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Biology and Genomics of *Lettuce necrotic yellows virus*

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ABSTRACT

Rhabdoviruses are important pathogens of humans, lifestock, wildlife, fish and plants. *Lettuce necrotic yellows virus* (LNYV) is the type species of the genus *Cytorhabdovirus* in the family *Rhabdoviridae*. LNYV has characteristic bacilliform, enveloped particles with a single-stranded, negative-sense RNA genome of 12,807 nucleotides contained within an infectious nucleocapsid core. The viral genome encodes six proteins, namely the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), 4b protein and large polymerase protein (L), the nucleotide and deduced amino acid sequences of which have recently been analysed in detail. LNYV naturally infects a narrow range of dicotyledonous and monocotyledonous host plants. It is transmitted in a persistent manner by aphids in which it also multiplies. This article reviews LNYV particle composition and structure, replication, purification, methods for identification and diagnosis, host range, symptoms, transmission and epidemiology. Recent advances in LNYV genome sequence analysis, genetic diversity and evolution, and potential transgenic control strategies are also discussed.

Keywords: cytorhabdovirus, genetically-engineered resistance, genetic variability, genome organisation, lettuce, nucleotide sequence, particle morphology, purification, taxonomy
Abbreviations: LNYV, lettuce necrotic yellows virus; **NCMV**, northern cereal mosaic virus; **PCR**, polymerase chain reaction; **SCV**, strawberry crinkle virus

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INTRODUCTION

Rhabdoviruses form a large group of important pathogens of humans, lifestock, wildlife, fish and plants. They are easily recognised by electron microscopy due to their characteristic bullet-shaped or bacilliform, enveloped particles. Rhabdoviruses (order *Mononegavirales*) have a monopartite, single-stranded, negative-sense RNA genome contained within an infectious nucleocapsid core. Plant-infecting rhabdoviruses have been taxonomically separated into the genera *Nucleorhabdovirus* and *Cytorhabdovirus*, based on their respective sites of replication and morphogenesis (Tordo *et al*. 2005; Jackson *et al*. 2005). The genus *Cytorhabdovirus* contains eight recognised virus species; the complete genomes of three cytorhabdoviruses have been sequenced, namely *Northern cereal mosaic virus* (NCMV), *Strawberry crinkle virus* (SCV) and *Lettuce necrotic yellows virus* (LNYV; Jackson *et al*. 2005).

LNYV is the type species of the genus *Cytorhabdovirus* in the family *Rhabdoviridae,* and the best studied of the cytorhabdoviruses. This review provides information on LNYV particle composition and structure, replication, purification, methods for identification and diagnosis, host range, symptoms, transmission and epidemiology. The article focuses in particular on recent advances in LNYV genome sequence analysis, genetic diversity and evolution. Initial attempts of implementing transgenic control strategies in lettuce including protein-mediated resistance and post-transcriptional gene silencing are also discussed.

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Invited Review

BIOLOGY OF LNYV INFECTIONS

Host range

Necrotic yellows disease of lettuce was first recognized in 1954 from Victoria, Australia (Stubbs and Grogan 1963). LNYV naturally infects both dicotyledonous and monocottyledonous plants and has a narrow host range in the *Compositae*, *Solanaceae*, *Chenopodiacae*, *Asteraceae*, *Liliaceae*, *Leguminosa*, *Amaranthacae*, *Alliaceae* and *Fabaceae*. Hosts include sowthistle (*Sonchus oleraceus*), *S. hydrophilus*, *Reichardia tingitana*, *Embergeria megalocarpa* (Randles and Carver 1971) and *S. kirkii* (Francki *et al*. 1989). Although lettuce (*Lactuca sativa* L.) is the main economically important host of LNYV (**Fig. 1A, 1B**), field infection has been diagnosed of garlic (*Allium sativum*) in central Victoria (Sward 1990), chickpea (*Cicer arietinum L.*) in south-east Queensland, and safflower (*Carthamus tinctorius*), *Lupinus albus*, *L. angustifolius*, *Medicago polymorpha* (Behncken, 1983) and peanut (*Arachis hypogaea* L.) (JE Thomas, pers. comm.) in Queensland. Experimentally, LNYV has been transmitted by the aphid *Hyperomyzus lactucae* to lettuce, prickly lettuce (*L. serriola* L.), *S. oleraceus* and *S. megalocarpus*. Host plants that can be infected by rub inoculation include *Nicotiana glutinosa*, *N. clevelandii, Petunia hybrida*, *Datura stramonium* L., *Spinacia oleraceus* L. var. 'Nobel', *Lycopersicon esculentum* L. var. 'Grosse Lisse' and *Gomphrena globosa* L. (Stubbs and Grogan 1963).

Symptomatology

LNYV causes a severe disease of lettuce in Australia and New Zealand (Stubbs and Grogan 1963; Fry *et al*. 1973). The symptoms resemble those caused by tomato spotted wilt virus, but are unlike those caused by lettuce mosaic virus. LNYV infection in lettuce generally progresses as follows: the healthy green shiny leaves become dull and faded, then begin to bronze, followed by necrosis, severe chlorosis, flattened growth, flaccidity and frequently plant death. Early infected seedlings are severely stunted (**Fig. 1A, 1B**). Infection prior to heart formation results in internal necrosis, and infection following seed stalk elongation causes single sided leaf and stalk necrosis and latex exuding from the necrotic areas of the stem (Stubbs and Grogan 1963).

