as components of the MCM complex (Tye, 1999); and the TOR targets S6K1 (CA10g10590) and eIF3h (CA03g03190), which promote translational reinitiation on polycistronic and uORF-containing mRNA during viral attack and auxin-mediated organogenesis respectively (Schepetilnikov *et al.*, 2013). S6K1 is involved in various processes promoting cap-dependent translation upon activation by TOR (Holz *et al.*, 2005; Silvera *et al.*, 2010). Thus the results seen herein show activation of anabolic processes and a lack of activation of defence pathways upon TSWV infection of susceptible pepper; this would fit with the central role TOR appears to play in antagonising defence (De Vleeschauwer *et al.*, 2017).

5.3 FUTURE WORK

In accordance with the results shown and discussed in Chapters 2 and 4, any future polysome profiling experiments to expand upon the results of this study

should use the methods of Mustroph *et al.* (2009a) in order to optimise RNA yield and retrieval, polysome profile peak resolution, and downstream validation of expression. In further experiments the cost, speed, and ease of expression analysis of individual candidate genes described herein should also be improved by the use of QRT-PCR.

In order to build upon the results of this study, polysome profiling should be performed at further time points in order to profile how the response changes over time, both before and after symptoms appear. This would help to identify and further verify genes that are important in promoting viral infection and susceptibility over the entire course of progression. A time-course experiment would also potentially identify genes and processes that may be temporally regulated during infection, as is the case in the results of Dorey *et al.* (1997), and may have not been observed as differentially expressed in this study, as well as investigating whether the increase in general translation observed at 6 hr post inoculation is consistent throughout infection. A similar experiment with larger replicate numbers over a time-course would also potentially provide appropriate data for performing co-expression analysis and modelling, which would show common patterns among sets of differentially expressed genes. This would allow much stronger inferences regarding common regulation and causative links between differentially expressed genes (Movahedi *et al.*, 2012).

In order to investigate which effects are specific to the *Tsw* resistance-breaking (RB) TSWV accession used in this study, the translational response could be profiled during infection with alternative accessions of TSWV and

other RNA viruses. As discussed in Chapters 1-3, the ability of TSWV to overcome the *Tsw* R gene in pepper is somewhat functionally linked to the RNA silencing suppressor (RSS) NSs and structurally linked to the NSs N-terminal domain (de Ronde *et al.*, 2013). The presence of NSs^{RB} instead of resistance-inducing NSs (NSs^{RI}) may have affected the results of the current study and it would be interesting to investigate how global translation and expression are affected differentially in NSs^{RB} and NSs^{RI}. Further to this, it would be useful to profile the RSS activity of RB and RI TSWV accessions using the methods of de Ronde *et al.* (2013), whereby silencing of GFP expression was monitored during transient expression of NSs^{RB} and NSs^{RI}, in order to show how RSS activity affects the susceptible response. This could also be performed with other TSWV knockout and domain mutants in order to further elucidate which viral factors are involved in enhancing translation of its genes.

Repeating this experiment with other tospoviruses, other viruses, and other pathogens and comparing the results to those of the current study would highlight which effects seen in this study are specific to TSWV, potentially demonstrating particular processes that are targeted by TSWV to allow and enhance infectivity. This would also help in providing more insights into how translation is affected during the pathogen response in general in peppers. It would also be interesting to perform the experiment in other susceptible hosts, in order to see which effects seen herein are conserved and which are unique to a given host.

This experiment only aimed to profile the response to TSWV infection in an organ distal to the point of infection. As *Tsw*-based resistance results in

HR upon induction, this response is entirely local and results in localised cell death and abscission of infected tissues (Moury *et al.*, 1997). It would be interesting to profile translation and expression during the response to TSWV around the point of infection and in distal organs in both a susceptible *Tsw⁻* and a resistant *Tsw⁺* host. As mentioned above, the results of Meteignier *et al.* (2017) show that activation of an NBS-LRR in *Arabidopsis* leads to a drop in TOR expression at the translational level; as the *Tsw* gene encodes an NBS-LRR, it would be interesting to investigate whether *Tsw*-activation has the same effect. As systemic acquired resistance (SAR) involves multiple events for signal transduction from the localised point of induction to distal tissues and the pepper germplasm currently contains no known SAR-based resistance to TSWV, investigating local and distal expression as well as defence hormone production and transport in *Tsw⁺* and *Tsw⁻* hosts may also demonstrate which SAR-related genes and processes are not induced during TSWV infection and may provide further candidates for more robust resistance (Choi Hyong and Hwang Byung, 2011).

One drawback to the current experiment was that, as of the time of writing, transcriptional start sites and mRNA un-translated regions (UTR) have not been mapped. Analysis of 5' and 3' UTR sequences of differentially transcribed and translated gene sets would have been useful in finding whether common UTR features are linked to expression pattern; these features may include sequence motifs, modification consensus motifs, uORFs, or secondary structure (Mignone *et al.*, 2002; Bodi *et al.*, 2012). This problem may also be addressed by repeating the experiment in a TSWV host with better

genomic resources that provide UTR sequences, such as *Arabidopsis* or tomato (Aoki *et al.*, 2010; Berardini *et al.*, 2015).

Another line of research to build from these results would be to use standard reverse genetic screening approaches to investigate how loss, knockdown, or overexpression of candidate genes highlighted herein affects disease progression, symptoms, viral spread, and susceptibility of pepper to TSWV infection. A mutant eIF4E1 allele conferring resistance against two potyviruses in tomato was found using a large-scale reverse genetic screen in an EMS-mutagenised TILLING (targeting induced local lesions in genomes) population; the translation initiation factors eIF4E1, eIF4E2, eIF(iso)4E, eIF4G, and eIF(iso)4G were used as candidates for mutation screening based on previous instances of these acting as effective recessive resistance genes (Piron *et al.*, 2010).

In order to profile the translome, an alternative method for future experiments would be ribosome footprinting, sometimes called Ribo-Seq or ribosome profiling. Ribosome footprinting is a technique that, similarly to the combination of polysome profiling and RNA-Seq described in this work, provides sequences of all ribosome-associated RNA (Mazzoni-Putman and Stepanova, 2018). Ribosome footprinting requires no ultracentrifugation and fractionation; as in polysome profiling, cell extracts are treated with chemical inhibitors of translational elongation, such as cycloheximide and chloramphenicol, in order to pause ribosomes bound to mRNA molecules. These extracts are then treated with ribonucleases to target and degrade any exposed single-stranded RNA that is not protected by a

ribosome, leaving only a pool of 18-34 nucleotides-long ribosome protected fragments (RPFs). RPFs are used to prepare a cDNA library, which can then be sequenced. This generates specific data about the position and distribution of ribosomes on each transcript, not merely whether ribosomes are bound to a transcript, which provides more insight into whether a given transcript is actively translated, which would be indicated by a uniform distribution of RPF sequences aligning to a single ORF, or merely has ribosomes bound to the 5' or 3' UTRs or otherwise stalled, which would be indicated by an overrepresentation of RPF sequences at a given site on a transcript (Dmitriev *et al.*, 2016).

In comparison to polysome profiling, ribosome footprinting provides a more nuanced view of translation as there is no assumption that the density of an mRNA molecule directly relates to its translational state. Work in yeast has shown that, in polysome profiling experiments, stress granules (cytoplasmic bodies formed of stalled mRNAs that form during acute stress) sediment in fractions denser than 80S (Jain, 2015; Lee and Seydoux, 2019). This contradicts a core assumption of polysome profiling, that mRNA denser than 80S are actively translated, and means that non-translated mRNA would be included in polysomal fractions. This may be mitigated by a lysate clarification; according to the results of Jain (2015), stress granules preferentially pellet by centrifugation at >18,000 g. The protocol of Mustroph *et al.* (2009a) includes a post-lysis 16,000 g clarification step but it is not clear that this is sufficient to remove stress granules from a plant lysate. In development of future polysome profiling protocols, the presence or absence

of stress granules in dense fractions extracted from plant tissue should be verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for stress granule marker proteins, such as OLIGOURIDYLATE BINDING PROTEIN 1 (UBP1) or RNA-binding protein 47 (RBP47) (Blancher and Jones, 2001; Weber *et al.*, 2008).

