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In this investigation we examined the molecular architecture and functions associated with the virion components of the *Beet yellows virus* (BYV), family *Closteroviridae*. The BYV virions are filamentous particles composed of two coat proteins, the major coat protein (CP), which encapsidates the majority of the genome, and the minor coat protein (CPm) that makes a small tail-like structure at one end of the virions. The bipolar architecture of closteroviruses is unique among the plant helical viruses, and suggests that CP and CPm evolved to fulfill different functions in the life cycle of closteroviruses. Current models propose that the CPm tails are specialized virion components that participate in the vector transmission and cell-to-cell movement of the virus. In addition to CP and CPm, the cell-to-cell movement of BYV requires the contributions of three highly conserved viral genes encoding a small 6.4-kDa hydrophobic protein (p6), a HSP70 homologue (HSP70h) and a 64-kDa protein (p64). Using nano-liquid chromatography/tandem mass spectrometry and biochemical analyses we demonstrate that HSP70h and p64 are associated with virions. This conclusion is based on the co-migration of HSP70h and p64 with BYV virions in sucrose density gradients, and on the co-immunoprecipitation of HSP70h, p64 and BYV capsid protein using anti-HSP70h or anti-p64 serum. The association of HSP70h and p64 with the virions is resistant to high concentrations of sodium chloride, which normally disrupt non-covalent protein interactions. Low concentrations of sodium

dodecyl sulfate (SDS) or lithium chloride (LiCl), and treatment with alkaline or acidic pH resulted in the simultaneous disassembly of virions and dissociation of HSP70h and p64. The N-terminal domain of p64 is exposed at the virion surface and is accessible to antibodies and mild trypsin digestion. In contrast, the C-terminal domain is embedded in the virion and is inaccessible to antibodies or trypsin. The C-terminal domain of p64 is shown to be homologous to CP and CPm. Mutation of the signature motifs of capsid proteins of filamentous RNA viruses in p64 results in the formation of defective virions, which are unable to move from cell to cell. These results reveal the dual function of HSP70h and p64 in tail assembly and BYV motility and support the concept of the virion tail as a specialized device for BYV cell-to-cell movement.

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Valerian Dolja was involved in the major experimental design of the research and provided scientific inspiration, interpretation and editorial advice on all chapters. Bryce W. Falk provided scientific advice, anti-CP antibodies and important technical support regarding purification of BYV virions. Dina V. Alzhanova generated the cDNA clones used to analyze the protein composition of virions produced by BYV variants with inactive HSP70h or p64 proteins. Catalin E. Doneanu was responsible for the nano-liquid chromatography/tandem mass spectrometry analysis of HSP70h and p64 in the lab of Douglas F. Barofsky. Eugene V. Koonin provided the protein sequence analysis of p64, CP and CPm.

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Chapter 1

Introduction

The study of virus structure has been an essential element of virology since the discovery of viruses in the early 1900s. The original definition of a virus was solely rooted on the structural characteristics of the virus particle: an infectious agent smaller than bacteria composed of protein and nucleic acid. Since then, the structures of virtually all known viruses have been described to various levels of detail. Advances in molecular biology coupled with powerful microscopy techniques have generated a wealth of knowledge related to the architecture of viruses. These methods have revealed molecular composition and morphology of virus particles, in certain cases at the atomic resolution. In addition, these studies have also contributed to a better understanding of molecular processes that affect all aspects in the life cycle of a virus. These processes include virus entry into cell, regulation of assembly and disassembly, avoidance of host's defense mechanisms, and virus dissemination.

The virions of most viruses are composed of a protein shell, or capsid, which is made of multiple subunits of coat proteins that surround and protect the viral genome. There are two major types of virion architecture with distinct geometric arrangement of the coat protein subunits, icosahedral and helical. The helical viruses are further subdivided into rod-shaped viruses with rigid particles and filamentous viruses with flexible particles. Variations to these themes, in viruses with increasing architectural complexities, are usually expressed in the form of minor structural components that complement the basic architecture defined by the major coat protein. In addition, the capsids of some viruses are enveloped by a lipoprotein layer acquired from cellular membranes.

The best described virus with helical architecture is the *Tobacco mosaic virus* (TMV), genus *Tobamovirus* (Mathews, 1991). The particles of TMV are composed of a single coat protein that encapsidates and protects the viral RNA genome. The protein

subunits are arranged into a helical array that makes a rod-shape structure with a central canal. The RNA molecule is packed inside this rod in the form of a tight helix that closely follows the helical arrangement of the coat protein subunits. The stability of these particles is supported by protein-protein and protein-RNA interactions between the coat protein subunits and the viral RNA. Interestingly, the canal remains exposed at both ends of the particles, which have a convex end at the 3' end of the RNA and a concave end at the 5' end. Nonetheless, the TMV virions are exceptionally stable and can survive for many years outside of the host cell.

Although automation is used by virtually all viruses for the assembly of virions, the elements that promote this process are unique among diverse viruses. The coat proteins of some viruses have been shown to be able to form empty capsids in the absence of the viral genome (McDonald *et al.*, 1976, Zhao *et al.*, 1995). However, the assembly of most viruses depends on specific sequences or signals present in the viral genome that promote the initial nucleation of coat protein subunits and subsequent elongation of the particles (Hull, 2001). The assembly of viruses with complex architectures sometimes requires the additional functions of cellular or viral chaperone proteins (Cobbold *et al.*, 2001; Sullivan *et al.*, 2002). Although the contribution of these components to virus assembly remains unclear, it has been proposed that their functions could be necessary for proper protein folding, posttranslational processing of virion proteins, coordination of assembly, or the cellular trafficking of virion components from sites of viral replication to sites of viral assembly. Some viruses also evolved to couple the assembly of their virus particles with other processes such as replication and translation (Novak and Kirkegaard, 1994; Nugent *et al.*, 1999). The coupling of assembly with viral replication enhances the specificity of assembly for viral templates, and has also been proposed to function as an early form of RNA proofreading, ensuring that only templates that are viable for replication are selected for assembly (Nugent *et al.*, 1999).

In the case of TMV, the coat protein and viral RNA are sufficient for the assembly of infectious particles. In fact, TMV can be reconstructed *in vitro* by mixing coat protein subunits and genomic RNA (Fraenkel-Conrat & Williams, 1955). The origin of assembly in TMV consists of a small stem loop element located ~ 1000 nt upstream from the 3' end of the RNA genome (Mathews, 1991). The initiation of assembly begins by the recruitment of coat protein complexes that make a double disc structure with an open central core. Specific interactions between the double discs and the origin of assembly trigger a conformational change that results in the re-arrangement of the coat protein subunits into a helical array. This results in the packaging of a small section of RNA into the central core of the helical disk. The elongation of the rod proceeds by the addition of double discs as the RNA is drawn through the central core of the growing particle.

With the exception of TMV, there is very little known about the assembly of plant filamentous viruses, which include economically significant and well characterized virus families: *Potyviridae*, *Potexviridae* and *Closteroviridae*. Unlike TMV, the assembly of virions in the family *Potyviridae* and genus *Potexvirus* appears to start at the 5' end terminus of the RNA (Sit et al., 1994; Wu and Shaw, 1998). Nonetheless, *in vitro* analyses of *Potato virus Y* (PVY) coat protein suggest that the assembly mechanisms could involve the formation of coat protein double disc structures (McDonald *et al.*, 1976). However, with the exception of *Potato virus X* (PVX), the *in vitro* assembly of flexuous virions have been largely unsuccessful (Kaftanova and Novikov, 1975). Therefore, it is possible that the efficient assembly of filamentous virions requires the contribution of unknown host factors or the activities of non-structural viral proteins.

One of the important functions of virions is transport of viruses within plants. The systemic infection of plants occurs via complementary pathways that involve the cell-to-cell movement of the virus from infected to healthy cells and its subsequent long-distance transport through the host's vascular system (Carrington *et al.*, 1996). One of

the distinguishing structural characteristics of plant cells is that their outer perimeter is enveloped by a thick rigid cell wall. This cell wall surrounds the plasma membrane and creates a physical barrier impenetrable to viruses. However, unlike animal and bacterial viruses, plant viruses never cross plasmamembranes. Instead, their movement follows the plant's symplastic network, which effectively joins the cytoplasm and endomembrane systems of virtually all cells in a plant. This network is composed of small pore structures that transverse the cell walls of adjacent cells, called plasmodesmata. Plasmodesmata normally support and regulate the transport of plant metabolites, small proteins and host RNAs, and in the context of a viral infection, they also support the cell-to-cell movement of plant viruses (Ehlers and Kollmann, 2001; Oparka and Roberts, 2001; Roberts & Oparka, 2003).

Plasmodesmata are composed of an outer sheath generated from the plasma membrane of neighboring cells that make a tube-like structure with an average diameter of 50 nm (Ehlers and Kollmann, 2001). The passage of molecules through the plasmodesmata is restricted by a desmotubule that extents throughout the entire length of the pore. The desmotubule originates from endoplasmic membranes and is coated with globular proteins that further restrict the effective size of molecules that can passively diffuse through the pores. The space between the desmotubule and plasma membrane creates a sleeve-like channel that joins the cytoplasms of adjacent cells, and it is through this small cytoplasmic sleeve that plant viruses are proposed to move. However, the effective diameter of this cytoplasmic channel normally restricts the passive diffusion of molecules with a mass greater than 1 kDa. Therefore, plasmodesmata pores are too small to allow the movement of plant viruses. To overcome this challenge, plant viruses evolved to encode specialized proteins that facilitate the transport of viruses across plasmodesmata, termed movement proteins (MP).

Original studies on the MP of TMV proposed that the major function of viral movement proteins was to increase the size exclusion limit (SEL) of plasmodesmata (Wolf *et al.*, 1989). This model was supported by investigations that used

microinjection techniques to show that the SEL of plasmodesmata in plants expressing MPs was much greater than the SEL of plasmodesmata in control plants. Although the MPs of many viruses possess similar gating activities, it is unlikely that this function alone is sufficient to allow the movement of viruses across plasmodesmata (Carrington *et al.*, 1996). The protein composition of plasmodesmata includes components normally associated with the trafficking machinery of the cell, such as myosin-like motor proteins and actin filaments, which suggest that the transport of macromolecules across plasmodesmata requires the functions of active cellular processes (White *et al.*, 1994; Blackman & Overall, 1998; Radford & White, 1998). Therefore, current cell-to-cell movement models propose that plant viruses evolved to take advantage of pre-existing cellular mechanisms that normally support the intercellular transport of proteins across plasmodesmata, and that the functions of MPs are likely to involve specific interactions with these components (Carrington et al., 1996; Haywood et al., 2002; Lee et al., 2003).

The two best described models for the cell-to-cell movement of plant viruses are provided by studies of TMV and cowpea mosaic virus (CPMV) (Carrington et al., 1996; Lazarowitz *et al.*, 1999). The cell-to-cell movement of TMV occurs by the translocation of MP-genome complexes across plasmodesmata and depends on the functions of a single 30-kDa MP (Citovsky *et al.*, 1992). The MP functions include modification of plasmodesmata, coating viral RNA, and interactions with microtubules, actin filaments, and ER membranes (McLean *et al.*, 1995; Oparka *et al.*, 2001; Reichel *et al.*, 1999). Although the mechanism by which the 30-kDa MP promotes the translocation of genomes across plasmodesmata remains unknown, the leading model suggests that it involves the recruitment of pre-existing cellular trafficking components (Haywood *et al.*, 2002; Lee *et al.*, 2003).

The second model applies to several families of the icosahedral RNA viruses and pararetroviruses and occurs via virus induced tubules through which mature virions are translocated across cells (Pouwels *et al.*, 2002; Thomas and Maule, 1999). The

tubules extend through plasmodesmata pores that have lost their desmotubules and are composed of MP and possibly other cellular components. The formation of the MP tubules requires the activities of the cellular endomembrane secretion system, and their proper localization to plasmodesmata depends on putative interactions with the cytoskeleton (Laporte *et al.,* 2003). Although the MP tubules are large enough to allow the passage of virions, their translocation across cells is likely to depend on dynamic MP-virion interactions, as suggested by the observed affinity of the MP to virions (Wellink and van Kammen, 1989).

Mounting evidence indicates that the cell-to-cell movement of the filamentous potexviruses does not fit any of the above mentioned models. Although assembly is a prerequisite for movement, potexviruses do not induce the formation of MP tubules. The cell-to-cell movement of PVX requires the contribution of a single coat protein and the functions of three MPs encoded by a highly conserved triple gene block, which directs the expression of two membrane bound proteins and a 25-kDa silencing suppressor protein (Chapman *et al.*, 1992; Krishnamurthy *et al.*, 2003). In addition, the p25 MP also encodes an ATPase domain, has RNA helicase activities, and is able to disassemble virions in a polar manner (Morozov *et al.*, 2003).

Virus species from the family *Closteroviridae* are distinguished from other helical viruses by their exceptionally flexible virions and large positive sense RNA genomes. Interestingly, closterovirus particles are composed of two coat proteins (Fig. 1.1), the major coat protein (CP), which encapsidates the majority of the genome, and the minor coat protein (CPm) that makes a small tail-like structure at one end of the virions (Agranovsky *et al.,* 1995). The bipolar architecture of closteroviruses suggests that CP and CPm evolved to fulfill different functions in the life cycle of closteroviruses. Current models propose that the primary function of CP is to protect the viral genome, and that the CPm tails are specialized virion components involved in the vector transmission and cell-to-cell movement of the virus (Dolja, 2003; Tian *et al.,* 1999).

The family *Closteroviridae* includes more than 30 plant viruses divided into three major genera, as determined by their genome structure and vector specificity. Closteroviruses have monopartite genomes and are transmitted by aphids, criniviruses have bipartite genomes and are transmitted by whiteflies, and ampeloviruses have monopartite genomes and are transmitted by mealybugs (Dolja *et al.*, 1994b; Martelli *et al.*, 1999; Karasev, 2000). The three best characterized members of the family *Closteroviridae* are the *Beet yellows virus* (BYV), *Citrus tristeza virus* (CTV), and *Lettuce infectious yellows virus* (LIYV) (Dolja, 2003).