In lupins, LNYV causes interveinal chlorosis and slight stunting (Behncken 1983). LNYV causes mosaic symptoms in safflower, chlorotic streaking and slight leaf distortion in *M. polymorpha* and necrotic tip burn and lethal wilt in chickpea (Behncken 1983). LNYV infection in peanut has only been observed in mixed infection with a poty-like virus; symptoms include leaf mottling, stunting and reduced yield (JE Thomas, pers. comm.). Similarly, LNYV has only been found in mixed infections in garlic and specific symptoms have not been attributed to it. LNYV-infected sowthistle plants appear symptomless. Symptoms in experimentally infected plants vary markedly. In prickly lettuce there is almost complete growth inhibition, though little mortality and lateral curvature, distortion, marginal reddening and mottling of leaves. Dune thistle shows scarcely visible mottle symptoms and slight stunting whereas petunia displays systemic vein clearing and occasional transient apical stem necrosis (Stubbs and Grogan 1963). Symptoms on *N. glutinosa* vary between virus isolates from a systemic mild crinkling, to vein clearing and systemic necrosis (Francki *et al*. 1989; **Fig. 1C, 1D**). Mechanical inoculation of lettuce and *S. oleraceus* with LNYV is difficult (Stubbs and Grogan 1963), but *N. glutinosa* can be infected readily via this route (Fry *et al.* 1973). There is no evidence of transmission of LNYV through seed in lettuce, *S. oleraceus* or *N. glutinosa* (Francki *et al*. 1989).

Epidemiology

The natural weed hosts of LNYV, especially sowthistle, play an important role in the epidemiology of the virus. In Australia, sowthistle is a ubiquitous weed that rapidly colonises bare or sparsely vegetated soil and plants can be found throughout the year. Disease incidence in lettuce crops can be reduced by 70% by eliminating adjacent sowthistle plants (Randles 1983). There is a steep decline in disease incidence over distance from an external weed source, with clustering of LNYV-infected plants at the crop's edge closest to the source (Coutts *et al*. 2004). It has been proposed that the sudden increase of LNYV outbreaks in Australia in the 1950's may have been caused by the release of the myxomatosis virus to control rabbit populations and the subsequent increase in sowthistle plants and therefore LNYV reservoirs due to reduced grazing pressure (Randles 1983).

LNYV is transmitted persistently in a circulative and propagative manner by the aphids species *Hyperomyzus lactucae* (L.) (Stubbs and Grogan 1963), and *H. cardu-*

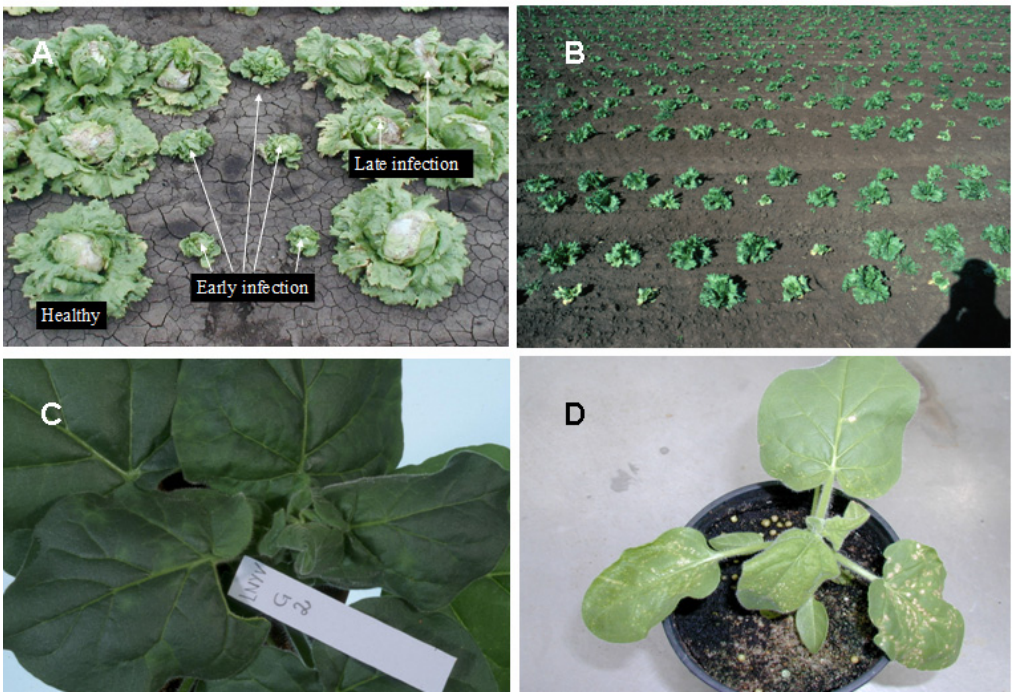

Fig. 1 Symptoms of LNYV on lettuce and *Nicotiana glutinosa*. (**A**) Iceberg lettuce plants healthy and infected at different growth stages, (**B**) field of infected lettuce seedlings (yellow) near Gatton, Queensland, (**C**) *N. glutinosa* infected with the mild G2 isolate and (**D**) the severe 318 isolate of LNYV.

