Distribution and multiplication of iris severe mosaic potyvirus in bulbous *Iris* in relation to metabolic activity

Implications for ISMV detection

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op dinsdag 8 november 1994
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen.

Publication of this thesis was
financially supported by
'Produktschap voor Siergewassen'.

The research described in this thesis was supported by the Bulb Research Centre Lisse, the Netherlands, which is financed by the Ministery of Agriculture, Nature Management and Fisheries, and the Dutch Flower bulb Industry.

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Vlugt, Cornelia Ida Maria van der

Distribution and multiplication of iris severe mosaic potyvirus in bulbous *Iris* in relation to metabolic activity: implications for ISMV detection/ Cornelia Ida Maria van der Vlugt. - [S.l. : s.n.] - Ill.
Thesis Wageningen. - With ref. - With summary in Dutch.
ISBN 90-5485-322-0
Subject headings: flower bulbs / potyvirus ; iris bulbs ; detection methods.

Stellingen

1. De slechte aantoonbaarheid van het ISMV in vers gerooide secundair geïnfecteerde irisbollen berust op een lage virusconcentratie en niet op een serologische verandering van het virus.

   Dit proefschrift.

2. De veronderstelling dat virus, eenmaal in het meristeem aangekomen, alle cellen vervolgens infecteert, is in tegenspraak met de ervaring dat juist meristeemweefsel geschikt uitgangsmateriaal is voor het verkrijgen van virusvrije planten.

   Baruch, E.R. and Quak, F. (1966). Virus-free plants of iris 'Wedgwood' obtained by meristem culture. *Netherlands Journal of Plant Pathology* 72: 270-273;
   Maule, A.J. (1991). Virus movement in infected plants. *Plant Sciences* 9: 457-473.

3. De beperkte resistentie tegen het tomatebronsvlekkenvirus in tabak, veroorzaakt door transgene expressie van het virale N-eiwit, staat in geen verhouding tot de immuniteit bereikt met het RNA van dit transgen.

   De Haan, P., Gielen, J.J.L., Prins, M., Wijkamp, I.G., Van Schepen, A., Peters, D., Van Grinsven, M.Q.J.M. and Goldbach, R. (1992). Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. *Bio/Technology* 10: 1133-1137;
   Pang, S.-Z., Bock, J.H., Gonsalves, C., Slightom, J.L. and Gonsalves, D. (1994). Resistance of transgenic *Nicotiana benthamiana* plants to tomato spotted wilt and impatiens necrotic spot tospoviruses: evidence of involvement of the N protein and N gene RNA in resistance. *Phytopathology* 84: 243-249.

4. De veronderstelling van Van Ree *et al.*, dat de verontreiniging van het synthetische peptide *Lol p* I (216-240) veroorzaakt wordt door onvolledige opheffing van de zijketenbescherming bij de afsplitsing van de vaste drager, is dubieus.

   Van Ree, R., Van Leeuwen, W.A., Van den Berg, M., Weller, H.H. and Aalberse, R.C. (1994). IgE and IgG cross-reactivity among *Lol p* I and *Lol p* II/III. Identification of the C-terminal of *Lol p* I, II, and III as cross-reactive structures. *Allergy* 49: 254-261.

5. Het door Lucas, Ding en Van der Schoot gepostuleerde model voor mRNA-transport door plasmodesmata voorziet in een verklaring voor een proces, waarvan niet is aangetoond dat het bestaat.

   Lucas, W.J., Ding, B. and Van der Schoot, C. (1993) Tansley Review No. 58. Plasmodesmata and the supracellular nature of plants. *New Phytologist* 125: 435-476.

6. De presentatie van de werking van cosmeticaproducten in reclameboodschappen is vaak niet alleen quasi-wetenschappelijk maar ook nog misleidend.

7. De glimmende en van foto's voorziene buitenkanten van de hedendaagse proefschriften doen ten onrechte vermoeden dat de bijbehorende promovendi meer te besteden hebben dan hun voorgangers.

8. De overlast door geluid van vliegtuigen rond Schiphol is veel groter dan op grond van de computermodellen in het integraal milieueffect rapport (IMER) Schiphol en omgeving wordt verondersteld.

   IMER Schiphol en omgeving, 1993; Hoorzitting Planologische kernbeslissing-Schiphol, Sassenheim 1994; Milieudefensie Nieuwsbrief 3-1994; Volkskrant 25 augustus 1994.

9. Uit het feit dat het verboden is om benzeen te gebruiken, in bijvoorbeeld laboratoria, en dat het vrijkomen van deze kankerverwekkende verbinding bij het benzine tanken wordt gedoogd, blijkt dat de wetgever te weinig echte bezorgdheid heeft voor gezondheid en milieu.

   Artikel 160a van het Veiligheidsbesluit fabrieken of werkplaatsen. (1977). *Staatsblad* 52; Benzine en diesel kunnen veel schoner.(1993). *Consumentengids* 7: 434-437.

10. Ballast zorgt voor diepgang.

11. Ter bevordering van de emancipatie van β-wetenschappers dienen er meer part-time onderzoeksbanen te komen.

Stellingen behorend bij het proefschrift:

Distribution and multiplication of iris severe mosaic potyvirus
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Implications for ISMV detection.

Wageningen, 8 november 1994 C.I.M. van der Vlugt

Voorwoord

Na jarenlange arbeid ligt hier dan eindelijk mijn proefschrift. Mijn promotie-onderzoek en het verwerken van de enorme berg gegevens heeft lang geduurd en heeft zich ook op vele plaatsen afgespeeld. Ik heb daarbij van vele kanten steun ondervonden en het is onmogelijk om iedereen persoonlijk te bedanken.

Met name wil ik hier mijn begeleiders noemen, te weten, Rob Goldbach, Piet Boonekamp, Toon Derks en Jeanne Dijkstra. Rob, door je drukke werkzaamheden was het soms moeilijk om je voor een bespreking van mijn project aan tafel te krijgen, maar was je er eenmaal, ook in Lisse, dan had mijn onderzoek de hoogste prioriteit. Ook ben ik je veel dank verschuldigd voor het snel retourneren van de manuscripten die ik jou ter beoordeling toezond. Piet, op je kamer in gebouw 10 kon ik altijd binnenvallen met alle resultaten en problemen die er waren. Als co-promotor heb je veel tijd gestoken in dit boek zodat veel teksten steeds beter gestroomlijnd werden. Toon, bedankt voor je adviezen voor allerlei praktische problemen maar ook voor het uitdragen van de manier waarop je met hart en ziel een groene-planteviroloog bent. Jeanne, jou wil ik bedanken voor je begeleiding bij vooral de start van dit project en hulp bij de microscopische analyses. Net als Toon heb je mijn manuscripten altijd zorgvuldig doorgelezen en zeer welkome suggesties gedaan. Dick Lohuis, bedankt voor de vele coupes die je van mijn bollen hebt gemaakt en gefotografeerd. Zonder dit werk en dat van de HBO stagiair Arnoud Marquart zou Hoofdstuk 4 nooit zo illustratief geworden zijn.

Verder wil ik alle medewerkers van het LBO bedanken, speciaal de afdeling Plantkwaliteit en de bewoners van gebouw 9. Tevens de MBO-stagiaires Karin Lameris en Daan Haket, die zich met name met de 40°C, warm water en ethyleen behandelde bollen hebben beziggehouden (Hoofdstuk 3). I also want to thank graduate student Delia Muñoz for her work on part of Chapter 8. Henk Gude en Simon Langeveld, bedankt voor jullie bijdragen in respectievelijk het bepalen van de ademhalingskarakteristieken (Hoofdstuk 6) en het bepalen van de taxonomische status van het ISMV (Hoofdstuk 5). Alle medewerkers van de vakgroep Virologie van de LUW en ook zij die er de afgelopen jaren gewerkt hebben, bij jullie was ik hartelijk welkom. Hoewel ik vaak slechts gedurende korte tijd aanwezig was, voelde ik me altijd direct thuis op mijn 'eigen' vakgroep. René van der Vlugt, bedankt voor de nuttige instructies om en nabij

de labtafel, het aanzetten of juist uithalen van diverse experimenten en de discussies over de potyvirussen.

Diana Prins, fijn dat je telkens weer je logeerbed ter beschikking stelde zodat ik niet na elke lange dag op 'viro' terug naar Lisse hoefde.

Ook wil ik hier de (ex-)leden van de Plantevirus-werkgroep (RUL) o.l.v. John Bol en in het bijzonder Huub Linthorst bedanken, voor de gastvrijheid en de assistentie bij het cDNA-werk toen ik aan het begin van mijn promotie-onderzoek stond. Dank zij de welwillendheid van de Bloembollenkeurings-dienst, met name Ton van Schadewijk, was het mogelijk dat ik al met mijn promotie-onderzoek kon beginnen terwijl ik nog niet officieel in dienst was van de LUW. Zo heb ik 'een seizoen extra' kunnen meenemen. Door de voort-durende steun van mijn omgeving en vooral van Arie heb ik dit proefschrift kunnen afronden. Het feit dat hij in zijn baan een mogelijkheid had voor opvang van ons kind heeft uiteindelijk gezorgd dat ik mij enige dagen per week volledig kon wijden aan het schrijven van de artikelen die uiteindelijk hebben geleid tot de afronding van mijn promotie-onderzoek.

Contents

CHAPTER 1

General Introduction

The genus *Iris*

Irises have been cultivated and used for centuries, not only as ornamentals but also as a source of perfumes, medicine and as a model for adornments (Köhlein, 1987). The name of the genus is derived from the female messenger of the gods in Greek mythology, Iris, who brought peace to the human race, and reflects the diversity of the colours of the rainbow (Cassidy and Linnegar, 1982). Irises are found throughout the temperate and subtropical zones of the Northern Hemisphere and more than 200 species are known (Köhlein, 1987). The major part of the *Iris* genus belongs to the group of rhizomatous plants, with the bulbous types being fewer in number. The rhizomatous types are primarily used in the garden. The bulbous species are produced commercially for use as cut flowers both in the greenhouse and outdoors. Some dwarf species are used for gardens and for forcing as potted plants (De Munk and Schipper, 1993). The delight in growing irises led to the foundation of e.g. American and British Iris Societies just after the First World War who were dedicated to the spreading of knowledge and interest in this 'loveliest of flowers' (Cassidy and Linnegar, 1982).

The most important group of iris for bulb-industry is the bulbous-iris containing group *Xiphium*. *Xiphium* irises originate from the western Mediterranean, i.e., Spain, Portugal, southern France, Morocco, Algeria, and Tunisia. Hybrids from crosses between species and varieties of this *Xiphium*-group are presently known as Dutch irises: *Iris x hollandica* Tub. They are characterised by a tall habitus and an early flowering period. A large assortment has been bred in the last century (Van Scheepen, 1991).

Dutch irises are grown in The Netherlands (772 hectares), France (103 hectares), United Kingdom (40 hectares), Israel (50 hectares), the United States of America (182 hectares) and Japan (118 hectares), (De Munk and Schipper, 1993).

Development and anatomy of the iris bulb

For commercial bulb production, Dutch irises are not propagated by seed. Propagation totally depends on annual asexual (clonal) daughter-bulb replace-

ment in the field. Since a bulb possesses a limited number of axillary buds, two to four new bulbs can be formed annually. Thus, it takes many years for breeders to produce a stock of sufficient quantity that can be tested for bulb productivity, disease resistance, and forcing abilities (De Munk and Schipper, 1993).

Blaauw (1935) has provided a detailed description of the growth and developmental cycle and De Munk and Schipper (1993) have recently written a well documented review on iris physiology. When iris bulbs are planted in autumn, the bulbs root immediately and, depending on the prevailing winter conditions, leaf emergence can be observed either in the fall or early spring. Flowering, if it occurs, is in the spring.

The new (daughter) bulbs are harvested in August/September after senescence of the leaves. Depending on the bulb size, or more precisely on its weight and temperatures used, a Dutch iris bulb may or may not flower. The periodicity of iris differs from other bulbous plants. In iris and lilies flowers are initiated after replanting while in tulips, daffodils and hyacinths flower development takes place shortly before or after lifting in early summer (Rees, 1992).

In a non-flowering iris plant, the apical bud of the mother bulb produces leaves, generally a maximum of three or four, and the bulb scale primordia. The leaf bases surround a new bulb. The iris bulb then contains, from the outside to the inside, 2 to 4 brownish membraneous layers, 3 fleshy white scales, and a central growing point. The scales and sprout are implanted on a basal plate, and thus connected to each other and the root primordia. When such a bulb is cut horizontally, it exhibits a round shape and therefore is called a 'Round Bulb'. In addition to this main bulb, a number of smaller bulbs can also be produced by axillary meristems of the mother bulb.

In a flowering plant, the apical bud of the mother bulb produces the leaves, at least five, the flower and a stem. Different stages of flower development in the central growing point are described by Cremer *et al.*, (1974). All the daughter bulbs are formed from axillary buds between the bulb scales and show a shape characterised by a flat side (bulbs formed against the flower stem). In addition, these bulbs, called 'Flat Bulbs', possess only one thin papery skin (tunic) and the scale edges do not overlap. Such features make this type of bulb highly susceptible to mechanical damage and fungal attacks. Although these bulbs will flower, their quality is commercially unacceptable. Thus as a general rule, only large sized 'Round Bulbs' are used for flower production.

By applying specific temperature treatments (combination of 9, 17 and 30°C Schipper *et al.*, 1988) to the bulbs before planting in the greenhouse, flowering is accelerated (forcing) or retarded, bulbs can be stored at 30°C for a longer period of

time, and flowers can be obtained all year round (Durieux and De Pagter, 1967).

Development of primordia of the central growing point into either primordial bulb scales, leaves or floral organs can be influenced by temperature treatment of the bulb during storage (Kamerbeek, 1962). When flower formation is not induced, a number of the primordia will turn into scale primordia (Kamerbeek, 1962).

To obtain flowering plants in a specific period, bulbs of the right cultivars should have a minimum size and temperature treatment should be applied strictly. Bulbs of critical size will produce a flower after completing the process of maturation and transition to a reproductive phase. The formation of more than three leaf primordia at relatively high temperatures is followed by flower induction at low temperatures (De Munk and Schipper, 1993). Bulbs grown under climatic conditions with moderate to low temperatures require a high temperature treatment after lifting to fulfill the heat requirement and to complete the maturation process (De Munk and Schipper, 1993; Durieux and Kamerbeek, 1974). Part of the heat requirement can be replaced by exposure to ethylene (Schipper, 1982; De Munk and Duineveld, 1986). Flower formation can be observed just before planting (De Munk and Schipper, 1993). When undersized bulbs are used, no flower will appear. Bulbs will show a typical three-leaved plant .

Although ethylene induces flower formation and stimulates the respiration of iris bulbs (Kamerbeek and Verlind, 1972), Marissen (1989) found that flower induction and alternative respiration are both influenced by ethylene but are not mutually dependent.

Diseases of iris

Bulbous iris can be affected by fungi of which most important are: *Fusarium oxysporum* f. sp. *gladioli*, (bulb rot), *Penicillium* spp. (bulb rot), *Pythium* spp. (bulb rot), *Rhizoctonia* spp. (bulb rot and leaf yellowing), by bacteria such as *Erwinia carotovora* pv. *carotovora*, causing soft rot, by nematodes such as *Ditylenchus destructor* (severe stunting of the plants) or *Pratylenchus penetrans*, and several viruses (Bergman, 1978; Schipper *et al.*, 1988). While fungal and bacterial attacks can be prevented or suppressed by climatic conditions or fungicides, the consequence of vegetative propagation of bulbs is virus transmission to progeny bulbs. This can be prevented by removal of virus infected plants/bulbs from the propagating stocks (rogueing) or by selecting the best ones.

Four potyviruses have been detected in bulbous iris, namely iris mild mosaic virus (IMMV), iris severe mosaic virus (ISMV), bean yellow mosaic virus (BYMV), and turnip mosaic virus (TuMV), (Asjes, 1979; Barnett, 1972; Brunt and Phillips, 1980; Derks *et al.*, 1980, 1985; Hammond *et al.*, 1985; Inouye and Mitsuhata, 1978). All these viruses are transmitted by aphids in a non-persistent manner. Unlike ISMV, the other three are all readily sap-transmitted to dicotyledonous diagnostic hosts; moreover, all three are serologically distinct from iris severe mosaic virus.