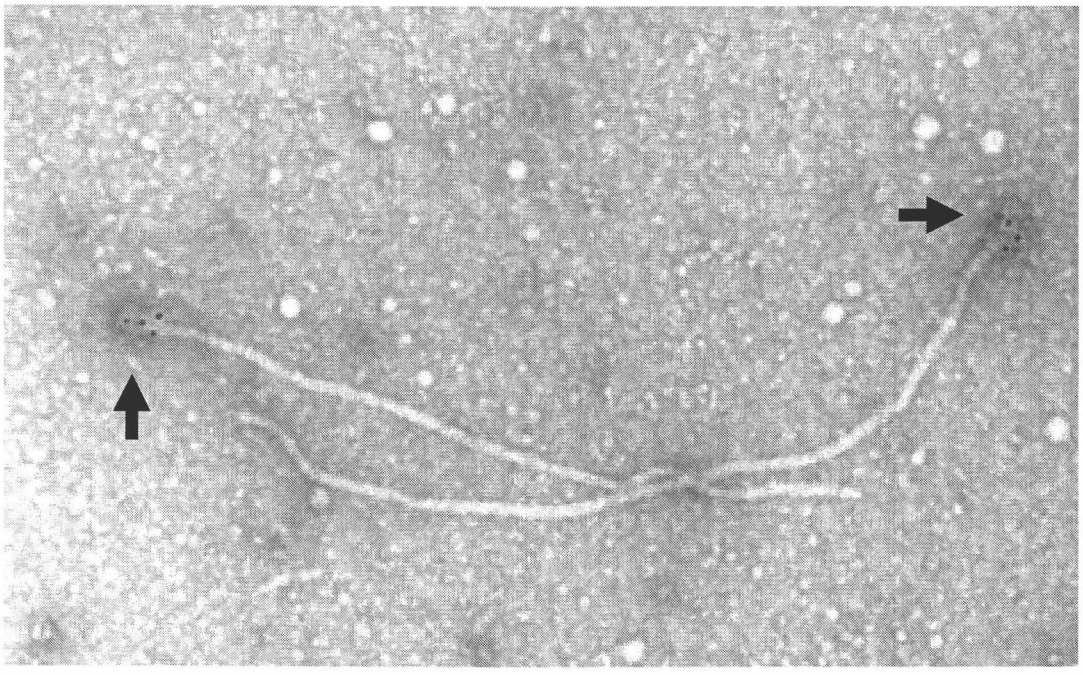

Figure 1.1. Electron micrograph of two BYV virions. Virion tails marked by arrows were immunogold labelled with anti-CPm antibodies and 10 nm gold conjugates.

BYV was first described in 1936 (Russel, 1970) and is the type member of the genus *Closterovirus*. BYV is geographically wide spread, is transmitted by aphids in a semi-persistent manner, and has a broad host range that includes major agricultural crop species such as sugar beet, red beet, and spinach (Dolja *et al.*, 1994b). Unlike other closteroviruses, BYV is not phloem limited and accumulates to relatively high levels

in both natural and experimental hosts (Dolja et al., 1994b). The BYV virions have a nominal length of 1200 nm and a diameter of 12 nm (Kassanis *et al.*, 1949). Although the helical symmetry of the coat protein subunits can be resolved by transmission electron microscopy (TEM), this technique reveals no obvious structural differences between the virion bodies and the CPm tails (Tollin and Willson, 1988; Tollin *et al.*, 1992). The bipolar architecture of BYV was determined by TEM examinations of virions immunodecorated with CPm antibodies, which revealed the presence of ~ 75 nm tail- like structures localized at one end of the virions. (Agranovsky *et al.*, 1995). This unique architecture has now been confirmed for other members of the family *Closteroviridae*, CTV and LYIV (Febres et al., 1996; Tian *et al.*, 1999).

The 15,500-nt BYV genome (Fig 2) is capped, has no poly A tail, and encodes 9 ORFs which direct the expression of at least 10 viral proteins (Agranovsky *et al.*, 1994; Karasev *et al.*, 1989; Peremyslov *et al.*, 1998). Expression of ORFs 1a and 1b occurs via translation of the capped mRNA genome and yields all of the proteins essential for RNA replication, which include a papain-like leader proteinase, a methyl transferase, RNA helicase, and RNA-dependent RNA polymerase domains (Agranovsky *et al.*, 1994; Peng and Dolja, 2000; Peremyslov *et al.*, 1998). The expression of non-replication proteins encoded by ORFs 2-8 occurs by synthesis of seven subgenomic mRNAs (Dolja *et al.*, 1994; He *et al.*, 1997). In spite of considerable variability in the composition and organization of genes outside of ORFs 1a and 1b, a block of five gene products is conserved among all members of the family *Closteroviridae* (Dolja *et al.*, 1994; Karasev *et al.*, 2000). In BYV, this block encodes a small 6-kDa hydrophobic protein, a HSP70 homolog, a 64-kDa protein, and both of the virion coat proteins CP and CPm (Agranosky et al., 1991). The rest of the 3' ORFs encode a 20-kDa protein that has been shown to enhance the systemic spread of BYV (Prokhnevsky *et al.*, 2002) and a 21-kDa protein, which has been recently characterized as a RNA silencing suppressor protein (Reed *et al.*, 2003).

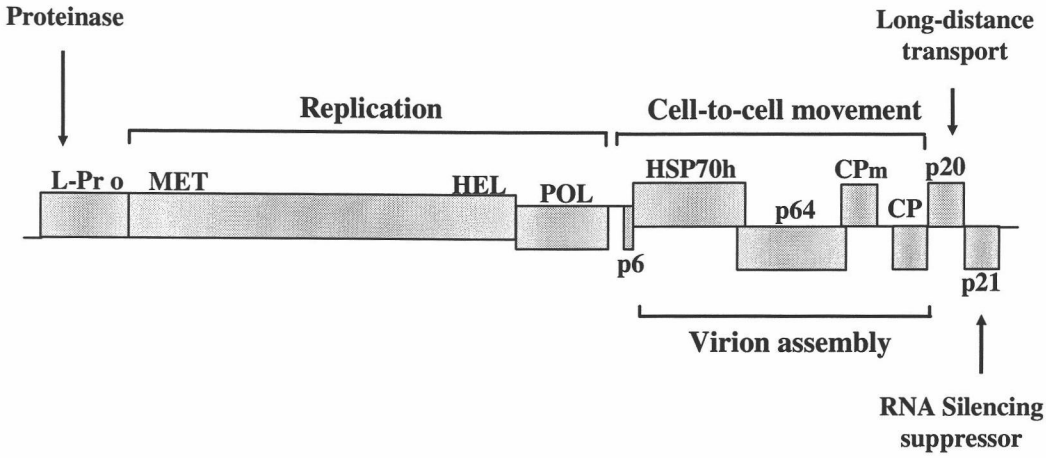

Figure 1.2 Map of the BYV genome. Diagram of the BYV genome showing the ORFs that code for leader proteinase (L-Pro), replication-associated proteins possessing putative methyltransferase (MET), RNA helicase (HEL), and RNA polymerase (POL) domains, 6 kDa-protein (p6), Hsp70-homolog (HSP70h), 64 kDa-protein (p64), minor capsid protein (CPm), major capsid protein (CP), 20 kDa-protein (p20), and 21 kDa-protein (p21).

Virtually all plant viruses encode specialized proteins whose functions are dedicated to the movement of viruses between cells, called movement proteins (MP). Recent genetic analysis have shown that the cell-to-cell movement of BYV requires the functions of five gene products, encoded by the highly conserved quintuple gene block, which direct the expression of p6, HSP70h, p64, CP and CPm (Alzhanova *et al.*, 2000). The association of these gene products with the cell-to-cell movement machinery of BYV was demonstrated using wild type (wt) and mutant BYV variants modified to express the green fluorescent protein (GFP). Inoculation of leaves with (wt) BYV-GFP variants produced multi-cellular infection foci a few days post inoculation, indicating the normal cell-to-cell movement of the virus. In contrast, BYV-GFP variants carrying mutations in any of the gene products encoded by ORFs 2-6 only produced unicellular infection foci as a result of the arrest of the virus to

initially inoculated cells (Alzhanova *et al.*, 2000). Therefore, the cell-to-cell movement machinery of BYV appears to be composed of three dedicated MPs and two structural proteins, CP and CPm. Although the mechanisms by which these viral components support the cell-to-cell movement of BYV remains unknown, none of the BYV movement associated proteins alone is sufficient to support the cell-to-cell movement of the virus, which suggest that these proteins function in a coordinated and interactive fashion.

The requirement for the participation of CP and CPm in the cell-to-cell movement of BYV suggests that assembly is a prerequisite for the cell-to-cell movement of BYV, and that the movement of the virus occurs by the translocation of virions between cells. Alternatively, it is also possible that assembly could simply be required for the intracellular movement of virions from sites of replication and assembly to plasmodesmata, followed by disassembly events and the subsequent translocation of the viral genome between cells. To date, TEM examinations of BYV-infected leaves have failed to capture BYV virions traversing plasmodesmata. Nonetheless, these analyses have shown the accumulation of virions in association with the plasmodesmata, which suggests that cell-to-cell movement of BYV involves specific interactions between virions and plasmodesmata (Esau *et al.,* 1971; Medina *et al.,* 1999). Interestingly, in the absence of CPm, CP alone was able to assemble virus-like particles (VLPs) and protect the entire BYV genome. However, these VLPs were unable to move from cell to cell (Alzhanova *et al.*, 2001).

Recently it has been shown that the assembly of CPm depends on the presence of an RNA stem loop localized at the 5' end of the RNA genome (Satyanarayana *et al.,* 2004). This signal alone is sufficient to promote assembly initiation and the encapsidation of viral RNA by CPm. However, in the absence of HSP70h, p64 and CP, the encapsidation of vRNA by CPm continues to the 3' end of the viral genome and results in the assembly of "CPm only" VLPs. The functions of HSP70h and p64 have been shown to limit the encapsidation of viral RNA by CPm to the first 650 nt at

the 5' end of the genome in the absence of CP. Therefore, it is possible that the contribution of HSP70h and p64 to the cell-to-cell movement of BYV could be due to their requirement for proper incorporation of CPm. Alternatively, HSP70h and p64 could contribute to both the assembly and cell-to-cell movement of the bipolar virions.

HSP70h is the only known virus encoded member of the superfamily of 70-kDa heat shock proteins, HSP70 (Agranovsky *et al.,* 1991). HSP70h encodes the N-terminal ATPase domain, which is conserved in all members of the superfamily of HSP70 proteins, and the less conserved peptide binding C-terminal domain, which typically determines the target specificities of diverse HSP70 proteins (Flaherty *et al.,* 1990; Zhu *et al.,* 1998). The contribution of HSP70h to the cell-to-cell movement of BYV has been supported by extensive genetic analysis, which showed that mutations in the ATPase or C-terminal domain of the protein completely abolished the cell-to-cell movement of BYV (Alzhanova *et al.*, 2000; Peremyslov *et al.*, 1999). TEM examination of infected plants immuno-decorated with HSP70h antibodies showed the association of HSP70h with both virions and plasmodesmata (Medina *et al.*, 1999). Furthermore, HSP70h partially complemented the cell-to-cell movement requirements of two unrelated movement-deficient viruses, *Potato virus X* (PVX) and barley stripe mosaic virus (BSMV), (Agranovsky *et al.*, 1998).

In contrast to HSP70h, the only known proteins that share sequence homology with p64 are other orthologue proteins encoded by viruses in the family *Closteroviridae* (Napuli *et al.*, 2003), and there is very little known about their biochemical functions.

Unlike other movement-associated proteins, the p6 protein is not required for virion assembly (Alzhanova *et al.*, 2001). It encodes a highly hydrophobic, type III, alpha-helical transmembrane domain that anchors the protein to the rough ER membrane (Peremyslov *et al.,* 2004). The N-terminal domain of p6 protrudes into to the lumenal side of the ER, and encodes a cysteine residue that is essential for the formation of p6 homodimers (Peremyslov *et al.,* 2004). The C-terminal domain is localized to the

cytoplasmic side of the ER membrane (Peremyslov *et al.,* 2004). Although the biochemical function of p6 remains unknown, mutations that inhibited the formation of dimers abolished the cell-to-cell movement of BYV, in spite of the normal localization of the protein to the rough ER (Peremyslov *et al.,* 2004). Similarly, the deletion of even a few amino acids from the C-terminal domain also resulted in the inhibition of virus movement (Peremyslov *et al.,* 2004).

Requirement of the CP and CPm for BYV cell-to-cell movement prompted us to ask if other movement associated proteins are also incorporated into virions. The cell-to-cell movement machinery of closteroviruses is composed of five genes that include both of the virion structural proteins, CP and CPm. Although the contribution of CP appears to be limited to the protection of the viral genome, there is evidence suggesting that CPm plays an active role in the cell-to-cell movement of closteroviruses. The functions of HSP70h and p64 are also required for the cell-to-cell movement of BYV and the assembly of CPm. The major goal of this study was to determine molecular composition of the BYV virions. It was found that in addition to CP and CPm, BYV virions incorporated HSP70h, p64 and p20 (Napuli *et al.,* 2000; Napuli *et. al.,* 2003). Although the detailed architecture of HSP70h and p64 in the BYV virions remains to be resolved, current biochemical evidence suggests that both of these proteins are associated with the CPm component of the bipolar virions. Therefore, with the exception of the small hydrophobic protein p6, it appears that closteroviruses evolved to include the proteins required for the cell-to-cell movement of the virus as integral components of a unique and specialized virion structure composed of CPm, HSP70h, and p64. This work contributes to the already diverse repertoire of cell-to-cell movement strategies used by plant viruses and describes the complex and unusual architecture of a closterovirus virion.

Chapter 2

Interaction between HSP70 Homolog and Filamentous Virions of the Beet Yellows Virus

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2.1 Abstract

An HSP70 homolog (HSP70h) encoded by the *Closterovirus*, *Beet yellows virus* (BYV), functions in viral movement from cell to cell. A previous study revealed that in infected cells, HSP70h co-localizes with the masses of BYV filamentous virions. Here we demonstrate that HSP70h forms a physical complex with BYV virions. This conclusion is based on the co-migration of HSP70h with BYV virions in sucrose density gradients, and on the co-immunoprecipitation of the HSP70h and BYV capsid protein using anti-HSP70h serum. The HSP70h-virion complex is stable at high concentrations of sodium chloride; its dissociation using sodium dodecyl sulfate, lithium chloride, or alkaline pH was accompanied by virion disassembly. However, the complex formation does not involve covalent bonds between HSP70h and virion components. Each BYV virion contains approximately 10 molecules of HSP70h. The possible role of HSP70h interaction with the virions in cell-to-cell movement of BYV is discussed.

2.2 Introduction

Molecular chaperones from the family of ~70-kDa heat shock proteins (HSP70s) are conserved through all kingdoms of unicellular and multicellular organisms. In eukaryotic cells, the specialized members of the HSP70 family are present in all cell compartments (Boorstein *et al.*, 1994). Some of the HSP70s are stress-inducible and play important roles in cell survival under heat shock and other stress conditions. Other HSP70s are expressed constitutively and are involved in protein folding in the cytoplasm, protein import into the endoplasmic reticulum, mitochondria, chloroplasts, or trafficking of the receptors and coated vesicles, etc. (Bukau and Horwich, 1998; Pilon and Schekman, 1999).

