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Sobemoviruses: Genomic Organization, Potential for Recombination and Necessity of P1 in Systemic Infection

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Declaration:

I hereby declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any degree or examination.

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Sobemoviirused: genoomi organisatsioon, rekombinatsioonipotentsiaal ja valgu P1 vajalikkus süsteemseks infektsiooniks

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TTÜ KIRJASTUS

To Oliver and Kaido

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INTRODUCTION

A type member of sobemoviruses - *Southern bean mosaic virus* (SBMV) - was the subject of biochemical and physiochemical studies already since 1940-s (Zaumeyer and Harter, 1943a; Zaumeyer and Harter, 1943b; Choe, 1955; Diener, 1965; Price, 1965; Ghabrial *et al*., 1967; Weintraub and Ragetli, 1970; Sehgal, 1973). Subsequently, it served as a prototype for small angle x-ray diffraction and neutron scattering applications (Abad-Zapatero *et al.*, 1980; Abad-Zapatero *et al.*, 1981; Krüse *et al.*, 1982) in scrutinizing organization of icosahedral virions. Also, SBMV was the model of virion stability investigations (Wells and Sisler, 1969; Erickson and Rossmann, 1982).

Consequently, the first fully sequenced sobemovirus was a cowpea strain of SBMV (SBMV-CP) - later classified as an independent species - *Southern cowpea mosaic virus* (SCPMV; Wu *et al.,* 1987; Hull and Fargette, 2005). Soon after, a similar genomic organization was found to be characteristic to *Rice yellow mottle virus* (RYMV; Ngon A Yassi *et al.,* 1994; Pinto and Baulcombe, 1995), *Lucerne transient streak virus* (LTSV, Jeffries *et al.*, 1995) and the bean strain of *Southern bean mosaic virus* (SBMV-B, later known as SBMV; Othman and Hull, 1995; Lee and Anderson, 1998). At the same time, the sequence analysis of *Cocksfoot mottle virus* (CfMV) showed substantial sequence similarity but different genome structure in the middle part of the genome when compared to the others (Mäkinen *et al*., 1995b; Ryabov *et al.*, 1996). In contrast to SBMV, SCPMV, LTSV and RYMV sequences, no individual ORF capable of directing the synthesis of a long (ca 100-kDa) protein was found in CfMV genome (Mäkinen *et al.*, 1995a). However, *in vitro* translations of the CfMV genomic RNA revealed a 100-kDa protein, characteristic to all sobemoviruses (Mäkinen *et al.*, 1995a). Analysis of the genome sequence information revealed a -1 ribosomal frameshifting signal that leads translation from one reading frame (ORF2a that encodes a serine protease, Pro, and a viral genome-linked protein, VPg) into an overlapping reading frame (ORF2b encoding a RNA dependent RNA polymerase, RdRp) enabling generation of the 100-kDa polyprotein. Surprisingly, similar frameshifting signals were also found in long ORF2 sequences (encoding Pro, VPg and RdRp) of SBMV, SCPMV and RYMV (Mäkinen *et al.*, 1995a). Later, re-sequencing of RYMV indicated that the genome of RYMV is actually "CfMV-like" (Fargette *et al.*, 2004). Afterwards, several new sobemoviruses were sequenced and announced as "SCPMV-like" (Lokesh *et al.*, 2001; Zhang *et al.*, 2001) and several as "CfMV-like" (Dwyer *et al.*, 2003; Callaway *et al.*, 2004; Sérémé *et al.*, 2008; McGavin and MacFarlane, 2008). All the other evolutionary related virus groups (polero-, enamo-, luteo-, tombus-, diantho-, panico- and umbraviruses) use -1 ribosomal frameshift to control the amount and expression of RdRp (Hull, 2002). Thus, "the SCPMV-like" organization turns out as unusual. The first objective of the current thesis was to re-sequence the middle part of the genome of SCPMV-like sobemoviruses to clarify their genomic organization.

Sobemoviruses are defined as an unclassified genus (Hull and Fargette, 2005). The reason for that is a modular nature of sobemovirus genomes – sequence comparisons and phylogenetic analyses have identified no similarities with other viruses at ORF1 of sobemoviruses, whereas the middle part of the sobemovirus genome (encoding Pro-VPg-RdRp) has similarities with that of the genera *Polerovirus* and *Enamovirus* belonging to the family *Luteoviridae*, and the 3' part of the sobemovirus genome encoding the coat protein (CP) is related to the genus *Necrovirus* belonging to the family *Tombusviridae* (Hull and Fargette, 2005). Moreover, there exists a potential natural hybrid between polero- and sobemovirus. *Poinsettia cryptic virus* (suggested to be renamed as *Poinsettia latent virus*, PnLV) has been described as a virus showing a close relationship to poleroviruses within its first part of the genome encoding the non-structural proteins, whereas its last part of the genome that encodes a viral capsid protein is rather similar to sobemoviruses. The 5' and 3' UTR-s of PnLV are characteristic to poleroviruses (aus dem Siepen *et al.*, 2005). Due to the modularity, i.e. the shuffling of genes and gene blocks, the key mechanism in the evolution of "the supergroup" luteo-sobemo-tombusviruses is supposed to be RNA recombination (Martin *et al.,* 1990; Gibbs, 1995). For CfMV, five different viral defective interfering RNAs (DI RNAs) corresponding to 35-40 nucleotides of the 5'-proximal end of genomic RNA linked with 850-950 nucleotides of the 3' terminus have been identified. That is considered to be a proof of the replicase-driven template switching mechanism needed for creation of the recombinant RNA molecules (Mäkinen *et al.*, 2000a). However, the phylogenetic analyses of sequenced sobemoviruses do not assure recombination events inside *Sobemovirus* genus (Lokesh *et al.*, 2001; Fargette *et al.*, 2004; Zhou *et al.*, 2005; Chare and Holmes, 2006; Stuart *et al.*, 2006; Fargette *et al.*, 2008; Sérémé *et al.*, 2008). Therefore, the second objective of the current study was to experimentally look for potential recombinants between two sobemoviruses – CfMV and *Ryegrass mottle virus* (RGMoV) – under little or no selection pressure (i.e. non-transgenic plants infected with wild-type viruses).

The third objective of this study was to start unraveling the functions of CfMV P1 – a highly divergent protein encoded by 5'-proximal ORF1 – that is not related to any other known protein.

ORIGINAL PUBLICATIONS

The present dissertation is based on the following publications, which will be referred to in the text by their Roman numbers.

I **Meier, M. and Truve, E**. (2007). Sobemoviruses possess a common CfMV-like genomic organization. *Archives of Virology* **152**, 635-640.

II **Meier, M. and Truve, E.** (2006). An attempt to identify recombinants between two sobemoviruses in doubly infected oat plants. *Environmental Biosafety Research* **5**, 47-56.

III **Meier, M., Paves, H., Olspert, A., Tamm, T. and Truve, E**. (2006). P1 protein of *Cocksfoot mottle virus* is indispensable for the systemic spread of the virus. *Virus Genes* **32**, 321-326.

ABBREVIATIONS

ARM	arginine rich motif
BSSV	*Blueberry shoestring virus*
CAYMV	calopo yellow mosaic virus
cDNA	complementary DNA
CfMV	*Cocksfoot mottle virus*
CfMV ε	CfMV 5′ translation element ε
CfMV-No	Norwegian isolate of CfMV
CfMV P1(-)	P1-defective variant of CfMV-No icDNA
CfMV RdRp(-)	RdRp-defective variant of CfMV-No icDNA
CMMV	*Cocksfoot mild mosaic virus*
CnMoV	*Cynosurus mottle virus*
CP	coat protein
crTMV	crucifer-infecting *Tobacco mosaic virus*
cv	cultivar
DI RNA	defective interfering RNA
DNA	deoxyribonucleic acid
dpi	days post infection
dsDNA	double-stranded DNA
eIF(iso)4G	isoform of the eukaryotic translation initiation factor 4G
GCFV	*Ginger chlorotic fleck virus*
GFP	green fluorescent protein
icDNA	infectious cDNA
ICTV	International Committee on Taxonomy of Viruses
IRES	internal ribosome entry site
IYMV	*Imperata yellow mottle virus*
LTSV	*Lucerne transient streak virus*
LTSV-Can	Canadian isolate of LTSV
MOI	moiety (or multiplicity) of infection, shows the number of virus particles per target cell
MOPS	3-N-morpholino propane sulfonic acid
MP	movement protein
ORF	open reading frame
P1	protein encoded by ORF1
P2	protein encoded by ORF2
P2a	protein encoded by ORF2a
P2b	protein encoded by ORF2b
P3	protein encoded by ORF3
PLYV	*Papaya lethal yellowing virus*
PnLV	poinsettia latent virus
Pro	protease
PTGS	post-transcriptional gene silencing
QTL	quantitative trait locus

R-domain	random domain
RdRp	RNA-dependent RNA polymerase
RGMoV	*Ryegrass mottle virus*
RGMoV-Jap	Japanese isolate of RGMoV
RoMoV	*Rottboellia yellow mottle virus*
rms	root mean square
rpm	revolutions per minute
RT-PCR	reverse transcriptase PCR
RuCMV	*Rubus chlorotic mottle virus*
RYMV	*Rice yellow mottle virus*
RYMV-Mg1	Madagaskar isolate 1 of RYMV
RYMV-Nig	Nigerian isolate of RYMV
RYMV-Tz3	Tanzaniam isolate 3 of RYMV
satRNA	satellite RNA
SBMV	*Southern bean mosaic virus*
SBMV-ALM	Almerian isolate of SBMV
SBMV-Ark	Arkansas isolate of SBMV
SBMV-B	bean strain of SBMV (now: SBMV)
SBMV-Col	Colombian isolate of SBMV
SBMV-CP	cowpea strain of SBMV (now: SCPMV)
SBMV-Mor	Moroccon isolate of SBMV
SBMV-SP	São Paolo isolate of SBMV
SCMoV	*Subterranean clover mottle virus*
SCPMV	*Southern cowpea mosaic virus*
SCPMV-WI	Wisconsin isolate of SCPMV
S-domain	surface (or shell) domain
SeMV	*Sesbania mosaic virus*
sgRNA	subgenomic RNA
siRNA	small interfering RNA
SMAMV	snake melon asteroid mosaic virus
SNMoV	*Solanum nodiflorum mottle virus*
SoMV	*Sowbane mosaic virus*
ssRNA	single-stranded RNA
TLS	tRNA-like structure
TNV	*Tobacco necrosis virus*
TRoV	*Turnip rosette virus*
UTR	untranslated region
VPg	viral genome linked protein
VTMoV	*Velvet tobacco mottle virus*
wt	wild-type

1. REVIEW OF THE LITERATURE

1.1. Genus *Sobemovirus*

In 1969, single-component-RNA beetle-transmitted viruses were proposed to classify into a southern bean mosaic virus group (Walters, 1969). Since 1995, this group was recognized as a genus *Sobemovirus* (sigla from *So*uthern *be*an *mo*saic *virus*) by the International Committee on Taxonomy of Viruses (ICTV) (Hull, 1995). The establishment of the genus was based on similarities in particle morphology, thermal stability, sedimentation coefficient and sizes of their coat protein subunits, size and type of their genomic RNA, distribution of virus particles within the cell, features in mode of vector transmission and their rather narrow host range. Hitherto, the genus *Sobemovirus* is not assigned to any family (Hull and Fargette, 2005).

At this time, 13 viruses are accepted as definite species and 4 viruses as tentative species for sobemovirus group by the ICTV (Hull and Fargette, 2005). The list of definite species involves completely sequenced viruses such as *Cocksfoot mottle virus* (CfMV), *Lucerne transient streak virus* (LTSV), *Rice yellow mottle virus* (RYMV), *Ryegrass mottle virus* (RGMoV), *Sesbania mosaic virus* (SeMV), *Southern bean mosaic virus* (SBMV), *Southern cowpea mosaic virus* (SCPMV), *Subterranean clover mottle virus* (SCMoV), and *Turnip rosette virus* (TRoV), but also several extensively studied but non-sequenced or partially sequenced viruses such as *Blueberry shoestring virus* (BSSV), *Solanum nodiflorum mottle virus* (SNMoV), *Sowbane mosaic virus* (SoMV) and *Velvet tobacco mottle virus* (VTMoV) (Table 1, p. 16). The list of tentative species involves *Cocksfoot mild mosaic virus* (CMMV), *Cynosurus mottle virus* (CnMoV), *Ginger chlorotic fleck virus* (GCFV) and *Rottboellia yellow mottle virus* (RoMoV) (Table 1). However, according to the recent sequencing data, CMMV is not a sobemovirus but a panicovirus (Ziegler *et al.*, 2009). CnMoV, GCFV and RoMoV have not yet been sequenced.

In addition, two recently fully sequenced viruses should be classified as sobemoviruses. Firstly, *Imperata yellow mottle virus* (IYMV) was found to be consistent with all criteria to assign it into the genus *Sobemovirus* (Sérémé *et al.*, 2008). Secondly, *Rubus chlorotic mottle virus* (RuCMV) was found to be a typical sobemovirus according to its genomic sequence (McGavin and MacFarlane, 2008). The partial sequence of SoMV (Gratsia *et al.*, 2006) shows 94 % identity with the corresponding region of RuCMV RdRp. Moreover, three other viruses presently not fully sequenced probably belong to the genus *Sobemovirus*. The physical and chemical properties of *Calopo yellow mosaic virus* (CAYMV) are similar to those reported for the sobemovirus group. CAYMV has been found to be antigenically related to SBMV and SCPMV but its host range partially differs from previous ones. A 609 bp fragment amplified from the region encoding virus coat protein has 81.8 % of similarity to the corresponding region of SBMV and 66.1 % to that of SCPMV (Morales *et al.*, 1995). The partial sequence of *Papaya lethal yellowing*

virus (PLYV) shows considerable similarity (about 44-51 %) to the polymerase, VPg and coat protein genes of sobemoviruses (Lima *et al.*, 2001). The 71 % of amino acid sequence similarity has been found between the RYMV RdRp and a putative protein-encoding fragment of *Snake melon asteroid mosaic virus* (SMAMV) (Lecoq *et al.*, 2005).

Some sobemoviruses are distributed throughout the world (CfMV, LTSV, RGMoV, SBMV, SCPMV, SoMV) whereas some are limited to only one continent (BSSV, RYMV, SNMoV, SCMoV, TRoV, VTMoV) or even to just one endemic region (SeMV) (Table 1, p. 16). Distribution is directly related to their host range which is relatively narrow, i.e. individual viruses (except SoMV) naturally infect plants from one family only (Table 1, p. 16). In some cases, global exchange of infected material and introduction of novel crops in existing or new agricultural areas have expanded virus areal. For example, SCMoV may have been introduced to Australia following European colonisation. The argument for its Mediterranenean origin is that SCMoV has a beetle vector there but not in Australia (Jones, 2004). Similarly, detection of CfMV in New Zealand is most probably of a foreign origin as its host plant cocksfoot is introduced there (Campell and Guy, 2001). In Spain, the first infections of SBMV are most probably introduced via seeds from infected plants (Verhoeven *et al.*, 2003). Similarly, SoMV has been found to be recently imported with seeds from Netherlands to Greece (Gratzia *et al.*, 2006). An outbreak of RYMV epidemy reported in last 40 years is related with intensification of rice cropping in Africa (Traoré *et al.*, 2009).

The main transmission source of sobemoviruses is mechanical wounding of host plants. The transmission study of RYMV shows that it is efficiently transferred from plant to plant by farming operations and by grazing donkeys, cows and grass rats. It can also spread by wind-mediated leaf contacts and via soil (Sarra, 2005). For SCMoV, it has been shown that the main source of transmission is grazing and trampling by livestock, crushing under vehicle wheels and by mowing for hay production, i.e. contact-transmission (Jones, 2004). In addition, insects, mostly the different species of leaf-eating beetles, transmit sobemoviruses in a semi-persistent manner. Also, aphids, leafminers, leafhoppers, or mirids can transmit sobemoviruses (Table 1, p.16). Several but not all sobemoviruses are seed-transmissible (Table 1, p.16).

Several sobemoviruses are economically important pathogens. RYMV causes one of the most damaging and rapidly spreading diseases of rice in Africa. As a result of RYMV infection, yield losses fluctuate between 10 and 100%, depending on plant age prior to infection, susceptibility of the rice variety, and environmental factors (Traoré *et al.*, 2009). PLYV, causing serious chlorosis, is responsible for severe disease of papaya in Northeast Brazil. SCMoV decreases clover seed and herbage production in Australia. Over time, SCMoV-infected pastures will become weedy and unproductive (Jones, 2004). A severe outbreak of CfMV was observed in the cocksfoot fields in Norway in the middle of 1980´s (Rognli *et al.*, 1995).

Table 1. Viruses of the genus *Sobemovirus* and their biological properties (modified from Meier *et al.*, 2008).