ellinus (Theob.) (Randles and Carver 1971). Neither of the vectors can breed on nor colonise lettuce; virus transmission occurs when migrating aphids probe lettuce plants while searching for suitable hosts. Both aphid species breed on *S. oleraceus*, *R. tingitana*, and *E. megalocarpa*. *H. carduellinus* has a limited geographical distribution making it a less important vector of LNYV (Randles and Carver 1971). In Australia, *H. lactucae* is normally anholocyclic on *S. oleraceus*, reproducing parthenogenetically throughout the year (Randles and Carver 1971; Martin 1983). Three primary interacting factors affect the population dynamics of *H. lactucae*: environmental conditions (temperature, rainfall, wind speed and direction), aphid density and food quality. The numbers of migrating aphids have a direct influence on the incidence of LNYV in lettuce crops (Randles 1983).

Aphidius sonchi was imported as a biological control agent of *H. lactucae* in 1981 and established throughout all states of Australia (Carver and Woolcock 1986). However, no studies have been conducted to determine if this had an effect on the incidence of LNYV infection in lettuce. The recent emergence of the brown sowthistle aphid *Uroleucon sonchi* as a pest of both lettuce and sowthistle and its dominance on sowthistle plants may also affect the dynamics of LNYV infections.

Transmission

LNYV is transmitted transovarially through at least two generations of *H. lactucae*, with about 20% of the progeny of a viruliferous aphid able to transmit LNYV, and 20% of these progeny able to transmit LNYV to the next generation (Boakye and Randles 1974). LNYV particles have been detected by electron microscopy in the salivary glands, brain, muscle, fat body, mycetomes, ovaries, and oesophagus of *H. lactucae*. Due to evidence of a temperature-dependent latency period, the persistence of the virus in the vector, and the transovarial transmission, it has been concluded that the virus is propagative in *H. lactucae* (Randles 1983).

The interaction of the vector with the two host species of interest, *S. oleraceus* and lettuce is vastly different. *S. oleraceus* is the only important food source for the aphid; its distribution determines the presence and abundance of *H. lactucae* and the size of the reservoir of LNYV. Immature inflorescences are vital to the reproduction of the aphid (Martin 1983). An increased reproduction rate in spring and autumn explains the increased migratory activity at these times. The effects of other abiotic factors, such as temperature and rainfall, appear to be less important (Randles 1983).

In contrast, lettuce is not a host of *H. lactucae*. Aphids removed from a source plant were found not to settle and feed on lettuce until starved for several hours (Boakye and Randles 1974). Subsequently, aphids only penetrate the epidermal cells and take up a negligible amount of sap. Failure to imbibe sap from lettuce would increase starvation in migrating aphids that alight in a lettuce crop, thus increasing probing and migratory behaviour. Such performance could account for the effective spread of LNYV in lettuce crops. LNYV outbreaks seem to be mainly the result of long-distance migratory alates (Randles 1983).

CLASSIFICATION AND TAXONOMY

Lettuce necrotic yellows virus (ICTV decimal code 62.0.4.0.005) is the type species of the genus *Cytorhabdovirus*, family *Rhabdoviridae*, order *Mononegavirales*, whose members are enveloped viruses with linear, nonsegmented, negative-sense, single-stranded RNA genomes (Tordo *et al*. 2005). Plant rhabdoviruses have been classified into two genera which are distinguished by their site of virion maturation: Cytorhabdoviruses like LNYV mature in the cytoplasm, while nucleorhabdoviruses mature in the nucleus of infected cells (Jackson *et al*. 2005). Field

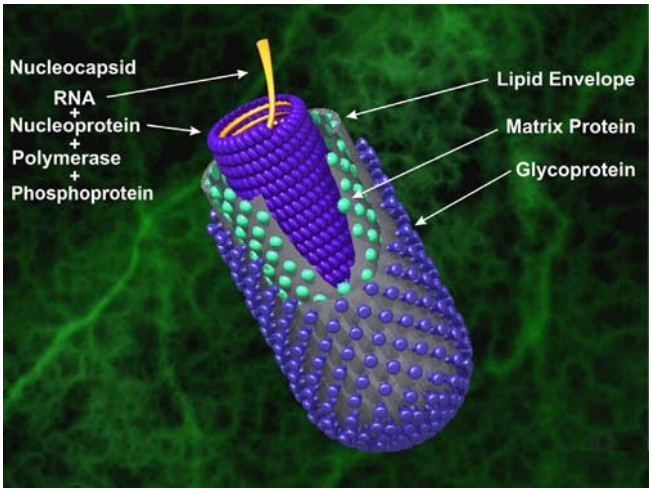

Fig. 2 Cartoon of an LNYV particle. Design by Paul Campbell.

isolates of LNYV can be distinguished by the severity of symptoms on the indicator host *N. glutinosa* (**Fig. 1C, 1D**), but no serological differences have been reported (Francki *et al*. 1989). The severe isolates SE3 and 318 have been used for most studies on the biological and molecular properties, respectively. Recently, two LNYV subgroups have been identified among isolates collected around Australia from 1985 to 2000 from lettuce, garlic, Bristly Oxtongue and sowthistle, based on nucleotide sequence identity of the nucleoprotein gene, but additional biological analysis is needed to determine if these subgroups represent distinct biological strains (Callaghan and Dietzgen 2005).