Narcissus latent virus (NLV), a carlavirus (Brunt and Phillips, 1980; Derks *et al.*, 1985; Brunt, 1977) formerly called iris mild yellow mosaic virus (IMYMV, Asjes, 1979) also infects bulbous iris and is transmitted by aphids (Brunt, 1977) but has particles which differ from those of iris severe mosaic virus in length, in serological reaction and in its ability to infect dicotyledonous hosts. Bulbous iris can also be infected by tobacco ringspot nepovirus, tobacco mosaic tobamovirus, tobacco rattle tobravirus (Asjes, 1979), and tomato spotted wilt and impatiens necrotic spot tospoviruses (Anonymous, 1979; Derks, *in press*). These last five viruses, however, have isometric or rod-shaped particles, infect a wide range of plant species and all can be quickly identified by their serological or other properties and most of these are transmitted by nematodes or thrips (Asjes, 1979).

In the Netherlands the following viruses cause problems in commercial growing of iris.

1. **IMMV.** Almost all commercially available stocks are infected with IMMV (Asjes, 1979; Brunt *et al.*, 1988). Leaf symptoms appear as a faint mosaic, which becomes more prominent on the flowerbud sheaths. The mosaic pattern is regular (Asjes, 1979). Flower symptoms, when present, are limited to a few small dark flecks in the fall (Hammond *et al.*, 1985).
2. **NLV.** Several cultivars are co-infected to a large extent with NLV. Leaves of iris show yellow spots, especially at the margins and tips, already soon after emergence. The mild yellow mosaic pattern is irregular with fairly conspicuous shades of yellowish-green. The severity of the symptoms of mild yellow mosaic is intermediate between mild mosaic and severe mosaic (Hammond *et al.*, 1985; Asjes, 1979). Later in the season the yellow spots turn into necrotic ones.
3. **ISMV.** ISMV causes in combination with IMMV a mild or severe mosaic in the leaves, consisting of pale-green and yellowish-green stripes and wider bands in irregular patterns extending upward from below soil level. Flowers show dark spots or stripes. Plants may be stunted and distorted and can die prematurely. (Asjes, 1979).

The other viruses occur only occasionally in bulbous iris in the Netherlands (Asjes, 1979; Brunt *et al.*, 1988).

Properties of ISMV

ISMV causes conspicuous chlorotic stripes and/or mosaic patterns in the leaves, and breaking in the flowers of sensitive cultivars of bulbous iris, rhizomatous iris and *Crocus vernus* (Brunt *et al.*, 1988). This results in considerable damage to iris plants. A severe mosaic was first described to occur in bulbous and rhizomatous iris by Brierley and McWhorter (1936) and in crocus by Van Slogteren (1958). The virus is partially characterised by Alper *et al.*, (1984), Barnett (1972), Brunt and Phillips (1980), Derks *et al.*, (1985) and Derks and Hollinger (1986). It is transmissible in the non-persistent manner by the aphids *Macrosiphum euphorbiae* and *Myzus persicae* and, less easily, by mechanical inoculation (Brunt *et al.*, 1988). Three strains of the virus have been recognised by the severity of the symptoms they induce in *Belamcanda chinensis* (Brunt *et al.*, 1988).

ISMV is a member of the Potyviridae family, showing characteristic filamentous and flexuous particles measuring circa 750 x 12 nm, and inclusions such as pinwheels, bundles and laminated aggregates (subdivision III, Christie and Edwardson, 1977) and is serologically distinguishable from morphologically similar viruses such as IMMV, BYMV and TuMV. As it is transmitted by aphids, it belongs to the genus *Potyvirus* and not to the other genera *Bymovirus* or *Rymovirus*, composed of fungal-borne and mite-transmitted potyviruses, respectively (Barnett, 1991).

The Potyviridae

Members of the Potyviridae usually occur wherever their principal host plants are grown, but they are especially prevalent in the tropical and subtropical regions. Most potyviruses have restricted or even very restricted host ranges, but the different viruses occur in a wide range of monocotyledonous and dicotyledonous plants (Hollings and Brunt, 1981). The potyviruses are the largest group of plant viruses collectively causing the greatest agricultural losses caused by virus infection.

They induce mosaic or mottle symptoms in leaves, many also induce colour-breaking in flowers, of which tulip breaking virus is a bright example, mottled and/or distorted fruits and seeds. Some potyviruses cause significantly more

severe disease symptoms at low temperatures (10-15°C) than under warm conditions (over 25°C) and may indeed be almost symptomless at higher temperatures (Matthews, 1991). E.g. on ISMV and IMMV infected iris plants produced by very late forcing (September/November in the Netherlands), severe mosaic symptoms can be extremely pronounced (Asjes, 1979).

Most potyviruses are transmitted in the non-persistent manner by aphids and epidemic levels of field spread often occur. Aphids acquire a potyvirus during short acquisition access periods (few seconds to few minutes) and transmit virus in equally short inoculation access periods. Potyviruses are also sap-transmissible, and some of them are carried in a small proportion of the seeds of some host species. The viruses are assumed to multiply and assemble in the cytoplasm.

The potyvirus particle consists of up to 2000 subunits of a single coat protein species arranged as a helix enclosing the genome (pitch 3-4 nm). The potyvirus genome contains a single-stranded plus-sense RNA of about 10,000 nucleotides with a genome-linked viral-encoded protein (VPg) at the 5'-end (Siaw *et al.*, 1985; Riechmann *et al.*, 1989; Murphy *et al.*, 1990) and a poly(A) tract at the 3'-end (Hari *et al.*, 1979). The genomes of several potyviruses have been completely sequenced, e.g. tobacco etch virus (Allison *et al.*, 1986), potato virus Y (PVY, Robaglia *et al.*, 1989), plum pox virus (Maiss *et al.*, 1989; Laín *et al.*, 1989), pea seed-borne mosaic virus (Johansen *et al.*, 1991) and tobacco vein mottling virus (Domier *et al.*, 1986). This enabled comparisons with other plant and animal RNA viruses on the molecular level, and revealed that the Potyviridae can be placed in the group of the picorna-like plant viruses (Goldbach, 1987; Domier *et al.*, 1987).

Translation of the potyvirus genome involves the synthesis of a large primary translation product ('polyprotein', MW circa 345 kDa) translated from a single open reading frame (Allison *et al.*, 1986). This polyprotein is autocatalytically cleaved by virus-encoded proteinases (Dougherty and Carrington, 1988; Dougherty *et al.*, 1989). So far, three viral proteinases have been identified (Verchot *et al.*, 1991). Cleavage results in seven to nine mature gene products (Dougherty and Carrington, 1988; Riechmann *et al.*, 1992; Fig. 1.1).

The (putative) functions and specific features for the proteins originating from the potyviral polyprotein after cleavage are :

-Protein P1, functioning as a proteinase (Verchot *et al.*, 1991) and possibly involved in cell-to-cell spread (Domier *et al.*, 1987),

-The helper-component protein (HC-Pro), with two major functions, i.e. in aphid transmission (Thornbury *et al.*, 1985) and as proteinase (Carrington and Herndon, 1992).

-Protein P3, occurring in the cell in association with CI protein (Rodriguez-Cerezo *et al.*, 1993).
-The 6K1 and 6K2 proteins, which are possibly involved in replication (Riechmann *et al.*, 1992).
-The cylindrical inclusion protein (CI) in characteristic bodies, located in the cytoplasm and which can be sub-grouped according to the predominant type (Christie and Edwardson, 1977). CI's consist of one type of 66-75 kDa protein. They contain a nucleotide binding motif (Domier *et al.*, 1987; Laín *et al.*, 1989; Robaglia *et al.*, 1989), have helicase activity (Laín *et al.*, 1991), and are proposed to be involved in viral RNA replication. Inclusion bodies formed by CI can be associated with the plasmodesmata (Lawson and Hearon, 1971; Calder and Ingerfeld, 1990) or with the cell membrane (Baunoch *et al.*, 1988, Martìn *et al.*, 1992). This suggests that, in some unknown manner, they may be associated with the intercellular transport of virus particles or the replication of the virus (Baunoch *et al.*, 1988; Calder and Ingerfeld, 1990).
-The small nuclear inclusion protein (NIa) is found frequently in the nucleus but also in the cytoplasm (Baunoch *et al.*, 1988; Martín *et al.* , 1992). It is the major viral proteinase (Dougherty and Carrington, 1988; Laín *et al.*, 1989). Moreover, its N-terminal represents the genome-linked protein (VPg), (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990).
-The large nuclear inclusion protein (NIb) contains all sequence motifs characteristic of viral RNA-dependent RNA polymerase and thus represents the putative potyviral polymerase (Domier *et al.*, 1987; Robaglia *et al.*, 1989).
-The coat protein, finally, encapsidates the viral RNA. A three amino acid sequence DAG or alike motif is located in the N-terminus of this protein and necessary for insect transmission (Atreya *et al.*, 1990, 1991; Gal-On *et al.*, 1992). Furthermore, coat protein sequences are used in the taxonomy of potyviruses (Shukla and Ward, 1988; Ward and Shukla, 1991).

The 3' non-translated region of the potyviral genome shows a high degree of

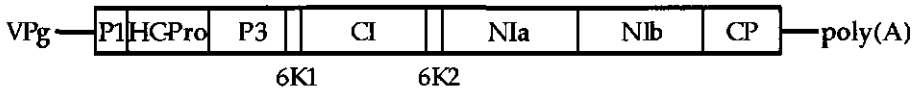

Fig. 1.1. Genomic organisation of the potyviral genome. Post-translational cleavage results in indicated viral polypeptides. See text for used abbreviations.

homology in strains of individual potyviruses and can be used as a means of differentiating species from strains (Frenkel *et al.*, 1992) but also within PVY to distinguish the various strains (Van der Vlugt *et al.*, 1993).

Virus control

As commercial iris stocks are still almost 100% infected with IMMV, inspection is focussed mainly on ISMV and NLV. Disease control in iris is carried out by close visual selection of ISMV- and NLV-infected plants in the field. Unfortunately, the early spring control usually takes place in cold weather. Furthermore, symptoms are easily overlooked in densely planted bulbs. In spring, too, incidental night frosts occasionally cause early death of the leaf tips, giving discolourations that can be confused with virus symptoms on leaves. Moreover, disease control can be hampered by the masking of NLV and ISMV when IMMV symptoms develop simultaneously (Asjes, 1979).

Nowadays it is possible to obtain virus-free iris stocks by meristem culture (Baruch and Quak, 1966; Hirata and Kunishige, 1982; Van der Linde *et al.*, 1988). Apical meristems of virus-infected plants may be free from virus in some instances. To obtain virus-free iris bulbs, the starting material should contain as little viruses as possible, preferably bulbs with iris mild mosaic virus only.

Meristems can be isolated and grown on an artificial medium into a plantlet (Baruch and Quak, 1966; Van der Linde *et al.*, 1988; De Munk and Schipper, 1993). Induction of bulbing in shoots can be achieved by e.g. interrupting the culture at 20°C with a chilling period of 4 weeks at 5°C (Van der Linde *et al.*, 1988), resulting in bulblet growth in about 50% of the shoots. Other methods are reviewed by De Munk and Schipper (1993). Prior to planting in soil, dormancy of the bulblets has to be broken by 6 to 10 weeks 30°C.

For commercial practice bulking-up is required. Bulb-scale explants of iris cultured in vitro produce adventitious shoots at the junction of the basal plate and the scales. When all scale segments of one bulb with a circumference of 9 cm can be used, it is possible in principle to obtain circa 50 bulblets from one iris bulb in 6 months (Van der Linde *et al.*, 1988).

Testing of the acquired virus-free irises showed that the bulb-yield can increase up to 50% (Rijnders and Schipper, 1990; Asjes, 1988). In forcing experiments, virus-free plants produced longer flower stems and higher quality cut flowers than the original commercial stocks (Hirata and Kunishige, 1982; Rijnders and Schipper, 1990).

Virus-free stocks were introduced to commercial growers several years ago and

initially propagated in gauze houses to provide protection from virus spread by aphids. With cultivation in the gauze houses indeed virus spread was under control. However, this was not the case in the field. Although strongly advised to grow virus-free (virus-tested) material in isolation, in practice this may be very difficult, as neighbouring growers may have virus-infected irises, narcissi, crocus, gladioli, ixia and crocosmia growing (Asjes, 1988). To maintain virus-free stocks, they must be sprayed with pesticides such as synthetic pyrethroids to kill aphids and mineral oil to limit virus spread by aphids (Asjes, 1985). In field trials, the speed of virus spread was unexpectedly high notwithstanding the weekly sprays with synthetic pyrethroids. However in an isolated field, stocks were unharmed when mineral-oil sprays were applied weekly (Asjes, 1988). Unfortunately, such mineral-oil sprays considerably reduce bulb yields, although other advantages may counteract these losses (as e.g. bulb yield of virus-free plants increases and better quality irises as cut flowers is achieved) (Asjes, 1988). Thus the ultimate aim of replacing all infected material with virus-free bulbs is still a remote prospect for irises.

Identification and routine detectability assays

Certification is required for an increasing number of bulbous crops especially now that virus-free bulbous crops have become available. Not only the fact that visual inspection of the plants poses problems, also primary as well as secondary infections are present in the bulb and are likely to be found in bulb testing. As the bulbs are the trade material, testing of these have the preference. Nowadays the bulbs can be indexed for the presence of specific viruses by using advanced laboratory assays, such as the enzyme-linked immuno sorbent assay (ELISA), which is sensitive, reliable and suitable for automation.

The laboratory assays, such as ELISA, necessary to detect viruses in the flower bulbs in the Netherlands are being developed mainly at the Bulb Research Centre in Lisse in cooperation with groups all over the world. For several viruses e.g. in tulip and lily, ELISA routine assays are carried out at the Dutch Flower Bulb Inspection Service and over 1,300,000 samples of bulbous crops are tested annually. Other research fields are being explored for development of new tests for identification as well as routine assays, e.g. nucleic acid hybridisation, polymerase chain reaction (PCR), and use of monoclonal antibodies (Mab) (Boonekamp *et al.*, 1990; Dekker *et al.*, 1993; Derks, 1985; Jordan and Hammond, 1991; Langeveld *et al.*, 1991; Van der Vlugt *et al.*, 1988).

Methods based on serology are usually focussed on the coat protein of the

virus.

The coat protein of potyviruses, encapsidating the viral RNA, contains a central core which is highly conserved among different potyviruses, in contrast to the N-terminus which is highly variable, both in length and in sequence and which contains the major virus-specific epitopes. Both the N- and C-terminal regions are exposed on the particles' surfaces (Shukla *et al.*, 1988). Usually polyclonal antisera are directed towards both the termini and the core. Especially epitopes of the N-terminus can be used for identification of individual viruses, while the core is used for potyvirus group-common identification. Mabs designed to the latter do indeed detect many potyviruses, but fail in detection of some bulbous infecting ones (Jordan and Hammond, 1991; Derks, 1992). Mabs however can be important for future routine assays, when rabbit antisera become less available. Local highly conserved regions in the coat protein and replicase nucleotide sequence were selected for the design of potyvirus specific (degenerate) primers for use in a combined assay of reverse transcription and polymerase chain reaction (RT-PCR) for diagnostic purposes (Langeveld *et al.*, 1991; Dekker *et al.*, 1993). This assay supported potyvirus-specific amplification including some of the Mab-negative bulbous potyviruses.