Translational efficiency (TE) was not determined from RNA-Seq results in the current experiment. The TE of a given gene is the ratio between its read counts in the translated pool and in the total RNA pool. TE ratios can be compared between genes within a data set with vastly disparate read counts as they are internally normalised. TE ratios for individual genes can also be compared between conditions or time points in order to determine which genes are changing their translational state throughout the process or response of interest. TE can be determined from any large scale expression data, such as that obtained using RNA-Seq or microarrays, derived from any technique that separates total or non-translated RNA from translated RNA, such as polysome profiling or ribosome profiling, and should be utilised in future experiments of this kind for large scale comparisons of how translational expression changes (Juntawong *et al.*, 2014; Krishnan *et al.*, 2014).

The functional roles of DE genes described herein on resistance and susceptibility to TSWV should be investigated through standard reverse genetic procedures. This may be achieved by directed mutagenesis of the candidate, such as by the use of CRISPR-Cas9: a technique whereby specific genomic sequences are disrupted via a synthetic guide RNA

(Doudna and Charpentier, 2014). Little information is available as to whether CRISPR-Cas9-mediated editing has been performed to generate mutant peppers; this may be due to the recalcitrant nature of pepper during tissue culture and plant regeneration (Kothari *et al.*, 2010). Alternatively, mutants of given genes may be found within TILLING populations, which has the advantage that it does not require culturing to obtain mature mutant plants (Tadele, 2016).

Where loss of function or knockout of a gene proves to be lethal to the plants, RNA interference (RNAi) or virus-induced gene silencing (VIGS) may be used instead to knock-down expression (Small, 2007; Purkayastha and Dasgupta, 2009). Overexpression may also be used to determine the role of a given candidate gene during infection (Datta *et al.*, 1999).

One would expect, if a given gene is involved in promoting infection, knockout, loss-of-function mutation, or reduced expression would enhance basal resistance, which would involve reduced symptom expression, virus accumulation, and systemic spread, while overexpression would increase susceptibility (Garcia-Ruiz, 2018). Conversely if a gene is involved in promoting defence pathways and disease resistance, knockout, loss-of-function mutation, or reduced expression would increase susceptibility and overexpression would enhance resistance (Peng *et al.*, 2011; MacQueen and Bergelson, 2016).

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7 APPENDICES

7.1 ABBREVIATED LISTS OF DIFFERENTIALLY EXPRESSED GENES

Below are abbreviated lists of differentially expressed genes ($\text{LogFC} > 3$ or < -3 ; $P < 0.05$; $\text{FDR} < 0.05$) in the comparison of RNA-Seq data derived from total, polysomal, and sub-polysomal RNA samples between TSWV- and mock-inoculated pepper plants.

7.1.1 Total RNA

Table 7.1. An abbreviated list of genes whose abundance shows a statistically significant increase ($\text{LogFC} > 3$ or < -3 ; $P < 0.05$; $\text{FDR} < 0.05$) in abundance in RNA-Seq data in comparisons between total RNA samples extracted from the first leaves of 6hr TSWV- and mock-inoculated 25 day old pepper plants.

Gene_ID	logFC	PValue	FDR	Annotation
CA01g01590	3.002138	3.81E-15	3.00E-13	NADPH oxidase
CA01g24400	3.102132	2.41E-17	2.58E-15	PREDICTED: transcription factor MYB12-like [Solanum tuberosum]
CA01g24460	3.130153	9.52E-05	0.000791	Unknown protein
CA01g29300	3.281466	1.81E-28	7.50E-26	Detected protein of unknown function
CA01g32810	5.314161	1.19E-16	1.20E-14	Acyltransferase 2 (Fragment)
CA01g32820	3.045721	4.02E-14	2.75E-12	Acyltransferase 2 (Fragment)
CA01g32940	4.563164	9.60E-09	2.32E-07	Acyltransferase 2 (Fragment)
CA01g33460	3.751393	1.08E-06	1.63E-05	CBF/DREB-like transcription factor 1
CA01g33480	3.298913	0.000126	0.001012	C-repeat binding facor
CA01g34000	3.13564	6.56E-24	1.60E-21	Nitrate transporter
CA01g34630	3.996749	2.32E-20	3.93E-18	MYB transcription factor MYB92
CA02g01890	3.13677	7.17E-08	1.42E-06	Rsmv23 protein
CA02g06440	3.241381	2.90E-09	7.93E-08	Putative At5g37260 (Fragment)
CA02g08660	5.842884	1.64E-08	3.79E-07	Pectate lyase, putative
CA02g18340	4.895452	0.000575	0.003639	1-aminocyclopropane-1-carboxylic acid oxidase
CA02g20380	3.376274	0.007778	0.031569	Acyl:coa ligase acetate-coa synthetase-like protein
CA02g23780	4.155755	8.63E-48	1.74E-44	Myosin heavy chain, putative
CA02g28820	3.595909	1.68E-08	3.88E-07	PREDICTED: probable mitochondrial chaperone BCS1-B-like [Solanum tuberosum]
CA03g02170	6.373339	0.008102	0.032627	photosystem I P700 chlorophyll a apoprotein A1 [Nicotiana glauca]
CA03g24550	3.32448	0.000182	0.001378	Cytochrome P450 A
CA03g32280	3.672298	2.55E-13	1.55E-11	PREDICTED: ethylene-responsive transcription factor ERF003-like [Solanum tuberosum]
CA03g32400	3.248385	1.78E-25	5.87E-23	F-box family protein
CA03g34540	3.232455	5.86E-11	2.27E-09	Auxin-responsive protein IAA1, putative
CA04g04080	3.534149	1.03E-30	5.38E-28	Phytoene synthase

CA04g15480	3.212006	2.34E-20	3.93E-18	PREDICTED: probable leucine-rich repeat receptor-like protein kinase At1g35710-like [Solanum tuberosum]
CA04g15970	3.089829	6.01E-10	1.87E-08	Hydrolase, putative
CA04g18280	3.661263	3.23E-09	8.69E-08	CYP81C6v2
CA04g19810	3.406917	6.46E-06	7.78E-05	Detected protein of unknown function
CA05g05850	3.68492	2.18E-25	6.91E-23	PREDICTED: zeatin O-glucosyltransferase-like [Solanum lycopersicum]
CA05g07270	3.018805	3.27E-08	6.93E-07	Polygalacturonase, putative
CA06g12640	3.410736	0.000136	0.001078	PREDICTED: phospholipase A1-1beta2, chloroplastic-like [Solanum tuberosum]
CA06g17720	3.153346	2.52E-46	4.52E-43	PREDICTED: calcium-binding allergen Ole e 8-like [Solanum tuberosum]
CA06g24300	3.377806	4.03E-54	1.08E-50	Flavone synthase II
CA07g04400	4.800003	2.85E-33	1.91E-30	Tetratricopeptide repeat-like superfamily protein, putative [Theobroma cacao]
CA07g11530	4.174479	5.49E-17	5.58E-15	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g11540	4.122956	4.31E-12	2.12E-10	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g19240	3.043879	1.09E-12	6.09E-11	Ubiquitin-conjugating enzyme variant, putative
CA08g09650	3.085095	8.58E-13	4.83E-11	Sulfotransferase family protein
CA08g10440	3.452371	0.000549	0.003503	Detected protein of unknown function
CA08g10450	4.698416	0.00136	0.007514	Calmodulin binding protein, putative
CA08g11580	3.971806	2.34E-37	2.51E-34	PREDICTED: broad-range acid phosphatase DET1-like [Solanum lycopersicum]
CA08g11590	5.430171	1.16E-13	7.41E-12	Serine/threonine-protein kinase bri1, putative
CA08g13530	3.561832	4.03E-24	1.03E-21	Ferric-chelate reductase, putative
CA08g16140	3.799017	4.95E-14	3.27E-12	Leucoanthocyanidin dioxygenase, putative
CA09g07540	7.0322	0.007516	0.030783	Photosystem I P700 apoprotein A2 (Fragment)
CA09g07550	8.97926	0.001323	0.007348	Photosystem I P700 chlorophyll a apoprotein A1
CA10g03640	3.561832	4.74E-46	7.66E-43	Quercetin 3-O-glucoside-6"-O-malonyltransferase
CA10g05630	3.972499	1.05E-17	1.21E-15	UDP-glucosyltransferase, putative
CA10g12130	4.306284	2.49E-26	8.38E-24	Flavonoid glucosyltransferase UGT73E2
CA10g16530	4.948926	9.00E-14	5.84E-12	Flavonoid 3-glucosyl transferase
CA11g00570	3.149055	4.64E-34	3.26E-31	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum tuberosum]
CA11g00600	3.01573	6.60E-27	2.42E-24	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum lycopersicum]
CA11g07810	3.278726	0.004332	0.019661	Photosystem I P700 chlorophyll A apoprotein (Fragment)
CA11g14880	3.574548	1.06E-15	9.35E-14	Putrescine hydroxycinnamoyl transferase
CA11g17090	5.283225	2.65E-28	1.04E-25	Unknown protein