VIRION MORPHOLOGY AND COMPOSITION

LNYV particles are bacilliform and measure about 227 nm × 68 nm in negative stained preparations or 360 nm × 52 nm in tissue sections. They consist of a lipid envelope with glycoprotein (G) projections arranged in a hexagonal lattice enclosing a nucleocapsid core coiled in a basic helix with a pitch of 4.5 nm (Francki *et al*. 1989; **Fig. 2**). The G protein (~78 kD) is glycosylated with a complex network of oligosaccharides containing N-acetylchitobiose, N-linked to asparagine residues. The carbohydrate binds to concanavalin A and can be removed by endoglycosidase F (Dietzgen and Francki 1988). The nucleocapsid is composed of a single-stranded, negative-sense RNA of ~12.8 kb complexed with the viral nucleoprotein (N, ~57 kD), phosphoprotein (P, ~38 kD) and polymerase (L, ~170 kD). The matrix protein (M, ~19 kD) links the G protein spikes to the nucleocapsid (**Fig. 2**). The location and function(s) of a sixth viral protein, termed '4b' have not yet been determined, but it is likely involved in cell-to-cell movement of nucleocapsids (Jackson *et al*. 2005). Virions have a sedimentation coefficient of approximately 940s and a buoyant density in sucrose solution of ~1.20 g/cm^3. Purified virus preparations contain an RNA-dependent RNA polymerase activity. Dissociation of the viral envelope with a non-ionic detergent such as Nonidet P40 or Triton X-100 releases the infectious nucleocapsid, whereas an ionic detergent such as sodium dodecyl sulphate dissociates both the envelope and the nucleocapsid releasing non-infective RNA (Francki *et al*. 1989).

IDENTIFICATION, PURIFICATION AND DETECTION

Visual identification of LNYV-infected plants requires experience because similar symptoms may be caused by tomato spotted wilt virus. *N. glutinosa* is a good biological indicator host, since severe isolates induce local necrotic lesions on mechanically inoculated leaves within 6-8 days, followed by systemic yellowing, leaf curling and stunting (Francki *et al*. 1989; **Fig. 1D**).

LNYV particles are sensitive to detergents and organic solvents such as butanol and chloroform (Francki *et al*.

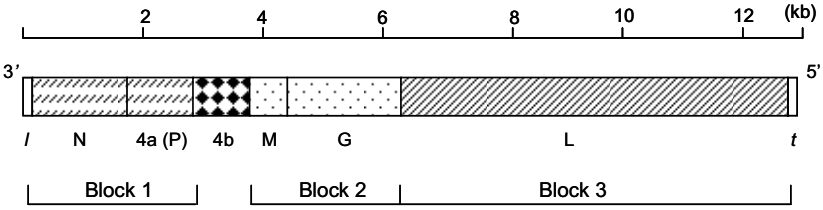

Fig. 3 Organisation of the LNYV genome. The names, location and relative sizes of LNYV genes are shown and gene blocks are indicated.

Table 1 Sizes of genes (open reading frame and flanking untranslated regions) encoded on the LNYV genome (3′ to 5′).

Gene Regions	N	4a (P)	4b	M	G	L
gene (nt) [1]	1530	1085	1046	631	1836	6332
5′ untranslated (nt)	78	81	38	55	33	78
ORF[2] (amino acids)	459	300	302	177	551	2067
3′ untranslated (nt)	78	101	102	45	150	53
GenBank accession No.	L30103	AF209035	AF209034	AF209033	AJ251533	AJ746199

[1] nt, nucleotides
[2] ORF, open reading frame

1989), widely used for the purification of non-enveloped plant viruses. Both *N. glutinosa* and naturally infected lettuce are good sources of virus for purification. A purification method involving celite filtration and calcium phosphate gel chromatography was first described by McLean and Francki (1967). This method was further improved and simplified, and a sucrose gradient ultracentrifugation step added if further purification was required (Francki *et al*. 1989).

Using purified virion preparations as antigen, specific, high-titered antisera were prepared in rabbits. Purified LNYV disrupted with detergent produces several precipitin lines in gel diffusion assays (McLean *et al*. 1971) and the antibodies react with the major structural proteins N, G and M in immunoblots (Dietzgen and Francki 1988). A double antibody enzyme-linked imunosorbent assay (DAS-ELISA) for detection of LNYV in infected leaf tissue and individual vector insects was significantly more sensitive (Chu and Francki 1982). Monoclonal antibodies to the N and G proteins have been used in immunoblots and indirect ELISA to study the fine structure of LNYV particles (Dietzgen and Francki 1988). LNYV can also be detected by reverse transcription polymerase chain reaction (RT-PCR) assay using N gene- specific primers (Thomson and Dietzgen 1995; Callaghan and Dietzgen 2005).