Virus	**Abbr.**	**Distribution**	**Natural host**	**Insect vector**	**Transmission**		**References**
					Mechanical	**Seed**	
Definitive species							
Blueberry shoestring virus	BSSV	USA (Maine, Michigan, New Jersey, Oregon, Virginia, Washington), Canada (New Brunswick, Nova Scotia, Ontario, Quebec)	*Vaccinium corymbosum, V. angustifolium*	*Illinoia pepperi* (aphid)	Yes	No	Ramsdell, 1979; Morimoto *et al.*, 1985; Jaswal, 1990; Hancock *et al.*, 1996
Cocksfoot mottle virus	CfMV	Europe (Denmark, France, Germany, Norway, Russia, UK), New Zealand, Japan	*Chionochloa rubra, Dactylis glomerata, Festuca novae-zelandiae, Poa anceps, P. cita, Triticum aestivum*	*Lema melanopus, L. lichensis* (beetles)	Yes	No	Serjeant, 1967; Upstone, 1969; Catherall, 1970; A´Brook and Benigno, 1972; Engsbro, 1978; Rabenstein and Schmidt, 1979; Mohamed 1980; Toriyama, 1982; Munthe, 1988; Ryabov *et al.*, 1996; Campell and Guy, 2001; Delmiglio, 2008
Lucerne transient streak virus	LTSV	Australia (Victoria, Tasmania), New Zealand, Canada	*Medicago sativa*	ND	Yes	No	Blackstock, 1978; Forester and Jones, 1979; Paliwal, 1983

Rice yellow mottle virus	RYMV	Africa (Benin, Burkina Faso, Cameroon, Chad, Cote d'Ivoire, Gambia, Ghana, Guinea, Guinea Bissau, Kenya, Liberia, Madagascar, Mali, Malawi, Mauritania, Mozambique, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, Tanzania, Togo, Uganda)	*Echinochloa colona, Ischameum rugosum, Oryza barthii, O. glaberrima, O. longistaminata, O. sativa, Panicum repens*	*Chaetocnema* spp., *Dicladispa gestroi, Dactylispa spp., Monomolepta spp., Oulema dunbrodiensis, Sesselia pusilla, Trichispa sericea* (beetles), *Conocephalus merumontanus* (grasshopper)	Yes	No	Bakker, 1974; Okioma *et al.*, 1983; Konaté 1997, 2002; Banwo *et al.*, 2001; Traoré *et al.*, 2001; Sarra 2005
Ryegrass mottle virus	RGMoV	Japan, Germany	*Dactylis glomerata, Lolium multiflorum*	ND	Yes	No	Toriyama *et al*., 1983; Zhang *et al.*, 2001
Sesbania mosaic virus	SeMV	India (Andra Pradesh)	*Sesbania grandiflora*	ND	Yes	ND	Singh and Srivastava, 1985
Solanum nodiflorum mottle virus	SNMoV	Australia (Queensland, New South Wales)	*Solanum nodiflorum, S. nitidibaccatum, S. nigrum*	*Epilachna sparsa, E. doryca australica, E. guttatopustulata, Psylliodes* sp. (beetles), *Cyrtopeltis nicotianae* (mirid)	Yes	No	Greber, 1981; Jones and Mayo, 1984; Greber and Randles, 1986; Gibb and Randles, 1991

Southern bean mosaic virus	SBMV	USA (Arkansas, California, Louisiana, Maryland), Central and South America (Brazil, Colombia, Mexico), Africa (Ivory Coast, Morocco), Europe (France, Lithuania, Spain), Asia (Iran)	*Phaseolus vulgaris*	*Ceratoma trifurcata, Epilachna variestis, Diabrotica undecimpunctata howardii* (beetles)	Yes	Yes	Zaumeyer and Harter, 1943a, 1943b; Yerkes and Patiño, 1960; Grogan and Kimble, 1964; Fulton *et al.*, 1975; Cupertino *et al.*, 1982; Tremaine and Hamilton, 1983; Morales and Castaño, 1985; Wang *et al.*, 1994; Lee and Anderson, 1998; Zitikaitė, 2001, Verhoeven *et al.*, 2003; Segundo *et al.*, 2004
Southern cowpea mosaic virus	SCPMV	USA (Wisconsin), Africa (Botswana, Ghana, Kenya, Nigeria, Senegal, Togo), Asia (India, Pakistan)	*Cassia toria, Pisum sativum, Vigna unguiculata*	*Ootheca mutabilis, Madurasia obscurella* (beetles)	Yes	Yes	Sheperd and Fulton, 1962; Sheperd, 1967; Lamptey and Hamilton, 1974; Singh and Singh, 1974; Shoyinka et al., 1979; Givord, 1981; Reddy and Varma, 1986; Gaikwad and Thottappilly, 1988; Bashir and Hampton, 1993; Gumedzoe *et al.*, 1997

Sowbane mosaic virus	SoMV	USA (California), Canada, Central and South America, Europe (Bulgaria, Czechia/Slovakia, Croatia, France, Greece, Hungary, Italy, Moldavia, Netherlands), Japan, Australia (Queensland, New South Wales, Victoria, Tasmania)	*Chenopodium* spp., *Atriplex subrecta, Spinacia oleracea, Vitis* sp., *Prunus domestica, Alisma plantago-aquatica, Danae racemosa*	*Myzus persicae* (aphid), *Liriomyza langei* (leafminer), *Circulifer tenellus* (leafhopper), *Cyrtopeltis nicotianae*, *Halticus citri* (mirids)	Yes	Yes	Costa *et al.*, 1958; de Silva *et al.*, 1958; Bennet and Costa, 1961; Kirkpatrick *et al.*, 1965;Bancroft and Tolin, 1967; Diaz and Waterworth, 1967; Kado, 1967; Teakle, 1968; Kado, 1971; Šaric, 1971; Šutić and Juretić, 1976;Guy, 1982; Horváth, 1993; Bos and Huijberts, 1996; Gratsia *et al.*, 2006
Subterranean clover mottle virus	SCMoV	Australia (New South Wales, South Australia, Tasmania, Victoria, Western Australia)	*Trifolium subterraneum, Trifolium* spp.	Not found	Yes	Yes	Francki *et al.*, 1983; Wroth and Jones, 1992; Helms *et al.*, 1993; Jones *et al.*, 2001
Turnip rosette virus	TRoV	UK (England, Scotland), Switzerland	*Brassica campestris, B. nigra*	*Phyllotreta nemorium* (beetle)	Yes	ND	Broadbent and Heathcote, 1958; Hollings and Stones, 1973; Thurston *et al.*, 2001
Velvet tobacco mottle virus	VTMoV	Australia (Northern Territory, Queensland, South Australia)	*Nicotiana velutina*	*Cyrtopeltis nicotianae* (mirid), *Epilachna* spp. (beetle)	Yes	No	Randles *et al.*, 1981; Randles and Francki, 1986; Gibb and Randles, 1988; Gibb and Randles, 1991

Tentative species							
Cocksfoot mild mosaic virus	CMMV	Europe (Czechia/Slovakia, Denmark, France, Germany, Norway, UK), Canada (Ontario), New Zealand	*Agrostis stolonifera, Bromus mollis, B. diandrus, Cynosurus cristatus, Dactylis glomerata, Festuca pratensis, Lolium perenne, L. perenne* x *L. multiflorum, Phleum pratense, Poa trivialis, Triticum aestivum*	*Myzus persicae* (aphid), *Lema melanopus* (beetle)	Yes	No	Hüth *et al.*, 1970; Paul et al., 1973/1974; Hüth and Paul, 1977; Torrance and Harrison, 1981; Paliwal, 1986; Guy, 2006
Cynosurus mottle virus	CnMoV	Europe (Germany, UK, Ireland), New Zealand	*Agrostis tenuis, A. stolonifera, Cynosurus cristatus, Lolium perenne, L. perenne* x *L. multiflorum*	*Lema melanopus* (beetle), *Rhopalosiphum padi* (aphid)	Yes	ND	A`Brook, 1972; Catherall *et al.*, 1977; Hüth and Paul, 1977; Mohamed, 1978
Ginger chlorotic fleck virus	GCFV	Africa (Mauritius), Asia (India, Malaysia, Thailand)	*Zingiber officinale*	Not found	Yes	ND	Thomas, 1986
Rottboellia yellow mottle virus	RoMoV	Nigeria	*Rottboellia cochinchinensis*	ND	Yes	ND	Thottappilly *et al.*, 1992
Species not yet recognized by ICTV							

Calopo yellow mosaic virus	CAYMV	Colombia	*Calopogonium mucunoides*	*Diabrotica balteata* (beetle)	Yes	Yes	Morales *et al.*, 1995
Imperata yellow mottle virus	IYMV	Burkina Faso	*Imperata cylindrica, Zea mays*	ND	Yes	No	Sérémé *et al.*, 2008
Papaya lethal yellowing virus	PLYV	Brasil (Pernambuco, Bahia, Rio Grande do Norte, Ceará, Paraíba)	*Carica papaya*	Not found	Yes	No	Loreto *et al.*, 1983; Lima *et al.*, 2001; Saraiva *et al.*, 2006
Rubus chlorotic mottle virus	RuCMV	UK (Scotland)	*Rubus* sp	ND	Yes	ND	McGavin and MacFarlane, 2008
Snake melon asteroid mosaic virus	SMAMV	Sudan	*Cucumis melo* var *flexuosus*	Not found	Yes	Yes	Lecoq *et al.*, 2005

1.2. Genome

All sequenced sobemoviruses have the polycistronic positive sense single-stranded RNA genomes approximately 4.0 - 4.5 kb in size. Sobemovirus genome lacks both 5'-terminal cap and 3'-terminal poly(A) tail (Hull and Fargette, 2005).

Eukaryotic mRNAs are usually monocistronic, their untranslated regions (UTRs) are capped at 5'-terminus, and polyadenylated at 3'-terminus. Both are essential for their efficient translation. Lack of cap and poly(A) indicates that the sobemoviral UTRs must somehow compensate these functions. The 5'-proximal end of sobemovirus genomic and subgenomic RNA (sgRNA) bounds covalently a viral genome-linked protein (VPg) that replaces 5' cap (Ghosh *et al.*, 1979; Ghosh *et al.*, 1981; Ke-Qiang *et al.,* 1982; van der Wilk *et al.*, 1998b; Mäkinen *et al.*, 2000b; Lokesh *et al.,* 2001; Hébrard *et al.*, 2008). The exact roles of phytoviral VPg-s are still unclear although interactions between eukaryotic translation initiation factors and viral VPg-s have been suggested to be involved in protein translation, in RNA replication and in cell-to-cell movement (Sadowy *et al.*, 2001). RYMV VPg was identified as a virulence factor (Hebrard *et al.*, 2006).

Although not much is known about the replication signals required for the initiation of sobemoviral RNA synthesis, a conserved 5'-proximal sequence ACAA is considered to play role in viral RNA replication by promoting or enhancing the binding of viral RdRp (Miller *et al.*, 1995). This motif is characteristic of the 5'-termini of sobemo-, polero-, diantho- and barnaviruses (Xiong and Lommel, 1989; Revill *et al.*, 1994; Miller *et al.*, 1995; Zavriev *et al.*, 1996; Revill *et al.*, 1998; Tamm and Truve, 2000b). It is also present upstream from translation initiation codon of CP in sobemovirus genome, indicating a possible 5'-terminus of sgRNA (Hacker and Sivakumaran, 1997). Differently from other sobemoviruses, such a motif is neither present at the 5'-proximal end nor upstream from the CP translation initiation codon in the genome of CfMV (Mäkinen *et al.*, 1995b) and it is also absent at the 5'-proximal end of IYMV (Sérémé *et al.*, 2008). Hypothetically, the different 5' sequence might provide a great advantage in certain cereals. Translational enhancer element CfMVε at the 5′ UTR was specifically highly successful in enhancement of the expression of reporter genes in barley suspension cells compared to other viral enhancer sequences (TMVΩ, CrTMV IRES, PVXαβ and 5′UTR of AMV RNA4) (Mäkeläinen, 2006). Notably, all these leader sequences promote efficient translation in wheat germ extract and in tobacco cells (Gallie *et al.*, 1987; Jobling and Gherke, 1987; Browning *et al.*, 1988; Gallie *et al.*, 1989; Smirnyagina *et al.*, 1991; Pooggin *et al.*, 1992; Ivanov *et al.*, 1997; Dorokhov *et al.*, 2002; Gallie, 2002a). TMVΩ has also shown to promote translation in maize and rice (Gallie *et al.*, 1989).

Both genomic and subgenomic RNAs of most of sobemoviruses contain a conserved polypurine tract GAAA shortly after the ACAA motif (Mäkinen *et al.*, 1995a; Ryabov *et al.*, 1996; Hacker and Sivakumaran, 1997; Tamm and Truve 2000b). Interestingly, a polypurine tract is found to be a key element of CrTMV IRES (Dorokhov *et al.*, 2002).

The vicinity of sgRNA transcription start site of SBMV, SCPMV, LTSV, RYMV and CfMV is predicted to fold into a hairpin loop (Hacker and Sivakumaran, 1997; Kokorev *et al*., 2007). Although the significance of that stem-loop is not known, it or its complement in minus-strand RNA is considered to play a role in sgRNA synthesis. In sequence of CfMV, another potentially stable stem-loop situated in the middle of CP encoding region has been found to be complementary to the one adjacent the transcription site of sgRNA. The hypothetical interaction between these loops has been proposed to be involved in transactivation of sgRNA synthesis (Kokorev *et al*., 2007).

Almost nothing is known about the signals essential for the initiation of synthesis of the genomic minus strand potentially provided by the 3'-terminus of the genomic RNA. The 3' UTRs of sobemovirus genomes show only marginal sequence conservation. A potential tRNA-like structure (TLS) has been attributed to the 3'-end of RYMV and CfMV by computer modelling (Ngon A Yassi *et al.*, 1994; Ryabov *et al.*, 1996) but no experimental data is available on that. No such secondary structure has been found at the 3' end of SBMV, SCPMV or SeMV in computational analysis (Wu *et al.*, 1987; Othman and Hull, 1995; Lokesh *et al.*, 2001). In general, a non-TLS heteropolymeric 3'-termini has been found customary among plant viruses (Dreher *et al.*, 1999).

Interaction of the UTRs is required for efficient translation (Gallie, 1998). However, 5' and 3' UTRs of CfMV had no synergistic effect on test mRNA translation in barley suspension cells. The reason of it was presumed to be a lack of the virus encoded proteins or a sequence buried somewhere in the middle part of the genome needed for bringing together the genomic termini (Mäkeläinen, 2006).

The genomic RNA of incoming sobemovirus particles is probably uncoated by the co-translational disassembly mechanism and followed by RNA replication. For example, the particles of SBMV can completely disassemble only after their RNA has initiated translation (Brisco *et al.*, 1985; Shields *et al.*, 1989).

Sobemovirus genome consists of four ORFs (Fig. 1).

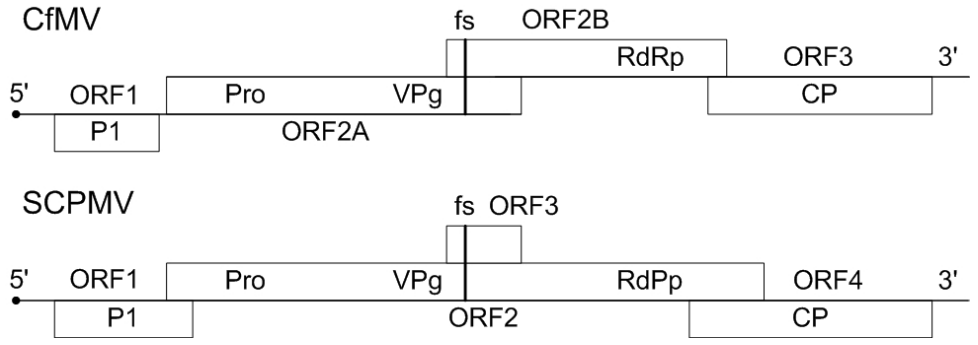

Figure 1. CfMV-like genomic organization is represented by CfMV genome according to Mäkinen *et al.*, 1995. SCPMV-like genomic organization is represented by SCPMV according to Wu *et al.*, 1987.

ORF1 as well as the downstream ORF2 is translated from the genomic RNA (Salerno-Rife *et al.*, 1980; Ke-Qiang *et al.*, 1982; Wu *et al.*, 1987; Tamm *et al.*, 1999; Zhang *et al.*, 2001). Initiation of translation of the genomic RNA is facilitated at least in case of CfMV by the translational enhancer CfMVε situated at 5' UTR. Deletion of 3' terminal part of CfMVε has no effect on translation yield but the first 10 nt-s of CfMVε (possibly forming a stem-loop) together with several downstream 6-23 nt stretches complementary to plant 18S rRNA, might be involved in translation initiation and binding of the 43S ribosomal preinitiation complex (Mäkeläinen, 2006).

Translation of ORF2 occurs by the process termed leaky scanning of 40S ribosomes that can bypass the start codon of ORF1 due to its suboptimal context (Hacker and Sivakumaran, 1997; Dwyer *et al*., 2003) and initiate translation from subsequent ORF2 (Sivakumaran and Hacker, 1998; Tamm *et al*., 1999). It was postulated that some sobemoviruses (referred as a "SCPMV-like" genomic organization) express their ORF2-encoded proteins from one in-frame ORF, whereas the others (named as "CfMV-like" sobemoviruses) express predominantly only a part of it whereas they use a –1 translational frameshift mechanism to extend translation for the expression of a "full-length" polyprotein (Fig. 1, p. 23)[1]. Studies on CfMV showed that the ribosomal frameshifting takes place with an efficiency of approximately 10-20 % (Luccesi *et al.*, 2000; Mäkeläinen and Mäkinen, 2005). The -1 ribosomal frameshift signal of CfMV consists of the heptameric slippery sequence UUUAAAC, followed by a simple stem-loop structure located 7 nucleotides downstream of it (Tamm *et al.*, 2009). Notably, the ribosomal frameshift signal that was discovered from the sequence of CfMV was found to be conserved for all the sobemoviruses (Mäkinen *et al.*, 1995a; Ryabov *et al.*, 1996; Lokesh *et al.*, 2001; Zhang *et al.*, 2001; Dwyer *et al.*, 2003; McGavin and MacFarlane, 2008; Sérémé *et al.*, 2008; Ozato *et al.*, 2009). In the case of SCPMV-like genomic annotations, the ribosomal frameshift signal was located upstream of the small internal coding region ORF3 having no stops between the –1 frameshift signal and the initiation codon of the putative ORF3. Moreover, the beginning of ORF3/putative P3 of SCPMV-like viruses and the beginning of ORF2b/putative P2B of CfMV-like viruses were found to be similar in sequence comparisons. Therefore it has been suggested that ORF3 may be expressed as a part of a smaller polyprotein via –1 ribosomal shift (Mäkinen *et al.*, 1995a; Dwyer *et al.*, 2003). However, the posterior part of ORF3/P3 sequences has been found to be very divergent (Dwyer *et al.*, 2003). No single protein has been attributed to ORF3 alone (Tamm and Truve, 2000b; Dwyer *et al*., 2003).