Not only diagnosis of the virus disease and study of the virus itself are prerequisites for development of a reliable assay. Also insight in the virus spread inside the plant, from a plant to its progeny, from one plant to another within one stock, and from one species, varying from ornamentals to weeds, to another is of major importance to be able to design a reliable test (Derks, 1988). Considerable effort is put into localisation of the virus inside the plants and its epidemiology. E.g. lily symptomless virus could be detected with ELISA in the top leaves of primarily infected plants, but not in all infected plants and during a restricted period only. This indicates that a leaf test usually reflects the virus situation of that stock in the preceding season. However, the actual virus presence is most reliably found in the bulbs and stem bulbils because both primary and secondary infections are detected (Derks, 1988; Van Schadewijk, 1986). In contrast tobacco rattle virus is found unequally distributed in the tulip bulbs just after lifting (Van der Vlugt *et al.*, 1988), whereas BYMV in gladiolus corms and ISMV in crocus corms and iris bulbs are below detection limits in ELISA (Stein *et al.*, 1986, Derks *et al.*, 1982; 1986). Such localised presence of a virus in a plant has also consequences for the spread to other plants, as vectors might have preference for certain plant parts, and even to the progeny, as daughter bulbs might escape virus infection.

Outline of the thesis

Several years ago virus-free iris bulbs were brought onto the market and now it is essential to test iris bulbs for the absence or presence of any virus occurring in iris.

IMMV is readily detected in leaves of secondarily infected plants and in primarily or secondarily infected Dutch bulbous irises from three weeks after lifting (Van Schadewijk *et al.*, 1988). NLV is also well detectable in the leaves, but it is difficult to detect in the bulbs. A test for the detection of this virus in bulbs is to be developed at the Bulb Research Centre. Like NLV, so far, it has been difficult to detect ISMV in iris bulbs by ELISA or electron microscopy during storage after lifting in late August until planting in October (Derks *et al.*, 1986).

The aim of the research described in this thesis was to gain insight in the distribution and multiplication of ISMV in the plant, especially the bulb, in relation to the metabolic activity and application of stress, and thus to be able to detect or exclude its presence reliably and to develop a sensitive test for this virus.

Stein *et al.*, (1986) have shown that BYMV detection improved in gladiolus corms during storage by cutting them. BYMV became detectable in the cut area dependent on storage time and temperature. Another possible way to improve detection might be storage of the bulbs at specific temperatures which, in case of tobacco rattle virus also led to an improved detection (Van der Vlugt *et al.*, 1988). Preliminary investigations of such wounding and storage methods applied to iris bulbs are described in Chapter 2.

Based on the promising results from this first investigation especially the wounding treatment was further investigated. As stress was being hypothesised to be the trigger for enhanced virus detection, an alternative stress stimulus, i.e. high-temperature treatment, was tested for its effect on ISMV titres in bulbs (Chapter 3).

To investigate whether the virus detection was improved by multiplication rather than by (antigenic) modification of the virus, viral RNA accumulation in stress-treated bulbs was followed, using a viral cDNA clone (Chapter 4).
Furthermore, localisation of the virus was studied in specific parts of the bulb. The cloning of viral cDNA also enabled investigations on the taxonomic position of ISMV (Chapter 5).

To gain further insight into the relationship between host and virus, a possible correlation between ISMV multiplication and host plant metabolism was tested by following the respiratory activity in the bulb upon stress treatment as applied for virus testing (Chapter 6).

The investigations described so far were carried out on secondarily infected bulbs. In Chapter 7 attention was given to primary infections. This would not only gain insight into virus translocation in iris and more specifically in the new bulb, but was also necessary to further test the stress-treatment based detection assays on their reliability.

Another potential problem for implementation of the developed test for routine use could be the existence of different strains or isolates of ISMV, part of which would escape detection. In Chapter 8 these were investigated and discussed in relation to the detection assays developed.

Finally a model is proposed for the behaviour of ISMV in the iris plant during the growing season and during bulb storage (Chapter 9).

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CHAPTER 2

Towards a rapid and reliable detection method for iris severe mosaic virus in iris bulbs*

SUMMARY

Detection of iris severe mosaic virus (ISMV) in ISMV-infected iris bulbs during storage, by either ELISA or electron microscopy has been problematic. We have applied different storage-temperature treatments and a cutting method as possible procedures to enhance ISMV detection in stored iris bulbs cv. Professor Blaauw. While at lifting ISMV could not be detected in the bulbs, gradually the virus became detectable when bulbs were stored at about 17°C. Better results were obtained when the cutting method was employed. Using ELISA, a 100% score was obtained for infected bulbs that were cut 1 month prior to testing. The virus could be detected only in tissue adjacent to the cut surface. These results offer good prospects for the development of a reliable detection method for ISMV in iris bulbs.

* This chapter has been published in a slightly modified form as : Van der Vlugt, C.I.M., Derks, A.F.L.M., Dijkstra, J. and Goldbach, R.W. (1988). Towards a rapid and reliable detection method for iris severe mosaic virus in iris bulbs. *Acta Horticulturae* 234: 191-198.

INTRODUCTION

Annually, approximately 700 million iris bulbs are produced in the Netherlands of which 70% is exported. To improve the quality of the iris-stocks, bulbs have to be indexed for the presence of viruses, amongst others iris severe mosaic virus (ISMV). This virus belongs to the potyvirus group and is serologically distinct from other iris-infecting members of this group, such as iris mild mosaic virus (IMMV) and bean yellow mosaic virus (BYMV). ISMV has been found to occur naturally in bulbous and rhizomatous irises and in *Crocus vernus*. In iris, ISMV causes distinct chlorotic stripes or mosaic patterns (Brunt *et al.*, 1988). It has been difficult to detect ISMV in iris bulbs by enzyme-linked immuno-sorbent assay (ELISA) or electron microscopy during storage after lifting in late August until planting in October. By contrast, IMMV is readily detected in Dutch bulbous irises during the same period (Van Schadewijk *et al.*, 1988). Stein *et al.* (1986) have shown improved detection of BYMV in gladiolus corms during storage by cutting them. The latter virus was detected in the cut area dependent on storage time and temperature. In this paper we report on the detection of ISMV by double antibody sandwich (DAS) ELISA and immuno-sorbent electron microscopy (ISEM) in secondarily infected iris bulbs by different temperature treatments during storage and by cutting the bulbs.

MATERIALS AND METHODS

Virus and antiserum

To avoid possible contamination with other iris-infecting viruses, in particular IMMV (Asjes, 1979), ISMV was propagated in *C. vernus* cv. Remembrance, and purified according to Derks and Vink-Van den Abeele (1980). Antiserum was prepared according to Derks *et al.* (1982), and tested for its specificity. The antibodies did not cross-react in ELISA with other iris-infecting potyviruses.

Bulb material

In mid June 1986, virus-free iris plants of *Iris x hollandica* cv. Professor Blaauw, grown in the field under a gauze, were inoculated with sap from ISMV-infected *C. vernus* cv. Remembrance. In 1987, this inoculated stock was grown again under a gauze in the field and healthy plants were removed. Bulbs from this infected stock and from a virus-free stock were lifted on 28 August and dried for three days. Thereafter, bulbs from both stocks were stored in different lots at 2,

5, 9, 13, 17, 23 and 30°C. Bulbs varying in weight from 7 to 35 g were equally distributed over the different temperature treatments. On 14 September bulbs stored at 30°C were treated with 500 ppm ethylene for 24 h as a standard procedure.

Sampling of bulb material

After 0, 1, 2, 3 and 5 months of storage, we collected 10 ISMV-infected bulbs and 3 non-infected bulbs from each temperature treatment, and tested them for the presence of ISMV by cutting a sample of 2.0 g from each bulb (denoted A, Fig. 2.1). The cut bulbs were immersed in water for 20 min to reduce infection with micro-organisms (mainly Penicillium spp.) during prolonged storage. They were then stored at room temperature for 1 month, sampled in portions of 2.0 g each (denoted B, C and O, Fig. 2.1) and tested.

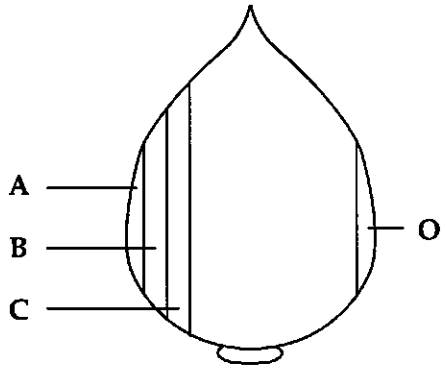

Fig. 2.1. Scheme of an iris bulb showing samples tested for the presence of ISMV. Sample A was cut off at different testing dates and the samples B, C and O after storing the cut bulbs at room temperature for 1 month.

ELISA

ELISA was performed according to Clark and Adams (1977), with some minor modifications. Wells of ELISA plates (Petra plastics type F) were filled with 200 μl fluid in the following procedure. Coating of the plates was with 1 μg γ-globulin per ml and the incubation time was 1 to 3 days at 6°C. The plates were washed several times with deionised water and finally once with 0.05% Tween 20 in water. For preparation of the antigen samples, 2.0 g of bulb material was ground in 10 ml of 66 mM Na-phosphate buffer pH 8.3 containing 130 mM NaCl, 3 mM KCl, 3 mM NaN_3. The starch and other coarse material was allowed to sediment for 0.5 to 2 h and the liquid was collected. Plates filled with the latter were incubated overnight at 6°C and washed again. The γ-globulins were conjugated according to Derks *et al.*, (1988). The suspension obtained was diluted with a 47 mM Na-K phosphate buffer pH 7.4 containing 130 mM NaCl, 3 mM KCl, 3 mM

NaN_3, 0.05% Tween 20, 0.4% normal horse serum. Plates with diluted conjugated γ-globulin suspension were incubated at 37°C for 3 h, and washed. Incubation with the substrate (p-nitrophenyl phosphate at a concentration of 0.5 mg/ml) was at 37°C for 1 h. The absorbance values at 405 nm were measured with a Titertek Multiskan or a Dynatech MR 600 spectrophotometer. Absorbance values of ISMV-free controls ranged from 0.00 to 0.09. Values above 0.15 were considered positive as they could be correlated with the presence of virus particles as observed in ISEM.

ISEM

This procedure was based on the methods of Derrick and Brlansky (1976) and Milne and Luisoni (1977) with some minor modifications. For trapping, grids with a pioloform-F carbon film were incubated with antiserum 1:20 diluted with 70 mM phosphate buffer, pH 7.0, at 37°C for 30 min, and washed in the same buffer for 5 min at room temperature. The grids were then floated, for 1 h at room temperature, on a droplet of antigen sample as prepared for ELISA, washed and subsequently floated on a droplet of the diluted antiserum at 37°C for 30 min. Thereafter, the grids were successively rinsed in buffer and in demineralised water, and then floated on a droplet of 2% uranyl acetate for about 2 min. Excess of staining fluid was absorbed by filter paper. The grids were then air dried and examined with a Philips 201 electron microscope.

RESULTS

Effect of the storage temperature treatment on the detectability of ISMV by ELISA

Immediately after lifting and drying, no ISMV could be detected in iris bulbs (Fig. 2.2). After storage for 1 month, there was a slight increase in the mean ELISA values at different temperatures, especially at 17°C (Fig. 2.3). At this temperature 3 out of 10 bulbs were found to be positive. Bulbs stored at 17°C for 2 and 3 months showed gradually increasing A405 values (data not shown) and a larger number of bulbs became positive. After a storage period of 5 months at 17°C, ELISA values were quite high (Fig. 2.4) and all bulbs were rated as positive (lowest A405 value 0.56). At 13 and 23°C also there was an increase, but less pronounced than that at 17°C (Fig. 2.4). At other temperatures, the number of positive bulbs was very low or zero.

The effect of cutting on the detectability of ISMV by ELISA

Sample B (Fig. 2.1) of cut bulbs, stored for another month at room temperature

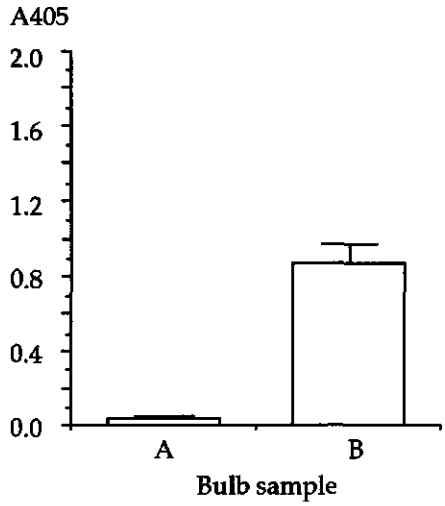

Fig. 2.2. Detection of ISMV by ELISA in samples A from infected bulbs, immediately after lifting and in B after storing the respective cut bulbs at room temperature for 1 month. Mean absorbance values at 405 nm (A405) with standard errors (n=10) are indicated.

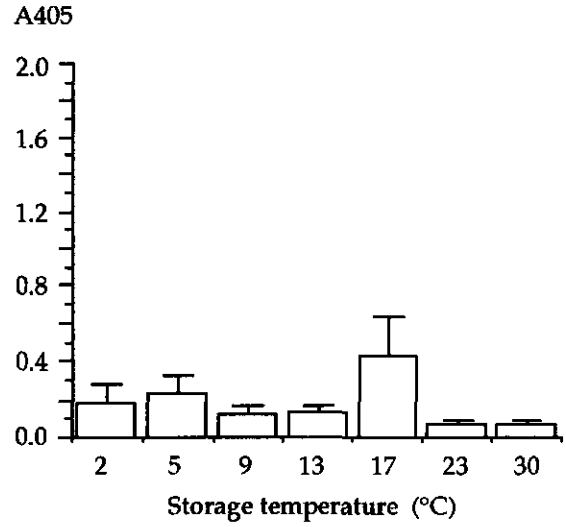

Fig. 2.3. Detection of ISMV by ELISA in samples A from infected bulbs stored at different temperatures for 1 month. Mean absorbance values at 405 nm (A405) with standard errors (n=10) are indicated.

after cutting, gave high readings and therefore a good detection of the ISMV in infected bulbs (Figs. 2.2 and 2.5): The cut bulbs gave a 100% score already after 1 month of storage, in contrast to the uncut bulbs giving only a 30% score when stored at 17°C, as seen earlier. Similar results were obtained with bulbs stored for 2 and 3 months prior to cutting (data not shown). However, when bulbs were stored for a longer period (for 5 months, Fig. 2.6), the effect became less pronounced.

Fig. 2.7 shows the ELISA values in different samples of cut iris bulbs. Bulbs were stored at 30°C for 1 month prior to the first sampling (A-parts).

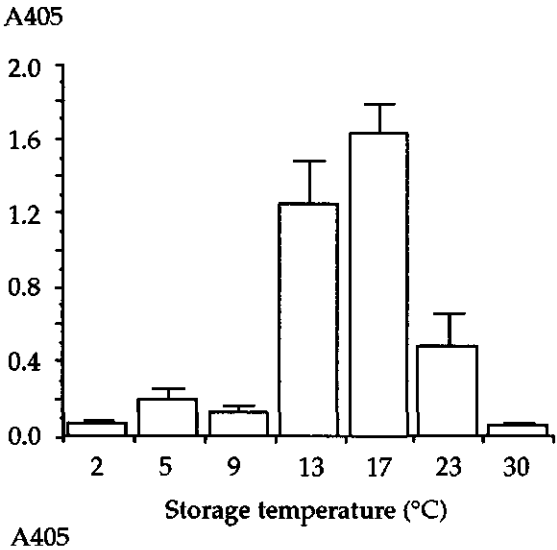

Fig. 2.4. Detection of ISMV by ELISA in samples A from infected bulbs stored at different temperatures for 5 months. Mean absorbance values at 405 nm (A405) with standard errors (n=10) are indicated.

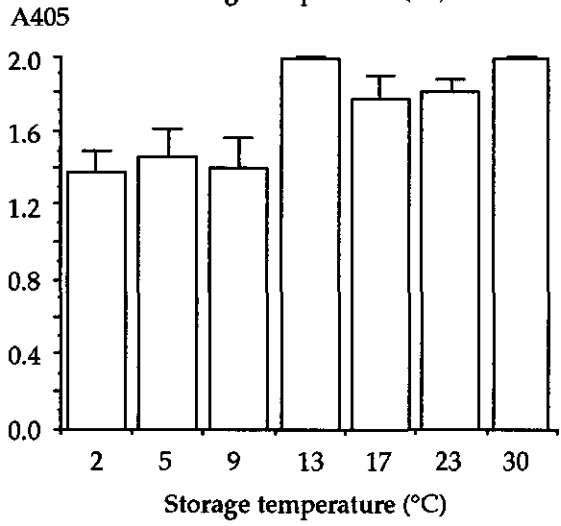

Fig. 2.5. Detection of ISMV by ELISA in samples B from infected bulbs stored at different temperatures for 1 month. Mean absorbance values at 405 nm (A405) with standard errors (n=10) are indicated.