GENOME

Structure and organisation

The negative-sense, single-stranded RNA genome of LNYV consists of 12,807 nucleotides (AJ867584). It encodes six genes in the order N- 4a (P)- 4b- M- G- L, flanked by untranslated, highly complementary 3′ leader (84 nt) and 5′ trailer (187 nt) sequences (Dietzgen *et al*. 2006; **Fig. 3**). All genes are separated by short intergenic regions that contain the highly conserved consensus sequence "GNU(C/U)(N)nACU", where (N)n stands for a variable number of nucleotides. Rhabdoviral genomes are organised in a conserved basic three gene block formation comprising the N and P genes (Block 1), the M and G genes (Block 2) and the L gene (Block 3). LNYV RNA encodes one additional gene (4b) located between blocks 1 and 2 (**Fig. 3**).

LNYV has a coding capacity of 90.4% which is average for rhabdoviruses. The sizes of LNYV RNA encoded genes, ORFs, 3′ and 5′ untranslated regions (UTR) and GenBank sequence accession numbers are listed in **Table 1**. The lengths of the UTRs vary from 33–81 nt for 5′ UTRs and 45–150 nt for 3′ UTRs. The lengths of the intergenic regions are 6 nt (3′ leader - N gene), 7 nt (N - P, P - 4b and 4b - M), 8 nt (M - G) and 31 nt (G - L). Analysis of the 5′ ends of LNYV mRNAs (except L gene mRNA) has been carried out using random amplification of cDNA ends (5′ RACE). This analysis suggests that LNYV mRNAs, like those of other rhabdoviruses are capped at their 5′ ends with $m^7G^{5'}ppp^{5'}$. In addition some clones of the N, M and G mRNAs had two additional nucleotides (GA) between the 5′ cap and the proposed transcription start sequence (Wetzel *et al*. 1994; Callaghan 2005). The additional dinucleotide corresponds to the sequence of the preceding intergenic region and may have arisen by polymerase slippage during transcription (Dietzgen *et al*. 2006).

Replication and transcription

Cytorhabdoviruses replicate in the cytoplasm of infected cells in association with masses of thread-like structures or viroplasms. Virus morphogenesis occurs in association with vesicles of the endoplasmic reticulum (Tordo *et al*. 2005). The genome serves as template for both replication and transcription. Replication occurs through an anti-genomic, positive-sense full length intermediate (Dietzgen 1995) from which negative-sense copies are made. Infectious nucleocapsids composed of RNA, N, P and L proteins possess readily detectable RNA-dependent RNA polymerase activity. *In vitro* transcription yields ssRNA products of 1.9, 1.4 and 0.85 kb that appear to correspond to the G, N and P mRNAs, respectively (Dietzgen 1995). Six polyadenylated RNA species complementary to the viral genomic RNA have been detected in infected plants by northern blot analysis (Dietzgen *et al*. 1989; Wetzel *et al*. 1994a). From their size range and complexity they are assumed to represent mRNAs encoding the viral proteins; complementary RNA 3 has been identified as N protein mRNA by reactivity of its product with a specific monoclonal antibody (Dietzgen *et al*. 1989).

SEQUENCE ANALYSIS AND PROPERTIES OF ENCODED PROTEINS

Besides *in silico* analyses of LNYV-encoded proteins, their biological functions have not yet been determined experimentally. However, similarities to the proteins of the better studied animal rhabdoviruses suggest they have similar functions and properties. Analysis of the major structural proteins of LNYV using discontinuous polyacrylamide gel electrophoresis (PAGE) identified five major proteins which correspond to the L (M_r ~190,000), G (M_r ~78,000), N (M_r ~57,000), P (M_r ~38,000) and M (M_r ~19,000) proteins (Dietzgen and Francki 1988). An additional gene, named "4b", was identified when multigenic clones were used to probe blots of LNYV mRNAs isolated from infected plants. This 4b gene contains an ORF that encodes a 33.7 kDa protein (Dietzgen *et al*. 2006).

Sequence analysis and location in the genome suggest that the 4b protein may be involved in cell-to-cell movement of LNYV nucleocapsids. The 4b gene is located between gene blocks 1 and 2 (**Fig. 3**), where the proposed movement protein genes of other plant rhabdoviruses are located (Jackson *et al*. 2005). Refined secondary structure

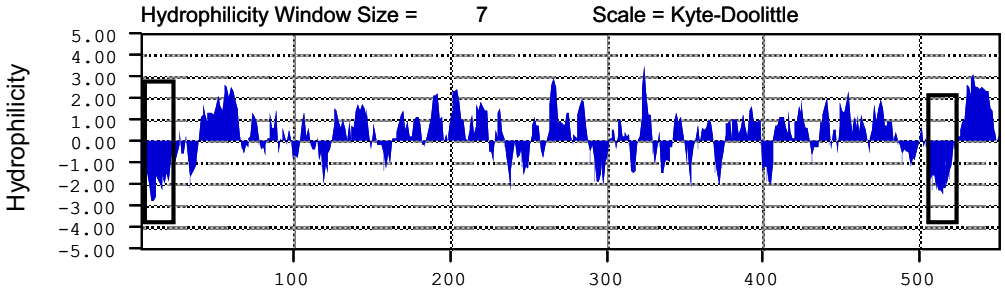

Fig. 4 Hydrophilicity plot of the LNYV glycoprotein. Hydrophobic regions at the amino and carboxy termini are framed.

predictions suggest that the 4b protein belongs to the 30 kDa superfamily of plant virus movement proteins (Melcher 2000). Furthermore, BlastP database searches have revealed that the predicted sequence of the 4b protein has significant similarities to the movement proteins of several trichoviruses and capilloviruses, family *Flexiviridae* (Dietzgen *et al*. 2006).