A sobemovirus coat protein (CP) is encoded by the genome 3'-proximal ORF but translated from its copy in subgenomic RNA (Rutgers *et al.*, 1980; Ghosh *et al.*, 1981; Chu and Francki, 1983; Morris-Krsinich and Forster, 1983; Morris-Krsinich and Hull, 1983; Kiberstis and Zimmern, 1984; Tamm *et al.*, 1999). The

[1] In this thesis, we will demonstrate that all sequenced sobemoviruses possess a common genomic organization characteristic of CfMV-like viruses (publication I).

sgRNA has been detected both in sobemovirus-infected tissues and in virus particles (Rutgers *et al.,* 1980; Salerno-Rife *et al.*, 1980; Weber and Sehgal, 1982; Mäkinen *et al.,* 1995b; Ryabov *et al.,* 1996; Bonneau *et al*., 1998; Tamm *et al*., 1999). In addition to the genomic and sgRNA, some sobemoviruses (LTSV, RYMV, SCMoV, SNMoV, VTMoV) can also encapsidate a viroid-like circular satellite RNA (satRNA) (Gould *et al.*, 1981; Gould and Hatta, 1981; Randles *et al.*, 1981; Tien *et al.*, 1981; Francki *et al*., 1983; Jones *et al.*, 1983; Paliwal, 1983; Jones and Mayo, 1984; Dall *et al.*, 1990; Davies *et al.*, 1990; Sehgal *et al*., 1993; Collins *et al.*, 1998). The sizes of sobemovirus-associated satRNAs range from 220 to 390 nt (Haseloff and Simons, 1982; Keese *et al.*, 1983; AbouHaidar and Paliwal, 1988; Davies *et al*., 1990; Collins *et al*., 1998). The 220 nt-s satRNA of RYMV is the smallest of all discovered satRNAs (Collins *et al*., 1998; Pinel *et al.*, 2003). In addition, CfMV is reported to encapsulate DI RNA molecules (Mäkinen *et al*., 2000a). The preliminary studies on CfMV DI RNA suggest that it could favour accumulation of CP (Kokorev *et al.*, 2007). Also, it has been suspected that a sequence lacking nt 231-509 amplified from RNA derived from SeMV particles was most probably arose from DI RNA (Lokesh *et al.*, 2001).

1.3. Gene products and their functions

1.3.1. P1

P1 is encoded by the 5'-proximal ORF1 of the genomic RNA of sobemoviruses. Translation of P1 occurs with poor efficiency due to the suboptimal context for ORF1 initiation (Dwyer *et al.*, 2003). Surprisingly, no considerable homologies have been found at nucleic acid as well as amino acid sequence levels between different sobemovirus P1s, making this region the most variable one in the genome of sobemoviruses (Ngon A Yassi *et al*., 1994; Mäkinen *et al*., 1995b; Othman and Hull, 1995; Tamm and Truve, 2000b). The molecular masses of sobemoviral P1s range between 12-kDa and 25-kDa (Tamm and Truve, 2000b).

P1s of RYMV and SCPMV are required for systemic infection but not for virus replication (Bonneau *et al*., 1998; Sivakumaran *et al*., 1998). SCPMV P1 has been also shown to be non-essential for viral assembly (Sivakumaran *et al*., 1998). RYMV P1 was first described as a pathogenicity determinant (Bonneau *et al*., 1998). Later, it was shown to act as a suppressor of RNA silencing in *Nicotiana benthamiana*, a non-host species for sobemoviruses. P1 of RYMV-Nig prevents a systemic spread but not a short-range movement of the silencing signal (Voinnet *et al*., 1999; Hamilton *et al.*, 2002; Himber *et al.*, 2003; Sarmiento *et al*., 2007). Differently, P1s of RYMV-Tz3 and RYMV-Mg1 isolates have been shown to affect also the spread of local silencing signal (Lacombe *et al*., 2010). The suppression of silencing was associated with slight reduction in 21 nt siRNA and severe reduction in 24 nt siRNA accumulation (Lacombe *et al*., 2010). Comparison of P1s of these two isolates revealed contrasting suppression strength. Interestingly, in addition to the ability to suppress silencing RYMV P1 was found to enhance the

short distance movement of silencing signal and even to increase the efficiency of the systemic spread of silencing (Lacombe *et al*., 2010). Therefore, it was suggested that the dual functions to suppress and activate silencing by RYMV P1 has a key role for a successful RYMV infection by maintaining equilibrium between efficient virus multiplication and preservation of the host integrity.

Recently, it was shown that also CfMV P1 acts as a silencing suppressor both at local and systemic levels in *N. benthamiana* as well as in *N. tabacum* (Sarmiento *et al*., 2007). When expressed transgenically in *N. benthamiana*, RYMV P1 affected the normal plant phenotype whereas CfMV P1 did not. Concurrently, both suppressors enhance the spread but not the accumulation of crucifer-infecting *Tobacco mosaic virus* (Siddiqui *et al*., 2008). P1 of CfMV binds ssRNA in a sequence-independent manner (Tamm *et al.*, 2000a) but it does not bind double-stranded small interfering RNAs (siRNAs; Sarmiento *et al.*, 2007). RYMV P1 nucleic acid binding activity remains to be demonstrated.

It was demonstrated that RYMV P1 acts as the suppressor also in rice. However, it was observed that P1 proteins originated from different RYMV isolates had a wide range in their ability to suppress silencing (Siré *et al*., 2008). The investigations on the functional diversity of silencing suppression of a set RYMV isolates elucidated that an ability to suppress silencing is not linked to RYMV pathogenicity or phylogeny but rather to the cell-to-cell movement ability of P1 (Siré *et al*., 2008). Variations in silencing suppression were correlated with a sequence variation of P1. Mutagenesis of P1 demonstrated a strong link between some amino acids and silencing suppression features – some conserved amino acids of the putative zinc-finger motif of RYMV P1, $C64(X)_2C67(X)_{24}C92(X)_2C95$, were involved in cell-to-cell movement and in the strength of the silencing suppression. In addition, one non-conserved position in RYMV P1 in the sequence of the isolate Tz3 (that shows the strongest silencing suppression of all isolates tested) was involved in determination of the strength of the silencing suppression (Siré *et al*., 2008).

Transgenic rice expressing P1 originated either from RYMV-Tz3 or RYMV-Mg1 displayed inflorescence developmental defects comparable to those previously described in a *dcl4-1* mutant[2] (Lacombe *et al*., 2010). Similarly to *dcl4-1* mutant, further analysis showed down-accumulation of the Os *TAS3* siRNA (21-nt conserved siRNA) and up-regulation of Os ARF genes that were previously described as Os *TAS3* siRNA targets. Also, as in *dcl4-1* mutant, there was no accumulation of transitional small RNAs generated from the *AK120922* transcript (process dependent on *OsDCL4*). No effect was observed to other endogenous small RNA pathways (miRNA and ca-siRNA) as well as in case of *dcl4-1* mutant

[2] *Dcl4-1* is a mutant rice line generated and studied by Liu *et al.*, 2007. It has a deletion in the 5' extremity of *OsDCL4*, a gene encoding the Dicer-like protein 4 (which is the major Dicer responsible for 21 nt transitional siRNA synthesis playing a key role for normal plant development in rice).

earlier (Lacombe *et al*., 2010). It was concluded that the expression of RYMV P1 in rice specifically affects a pathway required for reproductive development.

The 5'-proximal half of the genome of sobemoviruses is similar to that of poleroviruses in their organization (Hull and Fargette, 2005). Considerably, although P0 of poleroviruses (encoded by the 5'-terminal ORF0) is the most divergent protein of the viral genome and shares no homology with any other known proteins (Mayo and Miller, 1999), P0s of poleroviruses have features common with sobemoviral P1s. Despite of their divergence, P0s of *Beet western yellows virus* (BWYV), *Potato leafroll virus* (PLRV), *Cucurbit aphid-borne yellows virus* (CABYV) and *Sugarcane yellow leaf virus* (SCYLV) all operate as suppressors of PTGS in 16c *N. benthamiana* model system (Pfeffer *et al.*, 2002; Mangwende *et al.*, 2009). However, P0s of poleroviruses have a conserved F-box-like[3] motif essential for a suppressor activity of P0 (Pazhouhandeh *et al.*, 2006; Baumberger *et al*., 2007; Bortolamiol *et al.*, 2007). F-box-like motifs have not been identified for sobemovirus P1 proteins.

1.3.2. Polyprotein

Although the presence of two different types of genomes has been postulated amongst the sobemoviruses, *in vitro* translation products of these viruses have similar molecular weights. This phenomenon has been explained by different translation strategies of the polyprotein both involving a –1 ribosomal frameshift event (Mäkinen *et al.*, 1995a; Tamm *et al.*, 1999; Dwyer *et al.*, 2003). A shorter, ca ca 70 kDa polyprotein was supposed to correspond to P2a or to frameshifted partial P2-P3, in case of CfMV-like or SCPMV-like viruses, respectively. A longer, ca 100-kDa polyprotein was supposed to correspond to frameshifted P2a-2b of CfMV-like viruses or to the full-length P2 of SCPMV-like viruses. Consequently, two different polyproteins with identical N-terminal part but dissimilar C-terminal part are translated from different sobemoviral genomic RNAs (Fig.1, p. 23).

According to *in silico* analysis, the N-termini of the sobemoviral polyproteins (except LTSV that lacks this region according to the sequence analysis) show the presence of strongly predicted transmembrane helixes (Ryabov *et al.*, 1996; Satheshkumar *et al.*, 2004b; McGavin and MacFarlane, 2008). This N-terminal hydrophobic region was proposed to target the polyprotein into cellular membranes that can possibly serve as a proteolytic processing site for polyprotein maturation. Alternatively, the membrane-anchoring domain of N-terminus is suggested to target the Pro-VPg-RdRp polyprotein into membraneous structures for viral minus strand synthesis (Nair and Savithri, 2010).

[3] The F-box protein family is the largest protein superfamily. F-box proteins are involved in plant hormone response and their related signal transduction pathways. F-box proteins are also involved into ubiquitin-mediated protein degradation complexes; they regulate lateral root formation, light signalling, circadian system and floral development. They probably also participate in stress response and regulation of leaf senescence (Yu *et al.*, 2007).

The N-terminal part of the polyprotein consists of a serine protease (Pro) and a viral genome-linked protein (VPg). The C-terminal part of full-length polyprotein consists of an RNA-dependent RNA polymerase (RdRp). The position of the VPg in between of the viral Pro and RdRp is unique for sobemo-, polero-, enamo- and barnaviruses (Revill *et al.*, 1998; van der Wilk *et al.*, 1998a; van der Wilk *et al.*, 1998b; Wobus *et al.*, 1998). It has demonstrated that sobemoviral polyprotein undergoes a proteolytic processing carried out by sobemovirus-encoded Pro itself (Satheshkumar *et al.*, 2004b).

During proteolytic processing, protease catalyzes the hydrolysis of specific peptide bonds located between two specific amino acid residues. The N-terminal sequencing of VPg-s of sobemoviral VPg-s attached to viral genomes indicated that the polyprotein must be processed between E/T for SBMV, between E/N for CfMV, and between E/S for RYMV (van der Wilk *et al.*, 1998; Mäkinen *et al.*, 2000; Hébrard *et al.*, 2008). No proteolytic processing of CfMV polyprotein(s) happens *in vitro* or in *E. coli* (Tamm *et al.*, 1999). Conversely, SeMV full-length polyprotein was shown to undergo proteolytic processing when expressed in *E. coli*. Analysis of cleavage site mutants confirmed the cleavage of E/T between Pro-VPg at E325/T326 and VPg-RdRp at E402/T403. A third, less efficient cleavage site of E/S at E498/S499 was found downstream (Satheshkumar *et al.*, 2004b). Comparison of ΔN70-Pro and ΔN92-Pro crystals identified identical parameters even though ΔN70-Pro was 22 amino acids longer than ΔN92-Pro. On mass spectroscopic analysis, both had the same mass of ~20,000 Da suggesting an internal cleavage at N-terminus (Gayathri *et al.*, 2006). Initially, a possible non-specific cleavage at the residues A134/V135 in both the mutants was suggested (Gayathri *et al.*, 2006). However, recent study on SeMV polyprotein processing has prooven cleavage at E132/S133 (Nair and Savithri, 2010). Besides, it was observed that cleavage at E132/S133 is crucial for an efficent processing of polyprotein, in particular for the cleavage at E498/S499 (Nair and Savithri, 2010). The *trans*-cleavage experiments of all the four cleavage sites of SeMV polyprotein suggested that cleavages at E132/S133 and E498/S499 did not occur *in trans*, i.e. these sites are only accesible *in cis* by auto-proteolysis (Nair and Savithri, 2010). All these four SeMV polyprotein cleavage sites were demonstrated to be utilized also in SeMV-infected sesbania plant (Nair and Savithri, 2010).

The analysis of CfMV-infected plant material showed that also the polyprotein(s) of CfMV is processed at several additional sites besides the verified E/N cleavage site between Pro-VPg (Mäkinen *et al.*, 2000b). Unexpectedly, the mature 12-kDa VPg was not detected with the antisera generated against VPg or P2a. Instead of that, a 24-kDa protein was observed. It was suggested that it represents either the N-terminal (Pro-VPg) or the C-terminal fragment (VPg-P2a C-terminus) of P2a, as the discrimination between these two was not possible with the antisera used. Also, a portion of potential cleavage intermediates with sizes of 18-kDa to 24-kDa was detected by P2a-antisera. Notably, the only protein detected with antisera generated against RdRp had the size of 58-kDa (instead of 100-kDa full-length translation product) indicating that the cleavage between RdRp and the

N-terminal part of full-length polyprotein must happen *in vivo*. A computational analysis identified a conserved site similar to the one found at the N-terminal cleavage site of VPg between the hydrophobic N-terminal region and Pro domain. No suitably positioned E/N site was identified downstream of VPg N-terminus (Mäkinen *et al.*, 2000b).

The structure of crystallized protease domain has been determined for SeMV at resolution of 2.4 Å. Remarkably, a comparison of the 3D-structure of SeMV protease domain with all the available entries in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) indicated that it is closer to the non-viral proteases than to the viral ones (Gayathri *et al.*, 2006). The structure of SeMV protease exhibits the characteristic features of trypsin fold with a well-formed active site and an oxyanion hole. SeMV Pro domain consists of two β barrels connected by a long inter-domain loop. Both active site and the substrate-binding cleft of SeMV Pro occur in between the two barrels and are fairly exposed to solvent (Gayathri *et al.*, 2006). The studies on sequence alignments have observed the similarities between the sobemoviral proteases and their relatives of polero-, enamo-, and barnaviruses (Gorbalenya *et al.*, 1988). The proposed consensus sequence for them all is $H(X_{32-35})[D/E](X_{61-62})TXXGXSG$, where H, D/E and S constitute a catalytic triad and X denotes any amino acid (Gorbalenya *et al.*, 1989; Koonin and Dolja, 1993). Also, the glycine and histidine residues downstream from the putative catalytic residues are suggested to be the site of substrate binding (Gayathri *et al*., 2006). Mutation analysis of the active site residues H181, D216, and S284 of SeMV Pro verified their crucial role in protease activity (Satheshkumar*et al*., 2004b). Mutation analysis of primary substrate-binding pocket called S1 binding pocket also showed the importance of downstream residues H298, T279, and N308 for protease activity (Gayathri *et al.*, 2006).

In vitro studies on SeMV Pro activity demonstrated that the uncleaved C-terminal VPg is crucial for both the *cis* and *trans* catalytic activity of SeMV Pro (Satheshkumar *et al*., 2005a). Tryptophan at position 43 of VPg was identified to mediate aromatic stacking interaction with the W271 of Pro domain (Satheshkumar *et al.*, 2005a; Nair *et al.*, 2008). Interestingly, also H275 of Pro was shown to be essential for protease activity. H275 is not a part of exposed aromatic stack in Pro but it lies close to the W271 and it faces the substrate-binding pocket (Nair *et al.*, 2008). It was concluded that most probably a release of free Pro domain from Pro-VPg during the proteolytic self-processing is changing the conformation of the protease in a way that it cannot cleave the substrate any more. Consequently, this would lead to the temporal regulation of the processing of the polyproteins (Nair *et al.*, 2008).

Although the region encoding VPg is similarly located in the genomes of sobemo-, polero-, enamo-, and barnaviruses, no significant similarity has been reported for the amino acid sequence or for the size of the VPg-s. The only conserved sequence element observed among them is a WAD or WGD or WNK motif followed by a D- or E-rich region (Mäkinen *et al.*, 2000b; McGavin and MacFarlane, 2008). VPg of RYMV has been identified as a virulence factor

(Hebrard *et al.*, 2006). It was shown to interact directly with rice eIF(iso)4G that mediates resistance against RYMV (Hébrard *et al.*, 2008). Further studies have identified five positions within a stretch of 15 amino acids in the central region of VPg to be associated with virulence. Amino acids 26 and 49 are major candidates to modulate the virulence among strains and variants of RYMV (Pinel-Galzi *et al.*, 2007). The residues 48 and 52 located at the C-terminus of RYMV VPg were proposed to participate in the interaction with two antiparallel helices of the eIF(iso)4G central domain. In avirulent isolates of RYMV, position 48 is occupied by conserved arginine whereas this site is polymorphic in virulent isolates (Hébrard *et al.*, 2006; Pinel-Galzi *et al.*, 2007). Interestingly, a reduced translation in the eIF4G depleted lysates indicated that translation from internally positioned CfMVε was eIF4G-dependent (Mäkiläinen, 2006).

The studies on VPg of SeMV indicated that it tends to be a disordered protein. It resembles the "natively unfolded"[4] proteins lacking both secondary and tertiary structures (Satheshkumar *et al.*, 2005a). Differently, the secondary structure predictions of RYMV VPg have suggested a high proportion (35%) of α-helices (Hébrard *et al.*, 2009). However, the regions with a propensity to be disordered are predicted in all sobemoviral VPg-s. Sobemoviral VPg-s can be distinguished according to their possible phylogenetic relationships. The group of VPg-s of RYMV/CfMV/RGMoV has been predicted to be disordered at the N- and C-termini. In the inner region, the consensus secondary structure prediction indicates the presence of α-helix followed by two β-strands and another α-helix whereas the disorder prediction shows a disordered state as well. For the group of VPg-s of SeMV/SBMV/SCPMV, the predicted disordered regions were shorter and a consensus for three β-strands was identified (Hébrard *et al.*, 2009). According to the *in silico* studies of sobemo-, poty- and caliciviral VPg-s, it was proposed that intrinsic disorder is a common feature of VPg-s and it confers on them the ability to bind to many different partners and to fulfill different functions in viral life cycle (Hébrard *et al.*, 2009). Their functions may imply interactions with eIF-s to enhance viral translation (calici- and potyviral VPg-s), they may contribute to host RNA shutoff due to a ribonuclease activity (potyviral VPg-s), they can be involved in viral replication via priming complementary strand synthesis (picorna- and polioviral VPg-s), they may have a role in plant cell-to-cell movement via interactions with eIF and microtubules (potyviral VPg-s), and they may be crucial in the processing of viral polyproteins (sobemoviral VPg-s) (Hébrard *et al.*, 2009).