Although viruses normally do not encode HSP70s, there are multiple examples of viruses relying on cellular HSP70s at different phases of their life cycles. For several animal viruses, the interaction with HSP70s appears to be involved in virion assembly that may take place in cytoplasm, nucleus, or endoplasmic reticulum, depending on the requirements of different viruses (Macejak and Sarnow, 1992; Cripe *et al.*, 1995; Xu *et al.*, 1998; Liberman *et al.*, 1999). The induction of a host HSP70 gene by diverse plant viruses suggested that corresponding protein may provide an important function in the infected cell, although it is not clear who is a beneficiary, the virus, or the host (Escaler *et al.*, 2000).

The *Closteroviridae* is a family of positive-strand RNA viruses (closteroviruses) that possess ~15-20 kb genomes (Bar-Joseph *et al.*, 1979; Dolja *et al.*, 1994b; Martelli *et al.*, 1999). These genomes are encapsidated into filamentous virions that are assembled from a major and minor capsid proteins (CP and CPm, respectively; Agranovsky *et al.*, 1995; Tian *et al.*, 1999). Although closteroviruses are remarkably divergent in their genetic organization, all of them possess a gene encoding an HSP70 homolog (HSP70h; Agranovsky *et al.*, 1991; Fazeli and Rezaian, 2000). Viruses of no other family described so far are reported to harbor an HSP70 gene.

Functional characterization of several closteroviruses revealed that HSP70h is not required for genome amplification (Klaassen *et al.*, 1996; Peremyslov *et al.*, 1998; Satyanarayana *et al.*, 1999). The *Beet yellows virus* (BYV) gene coding for HSP70h is expressed early in infection and to relatively low levels (Hagiwara *et al.*, 1999). We demonstrated recently that HSP70h is essential for BYV movement from cell to cell (Peremyslov *et al.*, 1999). Similarly to movement proteins of many other plant viruses (Carrington *et al.*, 1996; McLean *et al.*, 1997; Lazarowitz and Beachy, 1999), BYV HSP70h was observed in association with the plasmodesmata (Medina *et al.*, 1999). It was also found that in the cytoplasm of BYV-infected cells, HSP70h co-localizes with the virion masses, suggesting that it may physically interact with the virions (Medina *et al.*, 1999). This possibility was supported by the finding of HSP70h in the partially

purified virions of the *Lettuce infectious yellows virus* (LIYV), a member of the genus *Crinivirus* in the family *Closteroviradae* (Tian *et al.*, 1999). In addition to HSP70h, a 6-kDa protein, a 64-kDa protein, CP, and CPm are required to potentiate the movement of BYV (Alzhanova *et al.*, 2000). These results suggested that BYV translocation may involve virion formation, as well as interaction between HSP70h and other components of the viral movement machinery.

We report here that the virions isolated from BYV-infected plants are physically associated with HSP70h. Formation of these HSP70h-virion complexes does not involve covalent bonds with any of the virion components. However, our attempts to dissociate complexes using various treatments resulted in at least partial virion disassembly. A hypothetical model of BYV translocation involving formation of the HSP70h-virion complexes is proposed.

2.3 Results

2.3.1 Characterization of the anti-HSP70h and anti-CP sera

In this work, we have utilized sera raised against BYV HSP70h and CP that were previously used for serologically specific electron microscopy (Medina *et al.*, 1999) and immunoblot analysis (Dolja *et al.*, 1998), respectively. The former antiserum was derived using the recombinant C-terminal fragment of the HSP70h superexpressed in *E. coli*, whereas the latter was raised against purified BYV virions. We felt it necessary to further characterize these antisera, since their performance in immunoprecipitation experiments was of critical importance for testing possible association of HSP70h with virions.

To this end, we generated ^{35}S-cysteine-labeled HSP70h and CP using *in vitro* translation of corresponding mRNAs in the wheat germ extracts. Each mRNA directed formation of the labeled product of expected mol. weight, that is ~65 kDa for the

HSP70h and ~22 kDa for CP (Fig. 2.1, lanes HSP70h and CP, respectively). These labeled products were immunoprecipitated using the homologous, heterologous, and pre-immune sera. As is seen in Fig. 2.1, each antiserum specifically immunoprecipitated the homologous antigen, whereas pre-immune sera did not precipitate any. These results confirmed the utility of anti-HSP70h and anti-CP sera for immunoprecipitation experiments. It should be noted that since anti-CP serum was raised against purified BYV virions, it may also recognize CPm and other proteins potentially present in virions. Accordingly, we used this antiserum only as a positive control for immunoprecipitating virions that contain ~95% of the CP (Agranovsky *et al.*, 1995).

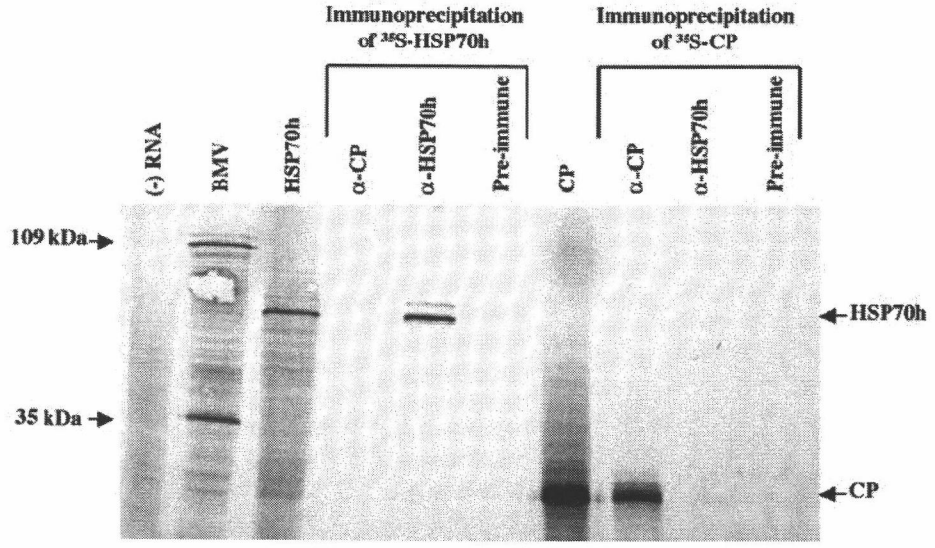

Figure 2.1 Immunoprecipitation of the ^{35}S-labeled HSP70h and capsid protein using anti-HSP70h (α-HSP70h), anti-capsid protein (α-CP), or preimmune sera. The products of *in vitro* translation reactions programmed with no RNA (negative control, -RNA lane), Brome mosaic virus RNA (positive control, BMV lane), HSP70h mRNA (HSP70h lane), and capsid protein mRNA (CP lane) were separated by SDFS-PAGE, blotted onto a nitrocellulose membrane, and subjected to autoradiography. The bracketed sets of three lanes show the analysis of the immunoprecipitation products using BYV ^{35}S-HSP70h and ^{35}S-capsid protein and antisera as indicated. The arrows mark the positions of corresponding BMV proteins (left) or BYV proteins (right).

2.3.2 HSP70h co-migrates with the virions in sucrose density gradient

To examine the possible association between HSP70h and virions, we employed centrifugation in sucrose density gradients and analysis by SDS-PAGE and transmission electron microscopy (TEM). The SDS-PAGE analysis showed that the virions purified from BYV-infected plants migrated in gradients to form a broad zone with peak in fractions 11-14 (Fig. 2.2B). TEM analysis revealed that the gradient fractions with the majority of BYV CP also contained apparently intact filamentous particles of the size and morphology characteristic of BYV (Fig. 2.2C; Bar-Joseph and Hull, 1974). The presence of the HSP70h in gradient fractions was tested using immunoblot analysis and an HSP70h-specific antiserum. As is obvious from comparison between the Figs. 2.2A and 2.2B, the distribution of the antiserum-reactive protein among the gradient fractions was very similar to that of BYV CP and virions. Moreover, the mobility of the detected protein in SDS-polyacrylamide gels was identical to the mobility of ^{35}S-cysteine-labeled HSP70h derived *in vitro* (Fig. 2.2A). Taken together, these results demonstrated that the HSP70h co-migrates with BYV virions in sucrose density gradients, and suggested that there is a physical association between the two.

2.3.3 Co-immunoprecipitation of the HSP70h and virions

Since it can not be excluded that the co-migration of the HSP70h and BYV virions in sucrose density gradients was coincidental, we used an independent experimental approach to provide further evidence for the existence of the HSP70h-virion complexes. The BYV virions isolated from the peak fractions of the gradient were incubated with either anti-CP or anti-HSP70h serum, and the resulting antibody-antigen complexes were precipitated with the aid of protein A-sepharose. As expected, the following analysis of the products immunoprecipitated using anti-CP serum revealed the presence of BYV CP (positive control; Fig. 2.3, lane 5). Conspicuously, the comparable amount of the CP was immunoprecipitated using anti-HSP70h serum

(Fig. 2.3, lane 6). A series of control reactions demonstrated that the appearance of the CP in this latter immunoprecipitated material was strictly dependent on the presence of HSP70h-specific serum (Fig. 2.3, lanes 1-4 and 7). The co-immunoprecipitation of the

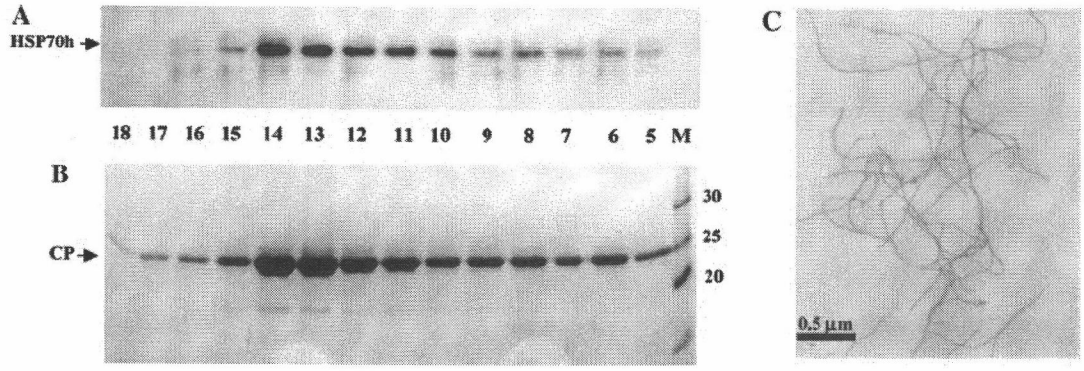

Figure 2.2 Co-migration of the HSP70h and virions in sucrose density gradient. (A) Immunoblot analysis of the gradient fractions (numbering from the bottom up) using anti-HSP70h serum; position of the ^{35}S-labeled HSP70h generated via *in vitro* translation is marked by the arrow. (B) Same gradient fractions separated by PAGE and stained with Coomassie brilliant blue. The position of the BYV CP is marked by the arrow; the numbers at the right indicate mol. weights of the protein markers (M). (C) Transmission electron micrograph of the BYV virions isolated from peak fractions of the gradient.

BYV HSP70h and CP from gradient fractions containing intact virions (Fig. 2.2C) by anti-HSP70h serum demonstrates the physical association of HSP70h with the virions. The identical result was obtained using the virions which were not fractionated in gradient, indicating that the HSP70h-virion complexes were formed prior rather than during the gradient centrifugation (data not shown). Collectively, the results of sucrose density gradient centrifugation and co-immunoprecipitation experiments provided strong evidence for the existence of HSP70h-virion complexes in the BYV-infected plants.

2.3.4 Stability and composition of the HSP70h-virion complexes

The fact that the antiserum-reactive protein detected in virion preparations possessed the mol. weight expected for monomeric HSP70h (Fig. 2.1A) suggested that HSP70h is not covalently linked to the virion components. However, if the putative covalent bonds were formed by disulfide bridges, they could dissociate during the sample boiling in the presence of β-mercaptoethanol prior to its electrophoretic analysis. To test this possibility, the parallel samples of the virions were treated by 2% SDS in or without the presence of 100mM DTT and analyzed using SDS-PAGE without the boiling in β-mercaptoethanol-containing buffer. The SDS treatment alone should result in complete dissociation of the virions (see below) without disrupting disulfide bridges, whereas combined treatment with SDS and DTT should disrupt both. Comparative immunoblot analysis of these parallel samples using antiserum to HSP70h revealed identical bands corresponding to monomeric HSP70h (data not shown). These results allowed us to exclude the involvement of covalent bonds in the formation of HSP70h-virion complex.

To characterize the complex stability under conditions of different ionic strength, we incubated the virions in the presence of various concentrations of NaCl. After the incubation, the virions were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum. The salt-induced dissociation of the HSP70h-virion complex was expected to result in decreasing amounts of HSP70h detected in the precipitated virions. As it is shown in Fig. 2.4, the complex remained stable under the concentrations of NaCl up to 2M.

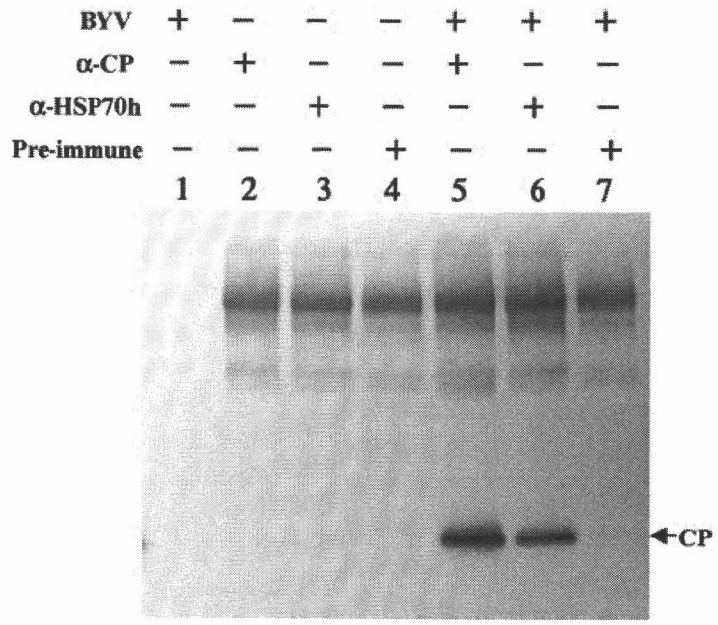

Figure 2.3 Co-immunoprecipitation of the HSP70h and BYV virions derived from the peak fractions of sucrose density gradients. The products of immunoprecipitation reactions were analyzed using immunoblotting and anti-CP serum. The principal components of the reactions corresponding to each lane are shown in the table above the photograph. Arrow marks the position of the BYV CP. The bands in the upper portion of the membrane correspond to rabbit antibodies present in immunoprecipitated material and recognized by the goat anti-rabbit serum conjugated to alkaline phosphatase.