CA12g02050	3.125578	4.84E-41	6.51E-38	Glucosyltransferase
CA12g12410	3.304262	5.83E-20	9.50E-18	Cytochrome P450 CYP736A54
CA12g16620	3.040511	1.44E-37	1.66E-34	UDP-glucose glucosyltransferase
CA12g19660	3.07127	3.43E-25	1.02E-22	Putative glycosyltransferase

Table 7.2. An abbreviated list of genes whose abundance shows a statistically significant decrease (LogFC>3 or <-3; P<0.05; FDR<0.05) in abundance in RNA-Seq data in comparisons between total RNA samples extracted from the first leaves of 6hr TSWV- and mock-inoculated 25 day old pepper plants.

Gene_ID	logFC	PValue	FDR	Annotation
CA01g01420	-4.15222	2.12E-21	3.98E-19	Unknown protein
CA01g03070	-4.3874	3.37E-34	2.59E-31	UDP-Glc-4-epimerase
CA01g11580	-4.03191	2.49E-06	3.42E-05	CXE carboxylesterase
CA01g15970	-3.19067	1.80E-08	4.10E-07	Detected protein of confused Function
CA01g24960	-4.21756	3.70E-34	2.72E-31	Sugar transporter ERD6-like protein
CA01g25760	-3.23415	7.76E-11	2.95E-09	PREDICTED: monothiol glutaredoxin-S3-like [Solanum tuberosum]
CA01g25990	-3.62374	0.006178	0.026235	DNA-directed RNA polymerase subunit alpha
CA01g26000	-3.8956	0.008004	0.032313	ribosomal protein S11 [Nicotiana tomentosiformis]
CA01g26320	-3.40391	2.48E-05	0.000249	Detected protein of unknown function
CA01g33100	-3.63272	2.73E-11	1.12E-09	PREDICTED: ABC transporter G family member 14-like [Solanum lycopersicum]
CA01g33440	-3.79064	1.83E-11	7.85E-10	3-hydroxy-3-methylglutaryl coenzyme A synthase
CA02g02820	-4.2052	2.78E-07	4.86E-06	Calcium-binding EF-hand family protein, putative [Theobroma cacao]
CA02g06030	-3.77434	1.75E-07	3.22E-06	PREDICTED: chaperone protein dnaJ 11, chloroplastic-like [Solanum lycopersicum]
CA02g06560	-3.20142	2.12E-17	2.31E-15	PREDICTED: transcription factor bHLH64-like [Solanum tuberosum]
CA02g09520	-4.73057	1.95E-17	2.14E-15	UV-induced sesquiterpene cyclase
CA02g09530	-3.15231	7.84E-06	9.18E-05	CYP71D20v2
CA02g09540	-3.29495	1.77E-08	4.05E-07	PREDICTED: premnaspirodiene oxygenase-like [Solanum tuberosum]
CA02g09570	-3.98691	1.24E-08	2.94E-07	CYP71D51v2
CA02g11040	-3.03756	1.72E-13	1.07E-11	PREDICTED: caltractin-like [Solanum tuberosum]
CA02g12030	-3.21655	2.45E-07	4.36E-06	PREDICTED: LRR receptor-like serine/threonine-protein kinase FLS2-like [Solanum tuberosum]
CA02g15240	-3.64037	1.14E-20	1.98E-18	PREDICTED: auxin-repressed 12.5 kDa protein-like isoform X1 [Solanum tuberosum]
CA02g15540	-5.80281	1.29E-19	1.95E-17	PREDICTED: homeobox-leucine zipper protein ATHB-52-like [Solanum tuberosum]
CA02g15910	-3.26148	4.96E-05	0.000455	PREDICTED: uncharacterized LOC101206260 isoform 3 [Cucumis sativus]
CA02g15940	-3.21442	4.77E-06	5.98E-05	Detected protein of unknown function
CA02g15970	-3.11818	2.18E-05	0.000222	PREDICTED: anther-specific protein TA-29-like [Solanum lycopersicum]
CA02g16010	-3.35688	2.84E-06	3.83E-05	Cyclin
CA02g16190	-3.43659	5.93E-05	0.000526	Detected protein of unknown function

CA02g21270	-3.16542	5.24E-17	5.35E-15	Unknown protein
CA02g26070	-3.31257	1.08E-08	2.59E-07	Unknown protein
CA03g00110	-3.11007	9.22E-20	1.44E-17	Beta-galactosidase
CA03g03540	-3.43576	8.35E-10	2.52E-08	ATP binding protein, putative
CA03g03750	-3.22791	4.41E-24	1.11E-21	DNA binding protein, putative
CA03g05640	-7.7261	1.17E-14	8.38E-13	S25-XP1 DNA binding protein
CA03g09890	-3.72848	3.73E-18	4.59E-16	Sec14p-like phosphatidylinositol transfer family protein, putative isoform 1 [Theobroma cacao]
CA03g15850	-5.78317	9.93E-15	7.19E-13	PME inhibitor
CA03g16270	-3.9145	9.28E-20	1.44E-17	Unknown protein
CA03g23550	-3.39605	3.97E-15	3.10E-13	Tumor-related protein
CA03g26130	-4.47981	2.65E-64	2.14E-60	PREDICTED: cytochrome P450 71A4-like [Solanum tuberosum]
CA03g26530	-3.39068	3.65E-12	1.82E-10	Detected protein of unknown function
CA03g33540	-4.25383	1.54E-23	3.61E-21	Detected protein of unknown function
CA03g36930	-4.06959	4.06E-10	1.30E-08	ACRE 132-like protein
CA04g02550	-4.49433	1.65E-06	2.36E-05	PREDICTED: stem-specific protein TSJT1-like [Solanum tuberosum]
CA04g07970	-3.36414	7.30E-25	2.00E-22	Unknown protein
CA04g10620	-3.45952	1.13E-11	4.98E-10	Pepper esterase
CA04g20830	-4.98383	1.81E-28	7.50E-26	Unknown protein
CA04g22730	-3.73419	6.29E-33	4.06E-30	BTB/POZ domain-containing protein
CA05g03160	-3.54997	6.77E-20	1.08E-17	ER glycerol-phosphate acyltransferase
CA05g03770	-3.01138	2.98E-35	2.67E-32	PREDICTED: MATE efflux family protein 6-like [Solanum lycopersicum]
CA05g09300	-5.67825	0.000513	0.003304	Cytochrome b
CA05g14230	-3.42583	5.72E-15	4.37E-13	Ethylene responsive element binding protein C3
CA05g18120	-3.56667	3.62E-17	3.80E-15	PREDICTED: ankyrin repeat-containing protein At5g02620-like isoform 1 [Solanum lycopersicum]
CA06g01390	-3.11455	7.40E-09	1.86E-07	Unknown protein
CA06g03090	-3.38082	7.24E-82	1.17E-77	F21O3.2 protein
CA06g16480	-3.55604	9.57E-05	0.000795	PREDICTED: transcription factor bHLH87-like [Solanum lycopersicum]
CA06g16490	-3.91493	1.02E-10	3.79E-09	PREDICTED: transcription factor bHLH87-like [Solanum lycopersicum]
CA07g01140	-3.47189	7.07E-19	9.92E-17	Auxin-induced protein 5NG4, putative
CA07g16060	-3.59238	1.46E-21	2.81E-19	Gibberellin 2-oxidase
CA07g17050	-3.35054	9.52E-07	1.46E-05	Epidermis-specific secreted glycoprotein EP1, putative