After storing these cut bulbs for 1 month at room temperature, the highest A405 values were obtained with sample B, those with samples C and O were invariably lower. With O samples of small bulbs (7-16 g), ELISA values were quite high but lower than those with B and C, whereas the values of O samples of large bulbs (17-35 g) did not exceed the level of uninfected bulbs. This shows a decrease in ELISA values with increasing distance from the site of cutting. To demonstrate that the high ELISA values with B and C resulted from a combined effect of cutting and subsequent storage and not from the position of the samples in the bulb, uncut infected bulbs were divided into 7 to 12 parts and each part tested

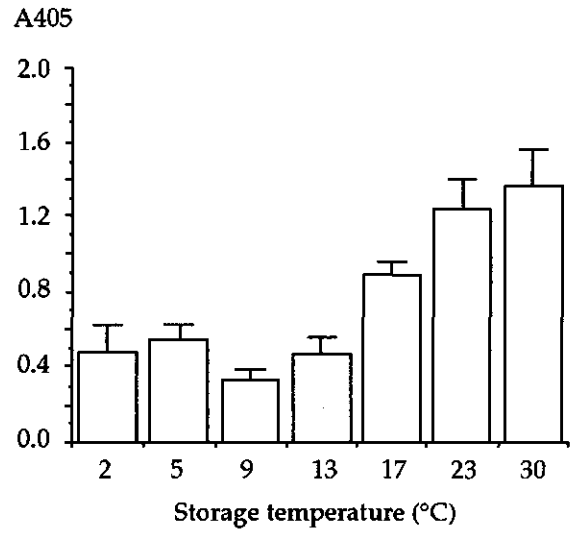

Fig. 2.6. Detection of ISMV by ELISA in samples B from infected bulbs stored at different temperatures for 5 months. Mean absorbance values at 405 nm (A405) with standard errors (n=10) are indicated.

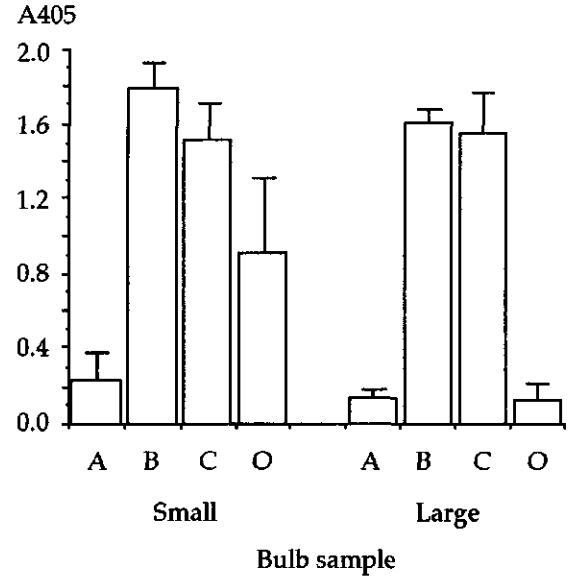

Fig. 2.7. Detection of ISMV by ELISA in samples A, B, C and O from small and large infected bulbs. Mean absorbance values at 405 nm (A405) with standard errors of 4 (small) and 6 (large) samples are indicated. Sample A was taken from bulbs stored for 1 month at 30°C and the cut bulbs were stored at room temperature for another month, whereafter samples B, C and O were tested (see Fig. 2.1)

separately. All slices of one bulb were found to have comparable A405 values (data not shown).

DISCUSSION

Iris bulbs are stored at different temperatures depending on their use. Optimal storage conditions used for commercial greenhouse forcing are a sequence of 1 month at 30°C, 2 months at 9°C and 2 weeks at 17°C. The 17°C period is known to be optimal for good flower production (Beyer and Slootweg, 1963). Storage at 17°C

(for 2-3 months) is also applied for planting material (Anonymous, 1970). In our experiments, each batch of bulbs was stored at one temperature only. We found that in infected bulbs stored at 17°C for several months, ISMV became gradually detectable. The improved detectability may be correlated with certain physiological activities brought about by the 17°C treatment. Our results clearly show that cutting and subsequent storage improved the detection of the virus to a much greater extent than a mere temperature treatment. After storage for 1 month the virus was readily detected in tissue adjacent to the site of cutting, but not in that farther away from it. Stein *et al.* (1986) observed a similar phenomenon with BYMV in gladiolus corms. Apparently, physiological activity induced by wounding stimulates processes leading to better detection of these viruses. From our results it can be concluded that the prospects are favourable for the development of a test for early detection of ISMV in iris bulbs. One should, however, bear in mind that the data presented here were from experiments with secondarily infected bulbs only.

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CHAPTER 3

Improved detection of iris severe mosaic virus in secondarily-infected iris bulbs*

SUMMARY

The influence of wounding and high-temperature treatment on the detection of iris severe mosaic virus (ISMV) in secondarily ISMV-infected iris bulbs was studied. Wounding of the bulbs just after lifting, followed by storage for 3 weeks at 17 or 20°C, increased the detectability of ISMV to 100% reliability. High-temperature treatment and consecutive storage at 17°C induced a similar improvement of detection. It is concluded that a certain degree of stress, such as wounding or high-temperature treatment, ultimately leads to an increase in viral antigens and thus to improvement of detection. It is hypothesised that the virus titre increases by the altered metabolism during the repair reactions as a response to stress applied to the bulbs.

* This chapter has been published in a slightly modified version as : Van der Vlugt, C.I.M., Derks, A.F.L.M., Boonekamp, P.M. and Goldbach, R.W. (1993). Improved detection of iris severe mosaic virus in secondarily-infected iris bulbs. *Annals of Applied Biology*, 122: 279-288.

INTRODUCTION

Annually, approximately 700 million iris bulbs are produced in the Netherlands of which 70% are exported. To improve the quality, bulb stocks are inspected visually for the presence of viruses. As already observed in other bulbous crops such as tulips and lilies, further improvement can be obtained by serological testing of the bulbs or scales. These tests are being developed for iris stocks. Three of the four viruses mainly infecting bulbous iris, namely iris mild mosaic virus (IMMV), iris severe mosaic virus (ISMV) and bean yellow mosaic virus (BYMV) are potyviruses. In the Netherlands, BYMV does not occur in irises grown for bulb production. IMMV can be reliably detected in infected iris bulbs after lifting (Van Schadewijk *et al.*, 1988) but, so far, ISMV (Brunt *et al.*, 1988) has been difficult to detect by enzyme-linked immunosorbent assay (ELISA) or electron-microscopy in freshly harvested iris bulbs. However, Stein *et al.*, (1986) showed improved detection of a potyvirus (BYMV) in infected gladioli corms by cutting and consecutive storage of this vegetative material, and testing of the cut surface area of the corm. Preliminary experiments with wounding of iris bulbs also showed a promising increase of ISMV detectability in the tissue adjacent to the cut surface area (Van der Vlugt *et al.*, 1988).
Here we report the influence of stress factors, such as wounding and high-temperature treatment and the involvement of recovery processes of the bulb, on the improvement of detection of ISMV in secondarily-infected iris bulbs during the storage period after lifting.

MATERIALS AND METHODS

Virus and antiserum

To exclude possible contamination with other iris-infecting viruses, in particular IMMV (Asjes, 1979), ISMV was propagated in *Crocus vernus* cv. Remembrance. The virus was purified as described for lily symptomless virus (Derks and Vink-Van den Abeele, 1980). Antiserum, the γ-globulin fraction and conjugate were prepared as described by Derks *et al.*, (1988). The antibodies did not cross-react in ELISA with other iris-infecting potyviruses.

Bulb material

In June, virus-free plants of *Iris x hollandica* cv. Professor Blaauw, grown in the field under gauze, were mechanically inoculated with leaf sap from Iris cv. Prof Blaauw, infected with the ISMV isolate from *C. vernus* cv. Remembrance.

These inoculated stocks were grown in successive years under gauze in the field. The secondarily ISMV-infected stock and a virus-free stock were lifted at the end of August and dried for about three days. Lots from both stocks were used for the experiments. For further propagation of stocks, the remaining bulbs were planted again in November each year. Despite growing under gauze and inspection of the plants during the growing season, usually a low percentage of IMMV was detected in the lifted bulbs from both stocks. In one of the years, a part of the stock of ISMV-infected bulbs grown in one gauze cage, was found to be 100% infected with IMMV. However, the presence of IMMV did not seem to influence ISMV titres or detection.

Treatments of bulbs were carried out in large storage rooms especially equipped for storing bulbs, unless otherwise indicated. To prevent spread of diseases by vermin, these rooms were gassed with cyanide every 2-3 weeks.
Bulbs varying in weight were equally distributed over the different treatments.

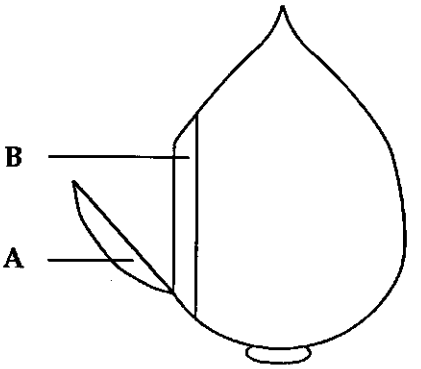

Fig. 3.1
Schematic view of cut iris bulb, part A and part B are indicated.

Bulb treatment : Wounding

Soon after lifting, a piece of about 2.0 g (denoted A, in Fig. 3.1) was removed from each bulb of virus-free and ISMV-infected stocks. Cut bulbs were immediately immersed in water for 20 min to reduce infection with micro-organisms (mainly *Penicillium* spp.) and dried, before storing at different temperatures. Uncut control bulbs of both stocks were also stored at these temperatures. For virus detection, a second section (part B, in Fig. 3.1) was taken from each bulb and assayed by ELISA.

Bulb treatment : High-Temperature

In the first season, bulbs from virus-free and ISMV-infected stocks were stored in different lots at 30°C and 17°C. During this period, bulbs were tested in ELISA

for the presence of ISMV. After 1, 2, 4 and 6 months different lots of bulbs were transferred from 30°C to 17°C and tested.
In the other seasons, bulbs were first stored in different lots at 30°C for about 4 weeks to give the bulbs time for acclimation. Towards the end of the 30°C treatment, some lots received an additional temperature treatment at higher temperatures of 40°C and 50°C. For the 40°C treatment, bulbs were placed in an air-ventilated incubator; for 50°C, bulbs were immersed in a water bath. At the end of the incubation period, these treated bulbs and the 30°C lots were all transferred to 17°C and tested by ELISA during the 17°C storage period.

ELISA

ELISA was performed according to Clark and Adams (1977), with some minor modifications as described by Van der Vlugt *et al.* (1988) and as follows: Incubation with the substrate (p-nitrophenyl phosphate at a concentration of 0.5 mg/ml) was at 37°C for 1 h in the first season, and in the other seasons, until the ISMV-standard control reached an absorption value at 405 nm (A405) of circa 0.75. Measured values were converted by {A405 (sample measured) / A405 (standard measured)} x 0.75, and the standard error of the mean was calculated (S.E.). This standard control consisted of a suspension of ISMV-infected leaf material, frozen in small aliquots and stored at -20°C. Just before use, such an aliquot was thawed and diluted 1/40 as a standard control. The absorbance values at 405 nm were measured with a Titertek Multiskan, a Dynatech MR 600 or an Anthos 2001 spectrophotometer. Absorbance values of ISMV-free controls always remained below 0.10. Values above 0.15 were considered positive as they could be correlated with the presence of virus particles as observed in immuno-sorbent electron-microscopy as described earlier (Van der Vlugt *et al.*, 1988).

RESULTS

Influence of wounding on the detectability of ISMV by ELISA

In preliminary experiments, it has been shown that cutting of freshly lifted iris bulbs and their subsequent storage at room temperature enhanced the detectability of ISMV in the cut surface area (Van der Vlugt *et al.*, 1988) This effect has now been further investigated.

The effects of different temperatures during storage were studied first. After lifting, at t=0, hardly any ISMV could be detected (Fig. 3.2A). Non sectioned bulbs stored for 5 weeks at 17-23°C (Fig. 3.2A) showed some increase, but detectability was not reliable.

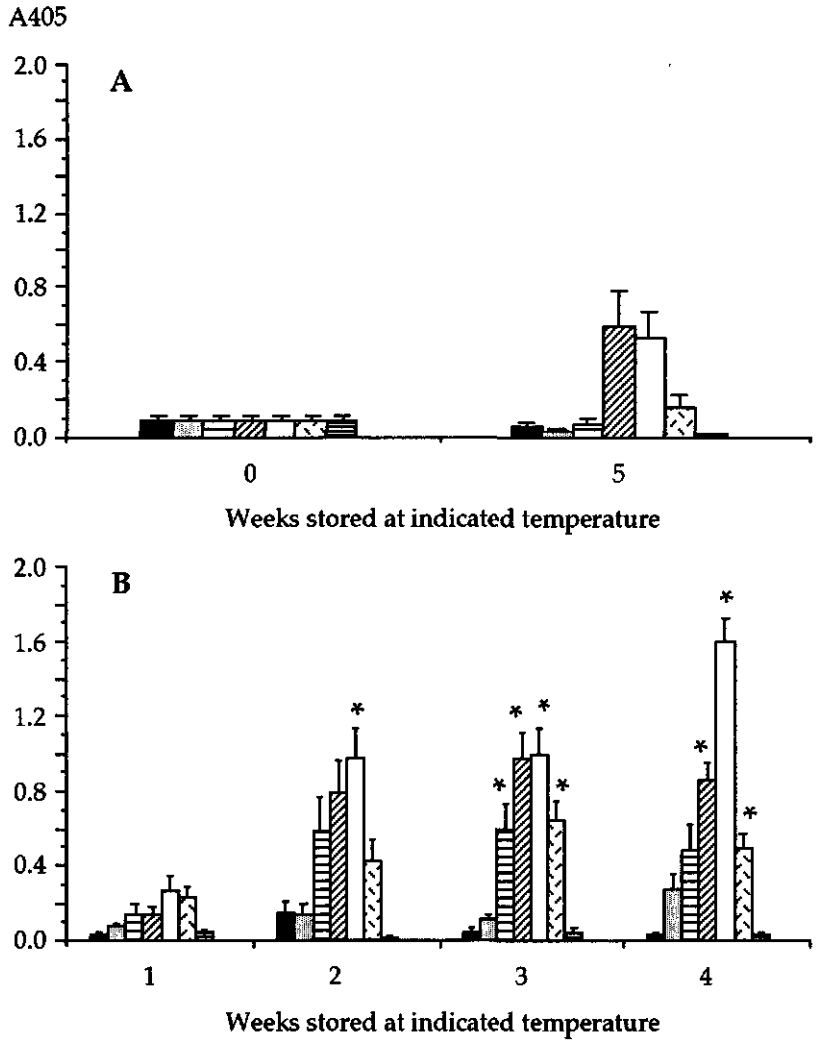

Fig. 3.2. Detection of ISMV (mean A405 and S.E.) in part B of uncut bulbs (n=10) just after lifting, and after 5 weeks of storage at temperatures between 5 and 30°C (3.2A), or part B of cut bulbs, stored for 1, 2, 3 and 4 weeks after cutting at 5°C (■), 9°C (▩), 13°C (▤), 17°C (▨), 20°C (□), 23°C (▦) and 30°C (▤), (3.2B). *: 100 % score of infected bulbs.