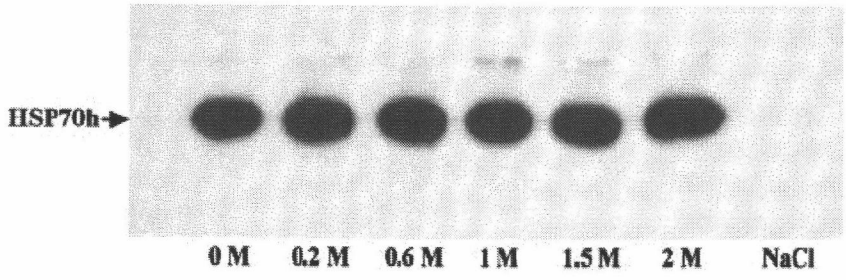

Figure 2.4 Stability of the HSP70h-virion complexes in the presence of NaCl. The BYV virions incubated in the presence of indicated concentrations of NaCl were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum.

In the next series of experiments, we used LiCl that is capable of dissociating protein-protein and protein-nucleic acid complexes including virions (Goodman, 1975). The treatment of the virions with increasing concentrations of LiCl resulted in gradual decrease in the amount of virions precipitated by ultracentrifugation (Fig. 2.5B), indicating virion destabilization. The immunoblot analysis demonstrated that the amount of the HSP70h present in treated virions was roughly proportional to the amount of precipitated virions (Figs. 2.5 A and B). Similar results were obtained when the virions were exposed to various pH. The decrease in the HSP70h amount detected in virions after incubation under alkaline (pH 11) or acidic (pH 4) conditions was approximately proportional to the amount of precipitated virions (data not shown). These results suggested that the HSP70h-virion complexes are relatively stable; their dissociation by LiCl or extreme pH was accompanied by virion disassembly.

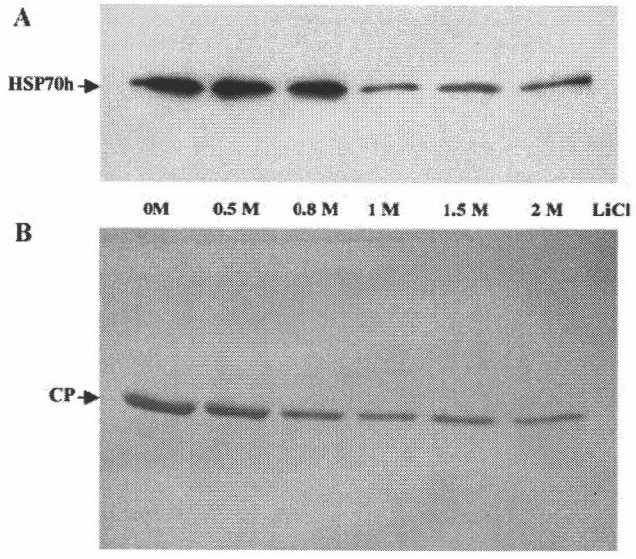

Figure 2.5 Stability of the HSP70h-virion complexes in the presence of LiCl. The virions incubated in the presence of indicated concentrations of LiCl were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum (A) or SDS-PAGE and Coomassie staining (B).

We have also examined the stability of the HSP70h-virion complex using SDS treatments. It was found that the treatment with 2% SDS results in virtually instantaneous virion disassembly, whereas this process proceeds much slower in the presence of 0.5% SDS (data not shown). The time-course experiment shown in Fig. 2.6 revealed the concomitant dissociation of the complex and the virions. However, it appears that the virion stability in the presence of 0.5% SDS is somewhat higher than that of the HSP70h-virion complexes. Indeed, the amount of the virions precipitated after 5 min treatment with 0.5% SDS is comparable to that at time zero, whereas the amount of HSP70h associated with the virions at this time point is much lower than at the beginning of treatment (compare Figs. 2.6 A and B). These results indicate that the stability of the HSP70h-virion complexes and the virions are different under the used experimental conditions.

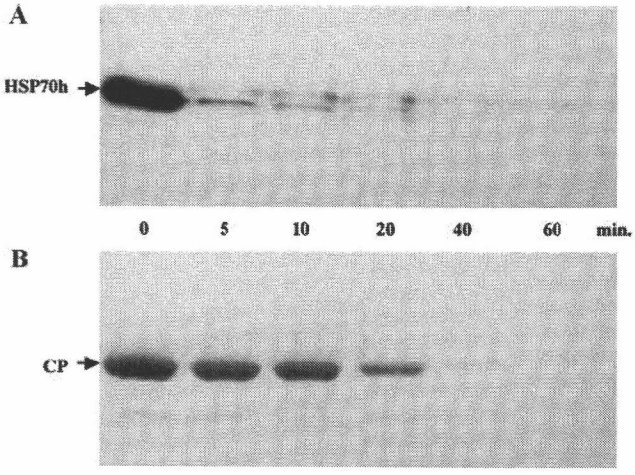

Figure 2.6 Time course analysis of the disassembly of the HSP70h-virion complexes in the presence of 0.5% SDS. The virions were incubated in the presence of SDS for the indicated periods of time, precipitated by ultracentrifugation and analyzed using immunoblotting and anti-HSP70h serum (A) or SDS-PAGE and Coomassie staining (B).

To determine the number of HSP70h molecules per virion, we separated virion proteins using SDS-PAGE. The approximate amount of HSP70h present in the sample was determined by comparing to standardized dilutions of a marker protein, bovine serum albumin (data not shown). Given that the intact BYV virion contains ~3,300 molecules of the CP (Bar-Joseph and Hull, 1974), we estimated that each virion is bound on average to ~10 molecules of HSP70h.

2.4 Discussion

Using two experimental approaches, the sedimentation in sucrose density gradients and the immunoprecipitation, we revealed that the virions isolated from BYV-infected plants contain one of the viral movement proteins, HSP70h. The HSP70h-virion complexes withstood the treatment by high concentrations of sodium chloride, whereas utilization of other dissociating agents resulted in concomitant disassembly of the complexes and virions. Our data indicate that the complexes are maintained by strong non-ionic interactions that does not involve covalent bonds between HSP70h and other components of the virion. Finding the HSP70h associated with virions of BYV (genus *Closterovirus*) and LIYV (genus *Crinivirus*; Tian *et al.*, 1999) suggests that this may be a feature shared by viruses in family *Closteroviridae.*

What is the role played by the HSP70h molecules that are attached to or present in the virions? Although HSP70h may be an integral virion component, it seems unlikely that its role is only structural or protective. Indeed, the RNA genomes of all rod-shaped and filamentous plant viruses except for closteroviruses are encapsidated into and protected by a single type of capsid protein. The major distinction of closteroviruses from other elongated plant viruses is the heterodimeric morphology of the virions with the minor capsid protein forming a short tail at one end of the virion (Agranovsky *et al.*, 1995; Tian *et al.*, 1999). It is possible that HSP70h aids in the assembly of the tailed BYV virions. On the other hand, high stability of the HSP70h-

virion complexes is suggestive of the HSP70h involvement in the phases of virus life cycle that follow encapsidation.

At least three of those additional functions are feasible with the existing data. First, HSP70h may be required for the virion disassembly at the onset of infection process. Second, finding of the HSP70h in the insect-transmissible virion preparations of LIYV is compatible with its role in vector transmission (Tian *et al.*, 1999). Third, HSP70h may mediate the intercellular translocation of the virions. This latter possibility is supported by the requirement for both HSP70h activity and virion assembly in order to potentiate BYV cell-to-cell movement (Peremyslov *et al.*, 1999; Alzhanova *et al.*, 2000). Moreover, association of HSP70h with the virions and plasmodesmata (Medina *et al.*, 1999 and this work) allowed us to propose that HSP70h functions via binding virions and aiding their translocation towards and through the plasmodesmata.

The requirements for cell-to-cell movement in distinct filamentous plant viruses reveal some intriguing analogies. Similar to BYV (genus *Closterovirus*), virions were implicated in the cell-to-cell movement of the members of the genera *Potexvirus* (Chapman *et al.*, 1992; Forster *et al.*, 1992; Santa Cruz *et al.*, 1998) and *Potyvirus* (Dolja *et al.*, 1994a; Rodriguez-Cerezo *et al.*, 1997). Movement machineries of each of these three virus genera include proteins possessing (putative) ATPase activity that may generate energy for virus translocation. Examples of such proteins are provided by HSP70h of BYV (Peremyslov *et al.*, 1999), 25-kDa protein of the *Potato virus X* (*Potexvirus*; Morozov *et al.*, 1999), and CI protein of the *Tobacco etch virus* (*Potyvirus*; Carrington *et al.*, 1998). These analogies suggest that the filamentous viruses share some of the mechanisms of their cell-to-cell movement. It will be interesting to see if the formation of complexes between virions and movement-associated ATPases takes place in *Potexvirus* and *Potyvirus* genera.

Although the gene encoding HSP70 homolog is found only in representatives of *Closteroviridae*, there is at least one example of unrelated virus that may utilize host

HSP70 for the cell-to-cell movement. It was found recently that the movement protein of *Tomato spotted wilt virus* (genus *Tospovirus*) is capable of binding viral nuclecapsid protein and plant DnaJ-like proteins (Soellick *et al.*, 2000). Since the members of DnaJ family function via interactions with HSP70s (Pilon and Schekman, 1999), it appears that the recruitment of these molecular chaperones in virion or nucleocapsid translocation may have evolved independently in very distinct families of plant viruses.

2.5 Materials and methods

2.5.1 Isolation, fractionation, and electron microscopy of the BYV virions

BYV virions were isolated from infected *Nicotiana benthamiana* plants 2 to 3 weeks post-inoculation, following the protocol developed for LIYV (Klaassen *et al.*, 1994). Sucrose density gradient centrifugation of BYV virions was conducted as described (Bar-Joseph and Hull, 1974). In brief, 1 to 2 ml of the partially purified virions (~1 mg/ml) were loaded onto 10-40% sucrose density gradients and centrifuged at 25,000 rpm for 4 hrs using Beckman SW40 rotor at 4^{o}C. The gradients were prepared by layering 10%, 20%, 30%, and 40 % sucrose solutions (5 ml, 7 ml, 8 ml, and 8 ml, respectively) containing TE buffer (10 mM tris and 1 mM EDTA; pH 7.6) in corresponding centrifuge tubes, and stored for 1 hr at the room temperature. Gradients were fractionated into 1 ml fractions by gravity flow.

TEM was conducted using the virions isolated from the peak gradient fractions. Formvar/carbon-coated grids were allowed to float on 25 µl drops of the 1:100 diluted virions for 10 min. The grids were rinsed 3 times in drops of the water and stained using 2% uranyl acetate for 10 min prior to viewing by TEM.

2.5.2 *In vitro* translation, immunoprecipitation, and immunoblot analysis

The ORFs coding for BYV HSP70h and CP were cloned into the pTL7SN plasmid downstream from an SP6 RNA polymerase promoter and a Tobacco etch virus leader region (Carrington and Freed, 1990) using *Nco* I and *Sma* I sites. These sites were introduced into the terminal regions of corresponding DNA fragments concomitantly with their generation via PCR. The *Sma* I-linearized plasmids and SP6 RNA polymerase were used to generate uncapped *in vitro* RNA transcripts as described (Peremyslov *et al.*, 1998). The resulting mRNAs were precipitated using LiCl, resuspended in water, and stored at -80^{o}C. *In vitro* translations of CP and HSP70h transcripts in the wheat germ extracts (Promega) were conducted in 100 µl reaction mixtures according to the manufacturer's protocol. The translation products were labeled using L-[^{35}S]-cysteine (Amersham). The Brome mosaic virus RNA and water were used as a positive and a negative controls, respectively.

The immunoprecipitation assays were conducted as described by Cripe *et al.* (1995) with some modifications. For the labeled translation products, the 200 µl reaction mixtures contained 180 µl of the IP buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 % Triton X-100), 20 µl of the products, and 1 µl of rabbit antisera against CP (Dolja *et al.*, 1998) or HSP70h (Medina *et al.*, 1999). Immunoprecipitations of the virions were conducted in 1 ml reaction mixtures containing 900 µl of IP buffer, 100 µl of BYV virions derived from the peak fractions of the gradients, and 1 µl of the rabbit antiserum against CP or HSP70h. The pre-immune sera were used as negative controls. The reactions with primary antibodies were incubated at 4^{o}C for 16 hrs with gentle mixing, followed by addition of 100 µl of 20 % protein A-Sepharose CL-4B (Pharmacia). After 4 hr incubation at 4^{o}C, the beads were loaded into 1 ml micro spin columns and washed 5 times with the IP buffer and 5 times with the TE buffer. Proteins were eluted by boiling the beads in protein dissociation buffer, and the

immunoblot analysis was performed as described (Dolja *et al.*, 1993) using anti-BYV serum at 1:1000 dilution.

The immunoblot analysis of HSP70h was conducted using a chemiluminiscent assay kit ECL Western Blotting System (Amersham) and anti-HSP70h serum at 1:2000 dilution. Secondary goat anti-rabbit antibodies conjugated to horseradish peroxide were used at 1:4000 dilution.

2.5.3 Examination of the stability and composition of the HSP70h-virion complexes

Preparations of BYV virions were used to determine the stability of the HSP70h-virion complexes in the presence of varying concentrations of the NaCl, LiCl, and SDS, as well as under alkaline or acidic conditions. For NaCl experiments, 100 µl of the virions (~1 mg/ml) were incubated on ice for 30 min in 0, 0.2, 0.6, 1, 1.5, and 2 M solutions of NaCl in 10 mM Tris-HCl, pH 7.4 (final volume of the mixture was 450 µl). The reaction mixtures were layered onto 150 µl of 20 % sucrose cushion in 1.5 ml tubes and centrifuged at 100,000 g in the Beckman TLA 100.3 rotor at 4°C for 2 hrs to pellet virions. The pellets were resuspended in 50 µl of 2X protein dissociation buffer, boiled for 10 min, and analyzed using SDS-PAGE or immunoblotting.

The LiCl experiments were conducted in general accord with the method described by Goodman (1975) for the isolation of Potato virus X CP subunits by LiCl dissociation. To determine the effects of LiCl on the HSP70h-virion complexes, reaction mixtures containing 100 µl of the BYV virions were incubated on ice for 1 hr in 0, 0.5, 0.8, 1, 1.5, and 2 M concentrations of LiCl (final volume of reactions was 600 µl). The virions were centrifuged and analyzed as described above.

The SDS experiments followed the method described by Mundry *et al.* (1991) for disassembly of Tobacco mosaic virus. 150 µl of the virions were mixed with 420 µl of

the buffer containing 50 mM tris-HCl, pH 7.4, 200 mM NaCl, and 2 % triton X-100. The 100 µl aliquot of the reaction mixture was transferred immediately into 900 µl of the protein dissociation buffer to represent a zero time point. After that, 10 % SDS was added to a final concentration of 0.5 %. The 100 µl aliquots of the reaction mixture were quenched in 900 µl of the same buffer at 5, 10, 20, 40, and 60 min after addition of SDS. The samples were centrifuged and analyzed as described above.