None of the sobemoviral RdRp-s has molecularly characterized yet. The RdRp has been identified *in silico* through a highly conserved GDD motif (SGSYCTSSTNX$_{19\text{-}35}$GDD) that is conserved amongst the RdRp-s of positive-strand ssRNA viruses (Gorbalenya *et al.*, 1988). According to sequence similarities, RdRp-s of sobemo-, polero-, enamo- and barnaviruses are classified as

[4] "Natively unfolded" proteins (also called intrinsically disordered proteins) form a unique class of proteins that exhibit their function in the absence of the ordered structure. These proteins are believed to adopt a rigid conformation stabilized *in vivo* upon interaction with natural substrates (Uversky, 2002).

a “sobemo-lineage” in the supergroup I of plus sense plant RNA viruses. RdRp-s of this group, as well as some of supergroup II (luteoviruses and viruses from the family *Tombusviridae*), apparently lack the conserved NTP-binding elements characteristic for viral helicases (Koonin, 1991; Buck, 1996). Antibodies generated against the putative RdRp of CfMV recognized a 58-kDa protein instead of a single large 100-kDa protein in CfMV-infected plants indicating that it must be cleaved out from the full-length polyprotein *in vivo*. The size of RdRp indicates that the cleavage must occur in the vicinity of the -1 ribosomal frameshifting site (Mäkinen *et al.*, 2000b).

1.3.3. Coat protein and virion structure

The virions of sobemoviruses have an icosahedral capsid with approximate diameter of 30 nm. The capsid consists of 180 molecules of a single 26-34 kDa CP which is encoded by 3´-proximally located ORF but is translated from sgRNA (Hull and Fargette, 2005). The 3D structures of SCPMV (Abad-Zapatero *et al.*, 1980), SeMV (Bhuvaneshwari *et al.*, 1995), RYMV (Qu *et al.*, 2000), CfMV (Tars *et al.*, 2003) and RGMoV (Plevka *et al.*, 2007) virions have been determined utilizing X-ray crystallography. Despite the fact that primary sequences of sobemoviral CP-s are quite different (the sequence similarities between the CP-s of previously mentioned sobemoviruses are 12-30 % with exception of 63 % similarity between SeMV and SCPMV) their 3D structure is nearly identical. Actually, it is a general observation that 3D structures of structural proteins are better conserved than their amino acid sequences (Rossmann and Johnson, 1989). The root mean square (rms) deviations between superimposed coordinates of Cα atoms of the respective sobemoviral CP residues are in general 1.4-1.5 Å (Plevka *et al.*, 2007). The structures of CP-s of SCPMV and SeMV are even closer (0.6 Å) and RGMoV seems to be slightly different from other sobemoviruses (1.8-1.9 Å). Instead, the 3D-structure of CP of RGMoV appears to be slightly more similar to CP of *Tobacco necrosis virus* (TNV from the family *Tombusviridae*, genus *Necrovirus*) than to these of sobemoviruses (Plevka *et al.*, 2007). According to the sequence similarities, CP-s of sobemoviruses are most closely related to those of necroviruses (Dolja and Koonin, 1991; Tamm and Truve, 2000b; Saeki *et al.*, 2001; Hull and Fargette, 2005). The sequence similarity between TNV CP and previously mentioned sobemoviral CPs is 15-27 % and rms deviation between the superimposed coordinates of Cα atoms of the respective residues of sobemoviral CPs is 1.4-1.5 Å (Plevka *et al.*, 2007). TNV is the only necrovirus that has been crystallized (Oda *et al.*, 2000).

A sobemovirus capsid is assembled according to T=3 icosahedral lattice symmetry (Fig. 2, p. 32). The CP monomers are chemically identical but they exist in three slightly different conformations, denoted as A, B and C subunits. The A, B and C subunits construct one icosahedral subunit. Altogether, there are 60 icosahedral subunits per sobemoviral T=3 particle. The A subunits interact at the icosahedral fivefold axes to form 12 pentamers while the pairs of B and C subunits

meet at icosahedral threefold axes to form 20 hexamers. The pentamers and hexamers differ significantly in shape — hexamers are generally planar and pentamers are substantially bent. The combination of hexamers and pentamers gives the particle its characteristic shape (Fig. 2).

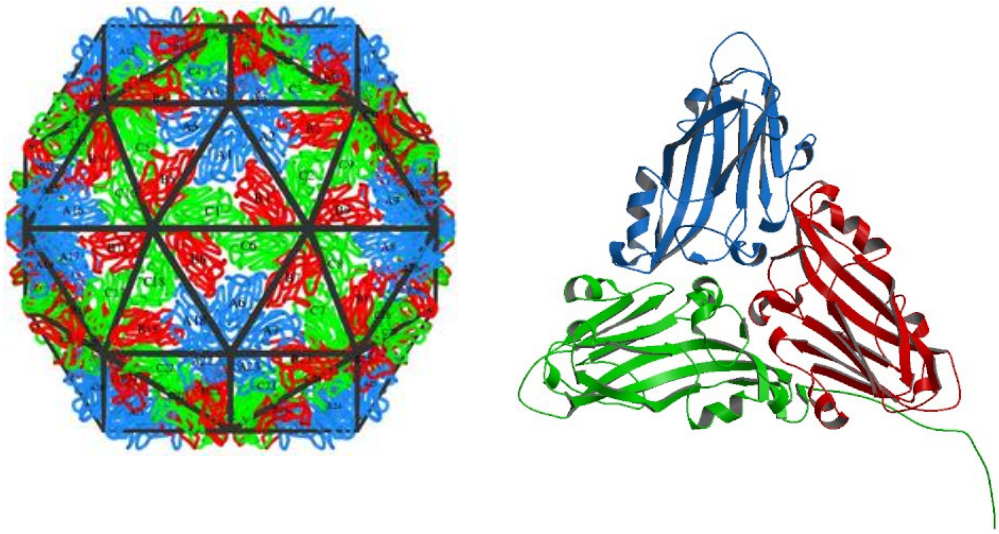

Figure 2. The structure of the sobemovirus particle. Chemically identical but structurally slightly different subunits are marked as follows: blue (A), red (B) green (C). On the left: 180 copies of CP monomers arranged in T=3 quasi-equivalent symmetry. On the right: Zoomed view to icosahedral subunit. Pictures representing CfMV capsid have been adapted from website: http://viperdb.scripps.edu.

Studies on TRoV, SCPMV, SeMV and RYMV particles demonstrate that the stability of the virions depends greatly on pH and the availability of divalent cations, Ca^{2+} and Mg^{2+} (Hsu *et al.*, 1976; Hull, 1977; Krüse *et al.*, 1982; Satheshkumar *et al.*, 2004a). Upon alkaline pH or removal of the cations, the virus particles swell and become less stable. A radius of RYMV and SCPMV particles increases about 7 % during the swelling process (Hull, 1977; Tama *et al.*, 2005). As the result of particle swelling, prominent protrusions are observed at the 5-fold axis, and small protrusions are also revealed at the quasi-3-fold axis. It is proposed that removing the Ca^{2+} ions results in electrostatic repulsions that trigger the swelling (Opalka *et al.*, 2000). Indeed, modeling of that process shows that separations between proteins interacting at the quasi-3-fold axis increase by 2 Å for RYMV and by 2.3 Å for SCPMV (Tama *et al.*, 2005). Calcium binding sites are located between the subunits AB, BC and CA. Sobemoviral particles have been shown to bind three Ca^{2+} ions per icosahedral subunit ABC, i.e. 180 Ca^{2+} ions per particle (Hermondson *et al.*, 1982). A structure-based alignment of the sobemoviral CP sequences has identified eight invariant amino acid residues – three of these (e.g. Asp 139, Asp 142 and Asn 234 for RGMoV CP) have involved binding of the

Ca^{2+} ions. Other five conserved amino acid residues have been suggested to be important for the backbone conformation (Plevka *et al.*, 2007). Mutation analysis of SeMV CP calcium binding sites demonstrates that cation-mediated interactions are mainly needed for particle stability but not for assembly (Satheshkumar *et al*., 2004a). The binding of calcium contributes in rigid packing of protein subunits into the viral particle (Sangita *et al.*, 2004; Satheshkumar *et al*., 2004a). Depending on the pH and the presence or the absence of calcium, RYMV particles have been demonstrated to exist in three forms. The unstable swollen form is present in the cytoplasm from where it is proposed to move into the vacuoles for compaction. It is proposed that a swollen particle might be an intermediate state before disassembly and after assembly of virions *in vivo* (Opalka *et al*., 2000; Satheshkumar *et al.*, 2004a). In infected rice plants, transitional and swollen forms were abundant during early infection (2 weeks post infection), whereas compact forms increased during later stages of infection (Brugidou *et al.*, 2002).

The assembly of SBMV and SeMV virions was proposed to be initiated by pentamer of dimeric units or 10-mer at the icosahedral 5-folds (Rossmann *et al*., 1983; Lokesh *et al.*, 2002; Satheshkumar *et al.*, 2005b). A single mutation W170K in SeMV has been found to result in stable dimers not assembling into particles (Anju *et al.*, unpublished results, referred by Pappachan *et al.*, 2008). It is presumed that packing of viral nucleic acid requires recognition of a specific region within a virus genome by viral CP. In case of SCPMV, a putative stem-loop sequence (mapped to the position corresponding to nt 1410–1438) within a conserved region encoding serine protease has been reported to bind CP (Hacker, 1995). However, it has not been demonstrated to nucleate SCPMV assembly.

In addition to external factors such as pH and divalent metal ions, sobemovirus particles are stabilized by protein-protein interactions between subunits and by CP-RNA interactions (Hull, 1977).

The monomers of sobemovirus CP possess an eight β-strand anti-parallel twisted sheet conformation known as a jellyroll β-sandwich or β-barrel topology, common in most non-enveloped icosahedral viruses (Richardson, 1981). In general, sobemoviral CP has two domains: N-terminal R (random) domain that is found to be completely disordered in subunits A and B but partially ordered in subunit C; and C-terminal S (shell) domain, which is the core building block of the virion (Abad-Zapatero *et al.*, 1980; Hermodson *et al.*, 1982; Rossmann *et al.*, 1983). It has been proposed that the disordered beginning of R domain interacts with RNA in the interior of the virus particle (Abad-Zapatero *et al.*, 1980; Erickson and Rossmann, 1982). The partially ordered N-terminal arm of C subunit is inserted between the interacting sides of subunits, making the contacts between subunits flat (i.e. tensed state). The contacts between the subunits that lack the inserted arms are bent (i.e. relaxed state). In that manner, the N-terminal arm acts as a molecular switch regulating the curvature of viral capsid and the assembly of T=3 particles. The removal of the R domain from CP results in formation of T=1 particles (composed of 60 identical monomers) only, with all of the bent contacts between subunits (Savithri and Erickson, 1983; Lokesh *et al.*, 2002).

The first half (ca 30-35 aa residues) of R domain contains a conserved arginine-rich motif (ARM) whereas the following region (the next 35 aa residues) is responsible for the formation of structure called β-annulus (which realizes only in case of partially ordered N-terminal arms of C-subunits). The analysis of SeMV CP has shown that deletion of the first part involving ARM results in formation of T=1 and pseudo T=2 particles (Lokesh *et al.*, 2002; Sangita *et al.*, 2004). On the contrary, deletion of the amino acid residues that constitute the β-annulus do not affect T=3 capsid assembly or stability (Satheshkumar *et al.*, 2005b). Moreover, the assembly of such kind of SeMV particles takes place without the formation of the β-annulus that, therefore, may be formed only as a consequence of the particle assembly (Pappachan *et al.*, 2008). However, SCPMV, SeMV and RGMoV have been found to be more sensitive to pH elevation than RYMV and CfMV. The difference is associated with the slight differences observed in the arrangement of β-annuli of the N-terminal arms of C-subunits (Opalka *et al.*, 2000; Qu *et al.*, 2000; Tars *et al.*, 2003; Plevka *et al.*, 2007). Amino acid residues forming the inter-subunit contacts are not conserved among sobemoviral CPs (Opalka *et al.*, 2000; Tars *et al.*, 2003).

R domain is rich in basic amino acid residues as arginine, lysine, proline, and glutamine, which have been considered to be responsible for coat protein contacts with RNA (Hermodson *et al*., 1982; Rossmann *et al.*, 1983). It has been shown that the first 30 aa residues (including ARM) of CP are determinant for a nonspecific *in vitro* RNA binding activity (Lee and Hacker, 2001). The overall charge of the ARM but not the arginine residues at specific positions is responsible for RNA binding in SCPMV and SeMV (Lee and Hacker, 2001; Satheshkumar *et al.*, 2005b). If only the arginine residues of ARM are replaced with glutamic acid residues, formation of empty T=3 particles (but with reduced stability) takes place. Hence, ARM is prerequisite for RNA interaction and encapsulation (Satheshkumar *et al.*, 2005b). The presence of RNA has been shown to enhance the overall stability of the virion (Satheshkumar *et al.*, 2005b). Studies on sobemoviral CP-RNA interactions do not demonstrate the requirement for specific interactions. Similarly to viral RNA, RNA of bacterial origin (23S rRNA or its degraded variants) is packed into SeMV particles when expressed in *E. coli* (Lokesh *et al.*, 2002). CP of CfMV shows rather a general affinity to bind any kind of ssRNA as well (Tamm and Truve, 2000a). Interestingly, it has been postulated that nonspecific electrostatic interactions might control both the genome length and conformation of all ssRNA/ssDNA viruses with highly basic peptide arms. As the result of mathematical modeling, a genome length was found to be linear in the net charge of CP peptide arms but not with the geometry and volume of the virion (Belyi and Muthukumar, 2006). Albeit the calculated genome packing density is similar within sobemovirus group, it varies significantly among different families of small single-stranded RNA viruses (Plevka *et al.*, 2007).

In addition to RNA-binding properties, the highly basic region of the N-terminus of SCPMV CP (involving ARM) withholds a potential to form α-helix and it has been shown to interact with artificial membranes *in vitro* (Lee *et al.*,

2001). Although the actual biological relevance of this membrane interaction is not known, several steps in the viral life cycle might be involved. Firstly, it is expected that a virus particle expands after the entry to the cell. This results in the externalization of R-domain that is believed to be essential for membrane-docking of the viral particle at the site of replication. Secondly, it has been speculated that viral movement depends on interactions with membranes (Lee *et al.*, 2001). The studies on RYMV (Brugidou *et al.*, 1995) and SCPMV (Sivakumaran *et al.*, 1998) prove that systemic virus movement is completely abolished in the absence of CP. CPs of CfMV (Fedorkin *et al.*, 2001) and TRoV (Callaway *et al.*, 2004) can complement the systemic movement of taxonomically distinct plant viruses. Systemic movement of SCPMV (Fuentes *et al.*, 1993) and RYMV (Opalka *et al.*, 1998) has been observed to be dependent on correct particle formation. Our studies on CfMV show that ARM is responsible for virus systemic movement (Olspert *et al.*, unpublished data). Thirdly, interaction with membranes may be also important for beetle transmission. It has been demonstrated that SCPMV enters the hemocoel of its beetle vector *Diabrotica undecimpunctata howardi* through the peritrophic membrane-lined midgut but not via the cuticle-lined foregut or hindgut (Wang *et al.*, 1994).

Based on sequence similarity with known amino acid motifs responsible for nuclear localization (Dingwall and Laskey, 1991), it has been supposed that the N-terminal region of CP contains a bipartite nuclear targeting signal (Ngon A Yassi et al. 1994; Mäkinen et al., 1995a) largely overlapping with ARM.

1.4. Distribution in cells and tissues, pathology

All sobemoviruses are found in cytoplasm[5]. SBMV (De Zoeten and Gaard, 1969), SoMV (Lombardo *et al.*, 1971), BSSV (Hartmann *et al.*, 1973), CfMV (Chamberlain and Catherall, 1976; Mohamed and Mossop, 1981), LTSV (Forster and Jones, 1979), VTMoV (Randles *et al.*, 1981), CnMoV (Mohamed and Mossop, 1981), SCMoV (Francki *et al.*, 1983), GCFV (Thomas, 1986) and RoMoV (Thottapilly *et al.*, 1992) virions have been detected in vacuoles. Studies with RYMV suggest that vacuoles become the storage compartments for virions in the course of infection. It is proposed that swollen and less compact virions exist in the cytoplasm, whereas vacuoles with acidic pH and higher Ca^{2+} concentration contain compact virions (Opalka *et al.*, 1998; Opalka *et al.*, 2000).

Sobemovirus particles accumulation into vacuoles is followed by the appearance of virus crystals and inclusions in cytoplasm (Opalka *et al.*, 1998, 2000). SBMV (De Zoeten and Gaard, 1969), SCPMV (Weintraub and Ragetli, 1970), SoMV (Lombardo *et al.*, 1971), BSSV (Hartmann *et al.*, 1973), CfMV (Mohamed and Mossop, 1981), SNMoV (Greber, 1981), VTMoV (Randles *et al.*, 1981), SCMoV (Francki *et al.*, 1983), GCFV (Thomas, 1986), PLYV (Kitajima *et al.*, 1992a; Kitijima *et al.*, 1992b), RoMoV (Thottapilly *et al.*, 1992), CAYMV

[5] There is no data available on cellular or tissue distribution of SeMV, IYMV and RuCMV.

(Morales *et al.*, 1995), RYMV (Opalka *et al.*, 1998, Brugidou *et al.*, 2002), RGMoV (Yao *et al.*, 2002) and SMAMV (Lecoq *et al.*, 2005) have been described to constitute crystalline arrays containing mature virions or cytoplasmic fibrils, some of which are enveloped in endoplasmic reticulum-derived vesicles. No inclusions have been found in cells infected with TRoV (Hollings and Stones, 1973), LTSV (Forster and Jones, 1979) or CnMoV (Mohamed and Mossop, 1981). However, aggregation of virus particles is considered to be a common characteristic of plant viruses. A formation of inclusions can be dependent on tissue type invaded. For example, crystalline arrays of RYMV have been observed in vascular tissues but not usually in mesophyll (Opalka *et al*., 1998; Brugidou *et al.*, 2002). Inclusions may be small and difficult to detect in the beginning of infection. Also, both abiotic (environmental conditions like light and temperature) and biotic (e.g. tolerance or sensitivity of host) factors can greatly influence inclusion development (Christie *et al.*, 1995). In case of CfMV, it has been reported that a formation of crystalline or paracrystalline arrays is in positive correlation with the amount of virus particles and the severity of symptoms (Chamberlain and Catherall, 1976).