CA07g19020	-3.28302	2.17E-08	4.85E-07	Detected protein of unknown function
CA07g20710	-3.99717	0.001258	0.007066	PREDICTED: F-box/kelch-repeat protein At3g23880-like [Solanum tuberosum]
CA07g20730	-4.33262	0.002983	0.01438	PREDICTED: F-box/kelch-repeat protein At3g23880-like [Solanum tuberosum]
CA07g20740	-4.4282	0.001916	0.009964	metal ion binding protein, putative [Ricinus communis]
CA07g21660	-3.88647	0.000236	0.001712	Unknown protein
CA08g02470	-4.44226	4.67E-09	1.21E-07	PREDICTED: C2 domain-containing protein At1g63220-like [Oryza brachyantha]
CA08g09160	-3.67692	0.00587	0.025206	cytochrome b6-f complex subunit IV (chloroplast) [Camellia taliensis]
CA08g09220	-4.44505	0.008861	0.035038	50S ribosomal protein L2, chloroplastic
CA08g15570	-3.01106	5.65E-06	6.89E-05	PREDICTED: subtilisin-like protease SDD1-like [Solanum tuberosum]
CA08g15580	-3.01119	5.64E-06	6.87E-05	Subtilisin-like serine protease
CA09g02800	-3.35771	3.88E-15	3.04E-13	ATP binding protein, putative
CA09g16570	-3.8876	1.32E-28	6.11E-26	Beta-galactosidase
CA09g16790	-3.44822	3.90E-32	2.10E-29	Myo-inositol oxygenase
CA09g17630	-3.02591	0.009432	0.036869	NBS-coding resistance gene analog (Fragment)
CA09g17650	-3.02039	0.010268	0.039395	Disease resistance protein BS2
CA09g17660	-3.02039	0.010268	0.039395	Disease resistance protein BS2
CA10g00490	-4.01555	5.59E-06	6.83E-05	SNF4b family protein [Populus trichocarpa]
CA10g03980	-3.86775	6.49E-22	1.34E-19	wound/stress protein precursor [Solanum lycopersicum]
CA10g15710	-4.15781	0.000681	0.004207	Tonoplast intrinsic protein bobTIP26-1
CA10g16710	-3.31974	1.80E-08	4.10E-07	Apoplastic invertase
CA10g19080	-3.39781	4.68E-29	2.22E-26	Detected protein of unknown function
CA11g01840	-4.25879	0.010389	0.039748	Catalytic, putative
CA11g10820	-3.68602	7.97E-20	1.26E-17	PREDICTED: bidirectional sugar transporter SWEET15-like [Citrus sinensis]
CA11g11330	-3.42877	3.50E-23	7.95E-21	Alpha-L-arabinofuranosidase/beta-D-xylosidase
CA11g18060	-3.99227	2.69E-12	1.37E-10	Serine carboxypeptidase III
CA11g18070	-3.09054	8.06E-11	3.05E-09	Serine carboxypeptidase III
CA12g00150	-4.65507	1.04E-60	5.62E-57	Tumor-related protein
CA12g05090	-4.00794	1.50E-08	3.50E-07	PREDICTED: cytochrome P450 71D7-like [Solanum tuberosum]
CA12g20490	-3.59776	1.15E-06	1.73E-05	Xyloglucan endo-transglycosylase (Precursor)
CA12g20510	-6.07405	4.89E-12	2.37E-10	Gypsy/Ty3 element polyprotein, putative
CA12g20520	-6.07405	4.89E-12	2.37E-10	Gypsy/Ty-3 retroelement polyprotein; 69905-74404

CA12g22650	-3.11694	3.82E-05	0.000361	PREDICTED: protein ECERIFERUM 1-like [Solanum tuberosum]
CA12g22670	-3.1687	2.10E-05	0.000216	PREDICTED: protein ECERIFERUM 1-like [Solanum lycopersicum]

7.1.2 Polysomal RNA

Table 7.3. An abbreviated list of genes whose abundance shows a statistically significant increase ($\text{LogFC} > 3$ or < -3 ; $P < 0.05$; $\text{FDR} < 0.05$) in abundance in RNA-Seq data in comparisons between polysomal RNA samples extracted from the first leaves of 6hr TSWV- and mock-inoculated 25 day old pepper plants.

Gene_ID	logFC	PValue	FDR	Annotation
CA01g01590	3.146861	8.25E-17	9.25E-15	NADPH oxidase
CA01g11080	3.075999	5.22E-09	1.34E-07	PREDICTED: methylsterol monooxygenase 1-1-like [Solanum tuberosum]
CA01g19770	3.398498	2.91E-28	1.00E-25	Fructose-bisphosphate aldolase
CA01g20620	3.943189	0.000194	0.001477	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic
CA01g20630	3.086294	0.000334	0.002364	ribosomal protein L20 (chloroplast) [Capsicum annuum]
CA01g20640	4.97359	5.84E-06	7.09E-05	clp protease proteolytic subunit (chloroplast) [Capsicum annuum]
CA01g20650	6.343798	1.13E-05	0.000125	PREDICTED: ATP-dependent Clp protease proteolytic subunit-like, partial [Fragaria vesca] subsp. vesca
CA01g20680	4.246534	6.94E-06	8.22E-05	cytochrome b6/f complex subunit IV [Ceratophyllum demersum]
CA01g20690	3.523701	0.000105	0.000877	ribosomal protein S11 [Nicotiana tabacum]
CA01g20700	6.702928	1.85E-05	0.000193	Polygalacturonase, putative
CA01g20720	3.08078	0.000122	0.000998	NADH-plastoquinone oxidoreductase subunit 2 [Coffea arabica]
CA01g24400	3.332398	1.43E-19	2.25E-17	PREDICTED: transcription factor MYB12-like [Solanum tuberosum]
CA01g24460	3.130536	9.45E-05	0.000802	Unknown protein
CA01g29300	3.273458	2.68E-28	9.41E-26	Detected protein of unknown function
CA01g32810	4.879008	1.13E-14	8.75E-13	Acyltransferase 2 (Fragment)
CA01g32820	3.618481	2.04E-18	2.66E-16	Acyltransferase 2 (Fragment)
CA01g32940	4.032176	3.21E-07	5.43E-06	Acyltransferase 2 (Fragment)
CA01g33460	3.852483	6.35E-07	9.83E-06	CBF/DREB-like transcription factor 1
CA01g33480	4.057319	7.22E-06	8.49E-05	C-repeat binding facor
CA01g33490	3.067586	0.000141	0.00113	C-repeat binding facor
CA01g34630	5.728217	7.70E-34	4.61E-31	MYB transcription factor MYB92
CA02g06440	3.381068	7.71E-10	2.40E-08	Putative At5g37260 (Fragment)
CA02g08660	9.334072	2.93E-11	1.22E-09	Pectate lyase, putative
CA02g18810	3.117138	3.28E-12	1.67E-10	Putative At5g37260-like protein (Fragment)