The pH experiments were conducted using the modified protocol of Pelcher and Halasa (1979) for alkaline degradation of virions of Tobacco mosaic virus. The BYV virions were dialyzed against 10 mM EDTA, pH 7.4 for 16 hrs to standardize the pH of the starting material. Alkaline buffer solution was prepared using 20 mM sodium tetraborate at the pH 11. Acidic buffer solution was prepared using 20 mM sodium citrate at the pH 4.5. To examine the stability of the HSP70h-virion complexes at varying pH conditions, the reaction mixtures containing 50 µl of the virions were mixed with 1 ml of corresponding buffer solution. The reactions were incubated for 1 hr on ice, then centrifuged and analyzed as described.

To determine the amount of HSP70h present in virions, the concentration of the total protein in virion preparation was measured using Bradford protein assay (BioRad Laboratories). The sample containing 10 µg of the total protein was separated using 12.5% SDS-PAGE; the samples containing 0.1, 0.2, 0.5, 1, and 1.5 µg of the bovine serum albumin were used as standards. After staining the gels with Coomassie brilliant blue, the approximate concentration of the HSP70h was determined by comparing the intensity of its band with that of the standards. The molar ratio of the CP to HSP70 was determined using the known mol. weight of each protein and the Avogadro number. The approximate number of HSP70h molecules per virion was calculated assuming that each BYV virion contains ~3,300 molecules of the CP (Bar-Joseph and Hull, 1974).

2.6 Acknowledgments

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Chapter 3

The 64-kDa Capsid Protein Homolog of *Beet Yellows Virus* Is Required for Assembly of Virion Tails

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3.1 Abstract

The filamentous virion of the Beet yellows closterovirus (BYV) consists of a long body formed by major capsid protein (CP) and a short tail, which is comprised of minor capsid protein (CPm) and the virus-encoded Hsp70 homolog. Using nano-liquid chromatography/tandem mass spectrometry and biochemical analyses, we show here that BYV 64-kDa protein (p64) is a fourth integral component of the BYV virions. The N-terminal domain of p64 is exposed at the virion surface and is accessible to antibodies and mild trypsin digestion. In contrast, the C-terminal domain is embedded in the virion and is inaccessible to antibodies or trypsin. The C-terminal domain of p64 is shown to be homologous to CP and CPm. Mutation of the signature motifs of capsid proteins of filamentous RNA viruses in p64 results in the formation of tailless virions, which are unable to move from cell to cell. These results reveal the dual function of p64 in tail assembly and BYV motility and support the concept of the virion tail as a specialized device for BYV cell-to-cell movement.

3.2 Introduction

Despite the enormous variation in molecular architecture, virions of all viruses share the primary function of encapsidation and protection of the virus genome. In addition, virions have more specialized roles at various steps of virus infection. Virions of bacteriophages and animal viruses mediate interactions with the cell surface receptors and subsequent entry and activation of the virus genome (Harrison, 2001). In particular, some bacteriophages have specialized molecular assemblies that puncture cells and/or eject the genome from the virion (Kanamaru *et al.*, 2002; Molineux, 2001; Simpson *et al.*, 2000). Virions of animal viruses, such as poxviruses, herpesviruses, and lentiviruses, are capable of translocation within and between the cells by hijacking cellular motility systems (Johnson and Huber, 2002; Ploubidou and Way, 2001).

For plant viruses, active cell-to-cell movement is one of the critical aspects of the life cycle. Therefore, all non-defective plant viruses evolved to encode dedicated movement proteins (MPs), which translocate the virus toward and through the plasmodesmata, the organelles responsible for intercellular communications in plants (Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999; Lucas and Wolf, 1999; Oparka and Roberts, 2001). Some plant viruses move from cell to cell in a non-virion form, whereas others require functional capsid proteins (CPs) and virion formation for their movement (Callaway *et al.*, 2001; Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999). In particular, CP-dependent movement has been described in members of the genera *Comovirus* and *Caulimovirus*. The icosahedral virions of these viruses are translocated intercellularly inside MP-induced tubules, which traverse plasmodesmal channels of neighboring cells (Kasteel *et al.*, 1997; Thomas and Maule, 1995). Among filamentous viruses, members of the *Potexvirus* and *Potyvirus* genera require functional CPs for the cell-to-cell movement (Chapman *et al.*, 1992; Dolja *et al.*, 1994). However, it remains unclear whether these viruses are actually translocated as virions (Lough *et al.*, 2000; Roberts *et al.*, 1998; Santa Cruz *et al.*, 1998).

A striking relationship between virion morphology and cell-to-cell movement has been revealed in *Closteroviridae*, a large family of plant positive-strand RNA viruses (Alzhanova *et al.*, 2001; Karasev, 2000). Unlike other filamentous viruses of plants, which have uniform structure, virions of closteroviruses consist of two distinct morphological units, a long body and a short tail (Agranovsky *et al.*, 1995; Tian *et al.*, 1999). The body and the tail both show helical symmetry of the protein subunits, which is typical of all elongated viruses, and consist of the homologous major and minor capsid proteins (CP and CPm), respectively. The CPm gene most likely evolved via tandem gene duplication in an ancestral closterovirus (Boyko *et al.*, 1992). In addition to CP and CPm, minor amounts of two other closterovirus proteins copurify with the virions and are thought to be involved in virion assembly or stabilization (Satyanarayana *et al.*, 2000; Tian *et al.*, 1999). One of these proteins, an Hsp70 homolog (HSP70h), has been recently shown to be an integral virion component

(Napuli *et al.*, 2000) that is specifically required for tail assembly (Alzhanova *et al.*, 2001). The role of another protein of ~60 kDa (p64), which is conserved among closteroviruses, so far remained obscure.

At least five proteins encoded in the ~16 kb genome of *Beet yellows virus* (BYV), the prototype closterovirus, are essential for cell-to-cell movement (Fig. 3.1A) (Alzhanova *et al.*, 2000; Peremyslov *et al.*, 1999). One of these is a small hydrophobic protein of ~6 kDa (p6). The others include the integral virion components CP, CPm, and HSP70h, as well as p64. These results indicated that the unusually complex BYV virions have a critical role in virus movement. Further studies revealed a strict correlation between the assembly of the tailed virions and the ability of the virus to move from cell to cell (Alzhanova *et al.*, 2001). Suppression of tail assembly by mutation of CPm or HSP70h resulted in the formation of tailless virions, which were competent for genome protection and infectivity assayed on single cells, but were defective in cell-to-cell movement. These results prompted the hypothesis that the closterovirus tail was a specialized movement device powered by the ATPase activity of HSP70h (Alzhanova *et al.*, 2001).

Here we identify p64 as the fourth integral component of the BYV virions and show that it is essential for tail assembly. We further show that p64 consists of a C-terminal domain that is homologous to CP and a unique N-terminal domain. Mutation of the signature motifs of the capsid proteins in the C-terminal domain of p64 abrogates cell-to-cell movement of BYV. These results indicate that the bipartite morphology and the complex movement function of the closterovirus virions evolved via triplication of the CP gene and acquisition of additional RNA sequences, which encode HSP70h and the N-terminal domain of p64.

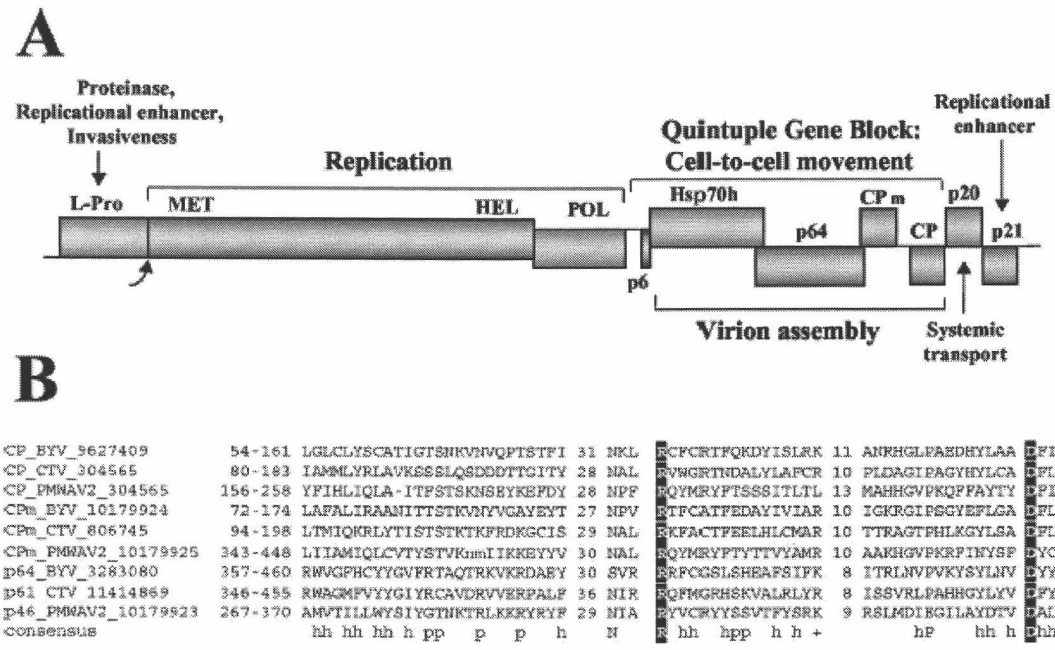

Figure 3.1 Diagram of the BYV genome and multiple alignment of the CPs, CPm's and the CP-like C-terminal domains of the ~60 kDa proteins of three closteroviruses (A) Diagram of the BYV genome showing the ORFs that code for leader proteinase (L-Pro), replication-associated proteins possessing putative methyltransferase (MET), RNA helicase (HEL), and RNA polymerase (POL) domains, 6 kDa-protein (p6), Hsp70-homolog (HSP70h), 64 kDa-protein (p64), minor capsid protein (CPm), major capsid protein (CP), 20 kDa-protein (p20), and 21 kDa-protein (p21). (B) Multiple alignment of the CPs, CPm's and the CP-like C-terminal domains of the ~60 kDa proteins of three closteroviruses. The alignment was constructed using the MACAW program and only the three conserved blocks are shown (see text). The consensus line shows the amino acid residues that are present in the majority of the proteins in each of the three groups: h indicates a hydrophobic residue, p indicates a polar residue and + indicates a positively charged residue. The two signature residues that are conserved in most of the coat proteins of elongated plant viruses are shown in white against a black background. The ranges of the aligned regions in the corresponding protein sequences are indicated in front of each sequence; the numbers between the blocks indicate the length of the variable spacers, which are not shown. Each protein is identified by its name, the virus name abbreviation and the Gene Identifier (GI number) from the NR database. PMWAV2, *Pineapple mealybug wilt-associated virus-2* (Melzer *et al.*, 2001).

3.3 Materials and methods

3.3.1 Protein sequence analysis.

The non-redundant (NR) protein sequence database at the National Center for Biotechnology Information (NCBI, NIH, Bethesda) was searched using the PSI-BLAST program (Altschul *et al.*, 1997). Additionally, the Conserved Domain Database (CDD) at the NCBI was searched using the RPS_BLAST program (Marchler-Bauer *et al.*, 2002). Multiple alignments of protein sequences were constructed using the MACAW program (Schuler *et al.*, 1991), with subsequent manual refinement. Protein secondary structure prediction was performed using the Jpred program (Cuff *et al.*, 1998).

3.3.2 Isolation, sedimentation, and stability of the virions.

The BYV virions were isolated from infected *Nicotiana benthamiana* plants and separated in 10-40% sucrose density gradients as described previously (Napuli *et al.*, 2000). Virion preparations were used to determine the stability of the p64-virion complexes in the presence of varying concentrations of LiCl. To this end, reaction mixtures containing 100 µl of BYV virions (1 mg/ml) were incubated on ice for 1 hr in 0, 0.5, 0.8, 1, 1.5, and 2 M concentrations of LiCl (final volume of reactions was 600 µl). The virions were pelleted using ultracentrifugation at 100,000 g for 1 hr at 4°C and their protein composition was analyzed as described below.

3.3.3 Nano-liquid chromatography tandem mass spectrometry.

Virion proteins were separated by electrophoresis in 12.5% SDS-PAG, a ~65-kDa protein band was visualized by staining with 300 mM $CuCl_2$ for 5 min, cut out, destained, and in-gel trypsin digestion was performed overnight at 37°C as described (Shevchenko *et al.*, 1996). The resulting tryptic peptides were separated by nano-liquid chromatography (nano-LC) using a 40 cm PicoFrit column (New Objective, Cambridge, MA) packed with 5 µm, 300 Å pore, Luna C_{18} silica gel particles

(Phenomenex, Torrance, CA) as described (Kennedy and Jorgenson, 1989). The nano-LC was conducted using exponential dilution to produce gradient separations (Doneanu *et al.*, 2001). To sequence tryptic peptides, on-line nano-LC/electrospray ionization tandem mass spectrometry was performed using an LC-Q ion trap mass spectrometer (Finnigan, San Jose, CA). The instrument was operated in the full scan mode with the spraying potential set to 2.5 kV (applied to the injector), the temperature of the heated inlet-capillary at 180°C, the capillary potential at 46 V, and the tube-lens offset-potential at 30 V. The maximum injection time was 50 ms. The instrument was set to acquire a mass spectrum between 400 and 2000 m/z followed by a tandem mass spectrometry (MS/MS) scan. For operation in the MS/MS-mode, the maximum injection time was increased to 500 ms, the isolation width was set to 2 Da, and the relative collision energy was set to 35% with a 30 ms activation time. Using the instrument's data dependent algorithm, the most intense ion from full scan spectrum was selected for MS/MS if its signal exceeded 2 x 10^4 counts. The peptide sequences were compared to the non-redundant NCBI database using Sequest search engine (Finnigan).