The matrix (M) protein of LNYV has an isoelectric point (pI) of 8.17 and is thought to be stabilised by disulfide bonds based on its altered electrophoretic mobility in polyacrylamide gels after treatment of virus preparations with 2-mercaptoethanol. The M protein of animal rhabdoviruses has been associated with virus budding from membranes, and is thought to provide a link between the transmembrane glycoprotein and the nucleocapsid. There is also evidence suggesting its involvement in the switch between transcription and replication of rhabdovirus genomes and the induction of cytopathic effects in infected cells.

Rhabdoviral phosphoproteins (P) are thought to play a pivotal role in the N protein-RNA complex interactions with the L protein (along with cellular factors), and to be involved in N protein multimerisation and P protein oligomerisation. The P protein is thought to be a non-catalytic co-factor and regulatory protein and is thought to exist in different phosphorylation states. Unlike nucleorhabdoviral P proteins which contain carboxy-terminal, bipartite nuclear localisation signals, LNYV lacks such signals. The LNYV P gene contains an additional overlapping ORF in a +1 reading frame that could potentially encode an 11.8 kDa protein (Dietzgen *et al*. 2006).

The nucleoprotein (N) has important structural and functional roles in encapsidation of genomic RNA, replication and transcription. However, there is little direct sequence homology of the N protein of LNYV with that of other rhabdoviruses (Wetzel *et al*. 1994b). Hydropathy plots of the N protein identified a region of hydrophilicity preceded by a region of short alternating hydrophobic-hydrophilic amino acid stretches, similar to features in animal rhabdovirus N proteins. Twenty-one potential phosphorylation sites within the N protein have also been identified by sequence analysis. The type of phosphorylation site was conserved across all LNYV isolates, suggesting that at least some of them may be functionally significant.

The G protein forms glycoprotein spikes which protrude from the rhabdovirus envelope. The mature LNYV G protein sequence has three potential glycosylation sites (Asn-X-Ser/Thr), two are centrally located and one is near the amino terminus (Callaghan 2005). In LNYV particles, purified from *N. glutinosa*, the G protein carries a network of *N*-acetylchitobiose *N*-linked to asparagine residues, but there is some indication that the type of glycosylation may be host-specific (Dietzgen and Francki 1988). Like other rhabdoviruses, the LNYV G protein contains an amino-terminal hydrophobic region (**Fig. 4**) that constitutes a signal peptide to target the polypeptide to the endoplasmic reticulum for cleavage and post-translational modification. The signal peptide consists of 25 amino acids and has the peptidase recognition sequence "VQG ↓V", where the arrow indicates the predicted cleavage site (Dietzgen *et al*. 2006). The carboxy terminus of the G protein has a predicted transmembrane domain (**Fig. 4**). Some of the 18 predicted cysteine residues from four plant rhabdoviruses could potentially be involved in the formation of disulfide bridges to stabilise their G proteins. Analysis of G protein sequences identified 11 conserved cysteine residues primarily located near the centre of the protein. Three other cysteine residues, located near the amino terminus, were conserved in the sequences of the two cytorhabdoviruses LNYV and NCMV (Callaghan 2005). Heptad repeats identified in G proteins of animal rhabdoviruses have been linked to the fusion between the virus particle and host cell membranes. These repeats take two forms, a-d and d-a, depending on the orientation of the amino acid residues within the repeats. Analysis of the LNYV G protein revealed 3 a-d heptad repeats (two of which are centrally located within the protein sequence and overlap) and one centrally located d-a repeat (Callaghan 2005). All of these repeats appeared three times concurrently. A number of both types of repeat were also found within the carboxy terminus, in the predicted transmembrane domain, though their location makes it unlikely that that they would be involved in membrane fusion activities. The LNYV G protein sequence is most similar to that of NCMV, but with only 23.2% sequence identity (Dietzgen *et al*. 2006).

The rhabdovirus L protein is an RNA-dependent RNA polymerase that is involved in viral RNA replication and transcription. The L gene of LNYV is most similar to that of the other sequenced plant rhabdoviruses with nucleotide sequence similarities of around 40% (Dietzgen *et al*. 2006). *In silico* analysis of the LNYV L protein suggests it may share a number of key activity domains with polymerases of several other rhabdoviruses, including a polymerase module, a loop structure for interaction with the triphosphate moiety of the RNA and an ATP-binding motif. The functionally important polymerase module has been used for phylogenetic analysis (see below) and contains the polymerase catalytic centre with the conserved "GDN" motif.