However, the efficiency of detectability increased significantly when bulbs were cut before storage (Fig. 3.2B). After 2 weeks at 20°C or 3 weeks at 13°C, 17°C or 23°C, the virus could be reliably detected. After 4 weeks, however, two bulbs stored at 13°C gave doubtful values, indicating possibilities of false negatives at

this temperature. Comparing the results obtained in various repeated experiments (Fig. 3.3, further data not shown) in subsequent years, it was concluded that storage of cut bulbs at 20°C gave optimal and reliable detectability of ISMV after 3 weeks, in the area of cutting.

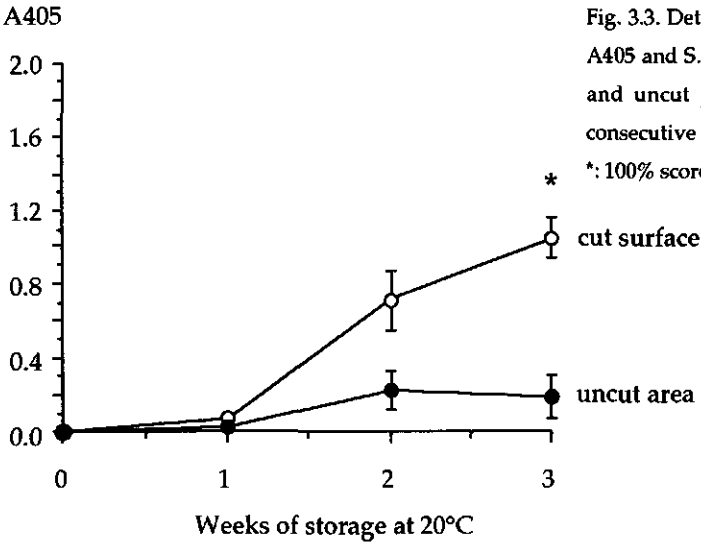

Fig. 3.3. Detection of ISMV (mean A405 and S.E.) in bulbs (n=10) cut and uncut just after lifting and consecutive storage at 20°C.
*: 100% score of infected bulbs.

As detection is enhanced after wounding, it is hypothesised that a stress stimulus is responsible for enhanced metabolic activity of the plant during the subsequent recovery period. Such metabolic processes are responsible for an increase of the virus titre. To confirm this hypothesis, high-temperature treatment as an alternative stress stimulus was tested for the effect on ISMV titres in bulbs.

Influence of high-temperature treatment on the detectability of ISMV by ELISA

Effect of variable periods at 30°C

In practice, a temperature of 30°C is used as a standard for storing bulbs over a long period of up to one year (so-called retarded bulbs). The influence of storage at 30°C on the detectability of ISMV after transfer to a metabolically more favourable temperature of 17°C was investigated.

In Fig. 3.4A and 3.4B the levels of ISMV detection are shown in control bulbs which were stored continuously at a single temperature. Storage at 17°C showed a gradual increase of ISMV detection, whereas detection remained low, around zero, when bulbs were kept at 30°C.

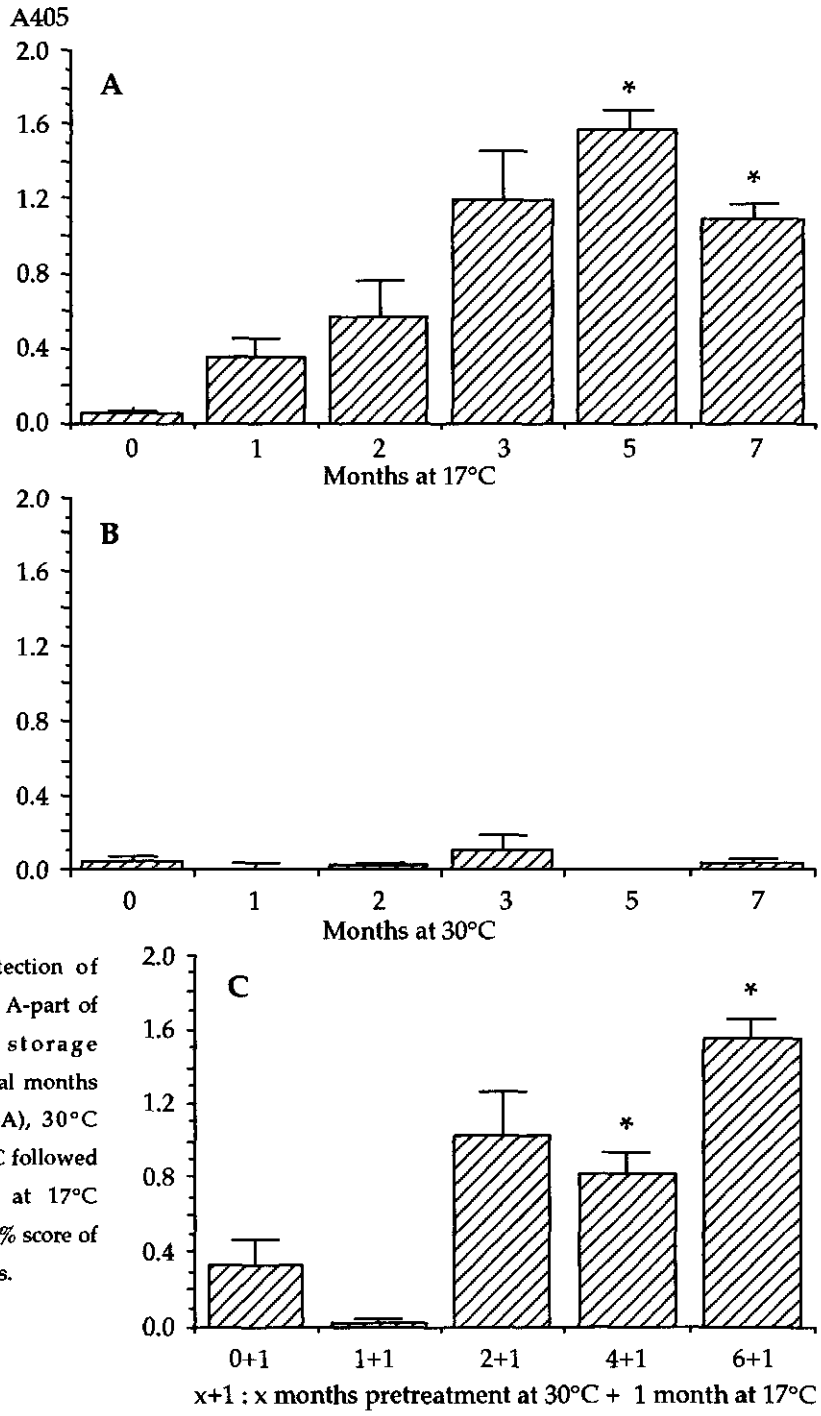

Fig. 3.4. Detection of ISMV in the A-part of bulbs after storage during several months at 17°C (3.4A), 30°C (3.4B) or 30°C followed by 1 month at 17°C (3.4C). *: 100 % score of infected bulbs.

In Fig. 3.4C, ELISA values are shown for bulbs first incubated at 30°C for prolonged periods, but always followed by 1 month at 17°C. Although all bulbs had been subjected to the same length of treatment at 17°C, the detection of ISMV varied. This indicates that the length of the 30°C incubation determines the development of the virus titre during subsequent storage at 17°C.

A short 30°C (1 month) treatment showed a negative influence on detectability, while a period of 2 months or longer at 30°C had a positive influence. Thus, in the latter treatment, the negative influence of a short 30°C period was overcome and the processes involved in improved detection were speeded up. A longer storage at 30°C probably involved stress and therefore had an enhancing influence on virus detectability during the recovery period at 17°C.

Effect of an additional 40°C or 50°C treatment

Subsequent experiments were carried out to investigate whether a temperature treatment higher than 30°C would further improve detection, by inducing higher levels of stress over a shorter period. The results in Fig. 3.5A show that a short 40°C treatment had a stimulating effect on the detectability of ISMV in the recovery period at 17°C. This 40°C treatment not only reversed the negative influence of a short 30°C incubation, but further improved the effect: After 4 weeks at 17°C, the reliability of ISMV detection improved to a 100% level. These data indicate that this 40°C treatment can be regarded as a stronger stress-stimulus than the 30°C treatment. Repeated experiments at 40°C in other years confirmed these results (data not shown). In the case of a treatment at 50°C (Fig. 3.5B), essentially the same phenomenon was observed, though less effective. Reliability of ISMV detection was reached after 6 and 8 weeks following the 1 and 3 h treatment at 50°C, respectively. After 1 h at 50°C, a better result was obtained than after 3 h at 50°C. A high-temperature treatment as stress-stimulus should thus be beneficial.

DISCUSSION

Development of tests for ISMV in secondarily infected iris bulbs

ISMV cannot be detected reliably in freshly lifted iris bulbs, possibly due to an initially low virus titre as detected by the ELISA procedure used, or a very localised presence of virus in the bulb. However, the detectability of ISMV increased during storage of the bulbs at temperatures circa 17°C and was reliable after about 5 months (Van der Vlugt *et al.*, 1988). In test procedures, however, such a long storage period is not adequate. Other specific treatments may

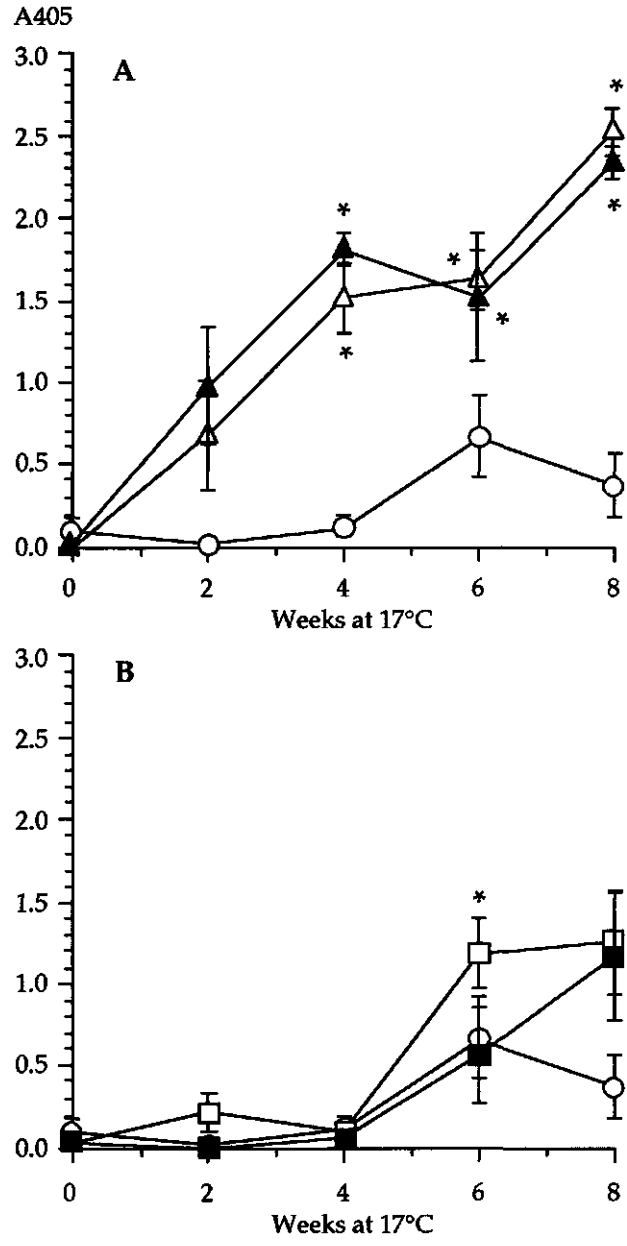

Fig. 3.5. Detection of ISMV in the A-part of bulbs treated after lifting, as follows:
31 days 30°C (—○—),
28 days 30°C plus 3 days 40°C (—△—),
25 days 30°C plus 6 days 40°C (—▲—) (3.5A),
or
31 days 30°C (—○—),
31 days 30°C plus 1 hour 50°C (—□—),
31 days 30°C plus 3 hours 50°C (—■—) (3.5B).

After these treatments bulbs were all transferred to the 17°C and samples were tested at intervals during this storage period. Mean A405 (n=5) and S.E. are indicated.
*: 100% score of infected bulbs.

increase virus titres and thus the detectability of ISMV. Following the procedure of Stein *et al.* (1986) bulbs were wounded, by cutting a slice from the side of the iris bulb, and consecutively stored at various temperatures. After wounding, the mean ELISA values gradually increased. This enhanced detectability showed 20°C

to be the optimal storage temperature after cutting. For a reliable test it is necessary that all infected bulbs are actually detected, which was the case after 2 to 3 weeks storage at 20°C after cutting. Comparing the results obtained during several years, the storage period necessary to reach reliable detection varied slightly, probably depending on the growing season. Thus, secondarily ISMV-infected bulbs can be reliably tested by wounding the bulbs after lifting and consecutive storage at 17°C or 20°C for at least 3 weeks. Such a short term test can be used, for instance, to check propagation material. Alternatively, when cutting is not desirable, for instance in the case of (very) small bulbs, it is possible to treat bulbs for 3 weeks at 30°C plus 6 days at 40°C and a subsequent storage at 17°C for 4 weeks. This test, however, requires a few more weeks.

For the year-round production of cut flowers, iris bulbs are stored at 30°C. After 4 months of storage at 30°C, these bulbs need not to be wounded, but can be stored immediately at 17°C for 1 month to obtain a reliable test result. It is concluded that secondarily infected iris bulbs can be tested reliably using various bulb treatments. Further investigations, concerning primarily infected bulbs, other iris cvs, different ISMV isolates, as well as the influence of wounding of the bulb on the detection of other viruses infecting iris, are needed before this test can be utilised for routine testing.

Improved detection of ISMV; a hypothesis.

Stein *et al.* (1986) observed an enhanced detectability of BYMV in gladioli corms, by wounding the corms and later testing the cut surface area. Preliminary experiments with cut iris bulbs and subsequent room temperature treatment (Van der Vlugt *et al.*, 1988) also showed that these treatments enhanced detection. A range of temperatures ranging from 5°C to 30°C showed an optimum at 20°C for enhancement of detection. Apparently, wounding stimulates processes leading to better detection of the virus, but it is obvious that this activity is only found around 20°C. The 17°C period is known to be necessary for good flower production (Beyer and Slootweg, 1963). Storage of the bulbs at 17°C for 2 to 3 months is also used for planting material (Anonymous, 1970). The improved detectability at 17°C and 20°C as found in earlier experiments (Van der Vlugt *et al.*, 1988), and as described in this paper (Figs 3.2 and 3.4), may therefore be correlated with physiological activities induced by the treatment at 17 or 20°C. Kamerbeek (1962) measured the respiration of iris bulbs during storage and found an optimum of respiration at 15°C (after six days of incubation in the range of 5°C to 30°C in iris cv. Van Vliet). This confirms that stored iris bulbs are physiologically most active at circa 17°C. Hence, enhanced detection of ISMV

appears to be correlated with (increased) physiological activity in the bulb. It is, therefore, hypothesised that the ISMV detectability is increased by applying a certain stress-factor, like wounding or high-temperature treatment. During the subsequent 17°C, it is supposed that the bulb starts repairing the damage caused by stress conditions, as this is a temperature at which the bulb is metabolically most active. These healing processes create conditions whereby the ISMV titre is enhanced, thus leading to improved detection.

Temperatures of 40°C or 50°C are too high for iris bulbs to survive for a longer period. An increase of respiration at these temperatures or after removal of the tunic from iris bulbs was found by Kamerbeek (1962), indicating that a rise in respiration can be considered as a reaction to stress. As ISMV detection was improved after both stress stimuli (i.e. wounding or 40/50°C temperature treatment), this supports our hypothesis. However, stimulation of detectability was found only if the appropriate temperature for recovery was applied. For healing, energy and metabolites are required. These can be delivered by respiration. Thus if the hypothesis holds, an increase of respiration should be found during the repair period.

It should be noted that the 50°C treatment gave less enhancement. It is possible that 50°C treatments, acting as an excess of stress caused by such a high temperature and/or oxygen depletion (water bath incubation), adversely affected the recovery mechanism. An indication of this is that the 3 h prolonged 50°C treatment enhanced detection less than the 1 h at 50°C. Thus dosage of stress seems to be rather important.