3.3.4 Analyses of the virion composition.

The PAGE and immunoblot analyses using anti-CP and anti-CPm antisera were conducted as described (Alzhanova *et al.*, 2001; Napuli *et al.*, 2000). The antisera to the N-terminal (amino acid residues 2-218) and C-terminal (residues 305-553) regions of p64 were raised using histidine-tagged, GST-fused recombinant proteins that were expressed in *E. coli* strain BL21 (DE3). To this end, corresponding coding regions were cloned as *Bam* HI-*Nsi* I fragments into the pGEX-2T plasmid (Pharmacia), which was modified to incorporate a *Nsi* I site followed by six histidine codons and a stop codon between the *Bam* HI and *Sma* I sites present in the original polylinker. Presence of the GST and histidine tags permitted purification of the recombinant products using glutathione or metal affinity resin. However, because the obtained products were insoluble, each of them was purified under denaturing conditions using only the

TALON metal affinity resin (Clontech) as recommended by the manufacturer. The proteins eluted from the resin were submitted to Cocalico Biologicals (Reamstown, PA) for generation of rabbit antisera. To characterize the specifities of the resulting antisera, the entire p64 ORF was cloned into the pTL7SN plasmid and used to generate the corresponding RNA transcripts as described (Medina *et al.*, 1999). The resulting p64 mRNA was translated in the wheat germ extracts (Promega) to yield L-[35S]-cysteine (Amersham)-labeled products that were used in immunoprecipitation assays (Napuli *et al.*, 2000). The immunoblot analyses of p64 were conducted using ECL Western Blotting System (Amersham) and anti-p64 sera at 1:2000 dilution. Secondary goat anti-rabbit antibodies were used at 1:4000 dilution.

Limited digestion of the BYV virions with the sequencing grade modified trypsin (Promega) was conducted in 20 mM Na-phosphate buffer, pH 7.4, for 1 hr at the room temperature. The reactions (total volume 175 µl) contained 1 mg/ml of the BYV virions and trypsin in 1:50, 1:100, or 1:200 wt/wt ratio to the total virion protein. After digestion, reactions were stoped by adding 5 µl of 100 mM PMSF, diluted to 400 µl, and virions were separated from digestion products by ultracentrifugation as indicated above.

3.3.5 Generation and characterization of the BYV mutants.

Site-directed mutagenesis and the plasmid pNB-4 were used to generate p64 mutants (Peremyslov *et al.*, 1998; Peremyslov *et al.*, 1999). The mutagenic primers 5'-CCGTTTCTGTTCGT**GC**CAGATTTTGTGGCAG and 5'-GTGAAATACTCCTATCTAAACGT-TGCCTATTACAGACACG (the mutated nucleotides are shown in bold) were used to replace Arg-416 and Asp-455 codons in p64 ORF with Ala codons to yield mutants $R_{416}A$ and $D_{455}A$, respectively. The *Nde* I-*Bam* HI fragments from the mutant pNB-4 variants were cloned into plasmid pBYV-4 to test for virion assembly in protoplast transfection experiments. Alternatively, these fragments were cloned into plasmid

pBYV-GFP for assaying virus cell-to-cell movement in plant inoculation experiments (Alzhanova *et al.*, 2001).

3.4 Results

3.4.1 The p64 protein of BYV contains a capsid protein-like domain.

In database searches performed using the PSI-BLAST program, the BYV p64 sequence showed statistically significant sequence similarity only to orthologs from several other closteroviruses, such as *Beet yellow stunt virus* and *Citrus tristeza virus* (CTV). However, when the orthologous sequences from all other sequenced closteroviruses (as determined by analysis of genome organization), which appeared in these searches with non-significant expectation (E)-values, were manually added to the position-specific scoring matrix, statistically significant similarity ($E < 0.005$) was detected between the C-terminal portion of p64 and closterovirus CPs and CPm's. When the sequences of these three groups of closterovirus proteins were aligned using the MACAW program, counterparts of the three prominent motifs, which have been detected previously in the capsid proteins of all filamentous plant viruses (Dolja *et al.*, 1991) and in closterovirus CPm's (Boyko *et al.*, 1992), were identified in p64 and its orthologs (Fig. 3.1B). The alignments of the two distal motifs centered around the invariant arginine and aspartate residues, respectively, were highly statistically significant ($E < 10^{-11}$). These motifs could be detected in all available sequences of closterovirus p64 orthologs (data not shown). In spite of the low sequence similarity (note that the alignment in Fig. 3.1B contains only two invariant residues), these observations strongly suggested that the C-terminal domains of the ~60-kDa proteins of closteroviruses are homologous to the closterovirus CPs and CPms (Fig. 3.2) and might have similar functions.

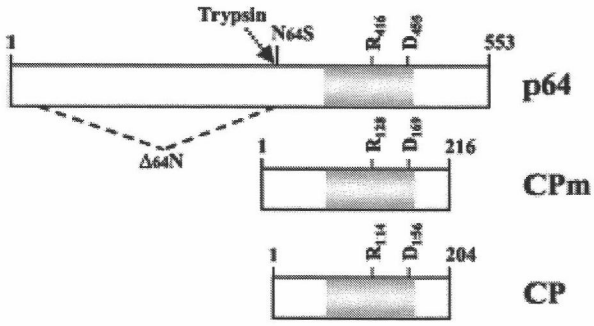

Figure 3.2 Diagrams of p64, CPm, and CP showing the homologous region. Diagrams of the p64, CPm, and CP showing the homologous region (gray; same as in Fig. 1B), as well as positions of invariant arginine (R_{416}) and aspartic acid (D_{455}) residues. An approximate position of the cleavage by trypsin (arrow) was used to arbitrarily delimit the N-terminal and C-terminal domains of p64. The premature stop codon in N64S mutant and the part of p64 ORF that is deleted in Δ64N mutant are also shown. The first and the last amino acid residues are indicated for each protein.

Examination of the N-terminal domains of the ~60-kDa proteins revealed a unique pattern of conservation, which was not detected in any other known protein family despite an extensive search of the CDD database. Secondary structure prediction suggested that this is a unique, predominantly α-helical domain (data not shown).

3.4.2 p64 is associated with virions.

Identification of the CP-like domain in p64 prompted us to ask if this domain enables the incorporation of p64 into BYV virions. The virions were extensively purified and subjected to protein analysis via PAGE. As shown in Fig. 3.3A, in addition to a bulky band corresponding to CP and CPm, a distinct minor band of ~65 kDa was present in the gel. To determine the identity of the protein(s) present in this band, it was excised and treated with trypsin, and the resulting peptides were separated and sequenced using nano-liquid chromatography/ tandem mass spectrometry. In total, 15 peptides shown in Fig. 3.3B were sequenced and compared to a data base. Among those, 11 peptides were derived from BYV p64, whereas the remaining four originated from

BYV HSP70h (Table 1). The total coverage for the p64 and HSP70h sequences was ~21% and 8%, respectively. These results demonstrated that the original ~65 kDa band contained a mixture of ~64 kDa p64 and ~65 kDa HSP70h. The latter protein was previously found to be an integral component of the BYV virions (Alzhanova *et al.*, 2001; Napuli *et al.*, 2000).

However, it could not be ruled out that p64 was merely a contaminant that co-purified with the virions. To test whether p64 was specifically associated with the virions, we separated the virions in a sucrose density gradient and analyzed each fraction for the presence of CP and p64 using the corresponding antisera. As shown in Fig. 3.4A, the peak fractions 12-15 that contained most of the CP and morphologically intact virions Napuli *et al.*, 2000), also contained most of the p64. Co-migration of the p64 with the virions is in agreement with their specific physical association.

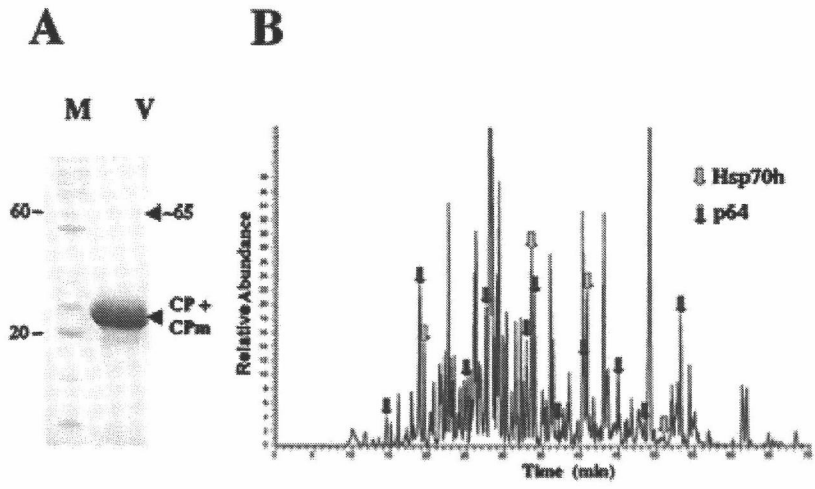

Figure 3.3 MS of virion proteins. (A) Analysis of the BYV virion proteins using SDS-PAGE. M, protein markers; V, virions. Positions of the 20-kDa and 60-kDa markers and a ~65-kDa band (arrowhead) are indicated. (B) Separation of the tryptic peptides derived from the ~65 kDa proteins present in virions using nano-liquid chromatography. Arrows mark the positions and origin of the 15 sequenced peptides.

Table 3.1 Tryptic peptides that were derived from ~65-kDa virion proteins and sequenced using nano-liquid chromatography/ tandem mass spectrometry

Peptide sequence[a]	Identity	Position[b]
ALISTACEAFK	Hsp70h	133–143
IDISFLK	Hsp70h	258–264
NDSPMLLVDCAAHNLSISSK	Hsp70h	379–398
VVADLHK	Hsp70h	514–520
FFGGR	p64	21–25
NFSDSTGESFVR	p64	58–69
EFSLLLTFPK	p64	70–78
LSDYNVSELNVVDVK	p64	100–114
FVSLIFK	p64	164–170
ALYDEFLK	p64	211–218
IPTINTHDSSTFLYK	p64	306–319
DNPELK	p64	348–353
FGVGFPPITRLNVPVKYSYLNVDYYR	p64	433–458
EVALQUAR	p64	488–494
NEVSPHAR	p64	511–518

[a]The sequences are shown in a single-letter code from N- to C-terminus.
[b]The positions within the 598 residue-long HSP70h and 553 residue-long p64 are shown.

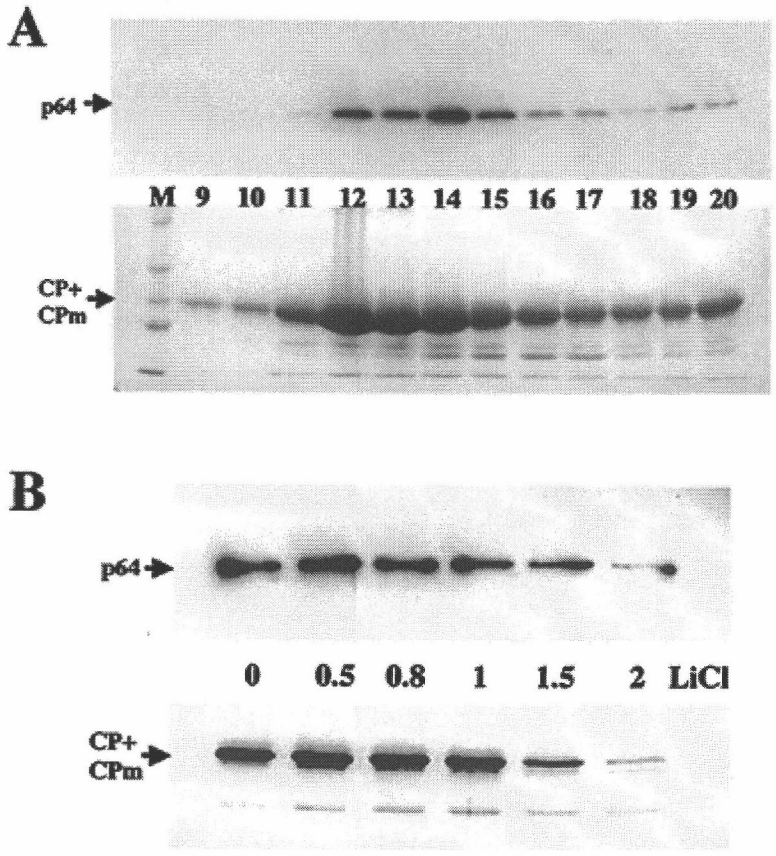

Figure 3.4 (A) Co-migration of the p64 and virions in sucrose density gradient. Top panel, immunoblot analysis of the gradient fractions (numbering from the bottom up) using antiserum to the recombinant N-terminal domain of p64. Bottom panel, same gradient fractions separated by PAGE and stained with Coomassie brilliant blue. M, protein markers. (B) Stability of the p64-virion complexes in the presence of LiCl. The virions incubated in the presence of indicated molar concentrations of LiCl were precipitated by ultracentrifugation and analyzed using immunoblotting and anti-p64 serum (top panel) or SDS-PAGE and Coomassie staining (bottom panel) as in (A). The minor band seen below the CP+CPm band likely represents a degradation product that occasionaly appears in some virion preparations [compare to Fig. 2.5 in chapter 2].

To determine if p64 could be selectively removed from the virions, we treated the virions with increasing concentrations of LiCl, collected them by centrifugation and analyzed their protein composition. As expected, this treatment resulted in partial disassembly of the virions; the extent of disassembly correlated with LiCl

concentration (Fig. 3.4B). Importantly, there was also a correlation between the amounts of CP and p64 in the virions that withstood disassembly (Fig. 3.4B). This suggested that p64 was tightly associated with the virions and could not be readily dissociated without virion disassembly. Similar results were obtained when the virions were treated for increasing time intervals with low SDS concentration or with 2M NaCl (not shown). Collectively, these results parallel those previously obtained for HSP70h (Napuli *et al.*, 2000) and indicate that p64 and HSP70h alike are tightly associated with the virions and are integral virion components.

3.4.3 Domain topology of p64 in BYV virions.

We were next interested in testing whether, as predicted by computational analysis, the C-terminal domain of p64 was functionally analogous to CP and was embedded in the virion. To assess the topology of p64 within the virion, we first tested the accessibility of p64 domains to antibodies specific to each domain. The Fig. 3.5A shows that each of these antibodies was able to specifically immunoprecipitate the isotope-labeled p64 translated in a cell-free system. When these antibodies were incubated with BYV virions, it was found that the antibody specific for the N-terminal domain of p64 but not the one specific for the C-terminal domain immunoprecipitated the virions (Fig. 3.5B). This result confirmed the specific physical association of p64 with BYV virions and the prediction that the C-terminal domain of p64 was functionally analogous to CP.

We also tested the accessibility of the p64 domains to limited trypsin digestion. The virions were treated with increasing concentrations of trypsin, precipitated by ultracentrifugation to remove the digestion products and subjected to immunoblot analysis. As seen in Fig. 3.6A, even treatment with most diluted trypsin resulted in virtually complete degradation of the N-terminal domain of p64. In contrast, the C-terminal domain of ~30 kDa was resistant to trypsin digestion and remained associated with the virions (Fig. 3.6B). Based on the mol. weight of this domain, the Lys_{325} was

selected arbitrarily as a likely site of cleavage by trypsin (Fig. 3.2). The difference in the apparent amount of the full-size p64 observed between Figs. 3.6 A and B is likely due to difference in the relative sensitivity of the used antisera. No change in the electrophoretic mobility of the CP was observed, indicating that the CP present in the virions was protected from trypsin digestion under the employed experimental conditions (data not shown). Thus, the results of immunoprecipitation and trypsin digestion were in agreement with the domain topology of p64 inferred by computational analysis.