GENETIC DIVERSITY AND EVOLUTION

LNYV is restricted to Australia and New Zealand. Analyses of the complete N gene sequences of ten isolates collected from five States of Australia between 1985 and 2001 provide some insights into the diversity of LNYV (Callaghan and Dietzgen 2005; unpublished data). Phylogenetic analysis revealed two distinct subgroups of isolates. Predicted stop codon usage, protein phosphorylation sites and sequence comparisons of both 3′ and 5′ UTR support these groupings. Nucleotide sequence identity of the N gene ORF within the subgroups exceeded 96%, while sequence variation between the subgroups was less than 4% at the amino acid level, but approximately 20% at the nucleotide level (Callaghan and Dietzgen 2005). This highlights the high level of synonymous versus non-synonymous nucleotide substitutions in the third codon position of the N gene ORF of these LNYV isolates. LNYV subgroups appear to have coexisted in space and time across Australia (**Fig. 5**). The last subgroup I isolate was collected almost 15 years ago and it remains to be determined if such isolates still persist today. It is also unknown if these subgroups correspond to biologically distinct strains.

Phylogenetic analyses of plant- and animal-infecting rhabdoviruses for taxanomic purposes have largely concentrated on the conserved polymerase module of the L protein

Fig. 5 Diagrammatic map of Australia showing collection sites of LNYV isolates. Isolates are identified by their number and year of collection. Subgroup I isolates are shown on grey and subgroup II on black background. Australian States and Territories shown are WA (Western Australia), NT (Northern Territory), SA (South Australia), QLD (Queensland), NSW (New South Wales), VIC (Victoria) and TAS (Tasmania).

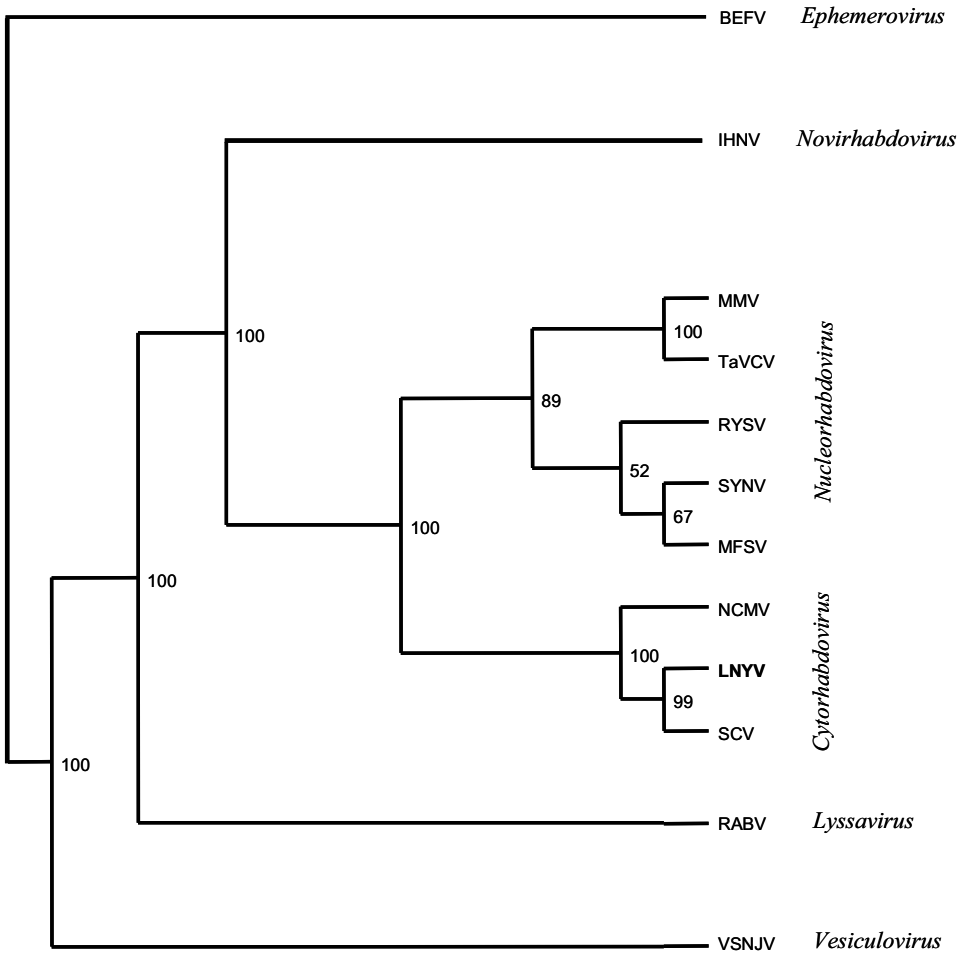

Fig. 6 Maximum parsimony phylogenetic tree based on alignment of rhabdovirus L protein polymerase module sequences. Bootstrap values of 100 replicas are show at branch nodes and genera are indicated in italics on the right. The viruses and GenBank accession numbers used in this analysis are bovine ephemeral fever virus (BEFV; AF234533), infectious hemopoietic necrosis virus (IHNV; X89213), maize mosaic virus (MMV; AY618418), taro vein chlorosis virus (TaVCV; AY674964), rice yellow stunt virus (RYSV; AB011257), sonchus yellow net virus (SYNV; L32603), maize fine streak virus (MFSV; AY618417), northern cereal mosaic virus (NCMV; AB030277), strawberry crinkle virus (SCV; AY005146), rabies virus (RABV; M31046) and vesicular stomatitis New Jersey virus (VSNJV; M29788).