Nuclear targeting signal of CP probably accounts for the presence of sobemovirus virions in the nuclei of mesophyll cells infected with SBMV (De Zoeten and Gaard, 1969), BSSV (Hartmann *et al.*, 1973), LTSV (Forster and Jones, 1979), SNMoV (Greber, 1981), CfMV (Chamberlain and Catherall, 1976; Mohamed and Mossop, 1981), VTMoV (Randles *et al.,* 1981), SCMoV (Francki *et al.*, 1983) and RYMV (Brugidou *et al.,* 2002). CnMoV has been occasionally found in the nuclei of its experimental host (wheat and oats) but not in naturally infected grasses (Mohamed and Mossop, 1981). It has been suggested that sobemoviruses can move into the nucleus as virions because SBMV can be visualized in the cytoplasm of young cells before being seen in nuclei. Virions of SBMV have been observed in cross-sections of nuclear pores in fixed blocks of infected tissue (De Zoeten and Gaard, 1969).

Sobemovirus particles are usually not detected in mitochondria and chloroplasts. However, BSSV particles have been observed to be associated with chloroplast membranes (Hartmann *et al.*, 1973). The chloroplasts of SBMV-infected (Weintraub and Ragetli, 1970) or RYMV-infected (Opalka *et al.*, 1998) cells have been reported to form occasionally finger-like extrusions. Further studies on RYMV showed that cytoplasmic integrity and structure of chloroplast and mitochondria are not affected in infected xylem parenchyma cells whereas in mesophyll cells the degenerative changes occur within chloroplasts when particles are not stored in vacuoles but appear in large quantities in cytoplasm (Brugidou *et al.*, 2002). Other cellular changes include proliferation of tonoplast membranes bulging into the vacuole in SoMV-infected (Lombardo *et al.*, 1971) or CfMV-infected (Chamberlain and Catherall, 1976) host cells. Organelle membranes, tonoplast and plasmalemma were destroyed and the remaining cytoplasm formed finger-like extrusions extending into the vacuole in cells severely affected by CfMV. In case of mild CfMV infection, increase in endoplasmic reticulum and

vacuolisation of cytoplasm was characteristic (Chamberlain and Catherall, 1976). RYMV infection results with disorganization of the middle lamellae of the cell walls of parenchyma and mature xylem cells (Opalka *et al.*, 1998).

Nothing is known about the subcellular localization of the nonstructural sobemovirus proteins.

Studies on tissue distribution of sobemovirus particles have revealed them in leaf mesophyll, epidermis and palisade cells, in guard cells of stomata, in vascular tissues (both in xylem and phloem) and in bundle sheath cells surrounding them, and even in meristem cells. Differently, sobemoviruses have been observed to be restricted to vascular tissues in roots (Hartmann *et al.*, 1973; Opalka *et al.*, 1998; Otsus, 2005). Distribution patterns of individual sobemoviruses differ to some extent.

SoMV has been reported to localize into mesophyll and xylem elements but not into phloem of infected quinoa leaves. In xylem tissue, it was found in parenchyma cells, in young tracheids and in differentiated tracheids of minor veins (Lombardo *et al.*, 1971).

The initial studies on BSSV detected it in leaf palisade cells and in xylem parenchyma, but not in mature xylem vessels or in phloem of highbush blueberry leaf and root tissues. In root tissues, BSSV was seen in varying amounts in almost all xylem parenchyma cells examined, usually in greater concentrations than in leaf xylem parenchyma (Hartmann *et al.*, 1973). Further studies have shown that BSSV localizes both in xylem and phloem although it was observed to be mainly related with xylem tissues (Urban *et al.*, 1989).

Similarly, RYMV particles have predominantly been detected in leaf mesophyll, bundle sheath cells, xylem parenchyma and vessels in rice (Opalka *et al.,* 1998; Brugidou *et al.*, 2002). In late infection stadia, RYMV particles have been occasionally observed also in phloem parenchyma cells and in sieve elements. It is suggested that the virus is transported between xylem cells by binding calcium from pit membranes into the composition of virion (Opalka *et al.*, 1998; Opalka *et al.*, 2000). In general, the high stability of virions is required for translocation via xylem because of the action of proteases during programmed cell death of tracheary element (Gergerich, 2002; Kozela and Regan, 2003).

SBMV and SCPMV have been found in bean or cowpea leaf epidermis, mesophyll and phloem companion cells mostly (Worley and Schneider, 1963; Fuentes and Hamilton, 1993). Similarly, CAYMV (presently considered as a strain of SBMV) has been reported to accumulate mainly in mesophyll and phloem of calopo leaf tissue (Morales *et al.*, 1995). When SBMV particles have been introduced directly into the xylem of bean stems, they migrated through the tracheids (and they were also able to traverse steam-killed parts) causing systemic symptoms (Schneider and Worley, 1959a; Schneider and Worley, 1959b; Gergerich and Scott, 1988). Systemic spread through xylem has been suggested to be characteristic for beetle-transmissible viruses (Gergerich and Scott, 1988).

CfMV has been reported to localize in cocksfoot leaf mesophyll and in phloem (Chamberlain and Catherall 1976; Rabenstein and Stanarius, 1984). Our results

demonstrate that both xylem and phloem are involved in CfMV infection (Otsus, 2005). However, the presence of CfMV in xylem occurs only during the late stadia of infection and CfMV is preferentially moving systemically through the phloem (Otsus, 2005). Interestingly, CfMV was always detected in few phloem-sided bundle sheath cells of minor veins during the first week of infection. By the end of the second week, when the first visible symptoms appear, there were a number of discrete infection loci in mesophyll surrounding vascular bundles. In vascular tissue, CfMV was observed in bundle sheath cells and in phloem parenchyma. In the third week (late stage of infection), CfMV had spread all over the inoculated leaf – it was abundantly found in mesophyll and in bundle sheath cells, and it was also localized both in phloem and xylem parenchyma. This pattern repeats in systemic leaves with a delay, except that no xylem localization is usually detected. In some systemic leaves, no virus was detected outside the vascular bundle. In straw and roots, CfMV was mainly detected in phloem companion cells and sometimes also sporadically in xylem (Otsus, 2005). Similarly to CfMV, a phloem spread has been found to be characteristic for a beetle-transmissible cowpea mosaic comovirus (CPMV) in cowpea plants. It has shown that upon beetle transmission, it rapidly reaches into phloem and uses phloem as its vascular movement route whereas upon mechanical inoculation, it moves from cell-to-cell until it loads into phloem minor veins. Subsequently, it unloads from phloem major veins like other viruses that are using phloem-transport (Silva *et al.*, 2002).

Virus infection causes major rearrangements on host physiology. According to the data gained from purification of RYMV-host protein complexes *in vivo* and *in vitro*, it is supposed that virus infection interferes in host metabolism (by binding host proteins involved in glycolysis, malate and citrate cycles), defence (by interacting with host proteins involved in elimination of reactive oxygen species but also with some pathogenesis-related proteins) and protein synthesis (by mating with host proteins involved in translation processes) (Brizard *et al.*, 2006). RGMoV has been reported to induce apoptotic cell death in yellow portions but not in green portions of infected oat leaves (Yao *et al.*, 2002).

The external outcome of sobemoviral infections varies from mild to severe chlorosis and mottling; also stunting, necrotic lesions, vein clearing and/or sterility have been documented (Hull and Fargette, 2005). However, some infections have been reported to be symptomless. For example, the infection of TRoV has been reported to be mostly without symptoms both in black mustard and field mustard (Thurston *et al.*, 2001; Pallett *et al.*, 2002). Also, LTSV may be transient or symptomless in lucerne (Blackstock, 1978; Forster and Jones, 1979).

1.5 Resistance

In general, production of reactive oxygen species and antioxidant metabolism are figured to be involved in symptom development and pathogenesis in plant-virus interactions (Riedle-Bauer, 2000; Hernandez *et al.*, 2001; Clarke *et al.*, 2002; Li and Burritt, 2003). Production of increased levels of reactive oxygen species are

thought to be involved in the regulation of localized cell death and the induction of defence genes (Riedle-Bauer, 2000) whereas up- and down-regulation of antioxidant enzyme activities are considered to play a role in generation of hypersensitive response to virus infection in plants (Fodor *et al.*, 1997, Mittler *et al.*, 1998). The measurements of reactive oxygen species and antioxidant enzymes of cocksfoot plants susceptible to CfMV and plants with acquired immunity[6] to CfMV, show completely different patterns in up- and down-regulation after inoculation of CfMV (Li and Burritt, 2003). In susceptible plants, H_2O_2 levels declined immediately after inoculation with CfMV and then gradually increased. Increase in H_2O_2 levels induced increased lipid peroxidation and the symptoms development. Conversely, recovered plants resistant to new infection showed only a brief increase in H_2O_2 levels, immediately after inoculation, with no significant increase in lipid peroxidation. Similarly, a decline in the oxidized forms of ascorbate and glutathione as well as a decline in the total glutathione pool in susceptible plants inoculated with CfMV were observed but no changes in the ascorbate or glutathione pools were observed in recovered plants. In susceptible plants, a decline in antioxidant enzyme activities (with the exception of catalase - its levels increased for the first 5 days after inoculation and then declined) were observed during the early stages of virus establishment, followed by increased activity in response to cellular damage. In recovered plants, only transient increases occurred in activities of some antioxidant enzymes (ascorbate peroxidase, dehydroascorbate reductase and monodehydroascorbate reductase) whereas the levels of other antioxidant enzymes measured (glutathione reductase, superoxide dismutase and catalase), did not change (Li and Burritt, 2003).

Natural resistance to sobemoviruses has been detected for CfMV in cocksfoot (Engsbro, 1978; Catherall, 1985; Rognli *et al.*, 1995), for CnMoV in *Cynosurus cristatus* (Catherall, 1985), for RYMV in *Oryza sativa* (Thottapilly and Rossel, 1993; Ghesquière *et al.*, 1997; Ndjiondjop *et al.,* 1999) and in *O. glaberrima* (Bakker, 1974; Thottapilly and Rossel, 1993; Paul *et al.*, 1995; Ndjiondjop *et al.*, 1999), for SBMV in beans (Zaumeyer and Harter, 1943) , for SCPMV in cowpea (Hobbs *et al.*, 1987), for SCMoV in subterranean clover (Ferris *et al.*, 1996; Wroth and Jones, 1992). RYMV infection was shown to be highly dependent on the viral dose inoculated (Sorho *et al.*, 2005).

The molecular mechanisms conferring resistance have been described only for RYMV in *Oryza* species. A monogenic recessive resistance trait *Rymv-1* (Ndjiondjop *et al.,* 1999) has been mapped on chromosome 4 (Albar *et al.*, 2003). *Rymv-1* has been identified to encode eIF(iso)4G (Albar *et al.*, 2006). *Rymv1-1* allelic variant is characteristic of susceptible varieties whereas three other allelic variants are related to different levels of resistance against RYMV. *Rymv1-2* has been found from *O. sativa* whereas *Rymv1-3* and *Rymv1-4* are two distinct resistance alleles in *O. glaberrima*, indigenous African rice species (Albar *et al.*,

[6] Plants fully recovered from a previous inoculation, have no detectable CfMV present and showed immunity to disease. Susceptible plants were not previously inoculated.

2006). All these resistance-conferring allelic variants are suggested to be a result of convergent evolution (Albar *et al.,* 2006; Traoré *et al.*, 2009). The difference between *Rymv1-1* and *Rymv1-2* lies in one amino acid substitution E309K in the central region of the eIF(iso)4G gene (Albar *et al.*, 2006; Rakotomalala *et al.*, 2008). Interaction of eIF(iso)4G with viral VPg is responsible for the high resistance trait (Hébrard *et al.*, 2006). Breakdown of the resistance conferred by *Rymv1-2* has been reported for some RYMV isolates (Traoré *et al.*, 2006; Pinel-Galzi *et al.*, 2007) due to non-synonymous mutations in RYMV VPg (Hébrard *et al.,* 2008). However, substitutions in RYMV VPg that were observed to enable overcoming of the *Rymv1-2* resistance did not operate in *Rymv1-4* plants (Albar *et al.*, 2006; Hébrard *et al.*, 2008). The interaction mechanisms involved in the rice/RYMV pathosystems are still unknown (Hébrard *et al.*, 2008). However, the 3D topology and the biochemical properties of virulence mutations both suggest a direct interaction between RYMV VPg and rice eIF(iso)4G encoded by *Rymv1*. It is proposed that the interaction domain of eIF(iso)4G with RYMV VPg is far from the domain interacting with eIF(iso)4A and therefore the functions of eIF(iso)4G have not been affected (Hébrard *et al.*, 2008). Besides *Rymv1* that expresses high resistance against RYMV infection but in limited number of cultivars, there are several other quantitative trait loci (QTLs) associated with partial resistance against RYMV mapped on chromosomes 1, 2, 7 and 12 (Ghesquière *et al.*, 1997; Albar *et al*., 1998; Ahmadi *et al.*, 2001). Expression of QTL_{12} has been reported to confer partial resistance via delayed movement of RYMV into mestome (bundle sheath cells) (Ioannidou *et al.*, 2000). Evaluation of genes from *eIF4E* and *eIF4G* multigenic families as potential candidates for partial resistance QTLs to RYMV in rice identified three other members of the *eIF4G* as good candidates while members of the family *eIF4E* seemed not to be involved in conferring resistance unlike described in several other studies on plant-virus interactions (Boisnard *et al.*, 2007).

Pathogen-derived transgenic resistance against RYMV has also been achieved by transforming plants with constructs expressing either RdRp (Pinto *et al.*, 1999) or CP (Kouassi *et al.*, 2006) sequences of the virus. Transgenic rice containing a large fragment of highly conserved region of RYMV RdRp has been reported to be moderately resistant to infection. Analysis of the most resistant line indicated that resistance was derived via posttranscriptional gene silencing (Pinto *et al.*, 1999; Voinnet *et al.*, 1999). Similarly, transgenic lines that were transformed with RYMV CP constructs producing untranslatable or antisense mRNA to trigger PTGS-mediated defense mechanisms were moderately resistant against RYMV (Kouassi *et al.*, 2006). Different results were gained when full-length CP or CP without the putative nuclear localization signal was expressed in transgenic plants. It appeared that expression of both these RYMV CP variants enhanced virus accumulation (Kouassi *et al.*, 2006). The mechanism underlying that process is unknown. It is supposed that RYMV CP can either suppress PTGS, enhance virus replication and cell-to-cell movement, or it may recruit specific host factors needed to enhance infection (Kouassi *et al.*, 2006).

Recently, it has been noticed that constitutive expression of *AtNPR1* (protein that is a key regulator of salicylic acid-mediated systemic acquired resistance in *Arabidopsis*) that confers resistance against fungi and bacterial pathogens, leads to a higher susceptibility to RYMV infection and higher sensitivity to salt and drought stress in rice (Quilis *et al.*, 2008). Remarkably, several viral RNA silencing suppressors have been shown to interfere with SA-mediated resistance (Ji and Ding, 2001; Alamillo *et al.*, 2006; Love *et al.*, 2007). Despite of higher susceptibility of *AtNPR1* transgenic rice to RYMV, no correlation between virus concentration and disease severity was observed. This phenomenon was explained by overreaction in defense response to viral infection due to *AtNPR1* overexpression and/or by lowered movement of RYMV into vascular tissues. Also, the increased expression of *OsRDR1* (that encode an RNA dependent RNA polymerase in *O. sativa*) in *AtNPR1* transgenic rice was suspected to reduce the accumulation of viral RNAs through the activation of RNA-silencing pathways (Quilis *et al.*, 2008). RDR1 proteins are known to mediate RNA silencing pathways regulating the defense against viruses (Baulcombe, 2004).

1.6. Phylogenetic relationships

The phylogenetic analyses have indicated that three sobemovirus species – SeMV, SBMV and SCPMV – which were earlier considered as the strains of SBMV are indeed very closely related to each other (Lokesh *et al.*, 2001; Zhou *et al.*, 2005; Stuart *et al.*, 2006; Fargette *et al.*, 2008; McGavin and MacFarlane, 2008; Meier *et al*., 2008; Sérémé *et al.*, 2008). Nucleotide identity shared between SeMV and SBMV is the highest one (71.5 %) within genus *Sobemovirus* (Sérémé *et al.*, 2008). Also, LTSV and SCMoV form a closely related pair (Zhou *et al.*, 2005; Stuart *et al.*, 2006; Fargette *et al.*, 2008; McGavin and MacFarlane, 2008; Meier *et al*., 2008; Sérémé *et al.*, 2008) with 61 % identity between their genomic sequences (Sérémé *et al.*, 2008). Recently sequenced RuCMV is clustered with these two according to the comparison of CP sequences (McGavin and MacFarlane, 2008). CfMV and RYMV have always been clustered together (Lokesh *et al.*, 2001; Zhou *et al.*, 2005; Stuart *et al.*, 2006; Fargette *et al.*, 2008; McGavin and MacFarlane, 2008; Meier *et al.*, 2008) sharing 46 % of nucleotide identities (Sérémé *et al.*, 2008). However, a newly sequenced IYMV is more close to RYMV (with 56.5 % idenity) than CfMV (Sérémé *et al.*, 2008). Nucleotide identity between IYMV and CfMV is 45.4 % (Sérémé *et al.*, 2008). In general, phylogenetic trees show a split between sobemoviruses infecting monocots (RYMV, IYMV and CfMV) and those that infect dicots (SBMV, SCPMV, SeMV, LTSV, SCMoV, RuCMV, TRoV). The only exception is RGMoV which is a monocot virus grouping together with dicot viruses in genome trees (Zhou *et al*., 2005; Fargette *et al.*, 2008; McGavin and MacFarlane, 2008; Sérémé *et al.*, 2008). However, RGMoV resembles the other monocot-infecting sobemoviruses on the bases of their codon usage patterns (Zhou *et al.*, 2005).

A set of 253 CP sequences of RYMV isolates has provided an approach to date virus diversification in sobemovirus group. The divergence time of RYMV has been found to be ca 200 years which spans the period of extension of the rice culture in Africa (Fargette *et al.*, 2008). The divergence time among sobemoviruses has been calculated to be approximately 500 - 3000 years. The divergence with related viruses has been calculated using RdRp sequences and it has been considered to be around 4000 years for sobemoviruses and MBV (*Mushroom bacilliform virus*, a sole member of the family *Barnaviridae*), 5000 years for sobemo-, barna- and poleroviruses, and 9000 years for sobemo-, barna-, polero- and luteoviruses (Fargette *et al.*, 2008). This timeline spans the domestification and spread of cultivated plants raising a hypothesis that the emergence of plant viruses is linked to the development of agriculture (Fargette *et al.*, 2008).