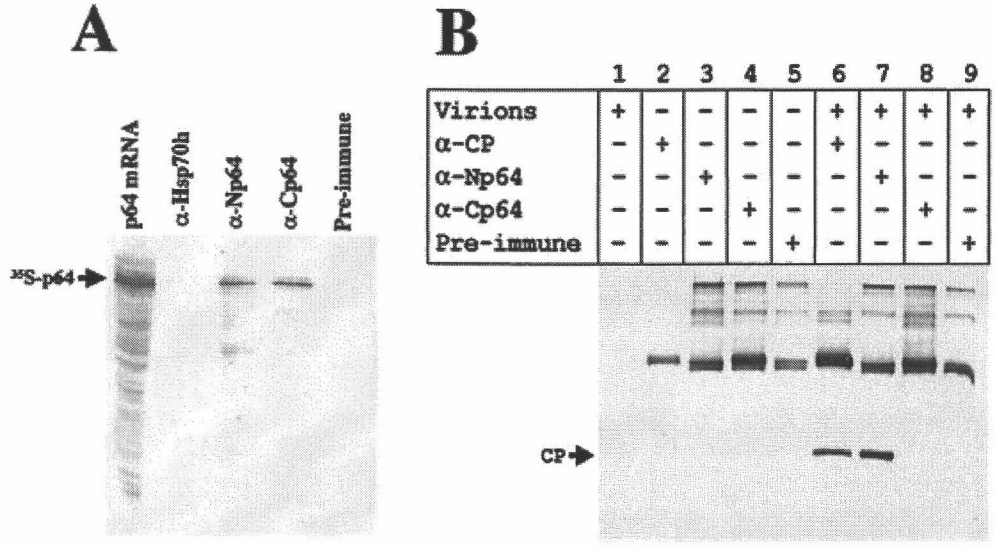

Figure 3.5 (A) Immunoprecipitation of the ^{35}S-labeled p64 using antisera raised against the recombinant N-terminal domain of p64 (lane α-Np64) or C-terminal domain of p64 (lane α-Cp64). Lane p64 mRNA, products of the *in vitro* translation reaction programmed with the p64 mRNA. The antiserum to HSP70h (lane α-HSP70h) and preimmune serum (lane Pre-Immune) were used as negative controls. The proteins were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and subjected to autoradiography. (B) Immunoprecipitation of the BYV virions using α-Np64 or α-Cp64 sera. The anti-CP (α-CP) and pre-immune sera were used as a positive and negative controls, respectively. The products of immunoprecipitation reactions were analyzed using immunoblotting and α-CP serum. The principal components of the reactions corresponding to each lane are shown in the table above the photograph. The bands in the upper portion of the membrane correspond to rabbit

antibodies present in immunoprecipitated material and recognized by the goat anti-rabbit serum conjugated to alkaline phosphatase.

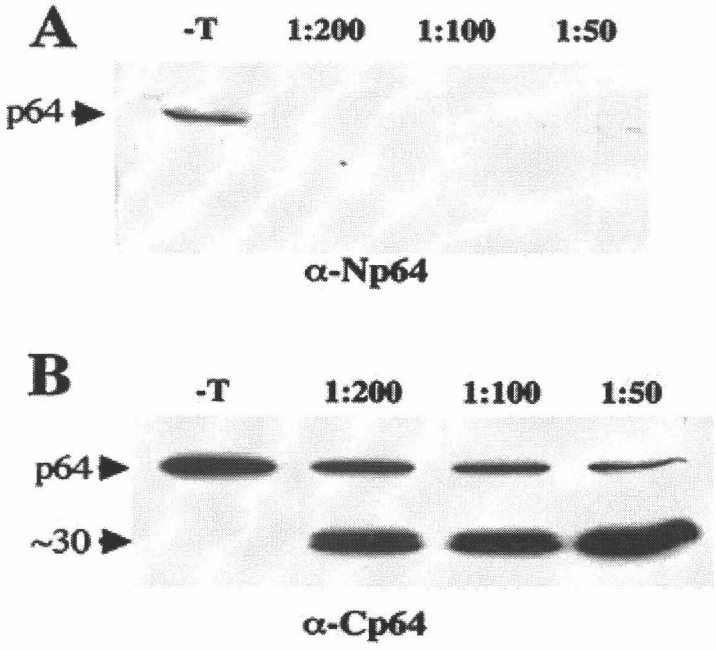

Figure 3.6 (A) and (B), relative resistance of the N-terminal and C-terminal domains, respectively, in the virion-associated p64 to a limited trypsin digestion. –T, no trypsin added (negative control), 1:200, 1:100, and 1:50; trypsin dilutions. The type of antiserum used for immunoblot analysis of the treated and precipitated virions is shown below each panel. The arrows mark the positions of the p64 and its ~30-kDa degradation product.

3.4.4 p64 is required for virion tail assembly and cell-to-cell movement of BYV.

The presence of p64 in virions prompted us to ask if p64 was required for the virion assembly and, if so, if there was a connection between the roles of p64 in assembly and cell-to-cell movement. Previously, we constructed two p64 mutants, each of which was defective in cell-to-cell movement (Alzhanova *et al.*, 2000). In the first of these mutants, Δ64N, most of the N-terminal domain was deleted, whereas the second mutant, N64S, expressed only the N-terminal domain (Fig. 3.2). Here, we designed

mutants $R_{416}A$ and $D_{455}A$, in which the invariant Arg-416 and Asp-455 residues in the CP-like domain (Fig. 3.1B) were replaced with Ala. As demonstrated previously for CPs of several filamentous viruses, mutations of these highly conserved amino acid residues completely abolished the assembly function of CP (Alzhanova *et al.*, 2001; Dolja *et al.*, 1991; Dolja *et al.*, 1994; Jagadish *et al.*, 1993).

Each of the four BYV mutants was transfected into tobacco protoplasts, and virions were isolated four days post transfection. To assess the presence of virion bodies and tails in the mutant virions, we used immunoblot analysis and antibodies specific for BYV CP and CPm, respectively (Alzhanova *et al.*, 2001). As shown in Fig. 3.7A, none of the four p64 mutations affected formation of the virion bodies: the amount of the CP found in mutant virions was similar to that of wild-type virions. However, in a sharp contrast to the wild type, the mutant virions contained no detectable CPm, indicating that the assembly of the tails was impaired (Fig. 3.7B). These results showed that p64 was required for the virion tail assembly and supported the functional importance of the conserved Arg-416 and Asp-455 in this process. Moreover, the lack of tail formation in the Δ64N and N64S mutants suggested that each of the two domains of p64 was required for its function in tail formation.

The cell-to-cell movement phenotypes of $R_{416}A$ and $D_{455}A$ mutants were tested in plant inoculation experiments using a GFP-tagged BYV variant (Peremyslov *et al.*, 1999). Screening of 12 leaves showed that, by 10 days post inoculation, the parental BYV-GFP formed 118 green fluorescent infection foci with a mean diameter of 4.4±2.4 cells. The mutant variants produced similar numbers of infection foci (130 and 99, respectively). However, all of these foci were unicellular, demonstrating that the $R_{416}A$ and $D_{455}A$ mutations abolished the cell-to-cell movement of the virus. Thus, each of the four tested p64 mutants was defective both in the ability to form virion tails and to move from cell-to-cell.

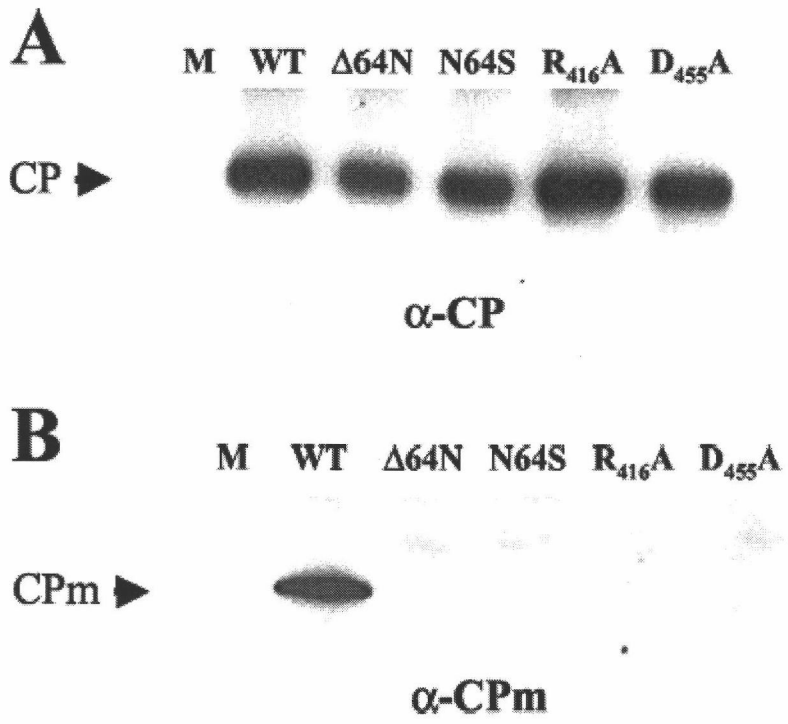

Figure 3.7 Protein composition of the virions assembled by four BYV variants possessing mutant p64. The virions were isolated from protoplasts transfected with the corresponding BYV variant and analized by immublotting using anti-CP (A) and anti-CPm (B) sera. M and WT, mock-transfected protoplasts and protoplasts transfected with the wild-type BYV, respectively. The mutants' names are shown above the panels, whereas the type of antiserum used in immunoblot analysis is shown below the panels.

3.5. Discussion

Among the plant positive-strand RNA virus families, *Closteroviridae* stand apart in having the largest genomes of 15-20 kb in contrast to the ~6 kb genomes of their cousins, *Tobamoviridae*. Our major goal in studying the biology of closteroviruses is to elucidate the unique functions and the underlying evolutionary mechanisms that account for the increase in the genetic capacity of closteroviruses. Some of the extra genes encode parts of the sophisticated RNA synthesis machinery of the closterovirus. In addition to the core replicase domains that are shared by all *Sindbis virus*-like RNA

viruses of plants and animals (Koonin and Dolja, 1993), closteroviruses encode unique domains that could account for their ability to replicate large RNAs and recognize an array of subgenomic promoters (Gowda *et al.*, 2001; Karasev, 2000). Moreover, efficient replication of closterovirus RNA requires two types of replication enhancers, the leader proteinases (Peng *et al.*, 2001) and the ~20-kDa proteins (Fig. 3.1A) (Peremyslov *et al.*, 1998; Reed *et al.*, 2002; Satyanarayana *et al.*, 2002).

This work, along with previous results (Agranovsky *et al.*, 1995; Alzhanova *et al.*, 2000; Alzhanova *et al.*, 2001; Napuli *et al.*, 2000; Satyanarayana *et al.*, 2000; Tian *et al.*, 1999), reveals another major function of the unique genes of closteroviruses by showing that proteins encoded by these genes contribute to the formation of morphologically and functionally complex virions. Closteroviruses have two gene blocks that are conserved within this family: the replication-related gene block and the so-called quintiple gene block or QGB (Fig. 3.1A) (Alzhanova *et al.*, 2000; Karasev, 2000). As we show here, p64 is the fourth of the five QGB-encoded proteins that is incorporated into the tailed BYV virions. Although each of the QGB proteins is essential for BYV cell-to-cell movement, only p6 is a BYV MP that is not a virion component (Alzhanova *et al.*, 2001).

How could this unique constellation of genes with a dual function in virion formation and cell-to-cell movement evolve? This work and previous analyses (Boyko *et al.*, 1992) showed that one of the underlying mechanisms was tandem gene duplication, which occurred at least twice to yield the coding regions for CPm and the CP-like domain of p64 (Fig. 3.2). Since the three CP-like domain-containing genes are present in all closteroviruses whose genomes have been sequenced so far, it appears that both duplications occurred prior to the divergence of these viruses from their common ancestor. Other important events in the evolution of closteroviruses apparently included acquisition of the coding regions for the HSP70h and the N-terminal domain of p64. In the former case, it appears obvious that an ancestral closterovirus had captured a cellular mRNA for Hsp70 (Agranovsky *et al.*, 1991), whereas the origin of

the upstream portion of the p64 gene remains obscure. In addition to being an MP and an essential virion component (Alzhanova *et al.*, 2001; Peremyslov *et al.*, 1999), HSP70h provides a docking site for the long-distance transport factor p20, which is required for the systemic virus spread through the plant vasculature (Prokhnevsky *et al.*, 2002). Although p20 is associated with the virions, unlike other virion proteins, it is not essential for the assembly or cell-to-cell movement. The evolutionary scenario for the tailed closterovirus virions can be interpreted as a hierarhical build-up of the virion functions from merely protecting the genome to driving cell-to-cell movement to mediating long-distance transport of the virus. It seems likely that, during closterovirus evolution, the selective advantage conferred on the virus by these increasingly complex devices for virus-host interaction was a driving force behind the evolution of the mechanisms for subgenomic RNA synthesis and its regulation and, accordingly, the overall increase of the genome size.

Identification of the third protein that is required for the tail assembly and cell-to-cell movement of BYV further advances the concept of the closterovirus tail as a specialized movement device. What could be the architectural and functional roles of p64 in BYV virions? The virion-embedded, CP-like domain of p64 might fit into the helical assembly of the CP and/or CPm subunits. Because p64 is required for tail assembly, it is likely to be physically associated with the tail. One of the possible functions of p64 is the formation of the connector between the body and the tail of closterovirus virions. Such connector proteins form collar regions between the head and the tail in some bacteriophages (Molineux, 2001; Simpson *et al.*, 2000). It seems likely that, in addition to an architectural role, the unique N-terminal domain of p64 might provide additional activities required for the cell-to-cell movement of BYV. The next challenge is to determine the exact molecular architecture of the closterovirus virions and to characterize the mechanistic contribution of each of the five virion proteins.

Although closterovirus virions are the most complex among non-enveloped plant viruses, recruitment of additional virion-associated proteins for cell-to-cell movement, systemic transport, or plant-to-plant transmission is rather common. Among the helical plant viruses, one example is provided by an RNA helicase-related MP of a *Potexvirus* that is capable of binding and modifying the virions (Atabekov *et al.*, 2000). Another example involves the VPg of a *Potyvirus* that is present at one copy per virion and is required for virus systemic transport (Schaad *et al.*, 1997). In a *Benyvirus* and a *Pomovirus*, a minor capsid protein generated via readthrough of the CP gene is incorporated at one end of the virion and is required for virion assembly and transmission (Cowan *et al.*, 1997; Schmitt *et al.*, 1992). Thus, evolutionarily diverse helical viruses have independently evolved an ability to utilize virions as a structural platform for the build-up of additional functional units.