So far we have been investigating the increase in detection of ISMV only by serological assays. To gain insight in the process of enhanced detectability, which might be due to replication of the virus or a better exposure of the virus antigens or transport of the virus to the area of stress, the localisation of this virus was studied in more detail by microscopical analysis and molecular hybridisation assays, which is reported elsewhere.

ACKNOWLEDGEMENTS

We thank Dr. (Mrs) J. Dijkstra for helpful discussions, Karin Lameris and Daan Haket for technical assistance.

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CHAPTER 4

Multiplication and distribution of iris severe mosaic virus in secondarily-infected iris bulbs after stress induced by heat and wounding*

SUMMARY

In freshly-lifted bulbs infected with iris severe mosaic virus (ISMV), virus was not always detected in the basal plate and rarely in bulb scale tissue. After exposing the bulbs to stress (wounding or high-temperature treatment) the sensitivity of virus detection was enhanced. The improved detection of viral antigen after local stress (wounding) coincided with an increase of viral RNA synthesis. When general stress (high-temperature treatment) was applied, the virus could be reliably detected in the basal plate, and usually in vascular bundles and surrounding tissue. Virus was detected in the upper part of the bulb scale when such tissues were detached from the basal plate. Thus, virus must have been present in the scales in localised spots, albeit at a very low concentration, and multiplication is likely to be the main factor involved in the improved sensitivity of viral detection. The distribution of ISMV in the bulb after local or general stress treatment is discussed.

* This chapter has been published in a slightly modified form as: Van der Vlugt, C.I.M., Lohuis, H., Dijkstra, J., Marquart, A., Goldbach, R.W. and Boonekamp, P.M. (1993). Multiplication and distribution of iris severe mosaic virus in secondarily-infected iris bulbs after stress induced by heat and wounding. *Annals of Applied Biology* 123: 601-610.

INTRODUCTION

In freshly-lifted iris bulbs, iris severe mosaic potyvirus (ISMV) usually remains undetectable in ELISA, unless the bulb is wounded or treated at high temperatures (Van der Vlugt *et al.*, 1988; 1993). It seems likely that the amount of viral antigen in freshly-lifted bulbs may be below a detection limit, possibly due to a low concentration or uneven distribution of the viral particles in the bulbs. There are two hypotheses that may explain the inability to detect virus in infected untreated bulbs shortly after lifting, and the improved detection after stress (i.e. wounding or high-temperature treatment). Stress factors may lead to improved multiplication in cells infected with a subliminal amount of virus. Alternatively, the treatments may cause an increase in the translocation of viral antigens. To test these hypotheses, the localisation of viral antigen and viral RNA have been assessed in freshly-lifted bulbs and subsequent exposure to stress treatment, with the aid of ELISA, tissue blotting, examination of stained tissue specimens by light microscopy, and hybridisation assays.

MATERIALS AND METHODS

Bulb treatments and tissue culture

Virus-free and ISMV-infected iris stocks (*Iris x hollandica* cv. Professor Blaauw) were planted in late November and grown in the field until lifting at the end of August. Bulbs were dried and one sample of bulbs was tested by ELISA for the presence of ISMV by division of whole bulbs into 10 to 14 scale parts and a basal-plate, all tissue pieces weighing circa 2.0 g each. Other samples of bulbs received a wounding treatment (by cutting or by removing a piece of about 2.0 g as indicated in Fig. 4.1) or a high-temperature treatment when bulbs were kept at 20°C or 30°C for prolonged storage (Van der Vlugt *et al.*, 1988; 1993).

To maintain bulbs without a basal plate and with large wounded surfaces for several weeks at 20°C, it was necessary to prevent tissue pieces from drying out. For this purpose the tissue-culture system devised for iris (Van der Linde *et al.*, 1988) was used. From ISMV-infected and virus-free bulbs, stored at 30°C for 3.5-5.5 months, the tunic and the protruding part at the base of the bulb were removed. The bulb was cut from top to bottom on the seam of the first outer bulb scale, into two halves. One half was used for immediate testing in ELISA (0.2 g/ml). The other half was surface sterilised as described by Van der Linde *et al.* (1988). This half was cut from top to bottom into longitudinal slices to make the pieces fit into tissue-culture tubes. Some of these bulb halves were also cut into

upper and lower parts (Fig. 4.1). All these bulb-parts were put into tubes containing circa 14 ml of iris tissue culture medium (Van der Linde *et al.*, 1988). The tubes were incubated in the dark at 20°C for 2 to 3 weeks.

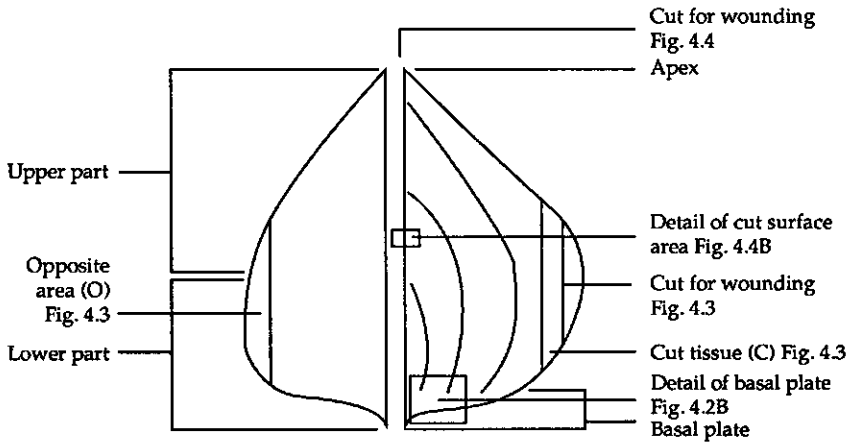

Fig. 4.1. Schematic representation of iris bulb, with indication of sampling area shown in subsequent figures.

Antisera

The ELISA procedure and ISMV coat protein antiserum used were as described earlier (Van der Vlugt *et al.*, 1988; 1993). The cylindrical inclusion (CI) protein was purified according to Dougherty and Hiebert (1980). A polyclonal rabbit antiserum was prepared by two intramuscular injections at a 2 week interval with 0.2 mg and 0.8 mg CI protein in Freund's incomplete adjuvant. Serum was obtained 1 month after the last injection. The γ-globulin-containing fraction of the antiserum (2 μg IgG/ml) was absorbed with 1% of acetone extracted healthy iris-leaf material for 1 h at 37°C before use.

Immunoblotting

SDS-polyacrylamide gel electrophoresis and blotting were performed essentially as described by Rybicki and Von Wechmar (1982) with the following modifications. Samples of bulb material were extracted in 20 mM Tris-HCl, pH 6.8 (0.7 g/ml) containing 16 mg/ml dithiothreitol (DTT), centrifuged for 10 min at 2,000 *g* and the supernatant diluted with an equal volume of sample buffer (20% glycerol, 0.015% bromophenolblue, 4% SDS). After electrophoresis, proteins were electroblotted onto a nitrocellulose (NC) membrane, in a continuous buffer system in a LKB semi-dry blotter according to the manufacturer's instructions.

The membrane was incubated at room temperature for 30 min with 5% (w/v) skimmed milk powder in phosphate buffered saline (PBS), treated with antiserum to CI protein followed by goat anti-rabbit antibodies conjugated to alkaline phosphatase (GAR-AP, Sigma) and stained with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) as described by Lin *et al.* (1990).

Preparation of RNA from iris bulbs

RNA was extracted essentially as described by Sambrook *et al.* (1989) with the following modifications. About 4 g of bulb tissue (frozen in liquid nitrogen) was ground to powder in a Bühler mixer, 4 ml of phenol/chloroform and 4 ml of extraction buffer (0.176 M glycine, 0.024 M NaOH, 0.176 M NaCl, 0.02 M EDTA and 2% N-lauroylsarcosine (Sigma)) were added and the suspension was shaken vigorously. After centrifugation for 10 min at 6000 *g* the aqueous layer was extracted again with phenol/chloroform. RNA was precipitated by adding 330 µl of 8 M LiCl to 1 ml of supernatant and the RNA-containing pellet was resuspended in 0.2 ml deionised water, then ethanol precipitated and the pellet finally dissolved in 40 µl deionised water. Samples (2 µl) were spotted onto Hybond-N (Amersham) and 5 min UV-cross-linked to the membrane using a transilluminator UV tube.

Riboprobe construction

Part of the ISMV-genome was cloned and sequenced (Van der Vlugt *et al.*, 1994). A *Hind*III fragment of 838 nucleotides located near the 3'-end of the polymerase gene was used for construction of riboprobe templates. This *Hind*III fragment was ligated in both orientations in the *Hind*III polylinker-site of the pTZ19R plasmid (Bio-Rad) containing a T7 promotor site upstream of the polylinker site to produce negative and positive sense riboprobes. These pTZ-plasmids were linearised by *Sma*I-digestion, proteinase-K treated and phenol/chloroform purified. These plasmids were transcribed into riboprobes for 2 h at 39°C using 1.5 µg of template DNA in a 25 µl reaction mixture, containing 0.4 mM rATP, rCTP and rGTP, 0.04 mM rUTP, 30 mM DTT, 40 mM Tris-HCl pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 25 U RNase inhibitor, 1.5 U T7-RNA-polymerase and 4 µl ^{32}P-α-labelled rUTP, as described by Sambrook *et al.* (1989).

Hybridisation assay

The hybridisation assay was done essentially as described by Sambrook *et al.*

(1989). The Hybond-N blots were prehybridised in 12.5 ml hybridisation buffer (3xSSC, 1% SDS and 1xDenhardt, 0.1 mg/ml calf-thymus DNA) for 5 hours at 65°C. Hybridisation took place overnight at 65°C with negative or positive-sense ^{32}P-UTP labelled riboprobes. Blots were washed for 20 min in 2xSSC and 0.1% SDS and 20 min in 0.1xSSC and 0.1% SDS at 65°C. Blots were exposed overnight to an X-ray film.

Tissue blotting on NC membranes (*in situ* blot)

Iris bulbs were cut vertically into three pieces. The freshly-cut surfaces were pressed onto 0.45 μm pore size NC membranes and the blots were processed as described by Lin *et al.* (1990) with some modifications. After drying, the blots were immersed in PBS containing 2% (w/v) sucrose and 5% (w/v) nonfat dry milk as a blocking agent, for 1 h at 37°C. This temperature and time were also used in following incubations. After the blocking step the NC membranes were incubated with antiserum against ISMV coat protein (1 μg IgG per ml PBS containing 0.05% Tween (TBS-0.5)), washed in a TBS-0.5 solution for 10 to 15 min and incubated with GAR-AP in TBS-0.5. To enhance the sensitivity, the membranes were then incubated with IgG prepared from rabbit normal serum (2 μg/ml TBS-0.5) followed by incubation with GAR-AP. After washing first in TBS-0.5 for 1 h and then in 0.1 M Tris substrate buffer for 30 min, the membranes were incubated at room temperature with 1.25 mg BCIP but without nitroblue tetrazolium salt in 7.5 ml of this substrate buffer. This procedure minimised non-specific background. In positive reactions the blot developed a blue colour.

Immunogold labelling and silver staining of sections

Thick sections

Iris bulbs were cut as described above. The cut pieces were fixed in cold Karnovsky fixative overnight. To ensure good penetration, the pieces were evacuated to circa 50 Torr during the first hour. These tissue pieces were cut with a razor-blade to produce sections approximately 1 mm thick. Immunogold labelling and silver staining were done as described by Van Lent and Verduin (1987) and Lohuis and Dijkstra (1992) with some modifications. The sections were washed in PBS for 1 h, rinsed twice for 5 min in deionised water and transferred to ethanol for 1 h. The ethanol treatment was found to improve the subsequent immunological reaction. After another wash in deionised water, the sections were kept in PBS overnight. In order to diminish non-specific reactions, the sections were incubated with PBS containing 2% (w/v) sucrose and 5% (w/v)

non-fat milk, incubated with anti-ISMV coat protein IgG, transferred into suspensions of 7-nm protein A-gold particles (A_{520nm} = 0.08) in TBS-0.5 and again incubated. A second fixation was done in 1% (w/v) glutaraldehyde in PBS. The immunogold-labelled sections were then silver stained.

Semi-thin sections

Semi-thin sections (approx. 1-3 μm) were prepared, immunogold labelled, silver stained and examined as described by Van Lent and Verduin (1987).

RESULTS

Virus distribution in bulbs immediately after lifting

Tissue sections of five freshly-lifted bulbs were tested by ELISA for the presence of viral antigen. By this method ISMV could be detected in the basal plate of some of the bulbs (Table 4.1), but not in the scale tissue. However, using *in situ* immunoblotting the antigen was sometimes detected in a cluster of cells in some scales (Fig. 4.1 and 4.2). Apparently these spots did not contain enough antigenic material to be detected with the sampling method used in ELISA. Healthy controls did not show any reaction.

Table 4.1. Detection of ISMV by ELISA in infected and virus-free bulbs immediately after lifting.

Bulb source	A405 of basal plate	Mean A405 of bulb scale parts*	S.E.
Infected 1	0.082	0.006 (10)	0.001
2	0.110	0.002 (11)	0.000
3	0.115	0.002 (14)	0.001
4	1.334	0.005 (12)	0.001
5	1.793	0.026 (14)	0.007
Virusfree	0.001	0.001 (10)	0.000

* Figures in parentheses are number of scale parts tested

ISMV-RNA was detected in bulb tissue extracts using a negative sense riboprobe (Fig. 4.3). Immediately after lifting, at the moment of cutting (time 0), no viral RNA could be detected in the infected bulbs (inf-C and inf-O). No negative sense of viral RNA could be detected using a positive sense riboprobe (data not shown). Hence, both serological and hybridisation data indicate that soon after lifting the virus is localised and present in low concentration in bulbs.

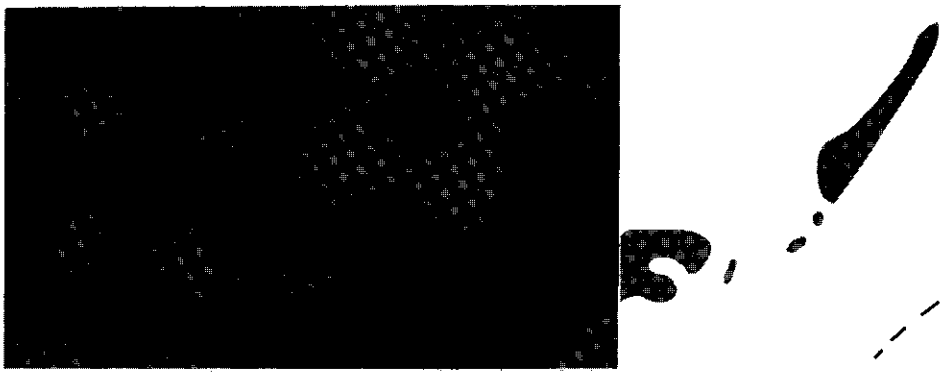

Fig. 4.2. An *in situ* immunoblot (A) and immunogold/silver staining of the basal part (B) of a longitudinal section of secondarily infected iris bulbs just after lifting (t=0), next to a schematic representation with patches indicating stained virus. Positive staining is found in the basal plate (bp) and in neighbouring cluster of cells, but hardly in the scale tissue. Other dark areas in B represent bulb tissue with its natural brown colour. Bar represents 3.5 mm in A and 0.3 mm in B.

Virus distribution after stress induced by wounding

Earlier work (Van der Vlugt *et al.*, 1988; 1993) has shown that ISMV can be detected by ELISA more reliably in tissues close to the cut surface and after storage of cut bulbs at 20°C. The present study shows that the detection of viral RNA was enhanced in tissues close to the cut bulb surface (Fig. 4.3). In the opposite part of the bulb (Fig. 4.1), most remote from the cut surface area, hardly any ISMV was detected, either by ELISA (Van der Vlugt *et al*, 1988) or by RNA hybridisation (Fig. 4.3). These data strongly suggest that the virus multiplied at the site of wounding, although the presence of negative-stranded viral RNA using a positive sense riboprobe could not be confirmed (data not shown).