CA02g20380	3.358545	0.008082	0.034141	Acyl:coa ligase acetate-coa synthetase-like protein
CA02g23780	4.730766	2.89E-62	1.17E-58	Myosin heavy chain, putative
CA02g26410	3.059127	7.15E-18	8.88E-16	Cinnamoyl-CoA reductase, putative
CA03g06220	3.579908	2.01E-10	7.11E-09	Basic helix-loop-helix protein
CA03g15030	3.336499	0.005237	0.02404	Photosystem II cp47 protein (Fragment)
CA03g24550	3.255304	0.00024	0.001784	Cytochrome P450 A
CA03g32280	3.492229	8.57E-13	4.86E-11	PREDICTED: ethylene-responsive transcription factor ERF003-like [Solanum tuberosum]
CA03g34540	3.695956	2.43E-13	1.53E-11	Auxin-responsive protein IAA1, putative
CA03g36940	3.227824	3.88E-09	1.03E-07	Unknown protein
CA04g02100	3.355258	2.34E-21	4.35E-19	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570-like [Solanum tuberosum]
CA04g04080	3.552979	4.94E-31	2.28E-28	Phytoene synthase
CA04g04320	3.434474	1.21E-15	1.09E-13	PREDICTED: LRR receptor-like serine/threonine-protein kinase GSO1-like [Solanum lycopersicum]
CA04g15480	3.852764	9.72E-27	2.85E-24	PREDICTED: probable leucine-rich repeat receptor-like protein kinase At1g35710-like [Solanum tuberosum]
CA04g15970	3.269028	8.33E-11	3.16E-09	Hydrolase, putative
CA04g18280	3.939216	3.60E-10	1.22E-08	CYP81C6v2
CA04g19810	3.108132	2.95E-05	0.00029	Detected protein of unknown function
CA05g05850	3.547054	5.91E-24	1.45E-21	PREDICTED: zeatin O-glucosyltransferase-like [Solanum lycopersicum]
CA06g12640	3.65738	5.31E-05	0.000488	PREDICTED: phospholipase A1-1beta2, chloroplastic-like [Solanum tuberosum]
CA06g17550	3.039483	9.04E-17	1.01E-14	Detected protein of unknown function
CA06g17720	3.096925	8.47E-45	1.37E-41	PREDICTED: calcium-binding allergen Ole e 8-like [Solanum tuberosum]
CA06g23860	4.432504	0.00662	0.029004	Unknown protein
CA06g24300	3.175111	7.16E-49	1.44E-45	Flavone synthase II
CA07g04400	4.906985	1.10E-34	6.85E-32	Tetratricopeptide repeat-like superfamily protein, putative [Theobroma cacao]
CA07g11530	4.507213	6.63E-19	9.56E-17	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g11540	4.020036	8.72E-12	4.03E-10	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g17080	3.464262	0.003656	0.017944	CYP72A58
CA07g17090	3.65973	0.007862	0.033393	CYP72A57
CA07g17100	3.408481	0.004888	0.022692	CYP72A58
CA07g17130	6.050388	0.001558	0.008728	CYP72A58
CA07g19240	3.108219	3.91E-13	2.36E-11	Ubiquitin-conjugating enzyme variant, putative
CA08g01270	3.530995	1.64E-11	7.16E-10	CMPG1b

CA08g10440	5.357533	9.71E-07	1.43E-05	Detected protein of unknown function
CA08g10450	7.29434	1.10E-05	0.000122	Calmodulin binding protein, putative
CA08g11580	4.198595	1.04E-40	1.20E-37	PREDICTED: broad-range acid phosphatase DET1-like [Solanum lycopersicum]
CA08g11590	5.449492	1.05E-13	7.01E-12	Serine/threonine-protein kinase bri1, putative
CA08g13530	3.236104	1.23E-20	2.11E-18	Ferric-chelate reductase, putative
CA08g16140	4.084435	1.20E-15	1.09E-13	Leucoanthocyanidin dioxygenase, putative
CA09g07680	3.705044	6.27E-06	7.50E-05	PREDICTED: myb-related protein Myb4-like [Solanum lycopersicum]
CA09g08840	5.867529	0.006462	0.028489	unnamed protein product (chloroplast) [Nicotiana undulata]
CA10g02980	3.620335	0.007148	0.030875	NRC1
CA10g03640	3.586029	1.21E-46	2.18E-43	Quercetin 3-O-glucoside-6''-O-malonyltransferase
CA10g05630	3.77696	2.00E-16	2.03E-14	UDP-glucosyltransferase, putative
CA10g12130	3.644449	3.54E-21	6.49E-19	Flavonoid glucosyltransferase UGT73E2
CA10g16530	5.297895	2.42E-15	1.99E-13	Flavonoid 3-glucosyl transferase
CA10g18200	3.17734	5.31E-10	1.71E-08	KED
CA11g00570	3.449162	1.04E-39	9.81E-37	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum tuberosum]
CA11g00600	3.062655	1.45E-27	4.78E-25	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum lycopersicum]
CA11g14880	3.283864	8.43E-14	5.74E-12	Putrescine hydroxycinnamoyl transferase
CA11g17090	6.655551	1.35E-38	1.09E-35	Unknown protein
CA12g02050	3.062141	1.09E-39	9.81E-37	Glucosyltransferase
CA12g07190	5.807617	0.000895	0.005491	NADH dehydrogenase subunit 2 (Fragment)
CA12g12410	3.158076	1.38E-18	1.89E-16	Cytochrome P450 CYP736A54
CA12g14160	4.754732	0.000583	0.003809	Photosystem I P700 chlorophyll a apoprotein A1
CA12g16620	3.223329	2.22E-41	2.76E-38	UDP-glucose glucosyltransferase
CA12g19660	3.496484	6.13E-31	2.75E-28	Putative glycosyltransferase
EF1a_AY496125	4.949768	0.00088	0.005426	Elongation factor 1-alpha (UniProt)

Table 7.4. An abbreviated list of genes whose abundance shows a statistically significant decrease ($\log FC > 3$ or < -3 ; $P < 0.05$; $FDR < 0.05$) in abundance in RNA-Seq data in comparisons between polysomal RNA samples extracted from the first leaves of 6hr TSWV- and mock-inoculated 25 day old pepper plants.

Gene_ID	logFC	PValue	FDR	Annotation
CA01g01420	-3.70915	1.24E-18	1.71E-16	Unknown protein
CA01g03070	-4.72033	3.51E-38	2.70E-35	UDP-Glc-4-epimerase
CA01g11580	-4.51822	2.72E-07	4.73E-06	CXE carboxylesterase
CA01g15970	-3.24124	1.16E-08	2.76E-07	Detected protein of confused Function
CA01g21040	-11.8391	0.009162	0.037788	F23N19.13
CA01g24960	-3.85043	4.25E-29	1.67E-26	Sugar transporter ERD6-like protein
CA01g25760	-3.293	3.80E-11	1.55E-09	PREDICTED: monothiol glutaredoxin-S3-like [Solanum tuberosum]
CA01g25770	-3.54748	1.06E-13	7.07E-12	PREDICTED: monothiol glutaredoxin-S10-like [Solanum tuberosum]
CA01g26320	-3.35776	2.99E-05	0.000293	Detected protein of unknown function
CA01g27100	-3.01564	3.17E-06	4.12E-05	PREDICTED: RING-H2 finger protein ATL73-like [Solanum lycopersicum]
CA01g33100	-3.92007	1.51E-12	8.28E-11	PREDICTED: ABC transporter G family member 14-like [Solanum lycopersicum]
CA01g33440	-3.99102	2.47E-12	1.29E-10	3-hydroxy-3-methylglutaryl coenzyme A synthase
CA01g33780	-3.07009	2.37E-27	7.37E-25	Nudix hydrolase
CA02g02820	-4.11965	4.48E-07	7.31E-06	Calcium-binding EF-hand family protein, putative [Theobroma cacao]
CA02g06030	-3.14684	5.91E-06	7.16E-05	PREDICTED: chaperone protein dnaJ 11, chloroplastic-like [Solanum lycopersicum]
CA02g09520	-5.06253	3.44E-19	5.04E-17	UV-induced sesquiterpene cyclase
CA02g09530	-3.56981	7.74E-07	1.17E-05	CYP71D20v2
CA02g09540	-3.31179	1.38E-08	3.27E-07	PREDICTED: premnaspirodiene oxygenase-like [Solanum tuberosum]
CA02g09570	-4.34418	1.08E-09	3.25E-08	CYP71D51v2
CA02g11040	-3.37605	3.31E-15	2.66E-13	PREDICTED: caltractin-like [Solanum tuberosum]
CA02g15240	-3.60585	2.27E-20	3.82E-18	PREDICTED: auxin-repressed 12.5 kDa protein-like isoform X1 [Solanum tuberosum]
CA02g15540	-4.96039	7.43E-16	6.93E-14	PREDICTED: homeobox-leucine zipper protein ATHB-52-like [Solanum tuberosum]
CA02g21270	-3.36268	2.14E-18	2.76E-16	Unknown protein
CA02g26070	-3.20737	2.63E-08	5.77E-07	Unknown protein
CA03g01640	-3.05156	0.000909	0.005564	Alcohol dehydrogenase, putative
CA03g03750	-3.6448	5.27E-29	2.03E-26	DNA binding protein, putative
CA03g05640	-5.96183	6.08E-11	2.38E-09	S25-XP1 DNA binding protein