RdRp sequences instead of CP sequences have been used in divergence calculation studies between sobemoviruses and their related viruses because of a mosaic structure of the sobemovirus genome. The 5'-terminal part of the sobemoviral genomes encoding non-structural proteins resembles those of enamo- and poleroviruses in its genomic organization. However, sobemoviral P1 sequences are not related to enamo- or poleroviral P0 sequences or to any other known sequences (Tamm and Truve, 2000b). While sobemoviral polyprotein (Pro-VPg-RdRp) shows sequence similarity to that of enamo- and poleroviruses (from the family *Luteoviridae*), sobemoviral CP is related to those of necroviruses (from family *Tombusviridae*) (Coutts *et al.*, 1991; Koonin and Dolja, 1993; Miller *et al.*, 1997; Tamm and Truve, 2000b; Stuart *et al.*, 2004). MBV, a single member of the family *Barnaviridae* has a genomic organization similar to sobemoviruses (except that its ORF1 is not situated at 5´ position characteristic to sobemoviruses but it largely overlaps with ORF2 in alternate frame). Its polyprotein (Pro-VPg-RdRp) sequence is related with those of sobemoviruses but its CP is distantly related to that of carmoviruses (from family *Tombusviridae*).

The viruses of the genera *Polerovirus* and *Enamovirus* are classified into the family *Luteoviridae* according to their homology with genus *Luteovirus* at the 3' parts of their genomes, whereas their 5' parts are related to sobemoviruses and clearly distant from luteoviruses. At the same time, the products of ORF1 (Pro) and ORF2 (RdRp) of viruses from genus *Luteovirus* are most similar to those of the viruses of the genus *Dianthovirus* but also to those of the genera *Carmovirus* and *Umbravirus*, all from the family *Tombusviridae* (Miller *et al.*, 1997). An extreme dichotomy in polymerase sequences indicates that possibly early recombination events have played an important role during the evolution of these genera (Martin *et al.*, 1990; Gibbs, 1995; Miller *et al.*, 1997). The most likely model suggests that recombination arose by strand switching near the subgenomic RNA start sites during RNA replication in cells co-infected with two parental viruses (Mayo *et al.*, 1989; Martin *et al.*, 1990; Mayo and Jolly, 1991; Gibbs and Cooper, 1995; Mayo and Ziegler-Graff, 1996; Miller and Rasochova, 1997; Miller *et al.*, 1997; Moonan *et al.*, 2000; Moonan and Mirkov, 2002; aus dem Siepen *et al.*, 2005; Silva *et al.*, 2008). Inconsistency between polero- and luteoviruses has been proposed to be a

result of ancient recombination between a polerovirus that was replacing its sobemovirus-like 5′ part of the genome with the one of dianthovirus-like (Miller *et al.*, 1997) as the subgenomic mRNA start sites of dianthoviruses and poleroviruses are known to be homologous (Miller *et al.*, 1995; Zavriev *et al.*, 1996).

Notably, the recombination seems to be intrinsic for the members of the family *Luteoviridae*, in which one can find lots of recombinant species (Mayo and Jolly, 1991; Rathejen *et al.*, 1994; Gibbs and Cooper, 1995; Miller *et al.*, 1995; Mayo and Ziegler-Graff, 1996; D´Arcy and Mayo, 1997; Miller and Rasochova, 1997; Moonan *et al.*, 2000; Smith *et al.*, 2000; Domier *et al.*, 2002; Miller *et al.*, 2002; Moonan and Mirkov, 2002; Liu *et al.*, 2007; Robertson and French, 2007; Silva *et al.*, 2008). Yet, there exists also a putative polerovirus-sobemovirus hybrid having a CP gene similar to the sobemoviruses but the rest of its genome being more similar to poleroviruses (aus dem Siepen *et al.*, 2005).

Based on the phylogenetic analysis, it has been concluded that sobemoviruses have evolved in the absence of recombination events (Lokesh *et al.*, 2001; Fargette *et al.,* 2004; Zhou *et al.*, 2005; Chare and Holmes, 2006; Fargette *et al.*, 2008; Sérémé *et al.*, 2008). However, a recent exhaustive search for recombinants of RYMV has identified few intraspecies recombination events in eastern Tanzania (Pinel-Galzi *et al.*, 2009). This region is the putative center of origin of RYMV with the highest diversity and a fully mixed spatial distribution of the strains (Traoré *et al.*, 2009). The first putative recombinant segment is a fragment of 300 nt encompassing the intergenic region between ORF1 and ORF2a and the 5′ end of ORF2a. The other putative 350 nt long recombinant segment matches the untranslated 3′ terminus of the genome. However, the number of recombinants was low, the recombinant fragments were short, and they occurred on terminal branches of the phylogeny. Recombinations are not detected in any of the ORFs analyzed individually (Pinel-Galzi *et al.*, 2009). Also, several DI RNA molecules cloned from CfMV-infected plants have been considered as an indication for replicase-driven template-switching mechanism (Mäkinen *et al.*, 2000a).

All the members of the family *Tombusviridae* cluster into a well supported group in phylogenetic analyses that has been considered as a sign of high homogenity but also as the result of extensive gene sharing among the genera (Stuart *et al.*, 2006). Different from a previous study, Xi *et al.* (2008) suggest that *Galinsoga mosaic virus* (GaMV) might originate from the recombination between a necro- and a carmovirus, as its RdRp gene and all small ORFs of GaMV cluster with necroviruses whereas its CP gene clusters into the carmovirus branch. *Melon necrotic spot virus* (MNSV), another carmovirus, also has been speculated to be a recombinant between necro- and carmoviruses (Xi *et al.*, 2008). Similarly, *Olive mild mosaic virus* (OMMV), considered before as a GP isolate of *Tobacco necrosis virus D* (TNV-D) that was not involved in the study carried out by Stuart *et al.* (2006), shows high similarity with TNV-D within its CP but with *Olive latent virus 1* (OLV-1) within its RdRp thus being a possible example of interspecies recombination in genus *Necrovirus* (Cardoso *et al.*, 2005).

Despite a weak sequence homology between sobemo-, polero- and necroviruses, it has been speculated that their CPs might have a common ancestor as their 3D-structures are similar (Terradot *et al*., 2001). This hypothesis bases on a model structure of *Potato leafroll virus* (PLRV) from genus *Polerovirus* assessed by Profiles-3D calculation, which obtained the best hits with SeMV, SCPMV and RYMV (Terradot *et al*., 2001). Comparisons of their CP sequences show 17 % identity and 33 % similarity between RYMV and PLRV (Terradot *et al*., 2001), 17 % identity and 33 % similarity between SCPMV and TBSV (Terradot *et al*., 2001), whereas there is 21 % identity and 41 % similarity between RYMV and SCPMV (Opalka *et al.*, 2000). Sequence alignments between the CPs of sobemoviruses (SeMV, SBMV, LTSV and CfMV) and a CP of *Tobacco necrosis virus* (TNV, a type member of the genus *Necrovirus*) show similarity ranges of 31 - 45 % (Saeki *et al.*, 2001). At the same time, CP similarities in genus *Necrovirus* range between 43 – 65 % and the overall similaritiy values of CPs of *Tombusvirus* and *Carmovirus* species from the family *Tombusviridae* ranges of 22 – 28 %. The TNV CP similarities with those of genera *Machlomovirus* and *Dianthovirus* from the family *Tombusviridae* are under 20 % (Saeki *et al.*, 2001).

2. AIM OF THE STUDY

The aim of this study was to extend our knowledge about the molecular biology of sobemoviruses.

For this purpose the following tasks were carried out:

1. Re-evaluation of the genomic organization of sobemoviruses.
2. Screening for recombinants occurring in plants co-infected by two sobemoviruses – *Cocksfoot mottle virus* (CfMV) and *Ryegrass mottle virus* (RGMoV).
3. Determination of a necessity of P1 of CfMV in virus replication and systemic infection.

3. MATERIALS AND METHODS

The methods used are described in detail in the articles presented in this thesis. Briefly, the methods used were as follows:

3.1. Plants and growing conditions

Oat cv. Jaak and barley cv. Kymppi were grown in a climate chamber (60 % relative humidity, 16 h light at 23 °C, 8 h dark at 16 ºC) (publications II, III).

Barley suspension culture line (cv. Pokko, VTT-G-93001) was grown at 22 °C in dark and subcultured weekly onto fresh solid (0.35 % Gelrite) modified B5-medium supplemented with 2,4-D (4 mg/l) (publication III).

3.2. Insect cell culture and growing conditions

Sf9 insect cell culture was grown at 28 °C in Sf900 II serum-free medium as recommended by Life Technologies (publication III).

3.3. Viruses

CfMV-No was in our lab collection. RGMoV (Japanese isolate, PV-307043) was obtained from MAFF GeneBank, SBMV-Col (Colombian isolate, PV-0100) was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ), SCPMV-WI (Wisconsin isolate, PV-114) and LTSV-Can (Canadian isolate, PV-454) were obtained from American Tissue Culture Collection (ATCC).

CfMV-No as well as RGMoV was propagated in oats cv. Jaak or barley cv. Kymppi. Infected plant material was frozen in liquid nitrogen and stored at -70 °C.

The inoculums were prepared by crushing the symptom bearing leaves from the stock of frozen material in a mortar with 1 ml 100 mM phosphate buffer (pH=7.0), supplemented with 0.5 % Celite per 1 g of leaf material. 50 µl of freshly prepared sap was rubbed onto the leaves of 2-leaf stage plants 10 days after sowing (publication II).

For recombination study, co-inoculation of CfMV-No and RGMoV was performed as described in publication II.

In case of engineered viruses, i.e. CfMV icDNA and its derivates, icDNA construct was linearized after the 3' terminus of viral sequence and used as templates for 5' capped RNA synthesis. *In vitro* transcription was carried out with T7 RNA polymerase (Fermentas) and template DNA was thereafter degraded by RQ DNase I (Promega). The viral RNA was purified using RNeasy kit (Qiagen) or phenol: chloroform extraction and ethanol precipitation (Sambrook and Russell, 2001) (publication I, II). Plant material was infected biolistically with *in vitro* transcribed 5' capped viral RNA bound onto 1.0 µm gold particles at 1100 psi using Biolistic PDS-1000 (Bio-Rad) equipment (publication III).

3.4. Primers

Primers used to amplify different sobemoviruses in publication I are described in table 2.

Table 2. The primers used to amplify different sobemoviruses (publication I).

Primer sequence	Corresponding to (nt)	Restrictase site added (shown in italics in the primer sequence)
5'-GACGACTCCTTGCCCC-3'	SBMV 1646-1661	-
5'-ACAAACCCCTGCGAACC-3'	SBMV 2333-2317	-
5'-GACGATGATGATTTACCCCTTCC-3'	SCPMV 1707-1729	-
5'-TCACACAAGCCCATCTCTACC-3'	SCPMV 2407-2387	-
5'-GGGCTGATGAGGAAACCA-3'	LTSV 1716-1733	-
5'-CCGCACCTTGTTGGC-3'	LTSV 2509-2495	-
5'-CGGACATACGTGAGCGGGAG-3'	RGMoV 3076-3095	-
5'-TGTGACGCGTACTAAAGAGCCA-3'	RGMoV 3529-3508	-
5'-CTCGGCACGCGCTGTCG-3'	RGMoV 2157-2141	-
5'-GCCCATTCATGCTCAACCCG-3'	RGMoV 1440-1459	-
5'-CT*GGTGACC*GTACTCCCTCGGC-3'	RGMoV 599-611	*Eco* 91 I
5'-AGA*GCCGGC*GCCTGGAAGAGCA-3'	RGMoV 2104-2092	*Pdi* I
5'-AGA*GCCGGC*AGGCAGATTCCGC-3'	RGMoV 2105-2117	*Pdi* I
5'-GA*CCTAGG*AGAGCACCGTGCCG-3'	RGMoV 3571-3559	*Xma* J I
5'-CAT*CCTAGG*TTAGTACGCGTCACAT-3'	RGMoV 3515-3530	*Xma* J I
5'-GA*GATTGGTATC*CCCCTACGCTAG -3'	RGMoV 4195-4184	*Bsa* B I

Primers used in publications II and III are described there.

3.5. DNA cloning

CfMV icDNA was engineered as described in publication III. Construction of the P1-deficient CfMV icDNA and the replication-deficient CfMV icDNA are also described in publication III. Construction of artificial viruses containing the elements both from CfMV and RGMoV was represented in publication II.
pUbi:EGFP and pUbi:P1:EGFP were constructed to express EGFP or CfMV P1:EGFP under the control of maize ubiquitin gene promoter. Recombinant bacmid bMON:P1 was created to express CfMV P1 under the control of polyhedrin promoter in Sf9 insect cells. Preparation of these constructs was described in detail in publication III.

All obtained clones were sequenced for validation.

3.6. Expression and analysis of CfMV P1 in insect cells

To gain a recombinant baculovirus stock, Sf9 cells were transfected with bacmid bMON:P1 using CellFECTIN liposome reagent (Life Technologies) following the manufacturer's instructions. For further studies, Sf9 monolayer cells were infected with recombinant baculovirus stocks at MOI=1. Cells were harvested after 24 to 96 h by mechanical detachment, centrifuged and washed twice with 1 x PBS at 2500 rpm for 5 min at 4 °C. Washed intact cells were homogenized using a loose-fit glass homogenisator on ice. The homogenate was fractionated according to two separate differential centrifugation protocols in parallel (Hockenbery *et al.,* 1990; Schreiber *et al.,* 1989).

30 µg of total protein from each fraction was analyzed on 15 % SDS-PAGE. For Western blot analysis, the primary antiserum against CfMV P1 (Tamm *et al.,* 1999) was used in 1:5000 dilution and goat anti-rabbit IgG conjugated to HRP (Calbiochem) was used as a secondary antiserum in 1:5000 dilution. The chemoluminescence reaction was triggered with SuperSignal Chemiluminescent Substrate (Pierce) and detected using Molecular Imager System GS-525 (BioRad) (publication III).

3.7. Expression and analysis of CfMV EGFP:P1 in oat leaves

Oat leaves were used for biolistic delivery of pUbi-EGFP or pUbi-P1:EGFP DNA at 1100 psi. After incubation for 24 to 48 hours in the dark, bombarded plant tissues were mounted into a mixture of 1:1 water and glycerol for microscopical examination. Fluorescence of GFP was visualised with Olympus IMT-2 inverted microscope with reflected light fluorescence attachment using XF-100 filter set (Omega Optical) and 40 x oil objective, NA 1.3. Recording was performed by digital CCD camera (CF 8/1 DX, KAPPA) and KAPPA ImageBase software (publication III).

3.8. RNA isolation and analysis

RNA from plant material was extracted with RNAEasy kit (Qiagen) or according to the protocol adapted from Logemann *et al.* (1987). The integrity of extracted RNA was checked in 6 % formaldehyde - 0.8 % agarose gel electrophoresis buffered with 1 X MOPS, pH=7 (publications II, III).

For replication analysis of CfMV P1(-) (publication I), RNA was extracted 20 h after bombardment from barley suspension culture cells as well as from oat leaves and analyzed for virus replication by negative-strand specific RT-PCR. To test the infectivity of CfMV P1(-), inoculated leaves and newly emerged systemic upper leaves were collected at 14 days post infection (dpi) for RT-PCR analysis.

For recombination study of CfMV and RGMoV (publication II), RNA was extracted from inoculated leaf material harvested at 7 dpi and from newly emerged

systemic leaves at 21 dpi. Possible recombinants were scouted by RT-PCR analysis.

SuperScript One-Step RT-PCR kit with Platinum Taq Polymerase (Invitrogen) was used for RT-PCR analysis in publications I and II. For virus replication analysis, only a negative-strand specific primer was used to start a reaction. After the synthesis of cDNA, the mixture of negative and positive strand specific primers were directly added into the reaction, followed by heat-activated PCR. For infectivity analysis from systemic leaves, both positive and negative-strand specific primers were supplemented in the beginning of reaction as suggested by the manufacturer.

To evaluate a specificity of primer pairs in RT-PCR analysis, 10-fold serial dilutions of *in vitro* transcribed RNA were mixed with RNA extracted from non-infected oat leaves (publications II, III).

For sequencing of samples of sobemoviruses obtained from international gene banks (publication I), RNA was extracted directly from lyophilized infectious plant material. First-strand cDNA syntheses were performed using Superscript III reverse transcriptase (Invitrogen). Dynazyme DNA polymerase (Finnzyme) was used for cDNA amplification.

All RT-PCR products were ligated into pTZ57R/T (Fermentas) for amplification and DNA purification. *Taq* terminator sequencing kit version 3.1 (Applied Biosystems) was used for sequencing reactions. The products were analyzed on Applied Biosystems 3130 sequencer.

Novel sequences obtained in this study were uploaded to the NCBI GenBank.

4. RESULTS AND DISCUSSION

4.1. CfMV-like genome organization is common for all sobemoviruses

Based on the determined structural differences in the central part of the genome, sobemoviruses have been subdivided into SCPMV-like and CfMV-like types of genomic organization.

Historically, the first fully sequenced sobemovirus was SCPMV in 1987. Analysis of sequencing data revealed four ORFs (Wu *et al*., 1987). Earlier, translation of virion-extracted RNAs in cell-free extracts yielded four major products for SCPMV– 105-kDa, 60-kDa, 28-kDa and a family of 3 proteins (21 to 25-kDa) (Ke-Qiang *et al*., 1982). Tryptic analysis of 60-kDa protein showed that it is a subset of 105-kDa protein (Ke-Qiang *et al*., 1982). Wu *et al.* (1987) suggested that they are encoded by long ORF2 whereas 60-kDa protein is derived from 105-kDa protein by proteolytic processing. 28-kDa protein was not translated from full-length RNA but sgRNA and it was shown to be a capsid protein (Ke-Qiang *et al*., 1982). 28-kDa protein size was found to be consistent with ORF4 that potentially encodes a 30.3-kDa protein (Wu *et al*., 1987). Wu *et al.* (1987) suggested that 21 to 25-kDa proteins might be a single protein that is post-transcriptionally modified – it can be encoded by 5′ small ORF1 consistent with translation of the 21 to 25-kDa protein from both genomic and 3′ degraded RNA. None of the translation products were found to correspond to small ORF3 situated in the middle of ORF2 in alternative reading frame (Wu *et al.*, 1987).