To establish precisely the sites of virus multiplication, immunogold-localisation studies were performed using thick sections of wounded scales (Fig. 4.1 and 4.4). Indeed, the presence of viral antigen in cut bulbs 16 days after wounding, was found to be restricted mainly to the cut surface and the basal plate (Fig. 4.1 and 4.4). It was not possible to conclude from these sections whether the virus had spread from the basal plate to the wounded area or amplified locally at the wounded area itself.

Virus distribution after general stress induced by heat

When, instead of local stress (wounding), general stress was applied by high-

temperature treatment, the sensitivity of virus detection by ELISA was found to be enhanced after the bulbs were allowed to recover at 17°C or 20°C (Van der Vlugt *et al.*, 1993). When such bulbs were sectioned and tested in ELISA, the highest concentrations of ISMV were found in the lower part of the bulb (Fig. 4.1; data not shown).

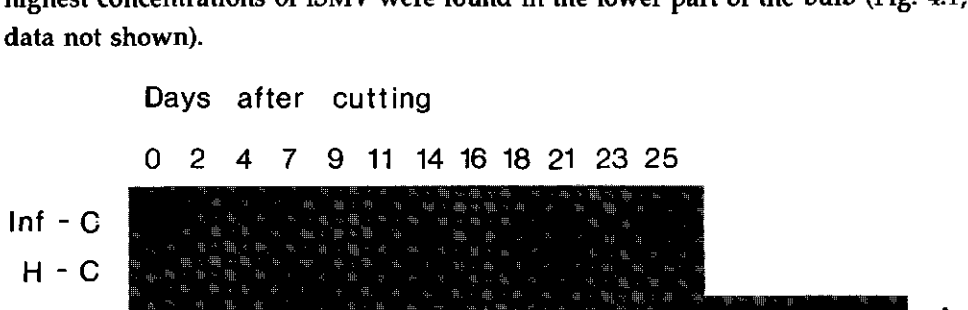

Fig. 4.3. Detection of +ss-RNA ISMV in cut (C) or opposite (O) area of infected (Inf) or healthy (H) cut bulbs in a time-course experiment after cutting, using a negative sense ^{32}P labelled riboprobe for hybridisation. Control, 1-4 are a dilution series of purified ISMV : 0.22, 1.12, 5.6 and 28 ng per spot, respectively, in duplicate.

In situ immunoblots of high temperature-treated bulbs showed that the basal plate was infected and also that there were infected patches of cells scattered all over the bulb scales (Fig. 4.5A). Such positively stained spots were identified as vascular bundles with surrounding parenchyma tissue by light and electron-microscopy in experiments in two different years (Figs. 4.5B, C and D). The presence of the CI-protein was tested by SDS-PAGE-immunoblotting of several upper bulb parts with low and high A405 values. The CI-protein was readily detected when ISMV could be found by ELISA in these samples but not when only very low A405 values were obtained from infected bulb tissue (data not shown). The correlation between the detection of coat protein and CI protein strongly suggests that the virus multiplied in the upper parts of the bulb as well.

Virus could not be detected in bulbs after storage for 3.5 to 5.5 months at 30°C. Six of these bulbs were dissected into upper and lower parts (Fig. 4.1). These tissue pieces were maintained on tissue culture medium for 2 to 3 weeks at 20°C, and then tested by ELISA. Both upper and lower tissue parts gave high A405 values (Fig. 4.6).

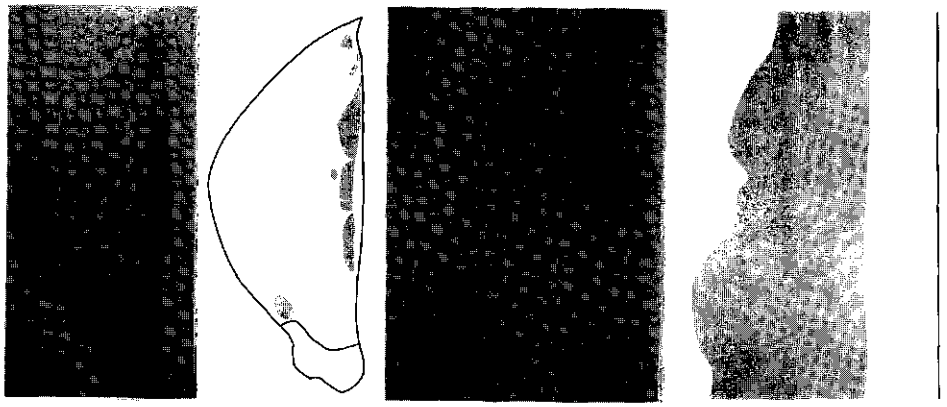

Fig. 4.4. Immunogold/silver staining of longitudinal section, perpendicular to the cut surface area (c), of an iris bulb secondarily infected with ISMV. The bulb had been cut just after lifting and was subsequently stored at 20°C for 16 days . An overview is shown in A (bar represents 2 mm) and a detail of the cut surface area in B (bar represents 17 μm), both next to schematic representations with patches indicating the stained virus. Other dark areas represent bulb tissue with its natural yellow/brown colour.

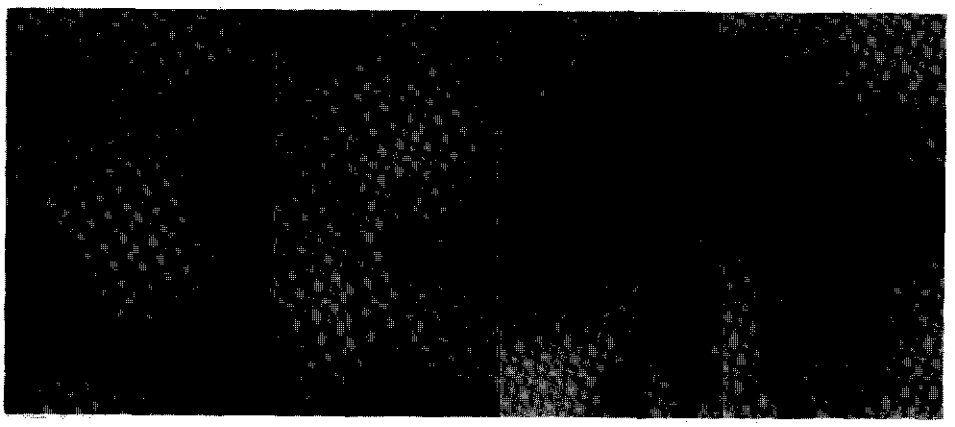

Fig. 4.5. Sections of secondarily-infected bulbs after long-term storage (7 or 8 months) at 30°C and consecutive storage at 20°C for 6 (A, B and C) or 12 days (D). In 6A an *in situ* immunoblot of the longitudinal section through the apex and bulb base is shown. Immunogold/silver stained details in longitudinal (B) and cross sections (C and D) show staining in the vascular bundles. Bar represents 2 mm in A, 10 μm in B, and 3 μm in C and D. bp=basal plate, pa=parenchyma, vb=vascular bundle and vbc=vascular bundle cells.

Similar high A405 values were obtained in tests on upper and lower parts of five bulbs which were not dissected before incubation at 20°C. Healthy bulbs, treated in the same way, gave A405 values equal or below 0.02. Because no transport of virus had been possible between the upper and lower part of bulbs split before the additional incubation, this suggests that subliminal amounts of virus were present in both parts of the bulb and were activated to multiply after the stress treatment.

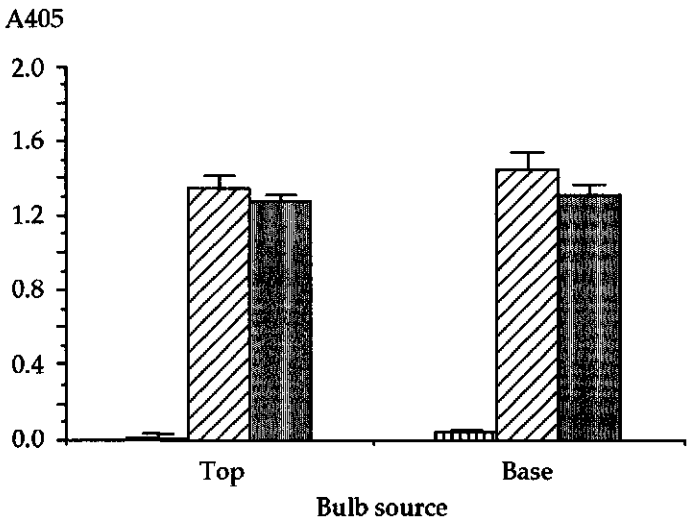

Fig. 4.6. Detection of ISMV by ELISA in upper and lowers parts of ISMV-infected iris bulbs after storage of the uncut bulbs for 3.5 to 5.5 months at 30°C. Every bulb was cut from top to bottom on the seam of the first outerscale, into two halves. One half of every bulb was then cut into an upper and lower part and tested directly in ELISA (▯). The other halves were incubated at 20°C for 2 to 2.5 wk and then tested in ELISA. One aliquot of these other halves (n=6) had been split into top and base parts, before the additional incubation (▨), and a second aliquot (n=5) of the halves after the additional incubation (▩).

DISCUSSION

Distribution of virus immediately after lifting

In the scales of freshly harvested bulbs, no viral antigen was found by ELISA and viral RNA could not be detected in the extracts of bulb scale tissue. This suggested that only subliminal amounts of virus particles were present. Similarly, a low concentration of bean yellow mosaic potyvirus was detected in gladioli corms in which the virus can only be detected using a modified

polymerase chain reaction test (Vunsh *et al.*, 1991; Rosner, *et al.*, 1992).

In the basal plate of some of the iris bulbs, viral antigen could be detected only when the basal plate was properly trimmed from the bulb scale tissue. This prevented dilution of the viral antigen by the remnants of non-reacting bulb scale tissue. The tissue-blotting and staining techniques were more suitable than ELISA for virus detection in localised areas of tissue, because small patches of viral antigen were sometimes detected in the bulb scale tissue as well.

Distribution of the virus after stress

After both types of stress treatments, the detection of viral antigen by ELISA was enhanced, as found in earlier experiments (Van der Vlugt *et al.*, 1993). This increase could be due to translocation or replication of the virus. If the virus is translocated after stress treatment, high ELISA values should have been found in some samples from freshly-lifted bulbs. From our results this possibility can be ruled out. The improved detection of viral antigen, accompanied by an increase in the amount of viral RNA, is indicative of active virus multiplication in the bulb part tested. These findings, as well as the increase in CI-protein, supports the hypothesis that the virus multiplies after stress treatment.

Site of multiplication of the virus after stress

Stress treatment may either increase replication of the virus in cells at the site of stress, and/or increase translocation from a site of replication, to the site of stress. Arguments for either assumption will be discussed.

An *in situ* immuno-stained tissue section after wounding and recovery (Fig. 4.4B) showed intense colour next to the dead, probably lignified cell layer. More distantly located cells with less colouring, are presumably less affected by the wounding. This suggests a direct influence of the stress on the replication of the virus in the wounded area itself. As we found a high virus titre only in the wounded area a few weeks after wounding, together with enhanced metabolic activity of the wounded tissue (see Chapter 6), virus multiplication may be dependent on the metabolic activity of the tissue during the recovery from stress.

In high-temperature treated bulbs, the detectability of the virus was better in the basal plate and lower part of the bulb than in the upper part. As with the wounding experiments, we assume that high-temperature treatment is a stress stimulus and has, therefore, an enhancing effect on the metabolism of the bulb during recovery, leading to multiplication of the virus. The basal plate plays an important role in the development of bulbs, forming a junction of vascular bundles and being a metabolically active area from which the root and leaf

primordia start growing (Kamerbeek, 1962).The observation that after stress the basal plate and the vascular bundles in the scale contain high levels of virus, suggests that stress induces multiplication of virus in the metabolically active basal plate and that the virus is translocated to the scales via the vascular tissue. However the separation experiment (Fig. 4.6) demonstrated that ISMV was already present in the upper part of the bulb before the stress treatment. In addition the increase in viral antigen after stress treatment was parallelled by an increase in the CI-protein. It is now known that the viral RNA of potyviruses is translated into one polyprotein which is presumed to be subsequently cleaved into equimolar amounts of viral proteins (Matthews, 1991; Baunoch, *et al.*, 1991). So far, CI proteins of potyviruses are not considered to spread through infected tissue, implying local virus replication in the upper part of the bulb. These findings indicated that translocation is not necessary for enhanced detection. It is concluded that stress-induced multiplication of the virus is the major mechanism underlying enhanced detection, although stimulation of translocation cannot be excluded.

ACKNOWLEDGEMENTS

We thank Dr. A.F.L.M. Derks for critically reading the manuscript.

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CHAPTER 5

Molecular cloning and sequence analysis of the 3'-terminal region of iris severe mosaic virus RNA*

SUMMARY

The 3'-terminal genomic region of the *Crocus vernus* cv. Remembrance isolate of iris severe mosaic potyvirus (ISMV) was cloned and sequenced. The nucleotide sequence determined was 2510 nucleotides long and contained a 3' non-translated region of 340 nucleotides, the coat protein (CP) gene and approximately 90% of the gene for the large nuclear-inclusion protein (NIb). Sequence analysis revealed that the ISMV coat protein is probably processed from the primary translation product using an unusual putative cleavage site consisting of an E/G dipeptide. The N-terminus of the ISMV coat protein is 15 amino acids long and the shortest found among potyviral coat proteins so far, the total coat protein being only 252 amino acids. Phylogenetic analysis of the CP sequences suggested that the ISMV is a typical, but taxonomically distinct potyvirus, not specifically related to other bulbous crops or monocotyledons-infecting potyviruses.

* Parts of this chapter have been published as : Van der Vlugt, C.I.M., Langeveld, S.A. and Goldbach, R.W. (1994). Molecular cloning and sequence analysis of the 3'-terminal region of iris severe mosaic virus RNA. *Archives of Virology* 136: 397-406.

INTRODUCTION

Potyviruses constitute an economically important group of pathogens (Matthews, 1991), causing frequent loss of yield in numerous agricultural crops worldwide. They are also responsible for most of the viral diseases in bulbous crops. Here we describe a study on iris severe mosaic virus (ISMV), a potyvirus naturally occurring in the monocotyledonous plants *Iris* and *Crocus* (Brunt *et al.*, 1988). The genomes of several potyviruses have been completely sequenced. The potyvirus genome contains a single-stranded plus-sense RNA of about 10,000 nucleotides with a viral encoded protein (VPg) at the 5'-end and a poly(A) tract on the 3'-end. The viral RNA encodes a polyprotein which is autocatalytically cleaved by virus-encoded proteinases (Dougherty and Carrington, 1988). So far, three viral proteinases have been identified (Verchot *et al.*, 1991). Cleavage results in at least 8 mature products. One or more functions of these proteins have been identified or proposed (Dougherty and Carrington, 1988).

To improve the quality of iris-stocks, bulbs have to be indexed for the presence of e.g. ISMV. This virus shows deviant behaviour compared to other bulbous-crop infecting potyviruses in terms of detectability by serology, which made it important to develop a molecular probe for reliable detection of its viral RNA (Van der Vlugt *et al.*, 1988; 1993). Moreover, molecular data on the coat protein gene of ISMV should clarify its taxonomic relationships with other potyviruses infecting bulbous crops or monocotyledons. In this paper, we describe the cloning and sequence determination of the 3' terminus of the ISMV genome. The amino acid sequences of the NIb protein and the CP were deduced from the nucleotide sequence and a non-translated region was localised at the 3'-terminus of the viral RNA. The CP sequence was compared with those of other potyviruses in relation to host specificity.

MATERIALS AND METHODS

Viral RNA isolation

For the λgt11 cloning, ISMV virions were purified from leaves of *Crocus vernus* cv. Remembrance according to Derks and Vink-Van den Abeele (1980). The virus was additionally centrifuged through a sucrose-gradient of 10 to 40% in a Beckman SW41 rotor for 2.0 h at 36000 rpm. Fractions containing virus were pooled and sedimented. Viral RNA was subsequently extracted as described by Zuidema *et al.*, (1989) and desalted on a G-50 Sephadex column (Sambrook *et al.*, 1989).