CA03g08140	-3.05912	2.18E-39	1.85E-36	PREDICTED: 2-hydroxyisoflavanone dehydratase-like isoform X1 [Solanum tuberosum]
CA03g13130	-3.85607	0.009522	0.039009	Maturase K
CA03g13150	-6.3646	4.15E-05	0.000392	ribosomal protein S11 (chloroplast) [Solanum lycopersicum]
CA03g13160	-5.25043	0.000191	0.001458	DNA-directed RNA polymerase subunit alpha
CA03g15850	-5.73746	1.38E-14	1.04E-12	PME inhibitor
CA03g15860	-3.02946	1.50E-23	3.61E-21	PREDICTED: 21 kDa protein-like [Solanum tuberosum]
CA03g16270	-3.29821	3.19E-15	2.57E-13	Unknown protein
CA03g23550	-3.46851	1.34E-15	1.18E-13	Tumor-related protein
CA03g26130	-4.39349	1.23E-62	6.64E-59	PREDICTED: cytochrome P450 71A4-like [Solanum tuberosum]
CA03g26530	-3.13823	8.27E-11	3.15E-09	Detected protein of unknown function
CA03g29020	-3.19515	3.97E-08	8.37E-07	Glutamine dumper 2, putative [Theobroma cacao]
CA03g33540	-3.26258	4.93E-17	5.77E-15	Detected protein of unknown function
CA03g36930	-4.22768	1.96E-10	7.00E-09	ACRE 132-like protein
CA04g02550	-4.63034	9.34E-07	1.38E-05	PREDICTED: stem-specific protein TSJT1-like [Solanum tuberosum]
CA04g07970	-3.29145	4.12E-24	1.02E-21	Unknown protein
CA04g10620	-3.32655	4.89E-11	1.96E-09	Pepper esterase
CA04g13980	-4.14871	0.005294	0.024252	PREDICTED: nuclear transport factor 2-like [Solanum tuberosum]
CA04g20830	-4.84256	2.28E-27	7.22E-25	Unknown protein
CA04g22730	-3.9493	6.91E-36	4.65E-33	BTB/POZ domain-containing protein
CA05g03160	-3.12559	1.41E-16	1.48E-14	ER glycerol-phosphate acyltransferase
CA05g03770	-3.26223	1.72E-40	1.86E-37	PREDICTED: MATE efflux family protein 6-like [Solanum lycopersicum]
CA05g14500	-3.26554	1.20E-14	9.15E-13	PREDICTED: uncharacterized acetyltransferase At3g50280-like [Solanum tuberosum]
CA06g01390	-3.30772	1.81E-09	5.19E-08	Unknown protein
CA06g03090	-3.25997	6.84E-77	1.10E-72	F21O3.2 protein
CA06g16480	-4.13597	1.43E-05	0.000154	PREDICTED: transcription factor bHLH87-like [Solanum lycopersicum]
CA07g01140	-3.50495	2.33E-19	3.56E-17	Auxin-induced protein 5NG4, putative
CA07g16060	-3.87688	2.43E-24	6.22E-22	Gibberellin 2-oxidase
CA07g17050	-3.60966	1.80E-07	3.29E-06	Epidermis-specific secreted glycoprotein EP1, putative
CA08g02470	-4.16304	1.74E-08	3.99E-07	PREDICTED: C2 domain-containing protein At1g63220-like [Oryza brachyantha]
CA08g15570	-3.26843	1.12E-06	1.62E-05	PREDICTED: subtilisin-like protease SDD1-like [Solanum tuberosum]
CA08g15580	-3.26885	1.11E-06	1.61E-05	Subtilisin-like serine protease

CA09g14360	-6.23929	0.003998	0.019325	Photosystem I P700 apoprotein A1 (Fragment)
CA09g16570	-4.1473	1.24E-31	6.47E-29	Beta-galactosidase
CA09g16790	-3.2935	1.62E-30	6.86E-28	Myo-inositol oxygenase
CA10g02510	-13.124	0.009169	0.037788	Serine/threonine-protein kinase
CA10g02520	-3.11392	0.00181	0.009958	orf124 gene product (mitochondrion) [Beta vulgaris] subsp. vulgaris
CA10g03980	-3.92103	2.05E-22	4.54E-20	wound/stress protein precursor [Solanum lycopersicum]
CA10g15710	-4.2049	0.0007	0.004462	Tonoplast intrinsic protein bobTIP26-1
CA10g16710	-3.45065	5.71E-09	1.45E-07	Apoplastic invertase
CA10g19080	-3.21217	8.32E-27	2.49E-24	Detected protein of unknown function
CA11g04440	-4.77123	5.14E-10	1.66E-08	NAP-like transcription factor
CA11g10820	-3.97667	2.77E-22	5.80E-20	PREDICTED: bidirectional sugar transporter SWEET15-like [Citrus sinensis]
CA11g11330	-3.43012	3.77E-23	8.82E-21	Alpha-L-arabinofuranosidase/beta-D-xylosidase
CA11g18060	-3.38971	1.09E-10	4.06E-09	Serine carboxypeptidase III
CA12g00150	-4.75829	7.03E-65	5.68E-61	Tumor-related protein
CA12g05090	-3.62327	2.82E-07	4.86E-06	PREDICTED: cytochrome P450 71D7-like [Solanum tuberosum]
CA12g20510	-4.37861	8.09E-08	1.58E-06	Gypsy/Ty3 element polyprotein, putative
CA12g20520	-4.37861	8.09E-08	1.58E-06	Gypsy/Ty-3 retroelement polyprotein; 69905-74404
CA12g22610	-3.56362	5.42E-06	6.62E-05	Fatty acid desaturase
CA12g22620	-3.56115	6.87E-06	8.15E-05	Fatty acid desaturase
CA12g22630	-3.66237	4.98E-06	6.18E-05	Microsomal omega-6-desaturase
CA12g22650	-4.08577	2.77E-07	4.80E-06	PREDICTED: protein ECERIFERUM 1-like [Solanum tuberosum]
CA12g22670	-3.92764	3.51E-07	5.91E-06	PREDICTED: protein ECERIFERUM 1-like [Solanum lycopersicum]

7.1.3 Sub-polysomal RNA

Table 7.5. An abbreviated list of genes whose abundance shows a statistically significant increase ($\text{LogFC} > 3$ or < -3 ; $P < 0.05$; $\text{FDR} < 0.05$) in abundance in RNA-Seq data in comparisons between sub-polysomal RNA samples extracted from the first leaves of 6hr TSWV- and mock-inoculated 25 day old pepper plants.