Soon after, sequences of three other sobemoviruses - SBMV (Othman and Hull, 1995), RYMV (Ngon A Yassi *et al*., 1994; Pinto and Baulcombe, 1995) and LTSV (Jeffries *et al.*, 1995) - followed. These viruses were annotated similarly. In contrast to these sequences, the sequencing data of CfMV indicated no individual ORF capable of directing the synthesis of 100-kDa protein (Mäkinen *et al*., 1995b). Instead of that, the middle part of the genome contained two overlapping reading frames called ORF2a and ORF2b. However, *in vitro* translations of the CfMV genomic RNA revealed four major products of 100-, 71-, 34- and 16-kDa (Mäkinen *et al*., 1995a). Analysis of sequence information revealed a -1 ribosomal frameshifting signal that leads ribosomes during the translation process from ORF2a into an overlapping reading frame ORF2b generating 100-kDa polyprotein. Moreover, similar frameshifting signals were also found in the sequences of SBMV, SCPMV and RYMV at the beginning of putative ORF3 region (Mäkinen *et al.*, 1995a).

In 2001, two new sobemoviruses – SeMV and RGMoV – were reported to possess SCPMV-like genomic organization (Lokesh *et al.*, 2001; Zhang *et al.*, 2001). In 2003, sequencing of SCMoV indicated the genomic organization similar to CfMV (Dwyer *et al.*, 2003). In 2004, a genomic organization of TRoV was shown to be alike CfMV (Callaway *et al*., 2004).

In 2004, 14 new isolates of RYMV were sequenced and found to have the CfMV-like genomic organization (Fargette *et al.*, 2004). Moreover, previously

published sequences of RYMV-Nig (Nigerian isolate) and RYMV-CI (Ivory Coast isolate) were re-sequenced. Deletion of an erroneous extra nucleotide U2244 from the former sequences established CfMV-like genomic organization also for those isolates (Fargette *et al.*, 2004).

Later, two novel SBMV isolates were found and partially sequenced (Verhoeven *et al.*, 2003; Segundo *et al.*, 2004). Interestingly, the alignment of these isolates [SBMV-Almerian, (SBMV-Alm) and SBMV-Morocco, (SBMV-Mor)] to previously published SBMV Arkansas isolate (SBMV-Ark; Lee and Anderson, 1998) reveals GG instead of GGG at position 2176-2178. If the putative extra nucleotide were to be removed from the full-length sequence of SBMV-Ark, a CfMV-like genomic organization would be established.

In current study, we re-sequenced the central part of SBMV-Col (Colombian isolate of SBMV), SCPMV-WI (Wisconsin isolate of SCPMV), LTSV-Can (Canadian isolate of LTSV) and RGMoV-Jap2 (Japanese isolate of RGMoV; named as Jap2 to distinguish it from previously published independent sequence that also originates from Japan) genomes. Three clones from each virus isolate were sequenced in forward and reverse directions.

Sequencing of SBMV-Col revealed the presence of GG instead of GGG at position 2176-2178 (publication I, Fig. 1) like in SBMV-Alm and SBMV-Mor when compared with the SBMV-Ark sequence. SBMV-Ark is known to cluster together with SBMV-Alm whereas SBMV-Col, SeMV and SCPMV form different clusters in phylogenetic analysis (Verhoeven *et al.*, 2003). Remarkably, the very recent sequencing data of SBMV Brazilian isolate (SBMV-SP) from São Paolo demonstrate CfMV-like genome organization (Ozato *et al.*, 2009). SBMV-SP clusters together with SBMV-Ark (Ozato *et al.*, 2009).

Sequencing of SCPMV-WI showed that G2232 (publication I, Fig. 1) was missing when compared with the published SCPMV sequence (Wu *et al.*, 1997). Sequencing of LTSV-Can revealed the extra nucleotides GC after position 2299 (publication I, Fig. 1) when compared with the LTSV sequence available from GenBank (Jeffries *et al.*, 1995). Sequencing of RGMoV-Jap2 revealed the GGGGGG instead of GGGGGGG at position 2203-2209 (publication I, Fig. 1; Balke *et al*., 2007) when compared with the RGMoV sequence published earlier (Zhang *et al.*, 2001). In accordance with these results, recent data on re-sequencing of SeMV verified the presence of C instead of CC at position 2177-2178 in the previously published sequence (Lokesh *et al.*, 2001; Lokesh *et al.*, 2006).

In all cases, the revision of the genomic sequence revealed a stop codon in the middle of ORF2 shortly after the –1 frameshift signal similarly to ORF2a of "CfMV-like" viruses. Also, the correction indicated a single continous ORF (ORF2b) in –1 frame like in CfMV-like viruses (publication I, Fig. 2). Earlier, considerable similarity was found between the N-terminal part of RdRp proteins encoded by "CfMV-like" viruses and P3 proteins of "SCPMV-like" viruses over the first 150 amino acid residues (Mäkinen *et al.*, 1995a; Dwyer *et al.*, 2003). Downstream from the point where a similarity was lost, it immediately continued between the C-terminal part of RdRp of "CfMV-like" viruses and the C-terminal

part of the RdRp of "SCPMV-like" viruses. Hence, the "similarity shift" was observed at the exact location of previous sequencing errors.

In conclusion, all the re-sequenced viruses previously described as SCPMV-like, exhibit a common, CfMV-like genomic organization (publication I, Fig. 3). We propose that the RdRp-s of all sobemoviruses are translated by a –1 ribosomal frameshift. Argument favoring the common CfMV-like genomic organization is that both the genomic organization and the primary structures of Pro-VPg-RdRp proteins of sobemoviruses are related to those of the polero- and enamoviruses that also exhibit the CfMV-like genome arrangement in the central part of their genome (Hull and Fargette, 2005).

4.2. No recombinants found between CfMV and RGMoV in doubly infected oat plants

Studies on mixed infections are pertinent for understanding the role of recombination in virus evolution. In the current study, the experiments were carried out under little or no selection pressure (i.e. non-transgenic plants infected with two wild-type viruses) selected to gain a varied pool of recombinants and to avoid a positive selection of escape-recombinations which may reflect the results of selective pressure rather than the mechanism of recombination itself (Aaziz and Tepfer, 1999).

To investigate the possibility of RNA recombination occurrence between two sobemoviruses, we co-infected oat and barley plants with CfMV and RGMoV. Although the natural co-infections of RGMoV and CfMV have not been reported, their distribution overlaps at least in Japan. Cocksfoot, wheat, barley and oats are among their common natural and experimental host species (Serjeant, 1964; Toriyama *et al.*, 1983; Mäkinen *et al*., 2000b).

At first, virus propagation and symptom development of both viruses in barley and oats was examined visually and then tested by RT-PCR using virus-specific primers.

Both viruses generated quite strong infection symptoms in oats. RGMoV was lethal for barley, whereas CfMV infection was nearly symptomless. RT-PCR analysis revealed 100 % infection with both viruses in oats but not in barley (Fig. 1, publication II). Therefore, oat plants were chosen for recombination experiments.

In the recombination study, 105 oat plants were co-inoculated with CfMV and RGMoV. Since the length of a replication cycle of CfMV and RGMoV is not known, co-inoculations were performed in five groups within three days (at "day 0", 25 plants were co-inoculated with both viruses, 40 plants were individually inoculated with RGMoV and 40 with CfMV; half of the individually inoculated plants were inoculated with the other virus at "day 1" and another half at "day 2") to provide the possibility of simultaneous replication and potential recombination between the two viruses. At "day 7", the inoculated leaves were collected for RT-PCR analysis to obtain an original pool of possible recombinant molecules and to

avoid loss of those with lower fitness. According to the tests, 99 of the 105 plants contained both viruses.

A replicase-driven template switching mechanism is considered to need secondary structures and homologous blocks for priming between the donor and acceptor strands (White and Morris, 1999). The sequence identity between the genomes of CfMV and RGMoV was found to be 48 % when aligned pairwisely by NCBI-BLAST2 (publication II).

Regions of the –1 ribosomal frameshifting signal and the putative subgenomic RNA promoter were selected to screen for recombination events as they probably possess secondary structure elements that have been suggested to be potential hot spots for RNA recombination. The –1 ribosomal frameshift signal characteristic of all sobemoviruses consists of a slippery sequence (UUUAAAC) and a stem-loop structure several nucleotides downstream from it (Tamm and Truve, 2000b). The position and secondary structure of the sobemoviral subgenomic promoter is not experimentally proven. Ryabov *et al.* (1996) have postulated that the start position of CfMV sgRNA might be 35 bases upstream from the first AUG codon of CP at position 3057. In this study, the subgenomic RNA promoter was expected to lie in the range of –150 and +150 nt from the CP translation start point, as characteristic of other plant viral subgenomic promoters (Miller and Koev, 2000). The reason for considering sgRNA promoter region as the potential hot spot is the presence of DI RNA molecules corresponding to 35-40 nucleotides of the 5'-proximal end of genomic RNA linked with 850-950 nucleotides of the 3' terminus in case of CfMV infection (Mäkinen *et al*., 2000a). That is considered to be proof of a replicase-driven template switching mechanism (White and Morris, 1999). Moreover, evolution of the "supergroup" of luteo-sobemo-tombusviruses indicates a recombinational hot spot between the gene blocks encoding Pro-VPg-RdRp and CP (Mayo *et al.*, 1989; Martin *et al.*, 1990; Mayo and Jolly, 1991; Gibbs and Cooper, 1995; Mayo and Ziegler-Graff, 1996; Miller and Rasochova, 1997; Miller *et al.*, 1997; Moonan *et al.*, 2000; Moonan and Mirkov, 2002; aus dem Siepen *et al.*, 2005; Silva *et al.*, 2008).

The alignment made by BLAST2 tool was used to design virus-specific primer pairs for RT-PCR analysis. The primers were designed to amplify the area between 1535 nt and 1967 nt in the sequence of CfMV (primers were named as Cfs5' and Cfs3') and between 1440 nt and 2157 nt in the sequence of RGMoV (primers were named as Rfs5' and Rfs3') in case of screening the –1 ribosomal frameshifting signal. In case of screening the putative subgenomic RNA promoter region, the primers were designed to amplify the area between 2818 nt and 3380 nt in the sequence of CfMV (primers were named as Csg5' and Csg3') and between 3076 nt and 3529 nt in the sequence of RGMoV (primers were named as Rsg5' and Rsg3').

Before using these primers for screening of recombinant molecules possibly arisen in co-infected plants, the specificity of these primers were checked by using RNA extracted from plant material singly infected with CfMV or RGMoV, or the artificial mixture of them both in RT-PCR (Fig. 2, publication II). No unspecific amplification was observed with primer pairs of Cfs5'+Cfs3', Csg5'+Csg3',

Rfs5'+Rfs3', Rsg5'+Rsg3', Csg5'+Rsg3', Rsg5'+Csg3' and Cfs5'+Rfs3'. Unfortunately, the primer combination of Rfs5'+Cfs3' amplified several non-specific fragments. Therefore, this primer pair was not involved in the recombination study.

To estimate the sensitivity of recombinant primer pairs Csg5'+Rsg3', Rsg5'+Csg3' and Cfs5'+Rfs3', several artificial CfMV-RGMoV chimerical virus templates were cloned (Fig. 3, publication II). These chimerical templates were used for *in vitro* RNA synthesis. The synthesized chimerical RNAs were diluted in tenfold series as low as 0.1 pg per reaction. Every reaction was mixed with 0.5 µg of RNA extracted from uninfected oat leaves to mimic a natural situation providing the diversity of cellular mRNA molecules as possible priming templates. The primer pairs Cfs5'+Rfs3' and Rsg5'+Csg3' had very good sensitivity – they detected down to 0.1 pg of the *in vitro* synthesized template RNA from the mixture of 0.5 µg total oat RNA whereas the primer pair Csg5'+Rsg3' detected the presence of recombinant template RNA in the mixture at a sensitivity level down to 100 pg only.

Next, the recombinant primer pairs Csg5'+Rsg3', Rsg5'+Csg3' and Cfs5'+Rfs3' were used to screen the pool of 105 co-inoculated plants. No recombinant molecules were detected. Altogether, approximately one quarter of the CfMV and RGMoV genomes were monitored for recombination events between two sobemoviruses. It remains unknown whether recombinations occured elsewhere between two genomes.

There can be various reasons for no detection of recombinant virus molecules. After co-infection of the host, the viruses must co-infect the same cell. Also, virus replication must happen at the same time to provide a possibility for template switching (Worobey and Holmes, 1999). The priming between the donor and acceptor strands as well as re-initiation of the dissociated replicase/nascent RNA complex has specific requirements (Suzuki *et al.*, 2003; Cheng *et al.*, 2005; de Wispelaere *et al.*, 2005). Studies on plant virus recombination demonstrate that the properties of the viral replicase and several host factors play a role in RNA recombination (Bousalem *et al.*, 2000; Desvoyes and Scholthof, 2002; Ohshima *et al.*, 2002; Panaviene and Nagy, 2003; Dzianott and Bujarski, 2004; Shapka and Nagy, 2004; Cheng *et al.*, 2005). It has been shown that host genes involved in RNA degradation were suppressing the generation of new viral RNA recombinants whereas the host genes contributing to the intracellular transport of proteins were identified as viral RNA recombination accelerators (Serviene *et al.*, 2005; Serviene *et al.*, 2006; Cheng *et al.*, 2006).

Our findings that no recombinants were identified between two sobemoviruses in doubly infected plants are in accordance with the results gained by phylogenetic analyses of sobemoviral sequences (Lokesh *et al.*, 2001; Fargette *et al.*, 2004; Zhou *et al.*, 2005; Chare and Holmes, 2006; Stuart *et al.*, 2006; Fargette *et al.*, 2008; Sérémé *et al.*, 2008). However, the newest exhaustive search for recombinants has found a few recombinations between close isolates of RYMV in Tanzania (Pinel-Galzi *et al*., 2009). The short recombinations were detected in the region near/at the

beginning of ORF2a and at the 3′ UTR of RYMV. Unfortunately, these segments were not involved in our screening for putative recombinants between CfMV and RGMoV.

Our study was the first experimental survey of recombinations between sobemoviruses.

4.3. P1 of *Cocksfoot mottle virus* is required for systemic infection in oat

P1 is encoded by the 5'-terminal ORF of the sobemoviral genomic RNA and it is the most divergent protein of sobemoviruses. Moreover, no sequence similarity has found between P1 proteins neither nucleic acid nor at the amino acid level (Ngon A Yassi *et al.*, 1994; Mäkinen *et al.*, 1995b; Othman and Hull, 1995). There are no conserved regions in P1 sequences offering a hint about their functions. Despite of the sequence difference both RYMV P1 and SCPMV P1 have been shown to be dispensable for viral RNA synthesis (Bonneau *et al.*, 1998; Sivakumaran *et al.*, 1998). Both RYMV P1 and CfMV P1 act as the supressors of a post-transcriptional gene silencing (PTGS) in non-host species of *Nicotiana benthamiana* and *N. tabacum* (Voinnet *et al.*, 1999; Himber *et al.*, 2003; Sarmiento *et al.*, 2007; Siddiqui *et al.*, 2008). Recently, RYMV P1 has been shown to function as a silencing suppressor in rice, the host plant of RYMV (Siré *et al.*, 2008; Lacombe *et al*., 2010).

The objective of current study was to start unraveling the functions of CfMV P1 in oats, the experimental host plant of CfMV.

At first, CfMV cDNA was amplified from RNA isolated from CfMV-NO infected oat plants. It was amplified as a cDNA copy and subcloned into a carrier plasmid under the control of T7 promoter. Viral 5'-capped RNA synthesized from cDNA copy was amplified *in vitro* and tested to be infectious in oat plants. CfMV infecious cDNA (icDNA) was used as a template to create a P1-deficient CfMV cDNA clone CfMV P1(-) by introducing a point mutation in order to convert the first AUG codon of ORF1 to UUG. Also, a replication-deficient CfMV cDNA clone CfMV RdRp(-) was generated by raising a stop codon into the beginning of RdRp-coding region. The 5'-capped RNAs were synthesized and biolistically delivered into the cells of barley suspension culture or into the oat leaves. Replication was tested after 24 hours by RT-PCR analysis. Differently from a replicase-deficient virus, it was observed that elimination of the expression of P1 does not abolish a virus replication neither in oat leaves nor in barley suspension culture (publication III, Fig. 1). However, although the P1 of CfMV was not strictly needed for virus replication, the absence of P1 expression lowered the virus accumulation so much that it was not detectable by Northern blot analysis.

Next, 5'-capped transcripts of CfMV P1(-) were biolistically delivered into the oat plants. The presence of CfMV was tested after 14 days by negative-strand specific RT-PCR analysis. It appeared that P1-defective virus was able to replicate as it was still present in inoculated leaves (of 49 plants out of 57). Systemic infection was detected only in case of a spontaneous transversion of mutated ORF1 initiation codon to wildtype (in 3 plants out of 57) that restored expression of P1 (publication III, Table 1). Consequently, the absence of P1 does not abolish CfMV

replication but systemic spread is inhibited. The same is true for RYMV and SCPMV (Bonneau *et al.*, 1998; Sivakumaran *et al.*, 1998).

Despite of a great diversity of sobemoviral P1, it has been speculated to function as a viral movement protein[7] (Othman and Hull, 1995; Mäkinen *et al.*, 1995b). Numerous studies have demonstrated that one of the functional hallmarks of MPs is their capability to bind single-stranded nucleic acids (Waigmann *et al.*, 2004). Therefore, the ability of CfMV P1 to bind ssRNA but not dsDNA (Tamm and Truve, 2000a) led to the suggestion that it may act as a virus movement protein. To test the movement ability, CfMV P1:EGFP and EGFP alone were expressed in oat leaves under the control of maize ubiquitin promoter independently from the context of other CfMV proteins. Expression was monitored during the next 48 hours using fluorescence microscopy. EGFP alone remained in single cells without detectable cell-to-cell movement whereas P1:EGFP showed limited cell-to-cell movement to neighboring cells within the epidermal tissue in 3 out of 100 individually bombarded cells (publication III, Fig. 2).