(Tordo *et al*. 2005). Phylogenetic trees clearly separate the members of the genera *Nucleorhabdovirus* and *Cytorhabdovirus*. LNYV clustered with the other sequenced cytorhabdoviruses NCMV and SCV, with an apparent closer evolutionary link to SCV (Dietzgen *et al*. 2006; **Fig. 6**).

TRANSGENIC CONTROL STRATEGIES

LNYV is a major problem in most Australian lettuce growing areas during dry years with economic losses to growers of 15-20%. Control options include removal of nearby sowthistle plants and insect control, but due to the possible long distance migration of the aphid vector, this has not always been effective (Randles 1983). There are no known sources of LNYV resistance in the lettuce gene pool. We have therefore attempted to introduce transgenic resistance to well performing Australian lettuce cultivars using a genetic engineering approach. A direct shoot organogenesis regeneration system was optimised for four grower-preferred commercial crisphead (iceberg) lettuce cultivars, and an *Agrobacterium tumefaciens*-mediated transformation system developed using the green fluorescent protein reporter gene

(Campbell 2003). The transformation and regeneration system proved particularly effective, with large numbers of stable transformants produced in 8 to 12 weeks, leading to transgenic lettuce seeds in as little as 5 to 8 weeks more than in field- grown lettuce.

The LNYV N gene was isolated and cloned into a plant gene expression vector in two variations, translatable and untranslatable. This approach allowed induction of virus resistance by one of two mechanisms: post-transcriptional gene silencing (Dietzgen and Mitter 2006) and protein-mediated resistance (Beachy *et al*. 1990). In total, 57 independent transgenic lines were regenerated, with both single and multiple copies of the genes integrated into the lettuce genome. Expression levels of the N protein varied between lines and in some lines the transgene was silenced (Campbell 2003).

Due to the low success rate (2%) of LNYV transmission by mechanical inoculation, resistance screening of transgenic lettuce plants was attempted using viruliferous *H. lactucae*. Since the strain of LNYV that the N gene was isolated from appeared not to be transmissible by aphids to lettuce, naturally LNYV-infected *H. lactucae* were sourced and used in both supervised feeding and cage trials. Unfortunately, no resistant lines could be clearly identified, but six lines were infected by the virus, and were classed as susceptible. However, the nucleotide sequence of the LNYV nucleoprotein gene of the challenge isolate (subgroup II) was found to be 18.7% different from the gene used in the resistance construct (subgroup I), so plants may not be protected against this particular strain. The presence of LNYV-specific small interfering (si) RNAs in infected *N. glutinosa* was confirmed, which demonstrates that LNYV RNA can act as an inducer and target of RNA-silencing (Campbell 2003). Therefore, the presence of such siRNAs in transgenic plants could provide molecular evidence that the plants will be resistant to LNYV. SiRNAs and other molecular indicators will now be used to identify likely LNYV-resistant lettuce lines for further biological testing.

The transgenic lettuce plants were also exposed to natural virus infections by aphids in two field trials in 1999/2000 and 2001/2002, however infection rates were only 2-10% - too low to determine with any degree of certainty which plants were resistant. The transformed plants also germinated well from seed, and in most cases the transgene in the progeny plants segregated predictably, though there were cases where none of the second generation progeny plants contained the transgene, suggesting that there may have been a negative segregation bias.

FUTURE DIRECTIONS

Studies of field isolates are continuing to delineate the diversity and evolution of LNYV in Australia and New Zealand. For reliable virus detection and control, it will be important to know if subgroup I isolates still exist today and if there is a correlation between subgroups and biological strains that are characterised by a unique host range and/or symptoms. The evaluation of transgenic control strategies will likely continue, initially by detailed molecular analysis of existing, potentially RNA-silenced lettuce lines. Novel strategies such as the deployment of artificial micro RNAs (Simón-Mateo and Garcia 2006) can be easily tested due to the availability of an optimised lettuce transformation and regeneration system.

The cloned sequences of the LNYV genome and its mRNAs will provide useful tools to verify the predicted functions and identify the intracellular locations of viral proteins expressed transiently or in transgenic plants as fusions with autofluorescent proteins (Goodin *et al*. 2002). Initially, the location and proposed function of the 4b protein in cell-to-cell movement will be of greatest interest. Laser scanning confocal microscopy of tagged viral proteins and microarray analysis of differentially expressed host genes will provide information on the interactions between viral proteins and between virus and host proteins to further elucidate the replication process and the involvement of host factors. Similarly, the molecular interactions between LNYV and its aphid vector are an area worth exploring to identify potential new targets to suppress virus propagation in the insect and to prevent transmission to insect progeny and plant hosts.

For future reverse genetics studies using "infectious clones", is important to realize that nucleocapsids are the minimal infectious units of rhabdoviruses, and that their formation is essential for each stage of the infection cycle. Therefore, application of genetics to study rhabdoviruses requires engineering recombinant DNAs that can express N, P and L proteins and genomic RNA that will lead to formation of infectious nucleocapsids. An experimental system that can generate infectious nucleocapsids can be expected to lead to a revolution in our understanding of the biology and pathology of LNYV and other economically important rhabdoviruses. Due to the stability of rhabdovirus genomes, it will also provide a basis for engineering gene vectors suitable for plant-based production of recombinant proteins for use in biotechnology and biomedicine.

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