Gene_ID	logFC	PValue	FDR	Annotation
CA01g24400	3.296434	1.09E-18	1.50E-16	PREDICTED: transcription factor MYB12-like [Solanum tuberosum]
CA01g29010	4.235785	8.75E-05	0.000718	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic
CA01g29300	3.60993	3.37E-33	3.40E-30	Detected protein of unknown function
CA01g32810	3.493767	9.54E-09	2.31E-07	Acyltransferase 2 (Fragment)
CA01g32820	3.506001	1.36E-17	1.59E-15	Acyltransferase 2 (Fragment)
CA01g32940	4.997161	1.99E-09	5.56E-08	Acyltransferase 2 (Fragment)
CA01g33460	3.011739	5.19E-05	0.000456	CBF/DREB-like transcription factor 1
CA01g34630	3.760823	1.49E-21	3.04E-19	MYB transcription factor MYB92
CA02g06440	3.011026	2.54E-08	5.53E-07	Putative At5g37260 (Fragment)
CA02g08660	4.11537	8.56E-06	9.53E-05	Pectate lyase, putative
CA02g08750	3.18971	1.23E-52	3.30E-49	Type 2 proly 4-hydroxylase
CA02g11140	3.134903	1.64E-07	2.89E-06	PREDICTED: cytochrome P450 71A2-like [Solanum lycopersicum]
CA02g20380	3.641828	0.004868	0.021349	Acyl:coa ligase acetate-coa synthetase-like protein
CA02g23780	4.318954	3.93E-54	1.59E-50	Myosin heavy chain, putative
CA02g24540	3.082494	0.000198	0.001446	DNA binding protein, putative
CA02g26410	3.015279	1.57E-17	1.80E-15	Cinnamoyl-CoA reductase, putative
CA02g28150	3.24532	3.65E-30	2.35E-27	Dof3 protein
CA02g30580	3.187725	2.27E-08	5.00E-07	Detected protein of unknown function
CA03g02150	6.716153	0.003424	0.015922	ribosomal protein S14 [Atropa belladonna]
CA03g06220	3.635056	1.12E-10	4.12E-09	Basic helix-loop-helix protein
CA03g24550	3.399705	0.000149	0.001137	Cytochrome P450 A
CA03g24570	3.034626	4.57E-07	7.19E-06	Cytochrome P450 A
CA03g34540	3.244933	5.35E-11	2.14E-09	Auxin-responsive protein IAA1, putative
CA04g02100	3.373975	4.24E-23	1.12E-20	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570-like [Solanum tuberosum]

CA04g03070	3.265056	1.01E-09	2.98E-08	ATP binding protein, putative
CA04g03880	3.174362	4.82E-39	5.56E-36	PREDICTED: probable LRR receptor-like serine/threonine-protein kinase At3g47570-like [Solanum tuberosum]
CA04g04080	3.357233	3.45E-28	1.59E-25	Phytoene synthase
CA04g04320	3.53967	2.16E-17	2.45E-15	PREDICTED: LRR receptor-like serine/threonine-protein kinase GSO1-like [Solanum lycopersicum]
CA04g06270	3.818332	4.05E-05	0.000367	Ycf68
CA04g15480	3.568568	2.40E-24	7.19E-22	PREDICTED: probable leucine-rich repeat receptor-like protein kinase At1g35710-like [Solanum tuberosum]
CA04g18280	4.026307	4.09E-10	1.33E-08	CYP81C6v2
CA04g19810	3.757787	8.41E-07	1.24E-05	Detected protein of unknown function
CA04g21000	3.035319	6.27E-05	0.000534	Jasmonate O-methyltransferase, putative
CA05g02030	3.130084	5.87E-09	1.48E-07	Kinase interacting protein 1
CA05g05850	3.49652	2.78E-23	7.49E-21	PREDICTED: zeatin O-glucosyltransferase-like [Solanum lycopersicum]
CA05g07270	3.946457	9.55E-12	4.43E-10	Polygalacturonase, putative
CA05g09300	3.779369	0.009575	0.037268	Cytochrome b
CA05g17570	3.22487	0.000381	0.002528	Triacylglycerol lipase, putative
CA06g15550	3.163916	2.59E-05	0.000249	Detected protein of unknown function
CA06g17550	3.284033	7.76E-19	1.11E-16	Detected protein of unknown function
CA06g17720	3.064863	2.05E-42	3.31E-39	PREDICTED: calcium-binding allergen Ole e 8-like [Solanum tuberosum]
CA06g24300	3.369812	1.10E-53	3.55E-50	Flavone synthase II
CA07g04400	4.547108	1.36E-30	9.55E-28	Tetratricopeptide repeat-like superfamily protein, putative [Theobroma cacao]
CA07g05330	4.676327	0.012124	0.044845	PREDICTED: histone H4-like [Solanum lycopersicum]
CA07g11510	3.276394	2.96E-10	9.91E-09	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g11530	4.395215	3.33E-18	4.30E-16	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g11540	4.042434	8.83E-12	4.15E-10	PREDICTED: mitogen-activated protein kinase kinase kinase A-like [Solanum tuberosum]
CA07g15330	3.179695	0.001391	0.007585	Polyprotein, putative
CA07g15350	4.110335	0.012289	0.045265	Os02g0253000 [Oryza sativa] Japonica Group
CA07g17080	4.194041	0.000766	0.00459	CYP72A58
CA07g17090	4.770409	0.001329	0.007316	CYP72A57
CA07g17100	4.765253	0.000307	0.0021	CYP72A58
CA08g10440	6.13672	6.97E-08	1.35E-06	Detected protein of unknown function
CA08g10450	7.378173	9.47E-06	0.000104	Calmodulin binding protein, putative
CA08g11580	4.330622	1.30E-42	2.33E-39	PREDICTED: broad-range acid phosphatase DET1-like [Solanum lycopersicum]

CA08g11590	5.257036	3.58E-13	2.26E-11	Serine/threonine-protein kinase bri1, putative
CA08g13530	3.206149	5.02E-20	8.62E-18	Ferric-chelate reductase, putative
CA08g16140	4.39836	2.38E-17	2.67E-15	Leucoanthocyanidin dioxygenase, putative
CA09g13830	3.932179	9.84E-12	4.54E-10	Unknown protein
CA10g03640	3.548274	4.26E-45	8.60E-42	Quercetin 3-O-glucoside-6''-O-malonyltransferase
CA10g05630	3.857463	6.44E-17	6.71E-15	UDP-glucosyltransferase, putative
CA10g10860	3.177763	4.85E-17	5.12E-15	PREDICTED: phosphatidylinositol:ceramide inositolphosphotransferase 2-like [Solanum lycopersicum]
CA10g12130	3.678097	7.87E-21	1.46E-18	Flavonoid glucosyltransferase UGT73E2
CA10g16530	5.541603	2.39E-15	2.11E-13	Flavonoid 3-glucosyl transferase
CA10g18200	3.148407	7.30E-10	2.24E-08	KED
CA10g18300	3.165855	1.34E-21	2.78E-19	PREDICTED: monoacylglycerol lipase ABHD6-like [Citrus sinensis]
CA11g00570	3.56841	9.19E-42	1.24E-38	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum tuberosum]
CA11g00600	3.847341	3.12E-40	3.87E-37	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum lycopersicum]
CA11g00610	4.029381	5.11E-14	3.63E-12	PREDICTED: probable serine/threonine-protein kinase At1g18390-like [Solanum lycopersicum]
CA11g14880	3.01082	4.69E-12	2.35E-10	Putrescine hydroxycinnamoyl transferase
CA11g17090	3.707203	2.62E-17	2.90E-15	Unknown protein
CA11g19540	5.062983	0.001863	0.00966	orf86 [Vitis vinifera]
CA12g16620	3.364462	3.48E-42	5.10E-39	UDP-glucose glucosyltransferase

Table 7.6. An abbreviated list of genes whose abundance shows a statistically significant decrease ($\log FC > 3$ or < -3 ; $P < 0.05$; $FDR < 0.05$) in abundance in RNA-Seq data in comparisons between sub-polysomal RNA samples extracted from the first leaves of 6hr TSWV- and mock-inoculated 25 day old pepper plants.