In parallel with our experiments, similar results have been gained with SCMoV P1 – the expression of GFP:P1 fusion protein was observed in the nucleus of single cells, cytoplasm and cell periphery of neighboring cells. It was found that there was limited spread of that fusion protein from one cell to another 36-48 hours after transformation. Finally, it was concluded that P1 of SCMoV cannot move independently from cell to cell, probably due to missing viral components (Fosu-Nyarko, 2005).

Both CfMV P1 and RYMV P1 have been shown to act as the suppressors of PTGS (Voinnet *et al.*, 1999; Sarmiento *et al.*, 2007). Interestingly, recent data on RYMV P1 show a connection between the silencing suppression and cell-to-cell movement abilities (Siré *et al.*, 2008). The single amino acid mutation C95S (in putative zinc finger motif) impairs the ability of RYMV P1 to suppress constitutive silencing of *uidA* in distal part of leaf but not in biolistic delivery spot suggesting that this mutation affects the cell-to-cell movement of RYMV P1 (Siré *et al.*, 2008). Whether PTGS restricts CfMV movement in P1(-) background, is not known at the moment.

Our attempts to localize CfMV P1 in wt CfMV-infected oat leaves by polyclonal antiserum generated against P1 (Tamm *et al.*, 1999) revealed expression of P1 neither by Western blot analysis nor by immunohistochemical staining (Tiismus, 2005). It remained unclear whether P1 was poorly accumulated in infected plants or the P1 antiserum was not suitable for the detection of P1 expressed in plant cells.

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[7] Plant viruses spread from an initially infected cell to neighbouring cells through plasmodesmata. This process is termed as local or cell-to-cell movement. The viral movement proteins (MPs) have a central role in cell-to-cell movement. During local movement, viral MPs either interact with the viral genomes to form nucleoprotein complexes that cross through plasmodesmata into adjacent cells or they become a part of virus-induced tubules that extend through the cell wall (presumably replacing plasmodesmata and serving as a conduit for the spread of virus particles).

Studies on phylogenetically related poleroviruses provide results that may explain that result. It has been shown that despite that BWYV P0 is dispensable for systemic infection (Ziegler-Graff *et al.*, 1996) the amount of P0 has clear effects on virus accumulation. Experiments on BWYV mutant with optimized context for ORF0 revealed deleterious effect to viral RNA accumulation. Moreover, appearance of the spontaneous mutations predicted to lower the efficiency of translation initiation at the P0 start codon was detected (Pfeffer *et al.*, 2002). Also, it was shown for PLRV that although P0 is undetectable in infected plants (van der Wilk *et al.,* 1997) it is necessary for virus accumulation (Sadowy *et al.*, 2001).

In current study, CfMV P1 was expressed at high level in Sf9 insect cell culture for subcellular localization studies using baculovirus vector system. Samples of total protein were collected at 24, 48, 72 and 96 h post infection. All samples were treated in parallel according to two differential centrifugation protocols to obtain subcellular fractions. Western blot analysis showed the presence of 12-kDa CfMV P1 in pellets of cell nuclei and membranes but not in the fraction of soluble cytoplasmic proteins. A further treatment of nuclear pellets with NP-40 or high salt did not dissolve any P1 into nuclear extract (publication III, Fig. 3). Thus, it was obvious that P1 of CfMV is coupled with cellular membranes and/or it is heavily aggregated when expressed in insect cells. Similarly, when recombinant CfMV P1 was expressed in *E. coli* – the majority of it remained aggregated and unsoluable (Tamm and Truve, 2000a) and therefore not appropriate for functional studies.

CONCLUSIONS

1. CfMV-like genomic organization is common for all sobemoviruses. Consequently, the expression and amount of sobemoviral RdRp is regulated by -1 ribosomal frameshifting similar to other evolutionary related viruses.

2. Albeit the phylogenetic analyses show that a recombinational shuffling of genes and gene blocks has played an important role in the formation of contemporary species of "the supergroup" luteo-sobemo-tombusviruses, no recombinations has been found between sobemovirus species. Accordingly, no recombinant virus molecules were found experimentally when 99 oat plants were co-infected with CfMV and RGMoV under no or little selection pressure.

3. P1 of CfMV is not required for virus replication but it is essential for the systemic infection of CfMV.

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PUBLICATION I

Sobemoviruses possess a common CfMV-like genomic organization

Meier, M. and Truve, E.

Archives of Virology (2007) **152**, 635-640.

PUBLICATION II

An attempt to identify recombinants between two sobemoviruses in doubly infected oat plants

Meier, M. and Truve, E.

Environmental Biosafety Research (2006) **5**, 47-56.

PUBLICATION III

P1 protein of Cocksfoot mottle virus is indispensable for the systemic spread of the virus

Meier, M., Paves, H., Olspert, A., Tamm, T. and Truve, E.

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ABSTRACT

Sobemoviruses

The vector and host range of sobemoviruses is quite narrow. Despite of that, some sobemoviruses seriously impact global agriculture – mainly due to their particle stability and effective transmission via mechanical wounding of host plants during farming operations. The best-known example is *Rice yellow mottle virus* (RYMV) that is the main cause for rice failure in Africa. The outbreaks of *Subterranean clover mottle virus* (SCMoV) are the main reason for decreases of herbage production of clover pastures in Australia, whereas *Cocksfoot mottle virus* (CfMV) has been reported to severely affect hay yields in Norway.

A genus *Sobemovirus* consists of small viruses with icosahedral capsids and a single-stranded polycistronic mono-component RNA genome. Their genome 5′ terminus bounds covalently a virus-encoded protein (VPg) and their 3′ terminus has not been polyadenylated. Sobemovirus genome has in size 4000-4500 nucleotides, bearing four ORFs only. The non-structural proteins (P1 and polyprotein) are translated from genomic RNA, whereas capsid protein (or coat protein, CP) is translated from sgRNA. A polyprein will be processed at least into three functionally different proteins. To date, there are 11 sobemoviruses which genome has been fully sequenced.

The genus *Sobemovirus* is not assigned to any family. A reason for this is a kind of mosaic nature of sobemovirus genome. The 5'-terminal part of the sobemoviral genomes encoding non-structural proteins resembles those of enamo- and poleroviruses from the family *Luteoviridae* in its genomic organization, whereas sobemoviral genome 3′ terminal part encoding CP is related to those of necroviruses from the family *Tombusviridae*. The reason for that is considered to be early recombination events that happened during the evolution of these genera.

Differently from enamo- and poleroviruses, the middle part of sobemovirus genome has been reported to have two different organizations – either "SCPMV-like" or "CfMV-like". Similarly to enamo- and poleroviruses, "CfMV-like" viruses can synthesize their polyproteins in two variants – the shorter one (that consists of VPg and Pro only) is translated from ORF2a whereas the longer one (consists of VPg, Pro and RdRp) needs -1 ribosomal frameshifting from ORF2a to ORF2b during translation. The efficiency of that -1 ribosomal frameshift has been measured to be 10-25 % in case of CfMV. "SCPMV-like" genomes were believed to be translated in-frame as long polyproteins (consisted of VPg, Pro and RdRp). Such appearance of two different strategies for RdRp synthesis in one virus family would be exceptional in virus world. However, a signal for -1 ribosomal frameshifting has also been found in genomes of "SCPMV-like" sobemoviruses. Moreover, it has been found to be conserved between all sobemoviruses. One possible function given, proposes that it may lead ribosomes into ORF3 that is shortly translated thereafter until the ribosomes meet stop codon.

The first goal of this thesis was to verify the existence or the non-existence of "SCPMV-like" genomic organization. A reason for that was the sequence homologies found in different reading frames between sobemovirus genomes with

"SCPMV-like" and "CfMV-like" organization. Also, it was known that RYMV that was announced as the virus with "SCPMV-like" genomic organization was recently re-sequenced and appeared to have "CfMV-like" genome. Thus, questionable genomic areas of *Lucerne transient streak virus* (LTSV), *Ryegrass mottle virus* (RGMoV), *Southern bean mosaic virus* (SBMV) and SCPMV were re-sequenced in current study. The results of the sequencing project showed clearly that the "SCPMV-like" genomic annotation is an artefact and it does not exist. Re-sequencing of *Sesbania mosaic virus* (SeMV) by Dr. Savithri confirmed that view. Thus, all sobemoviruses harbour similar genomic organization, previously known as "CfMV-like", also common for evolutionarly related enamo- and poleroviruses. Hence, all sobemoviruses regulate the synthesis of RdRp via -1 ribosomal frameshift.

The second goal of this thesis was to estimate recombination ability between two sobemoviruses (CfMV and RGMoV, *Ryegrass mottle virus*) in co-infected test plants. RT-PCR affirmed the co-infection of 99 test plants. Two possible evolutionary "hot spots" were screened for possible recombination events. Several previous studies have shown that both a strong secondary structure and a sequence homology are required for recombination events. The first possible "hot spot" area was supposed to be localized adjacent to -1 ribosomal frameshift area that has both of these qualities. Secondly, recombination events were screened from sgRNA promter vicinity. The region (encoding non-structural proteins) preceding sobemovirus sgRNA promoter is similar to enamo- and poleroviruses, whereas the subsequent sequence (encoding CP) is more similar to necroviruses. This is considered as a clue to an early evolutionary recombination event. RT-PCR used in the current study was extremely sensitive – it was able specifically amplify 0.1-100 pg of artificially synthesized recombinant template from reaction mixed with 0.5 µg RNA purified from non-infected plants. However, no recombinants were found from doubly infected test plants. We cannot exclude that recombination might have happened between these two viruses in non-screened genomic regions.

Finally, we started unraveling the functions of CfMV P1. As sobemoviral P1 proteins are highly divergent - they are not related to any other known protein – their functions cannot be presumed according to sequence homologies. In this current study, P1 translation initiation codon of infectious clone of CfMV was mutated to abolish P1 translation but not to harm virus RNA structure. Analysis of infected plants showed that P1-deficient virus was able to replicate in inoculated leaves but not able to infect plants systemically. However, 3 of 57 plants that were inoculated showed systemic infection caused by a spontaneous transversion of mutated ORF1 initiation codon to wildtype. Hence, it was concluded that P1 is required for systemic infection.

Recently, it was demonstrated by our group that CfMV P1 functions as a suppressor of systemic silencing. Such knowledge is also gained in studies of RYMV P1 (that however has no sequence homology with CfMV P1). Recent mutation analysis of RYMV P1 showed that cell-to-cell movement ability and the strength of silencing suppression have related to each other and needed for infectivity.

KOKKUVÕTE

Sobemoviirused

Sobemoviirustele on iseloomulik kitsas vektorputukate ja peremeestaimede ring. Sellest hoolimata on mõned sobemoviirused tõsiseks probleemiks globaalsele põllumajandusele – seda põhiliselt tänu viiruspartiklite stabiilsusele ja heale edasikandumisvõimele mehhaaniliste vigastuste kaudu. Näiteks, riisi kollalaiksuse viirus (RYMV) on põhiline riisiikalduste põhjustaja Aafrikas. Austraalias ohustab ristikupõlde maaristiku laiguviirus (SCMoV) ning Norras on tulnud ette keraheina laiguviiruse (CfMV) poolt põhjustatud heinasaagi drastilist vähenemist.

Sobemoviiruste perekonda kuuluvad väikesed ikosaeedrilise kapsiidi ning positiivse polaarsusega polütsistroonsed üheahelalised ühekomponentsed RNA viirused. Sobemoviiruste genoomi 5´ otsa on kovalentselt seondunud viiruse enda poolt kodeeritud valk VPg ja nende genoomi 3´ ots on polüadenüleerimata. Sobemoviiruste genoomi suurus jääb vahemikku 4000-4500 nukleotiidi, sisaldades nelja avatud lugemisraami. Tänaseks on teada 11 sobemoviiruse genoomi järjestused. Mittestruktuursed valgud nagu P1 ja polüproteiin transleeritakse genoomselt RNAlt, samal ajal kui viiruse kattevalgu translatsiooniks on vajalik subgenoomse RNA (sgRNA) süntees. Polüproteiin protsessitakse peale sünteesi vähemalt kolmeks erineva funktsionaalsusega valguks.

Sobemoviiruste perekonna süstemaatiline kuuluvus on määratlemata, kuna genoomi osa, mis kodeerib mittestruktuuseid valke (v.a P1, mis on täiesti unikaalne), on järjestuselt lähedane enamo- ja poleroviirustele luteoviiruste sugukonnast, kuid genoomi osa, mis kodeerib viiruse kattevalku, on lähedane hoopis nekroviirustele tombusviiruste sugukonnast. Sellise genoomi mosaiiksuse põhjuseks arvatakse olevat rekombinatsioon eellasviiruste vahel.

Erinevalt enamo- ja poleroviirustest arvatati senini, et sobemoviiruste genoomi polüproteiini kodeeriv osa võib olla kahe erineva struktuuriga – nn. lehmaherne lõunamosaiigi viiruse (SCPMV) või CfMV-tüüpi. CfMV-tüüpi viirustele (samuti kui polero- ja enamoviirustele) on iseloomulik, et polüproteiin sünteesitakse kahes variandis – lühem variant sisaldab vaid VPg ja seriinproteaasi (Pro) domääne, mis transleeritakse ORF2a-lt ning pikema variandi sünteesiks peab toimuma -1 ribosomaalne raaminihe raami ORF2b, mis kodeerib RNA-sõltuvat RNA polümeraasi (RdRp). Katsed CfMV-ga on näidanud, et raaminihke efektiivsus on 10-25% sõltuvalt reaktsiooni tingimustest. SCPMV-tüüpi genoomilt arvati transleeritavat kogu polüproteiin ühest raamist, mis ei võimalda viirusel reguleerida RdRp hulka. Selline kahe erineva RdRp sünteesi strateegia esinemine ühes viiruste perekonnas oleks täiesti unikaalne. Seda veelgi enam seetõttu, et -1 raaminihke signaal on konserveerunud ka SCPMV-tüüpi viiruste genoomis. Ühe mudeli kohaselt võiks raaminihe toimuda ka nendel viiruste translatsioonil, jätkudes lühikeses lugemisraamis ORF3, alternatiivina täispika polüproteiini sünteesile.

Käesoleva töö üheks eesmärgiks oli kontrollida, kas selline unikaalne SCPMV-tüüp ikkagi eksisteerib, kuna sobemoviiruste genoomsete järjestuste võrdlused ilmutasid märke järjestushomoloogiatest erinevates lugemisraamides. Samuti oli teada, et RYMV, mis algselt arvati olevat SCPMV-tüüpi, osutus ülesekvenee-

rimisel CfMV-tüüpi genoomi ülesehitusega viiruseks. Seetõttu sekveneeriti käesoleva projekti käigus üle lutserni transientse triibuviiruse (LTSV), raiheina laiguviiruse (RGMoV), põldoa mosaiigiviiruse (SBMV) ja SCPMV genoomide osad, mille struktuuri suhtes oli kahtlusi. Sekveneerimisprojekti tulemusena selgus, et tõepoolest, varasem SCPMV-tüüpi annotatsioon on artefaktne. Seega on kõik sobemoviirused oma genoomilt CfMV-tüüpi, sarnaselt evolutsiooniliselt lähedaste enamo- ja poleroviirustega, ning reguleerivad RdRp sünteesi -1 ribosomaalse raaminihke abil.

Käesoleva töö teises osas nakatati testtaimi samaaegselt kahe sobemoviirusega, et hinnata nende rekombineerumisvõimet. RT-PCR kinnitas 99 taime nakatumist mõlema viirusega. Rekombinatsioone otsiti kahest oletatavast evolutsioonilisest „kuumast kohast“. Mitmed varasemad tööd on näidanud, et rekombinatsioonide toimumiseks on vajalik nii homoloogne järjestus kui ka tugev sekundaarstruktuur. Esiteks otsiti rekombinatsioone -1 ribosomaalse raaminihke signaali ümbruskonnast, mis sisaldab tüviaas sekundaarstruktuuri ja on sobemoviiruste seas äärmiselt konserveerunud, pakkudes võimalust homoloogiliseks rekombinatsiooniks. Teiseks otsiti võimalikke rekombinatsioone sgRNA promootori piirkonnast, kuna sobemoviiruste genoom sarnaneb sgRNA promootorile eelnevas piirkonnas (mis kodeerib mittestruktuurseid valke) enamo- ja poleroviirustele, kuid sgRNA promootorile järgnevas piirkonnas (mis kodeerib viiruse kattevalku) nekroviirustele. Käesolevas töös kasutatud RT-PCR meetod osutus ülitundlikuks, suutes tuvastada 0.1-100 pg kunstlikult sünteesitud rekombinantset RNAd reaktsioonisegust, millele oli rekombinantse RNA mimikeerimiseks lisatud 0.5 µg nakatamata taimedest eraldatud RNAd. Sellest hoolimata, topeltnakatatud taimedes rekombinantseid viiruseid ei tuvastatud. Ei saa siiski välistada, et rekombinatsioonid võisid toimuda genoomi piirkondades, mida ei vaadeldud.

Töö viimases osas alustati CfMV P1 valgu rolli selgitamist viiruse elutsüklis. Kuna sobemoviiruste P1 valgud on äärmiselt unikaalsed, siis ei ole homoloogiate põhjal võimalik järeldada, millist rolli need võiksid täita. Käesolevas töös punktmuteeriti CfMV P1 translatsiooni initsiatsioonikoodon, et välistada P1 translatsioon, samal ajal võimalikult vähe kahjustades genoomse RNA struktuuri. Selliselt muteeritud viirusega nakatatud taimede analüüsist selgus, et P1 mitteekspresseeriv viirus on nakatatud lehes võimeline replitseeruma, kuid ei suuda taime süsteemselt nakatada. Kolmel juhul 57-st leiti, et viirus oli ka süsteemselt levinud, kuid saavutanud selle initsiatsioonikoodoni tagasimuteerumise kaudu. Seega järeldati, et P1 valk on vajalik CfMV süsteemseks levimiseks. Hiljuti on meie rühma poolt kindlaks tehtud, et CfMV P1 on oma funktsioonilt süsteemse vaigistamise supressor. Sama on teada ka RYMV P1 kohta, kuigi need kaks valku ei oma järjestushomoloogiat. RYMV P1 hiljutine mutatsioonanalüüs on näidanud, et P1 valgu rakust rakku liikumise võime ja vaigistamise supressiooni tugevus on omavahel seotud ning vajalikud viiruse infektsioonilisuseks.

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Tunnustused: 2009 Eesti Riigi Teaduspreemia põllumajanduses

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