3.6 Acknowledgments

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Conclusion

All plant viruses are effective pathogens in spite of significant differences in the size, organization, and composition of their genomes. There are many examples of "minimalist" plant viruses, like TMV, which have been extensively studied. The genomes of such viruses encode a small number of genes that fulfill all of the virus life cycle requirements. In TMV, these genes encode replication functions, a coat protein, and a movement protein. While viruses like TMV are characterized by the elegant simplicity of their genomes, other viruses like BYV evolved to become more complex. In recent years, the generation of cDNA clones has permitted the characterization of the genetic functions encoded by the large BYV genome. This advancement has provided interesting insights into the evolution of this positive-strand RNA virus and its unusually large genome.

The increased complexity of BYV affects all aspects of the virus life cycle, and has been associated with genetic functions involved in virus replication, assembly, and transport (Dolja, 2003). Interestingly, most of these functions are clustered into two discrete gene modules. The RNA replication module of BYV is conserved in all alphavirus-like viruses, and harbors the methyltransferase, RNA helicase, and RNA-dependent RNA polymerase domains, which are the hallmark of this superfamily of animal and plant viruses (Koonin and Dolja, 1993). The evolutionary innovation of the replication machinery of BYV and other closteroviruses includes large interdomain regions in the core replicase with unknown functions, a leader proteinase that is essential for efficient RNA amplification, and expression of the RdRp via a +1 translation frameshift mechanism that is not found in other positive-strand RNA viruses (Agranovsky *et al.*, 1994; Peng and Dolja, 2000; Peremyslov *et al.,* 1998).

The second conserved module is the quintuple gene block, which in BYV encodes the p6 protein, HSP70h and p64, as well as two coat proteins, CP and CPm. Initial

genetic characterization of the BYV cell-to-cell movement requirements demonstrated that each of these genes is essential but not sufficient to perform this critical function (Alzhanova *et al.,* 2000). The fact that each of the known structural BYV proteins participates in virus movement prompted us to ask a more broad question, what are the relationships between virus movement and assembly? The importance of this question was emphasized by the findings that the BYV virions were found in association with plasmodesmatal necks, that antibodies to HSP70h labeled both virion masses and plasmodesmata, and that HSP70h and p64 were detected in isolated virions of the BYV relative, LIYV (Medina *et al.,* 1999; Tian *et al.,* 1999).

The specific goals of this investigation were to examine the possible association of HSP70h and p64 with virions and to gain insights into the nature and significance of such an association. Using immunoblot analysis and anti-HSP70h or anti-p64 antibodies, we detected each of these proteins in preparations of purified BYV particles. In addition, SDS-PAGE analysis of virions revealed the presence of a minor band with a molecular weight similar to that of both HSP70h (~65 kDa) and p64. This band was extracted from the gel and analyzed by MS/MS. Strikingly, the resulting peptide sequences matched known sequences for both HSP70h and p64. To determine if HSP70h and p64 are physically associated with virions, purified preparations of BYV were analyzed using 10-40% density sucrose gradients and each fraction was examined by immunoblot analyses. Because HSP70h and p64 peaked in the same sucrose gradient fractions as intact virions, we reasoned that these proteins are either attached to virions, or represent integral virion components. To rule out the possibility of incidental co-migration of HSP70h and p64 with virions, we demonstrated that anti-HSP70h or anti-p64 antibodies effectively immunocapture BYV virions. To further characterize the strength of HSP70h and p64 association with the virions, we used treatments with varying concentrations of NaCl, LiCl, or detergents, as well as exposure of virions to high or low pH. None of these treatments were able to specifically release HSP70h and p64 from virions without dissociation of the virions

themselves. These results strongly suggested that HSP70h and p64 are integral virion components.

Additional important insights into the nature of p64 association with the virions were obtained using immunochemical and biochemical techniques. We found that the antibodies specific to the N-terminal, but not the C-terminal, domain of p64 immunoprecipitate virions. This result suggested that the former protein domain is exposed at the virion's surface, whereas the latter one is embedded into virion structure and is inaccessible to antibodies. This conclusion was further supported by data on virion treatment with low concentrations of trypsin. We found that this limited proteolysis completely digested the N-terminal p64 domain. In contrast, the C-terminal, ~30 kDa domain remained undigested and associated with the virions. Similar experiments with HSP70h resulted in rapid protein degradation, suggesting that HSP70h is likely to be exposed on the virion's surface.

Our studies have demonstrated that in addition to CP and CPm, BYV virions incorporate p64 and the viral molecular chaperone HSP70h. It is likely that p64 is embedded within the virion structure, whereas HSP70h appears to be exposed. Our repeated attempts to determine where these proteins are located relative to the tail and body of the BYV virions, by immunogold labeling failed. This was possibly due to the fact that low amounts of HSP70h and p64 are associated with purified virions. Others in the lab were able to isolate virion tails using virion fragmentation by sonication followed by buoyant density gradient centrifugation which allowed the analysis of their protein composition. It was found that in addition to CPm, tails contained both HSP70h and p64. It was also demonstrated that the tail encapsidates a 5'-terminal part of the viral RNA of ~650 nts in length (Peremyslov *et al.,* 2004; Satyanarayana *et al.,* 2004).

An additional series of experiments revealed that the ~20 kDa viral protein p20 is also associated with BYV virions. This protein has been shown to enhance the long-

distance transport of BYV (Prokhnevsky *et al.,* 2000). Like HSP70h and p64, p20 is also associated with the CPm tails (Peremyslov *et al.*, 2004). Atomic force microscopy showed that wild type virion tails possess a complex, three-segment structure (Fig. 4.1). Examination of p20 deficient virion tails revealed that the tip segment was lacking, suggesting a role for this protein in the assembly of this structure (Peremyslov *et al.,* 2004).

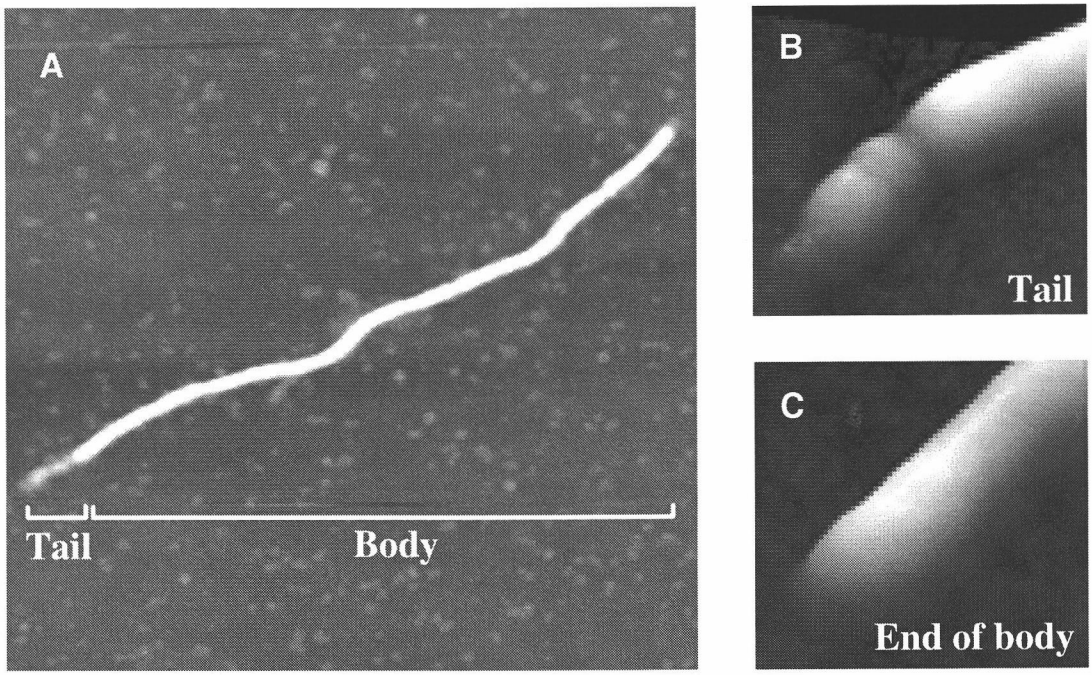

Figure 4.1. Morphology of BYV virions. (A) Atomic force microscopy image of a BYV virion. The white appearance of the body and the grey appearance of the tail are due to a larger depth of the body compare to that of the tail. (C and D) 3D reconstruction of the virion ends obtained using atomic force microscopy. Courtesy of Igor Andreev and Michael E. Taliansky.

What is the principal function of virion tails? It is clear that CPm provides protection for the 5'-terminal part of the viral genome. However, it is well known that the vast majority of plant helical viruses encapsidate their entire genome with a single capsid protein. As we have shown previously, inactivation of CPm results in the assembly of

"CP only" virus like particles (VLPs) that encapsidate the entire BYV RNA (Alzhanova *et al.,* 2001). The fact that each of the four BYV tail proteins are required for either cell-to-cell movement or long-distance transport suggests that the virion tails of the closteroviruses may have evolved as a specialized device for virus spread within infected plants. Unequivocal proof of this hypothesis requires genetic separation of the structural and transport roles of the tail proteins, which is yet to be provided. However, this proposition is indirectly supported by the autonomous targeting of BYV HSP70h to actin microfilaments and plasmodesmata (unpublish data). Because BYV transport through plasmodesmata occurs in association with microfilaments, it seems likely that HSP70h chaperones virions along this pathway.

In addition to novel information on the molecular composition of the closteroviral particles, this work highlights important trends in closteroviral evolution. The conservation of a quintuple gene block among all closteroviruses suggests that it was present in a common ancestor of this large and genetically diverse virus family. On the other hand, no other plant viruses with the helical virion morphology possess such complex virions whose formation requires acquisition of four additional structural genes. The possible evolutionary cause for origins of the quintuple gene block lies in the unusually large size of the closteroviral genomes. Transport of these genomes within plants may have required 'invention' of the specialized tail device.

This and other research provides support for the following tentative scenario of quintuple gene block evolution. Computer analysis of the amino acid sequences revealed that closteroviral CPs, CPms, and p64s share a conserved core domain found in all capsid proteins of the plant viruses with filamentous morphology (Napuli *et al.,* 2003). Moreover, closteroviral CPms are more closely related to closteroviral CPs than to CPs of other viruses that belong to the same protein family (Koonin and Dolja, 1993). This strongly suggests that closteroviral CPms and p64s evolved via a gene triplication event followed by functional diversification of the additional CP-derived genes. The fact that CPm and p64 genes are localized right upstream from a CP gene

is in line with this idea. Our demonstration that the CP-like, C-terminal domain of p64 is indeed incorporated into core virion structure implies that the architectural function of this domain was preserved in evolution.

Because Hsp70s are found in all cells, but in no other viruses except for closteroviruses, it seems likely that the Hsp70 gene has been acquired by a closteroviral ancestor via recombination with the cell mRNA encoding Hsp70. Subsequent evolution of this protein resulted in development of beneficial virus transport properties, such as targeting to actin-myosin motility systems and plasmodesmata. Cellular Hsp70s are molecular chaperones that participate in assembly of multiprotein complexes, but do not become integral components of these complexes upon their maturation (Bukau and Horwich 1998; Pilon and Schekman, 1999). As we have demonstrated, BYV HSP70h violates this rule, because it is incorporated into mature, movement-competent virus particles.

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J Gen Virol. **79**(Pt 5):1289-98.

Appendix

Appendix A

Abstracts of Second Author Publications

In addition to chapter 1 and 2 I was also coauthor in three other publications related to the assembly, cell-to-cell movement and long distance transport of BYV. I have included the abstracts for these publications in this appendix.

EMBO J. 2001 Dec 17;20(24):6997-7007.

Cell-to-cell movement and assembly of a plant closterovirus: roles for the capsid proteins and Hsp70 homolog.

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Diverse animal and plant viruses are able to translocate their virions between neighboring cells via intercellular connections. In this work, we analyze the virion assembly and cell-to-cell movement of a plant closterovirus and reveal a strong correlation between these two processes. The filamentous virions of a closterovirus possess a long body formed by the major capsid protein (CP) and a short tail formed by the minor capsid protein (CPm). Genetic and biochemical analyses show that the functions of these virion components are distinct. A virion body is required primarily for genome protection, whereas a tail represents a specialized device for cell-to-cell movement. Furthermore, tail assembly is mediated by the viral Hsp70 homolog (Hsp70h) that becomes an integral part of the virion. Inactivation of the ATPase domain of Hsp70h results in assembly of tailless virions that are incapable of translocation. A dual role for the viral molecular chaperone Hsp70h in virion assembly and transport, combined with the previous finding of this protein in intercellular channels,allowed us to propose a model of closteroviral movement from cell to cell.

J Virol. 2002 Nov;76(21):11003-11.

Interaction between long-distance transport factor and Hsp70-related movement protein of Beet yellows virus.

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Systemic spread of viruses in plants involves local movement from cell to cell and long-distance transport through the vascular system. The cell-to-cell movement of the Beet yellows virus (BYV) is mediated by a movement protein that is an Hsp70 homolog (Hsp70h). This protein is required for the assembly of movement-competent virions that incorporate Hsp70h. By using the yeast two-hybrid system, in vitro coimmunoprecipitation, and in planta coexpression approaches, we show here that the Hsp70h interacts with a 20-kDa BYV protein (p20). We further demonstrate that p20 is associated with the virions presumably via binding to Hsp70h. Genetic and immunochemical analyses indicate that p20 is dispensable for assembly and cell-to-cell movement of BYV but is required for the long-distance transport of virus through the phloem. These results reveal a novel activity for the Hsp70h that provides a molecular link between the local and systemic spread of a plant virus by docking a long-distance transport factor to virions.

J Virol. 2003 Mar;77(5):2843-9.

Leader proteinase of beet yellows virus functions in long-distance transport.

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The 66-kDa leader proteinase (L-Pro) of the Beet yellows virus (BYV) possesses a nonconserved N-terminal domain and a conserved, papain-like C-terminal domain. Previous work revealed that the N-terminal domain functions in RNA amplification, whereas the C-terminal domain is required for autoproteolysis. Alanine-scanning mutagenesis was applied to complete the functional analysis of L-Pro throughout the virus life cycle. This analysis indicated that the C-terminal domain of L-Pro, in addition to being required for proteolysis, also functions in RNA amplification and that these two functions are genetically separable. Examination of the role of L-Pro in BYV cell-to-cell movement revealed that none of the 20 examined replication-competent mutants was movement defective. In contrast, six of the L-Pro mutations affected the long-distance transport of BYV to various degrees, whereas three mutations completely abolished the transport. Because these mutations were located throughout the protein molecule, both domains of L-Pro function in virus transport. We conclude that in addition to previously identified functions of L-Pro, it also serves as the BYV long-distance transport factor.