Gene_ID	logFC	PValue	FDR	Annotation
CA01g01420	-3.78129	1.30E-18	1.76E-16	Unknown protein
CA01g03070	-3.96812	2.78E-29	1.49E-26	UDP-Glc-4-epimerase
CA01g11580	-4.50791	2.76E-07	4.62E-06	CXE carboxylesterase
CA01g24960	-3.27294	1.71E-23	4.84E-21	Sugar transporter ERD6-like protein
CA01g25770	-3.12111	1.35E-08	3.15E-07	PREDICTED: monothiol glutaredoxin-S10-like [Solanum tuberosum]
CA01g26320	-3.07483	0.000127	0.000985	Detected protein of unknown function
CA01g27100	-3.19364	1.00E-06	1.44E-05	PREDICTED: RING-H2 finger protein ATL73-like [Solanum lycopersicum]
CA01g33440	-3.86132	1.42E-11	6.26E-10	3-hydroxy-3-methylglutaryl coenzyme A synthase
CA02g02820	-4.70835	5.86E-08	1.15E-06	Calcium-binding EF-hand family protein, putative [Theobroma cacao]
CA02g06030	-3.8972	8.10E-08	1.56E-06	PREDICTED: chaperone protein dnaJ 11, chloroplastic-like [Solanum lycopersicum]
CA02g09520	-4.90968	8.11E-18	1.01E-15	UV-induced sesquiterpene cyclase
CA02g09530	-4.69714	3.66E-08	7.60E-07	CYP71D20v2
CA02g09540	-3.26633	2.96E-08	6.31E-07	PREDICTED: premnaspirodiene oxygenase-like [Solanum tuberosum]
CA02g09570	-4.03362	2.29E-08	5.03E-07	CYP71D51v2
CA02g12050	-3.51155	2.14E-20	3.80E-18	Light harvesting chlorophyll a/b-binding protein (Precursor)
CA02g12070	-3.23464	1.72E-15	1.55E-13	Chlorophyll a/b binding protein
CA02g15540	-5.55417	1.54E-18	2.07E-16	PREDICTED: homeobox-leucine zipper protein ATHB-52-like [Solanum tuberosum]
CA02g16200	-3.03441	8.40E-08	1.61E-06	Detected protein of unknown function
CA02g21880	-3.22691	9.56E-18	1.15E-15	Detected protein of unknown function
CA02g26070	-3.08821	8.93E-08	1.69E-06	Unknown protein
CA03g00110	-3.20298	1.29E-20	2.34E-18	Beta-galactosidase
CA03g01690	-3.24249	1.48E-07	2.64E-06	PREDICTED: uncharacterized mitochondrial protein AtMg00810-like [Glycine max]
CA03g03750	-3.61041	8.03E-29	3.93E-26	DNA binding protein, putative
CA03g05640	-5.69587	4.53E-10	1.47E-08	S25-XP1 DNA binding protein
CA03g09890	-3.20067	3.04E-16	2.96E-14	Sec14p-like phosphatidylinositol transfer family protein, putative isoform 1 [Theobroma cacao]
CA03g13270	-3.39059	0.007902	0.031926	NADH dehydrogenase subunit 2 (Fragment)
CA03g15850	-5.33656	3.12E-13	1.99E-11	PME inhibitor

CA03g16270	-3.80392	2.18E-19	3.48E-17	Unknown protein
CA03g26130	-3.84749	7.19E-49	1.66E-45	PREDICTED: cytochrome P450 71A4-like [Solanum tuberosum]
CA03g26530	-3.63533	1.57E-13	1.04E-11	Detected protein of unknown function
CA03g33540	-3.01037	1.22E-14	9.76E-13	Detected protein of unknown function
CA03g36930	-4.22435	2.13E-10	7.37E-09	ACRE 132-like protein
CA04g02550	-3.48478	9.83E-05	0.000793	PREDICTED: stem-specific protein TSJT1-like [Solanum tuberosum]
CA04g07970	-3.02506	5.15E-21	1.01E-18	Unknown protein
CA04g20830	-4.21306	3.06E-22	6.87E-20	Unknown protein
CA05g03160	-3.05594	6.85E-15	5.76E-13	ER glycerol-phosphate acyltransferase
CA05g14500	-3.17176	1.49E-12	8.10E-11	PREDICTED: uncharacterized acetyltransferase At3g50280-like [Solanum tuberosum]
CA05g15490	-3.36926	3.95E-14	2.85E-12	PREDICTED: abscisic acid receptor PYL4-like [Solanum tuberosum]
CA06g01390	-3.32475	5.20E-09	1.34E-07	Unknown protein
CA06g03090	-3.30299	6.31E-75	1.02E-70	F21O3.2 protein
CA06g16480	-3.70722	8.03E-05	0.000666	PREDICTED: transcription factor bHLH87-like [Solanum lycopersicum]
CA07g01140	-4.06834	2.29E-23	6.28E-21	Auxin-induced protein 5NG4, putative
CA07g16060	-3.80515	3.13E-22	6.93E-20	Gibberellin 2-oxidase
CA08g02470	-4.8719	1.21E-09	3.53E-08	PREDICTED: C2 domain-containing protein At1g63220-like [Oryza brachyantha]
CA08g15560	-4.19636	1.82E-13	1.19E-11	PREDICTED: protein ROOT PRIMORDIUM DEFECTIVE 1-like [Solanum tuberosum]
CA08g15570	-6.12093	2.74E-15	2.38E-13	PREDICTED: subtilisin-like protease SDD1-like [Solanum tuberosum]
CA08g15580	-6.12113	2.72E-15	2.37E-13	Subtilisin-like serine protease
CA09g02800	-3.03515	7.09E-13	4.16E-11	ATP binding protein, putative
CA09g13400	-3.53406	0.007081	0.029149	Hypothetical chloroplast RF1
CA09g16570	-3.99492	7.76E-29	3.92E-26	Beta-galactosidase
CA10g01270	-4.65696	0.00055	0.003476	Sodium/hydrogen exchanger
CA10g19080	-3.52015	1.16E-30	8.92E-28	Detected protein of unknown function
CA11g04440	-3.49992	1.67E-06	2.29E-05	NAP-like transcription factor
CA11g10820	-3.10416	3.81E-14	2.77E-12	PREDICTED: bidirectional sugar transporter SWEET15-like [Citrus sinensis]
CA11g11330	-3.27403	1.76E-21	3.55E-19	Alpha-L-arabinofuranosidase/beta-D-xylosidase
CA11g18060	-3.13845	6.02E-09	1.51E-07	Serine carboxypeptidase III
CA12g00150	-4.82425	2.69E-65	2.17E-61	Tumor-related protein
CA12g03180	-4.81079	1.18E-08	2.78E-07	Protein TRANSPARENT TESTA

CA12g03190	-4.16291	6.34E-10	1.98E-08	Multidrug resistance pump, putative
CA12g05090	-4.22017	3.74E-09	9.86E-08	PREDICTED: cytochrome P450 71D7-like [Solanum tuberosum]
CA12g14160	-4.19457	0.001886	0.009746	Photosystem I P700 chlorophyll a apoprotein A1
CA12g16920	-3.07165	1.28E-30	9.40E-28	PREDICTED: protein MIZU-KUSSEI 1-like [Solanum tuberosum]
CA12g20510	-3.96459	1.69E-06	2.31E-05	Gypsy/Ty3 element polyprotein, putative
CA12g20520	-3.96459	1.69E-06	2.31E-05	Gypsy/Ty-3 retroelement polyprotein; 69905-74404
CA12g22610	-4.68927	1.53E-08	3.49E-07	Fatty acid desaturase
CA12g22620	-4.71696	1.85E-08	4.11E-07	Fatty acid desaturase
CA12g22630	-4.05449	1.87E-06	2.53E-05	Microsomal omega-6-desaturase
CA12g22670	-3.57989	2.71E-06	3.49E-05	PREDICTED: protein ECERIFERUM 1-like [Solanum lycopersicum]