For polymerase chain reaction (PCR)-cloning, ISMV virions were purified from 10 g freeze-dried *Iris x hollandica* cv. Prof. Blaauw (infected with the above mentioned ISMV isolate from *C. vernus* cv. Remembrance). Infected leaf material was ground in 3 volumes of extraction buffer consisting of 0.05 M K_2HPO_4, pH 7.6, 0.01 M EDTA, 1% Na_2SO_3, 5% ethanol, filtered through cheesecloth and centrifuged in a Sorvall GSA rotor for 30 min at 8500 rpm. Triton X-100 was added to the supernatant to a final concentration of 1% and the solution was stirred at 6°C for at least 1 h. Virus particles were then collected by centrifugation through a 20% sucrose cushion in an SW 28 Beckman rotor for 5 h at 24000 rpm. Pellet was resuspended in 0.1 x extraction buffer and dialysed overnight.

The virus suspension was adjusted to 0.25% SDS and phenol/chloroform extracted to isolate the viral RNA. The viral RNA was further purified through a G-50 Sephadex column and precipitated with alcohol (Sambrook *et al.*, 1989) and resuspended in 25 µl water.

cDNA-cloning in λgt11

Oligo(dT) primed cDNA synthesis on viral RNA was performed essentially as described by Gubler and Hoffman (1983). Double-stranded cDNA was treated with *Eco*RI methylase, ligated to *Eco*RI linkers, *Eco*RI-digested and ligated to *Eco*RI-cleaved λgt11 (Promega) arms. Packaged recombinant phage DNA was plated on *Escherichia coli* strain Y1090. The library was screened according to Young *et al.* (1985) and Schaller *et al.* (1985), using 20% normal horse serum (for blocking) and 0.07 mg/ml purified antibodies of the ISMV-antiserum (SS216 titer serum: 1/160) directed to purified virions (Derks *et al.*, 1988). Horse-radish peroxidase conjugate was used as a second antibody. DNA was isolated from the positive plaques and subcloned into plasmid pTZ-19R (Bio-Rad).

Primers

Potyvirus group-specific primers U341 and D341 were derived from a conserved region in the core of the potyvirus coat-protein as described by Langeveld *et al.* (1991). The oligo(dT) plus (5'-TGCTCTAGAACTAGTGGATCC-(T)10-G/A/T/C-3') and 5 other primers (5'-GAGAGAACATATACGCC-3' (VLU4); 5'-GCACCATACTTAGCAGAG-3' (Ul1); 5'-CATAGCAACACAACAGC-3' (VLU1); 5'-ACGCGAACGTCGGAGCG-3'(VLU2); 5'-AGCGAGTGAATGGAAG-C-3' (VLU3) as indicated in Fig. 5.1) were obtained from Pharmacia.

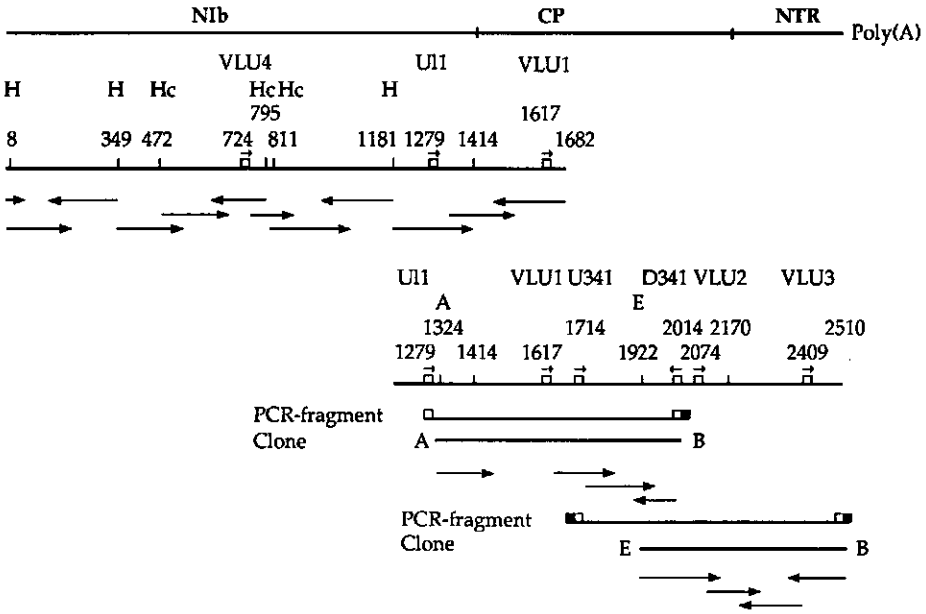

Fig. 5.1. Cloning and sequencing strategy of the 3'-terminal part of ISMV.
Restriction sites : H = *Hind*III, Hc = *Hinc*II, A = *Acc*I, E = *Eco*RI, B = *Bam*HI. Sequencing primers: VLU1, VLU2, VLU3, VLU4. Cloning and sequencing primers : Ul1, D341, U341. Cloning primer: oligo(dT)plus. ■ = restriction site box, □ = primer site box.

RT-PCR

2.5 µl Viral RNA was annealed to 25-65 pmol of downstream primer (D341 or oligo(dT)-plus) for 5 min at 65°C and 5 min at room temperature. The reverse transcription was performed using 100 units RT-Superscript (Gibco BRL) in 50 mM Tris-HCl pH 8.3, 65 mM KCl, 3 mM $MgCl_2$, 2.5 units of RNasin (Boehringer), 20 mM DTT, 0.2 mM dNTP in a 10 µl reaction for 1 h at 37°C. For amplification, this reaction mixture was supplemented with 25-50 pmol of upstream primer (Ul1 or U341), 1 unit of Taq polymerase (Promega) in a 50 µl volume containing final concentrations of 60 mM KCl, 20 mM Tris-HCl, 0.1% Triton X-100, 0.6 mM $MgCl_2$ and 0.2 mM dNTP. PCR was started with incubation at 94°C for 6.5 min followed by 30 cycles of : 60 sec to 54°C, annealing for 60 sec at 54°C, synthesis for 130 sec at 72°C and denaturation for 60 sec at 94°C in a Perkin Elmer Cetus Thermocycler.

Cloning of the PCR-fragments

After amplification the DNA-fragments were proteinase-K treated and phenol

purified. The Ul1-D341 amplified fragment was digested with *Acc*I and *Bam*HI and the U341-oligo(dT) amplified fragment with *Eco*RI and *Bam*HI overnight at 37°C and cloned into pSK plasmids (Stratagene; Sambrook *et al.*, 1989).

Sequencing

The DNA fragments were sequenced by the method of Sanger *et al.* (1977), and Zhang *et al.* (1988) using subclones if necessary or specific primers if available. For computer analysis the GCG-programmes were used as described by Devereux *et al.* (1984).

RESULTS

cDNA-cloning and sequencing

Double-stranded cDNA made from purified ISMV RNA was cloned into λgt11. The library was screened with rabbit antiserum raised against purified ISMV particles and one positive clone was isolated. Restriction fragments of this clone were subcloned and sequenced. The translated nucleotide sequence of this clone revealed homology with part of the nuclear inclusion protein b (NIb) and part of the coat protein (CP) of other known potyviruses (Fig. 5.1). To obtain the remaining extreme 3' part of the viral genome, including the 3' non-translated region, two DNA fragments were amplified from viral RNA by PCR, using a specific primer complementary to the C-terminal part of the NIb cistron (Ul1) or an oligo(dT) primer and a general primer made complementary to the core (D341 or U341) of the coat protein (Fig. 5.1).

These DNA fragments were digested (Fig. 5.1) using *Bam*HI for the 3'-ends and *Acc*I and *Eco*RI for the 5'-ends. The *Acc*I and *Eco*RI shortened the PCR fragments due to internal restriction sites. The restriction enzyme fragments were cloned into appropriate pSK-vectors.

Four independent clones of each digested PCR-fragment, were isolated and sequenced to reveal possible Taq polymerase incorporation artefacts (Innis *et al.*, 1990; Fig. 5.1). The sequences of the four clones indeed revealed inconsistencies at only four positions, at position 1338 (Fig. 5.2) (1xT and 4xA), at position 1818 (1xG and 3xA) at the third position of the codon, at position 1945 (1xG and 7xA), at the first position of the codon, and at position 2376 in the non-translated region (1xT and 3xG). The sequenced overlap of the λ-clone and the PCR-fragment were identical, as well as the overlapping sequence of the two PCR-fragments.

```
   1  GAATCAAAAGCTTTTTTTGAGCCACTATGTGGTTTTTATGGGAAGAGCAACTTAAATAAA
      E  S  K  A  F  F  E  P  L  C  G  F  Y  G  K  S  N  L  N  K

  61  GCAGCATGTGTCAAGGATTTCACTAAGTATGCCTCTGAAATTGTCGTTGGCAAAGTCGAT
      A  A  C  V  K  D  F  T  K  Y  A  S  E  I  V  V  G  K  V  D

 121  GTGGATGTATTTGACAGAGCTTTTCAAAACACGTACACGATTCTTTCACGAGCAGGAATC
      V  D  V  F  D  R  A  F  Q  N  T  Y  T  I  L  S  R  A  G  I

 181  AAGGAATGCAATTATGTTAACGACACAACTGAAATATTCAATAGTCTTAACATGAAAGCA
      K  E  C  N  Y  V  N  D  T  T  E  I  F  N  S  L  N  M  K  A

 241  GCCGTTGGTGCGCTGTATGGTGGGCAAAAGAAAGATTATTTCGCACACTATACGGATGAA
      A  V  G  A  L  Y  G  G  Q  K  K  D  Y  F  A  H  Y  T  D  E

 301  GATAAGGAAAACATAATTAAAGAGAGTTGTGAACGTTTATTTCTTGGGAAGCTTGGTATT
      D  K  E  N  I  I  K  E  S  C  E  R  L  F  L  G  K  L  G  I

 361  TGGAATTGTTCTCTGAAGGCTGAACTGCGATCAAAGGAAAAGATTGAACAAAATAAAACA
      W  N  C  S  L  K  A  E  L  R  S  K  E  K  I  E  Q  N  K  T

 421  CGAACATTTACCGCGGCACCACTTGAAACATTACTTGGTGGCAAAGTTTGTGTTGACGAT
      R  T  F  T  A  A  P  L  E  T  L  L  G  G  K  V  C  V  D  D

 481  TTTAACAATCAATTTTATGAATGCAATCTCAGGGGACCGTGGACTGTTGGAATTTCGAAA
      F  N  N  Q  F  Y  E  C  N  L  R  G  P  W  T  V  G  I  S  K

 541  TTTTATGGTGGATGGAACAGAATGTTGCAACAGTTGCCAGATGGTTGGATATATTGCGAC
      F  Y  G  G  W  N  R  M  L  Q  Q  L  P  D  G  W  I  Y  C  D

 601  GCCGATGGATCTCGATTCGATAGTTCATTAACACCATTCCTAATCAATGCGGTTTTGCGC
      A  D  G  S  R  F  D  S  S  L  T  P  F  L  I  N  A  V  L  R

 661  ATGCGTTTAAAATTCATGGAGTCATGGGATATCGGAGAAATAATGTTGAGGAATTTGTAC
      M  R  L  K  F  M  E  S  W  D  I  G  E  I  M  L  R  N  L  Y

 721  ACCGAGAGAACATATACGCCAATAGCAACACCAGATGGTAGCGTCGTTAAGAAATTTAAA
      T  E  R  T  Y  T  P  I  A  T  P  D  G  S  V  V  K  K  F  K

 781  GGCAATAACAGTGGTCAACCTTCTACAGTTGTTGACAATACTATGATGGTTATGTTCACT
      G  N  N  S  G  Q  P  S  T  V  V  D  N  T  M  M  V  M  F  T

 841  ATGCAATACGCTCTGCTGAGAACAGGTGTATCTTTTGAAGATCAGAGTGATTCTATTGTG
      M  Q  Y  A  L  L  R  T  G  V  S  F  E  D  Q  S  D  S  I  V

 901  TACCTGGCGAACGGAGATGACTTACTAATCGCAGTTCGACCTGATCTTGAGAACATTTTG
      Y  L  A  N  G  D  D  L  L  I  A  V  R  P  D  L  E  N  I  L

 961  GATGATTTTCAAAGTATTTTTGATGAACTTGGGTTAAACTACGATTTTACGTCAAGGACA
      D  D  F  Q  S  I  F  D  E  L  G  L  N  Y  D  F  T  S  R  T

1021  CGAAGCAAGGAAGAAGTTTGGTTTATGTCTCACCGTGGGAAGTTAATAAACAATATGTAC
      R  S  K  E  E  V  W  F  M  S  H  R  G  K  L  I  N  N  M  Y

1081  ATCCCAAAACTTGAACCAGAAAGAATCGCCGCGATTCTTGAATGGGATAGGAGCACACTT
      I  P  K  L  E  P  E  R  I  A  A  I  L  E  W  D  R  S  T  L

1141  CCCGAAAATCGTCTTGAGGCAATTTGCGCAGCTATGATTGAAGCTTGGGGTTATAATGAT
      P  E  N  R  L  E  A  I  C  A  A  M  I  E  A  W  G  Y  N  D

1201  CTCATATACCACATTCGCAAATTCTATAAGTGGGTTCTAGAGCAACACCCTTACTCAGAA
      L  I  Y  H  I  R  K  F  Y  K  W  V  L  E  Q  H  P  Y  S  E

1261  CTAGTCGCACAAGGCAAAGCACCATACTTAGCAGAGACAGCTCTGACAAAGTTGTACACT
      L  V  A  Q  G  K  A  P  Y  L  A  E  T  A  L  T  K  L  Y  T

1321  GACGTCGACGCAAATGAACAGCAAATTGAAGAATATGTTAAGGCATTCGACAATATGCAT
      D  V  D  A  N  E  Q  Q  I  E  E  Y  V  K  A  F  D  N  M  H
```

```
1381  GATTTTAATCCTATAATCGATGTGCACTTTGAAGGAGATAGCGAGGACGCTGCAAGACTC
      D  F  N  P  I  I  D  V  H  F  E//G  D  S  E  D  A  A  R  L

1441  GCTAGAAATGCAACACGAGACAGAGACGTGACTGCTGGAACGAGTGGAACATTCACTGTG
      A  R  N  A  T  R  D  R  D  V  T  A  G  T  S  G  T  F  T  V

1501  CCAAGAATGAAACCTTTATCAAACAAGATGAAGTTACCAAAAGTGAATGGAACATCTATC
      P  R  M  K  P  L  S  N  K  M  K  L  P  K  V  N  G  T  S  I

1561  ATGAATCTGGATCATTTACTGGTTTATAAACCAGAACAAGAAAAATTGTTCAACACCATA
      M  N  L  D  H  L  L  V  Y  K  P  E  Q  E  K  L  F  N  T  I

1621  GCAACACAACAGCAATTTGAGCATTGGTTTGAACGACTCAAGGAATCATATGATAAGAAC
      A  T  Q  Q  Q  F  E  H  W  F  E  R  L  K  E  S  Y  D  K  N

1681  GATGAAGAAATGAAAATTATTTTAAACGGTCTTATGGTATGGTGCATTGAGAATGGAACT
      D  E  E  M  K  I  I  L  N  G  L  M  V  W  C  I  E  N  G  T

1741  TCTCCAAATTTAACAGGAAACTGGGTTATGATGGATGGAGAAGAGCAAGTGGAGTTTCCT
      S  P  N  L  T  G  N  W  V  M  M  D  G  E  E  Q  V  E  F  P

1801  TTGGCTCCAGTATTACAACACGCACAGCCTACATTTAGGCAAATCATGGCACATTTCAGT
      L  A  P  V  L  Q  H  A  Q  P  T  F  R  Q  I  M  A  H  F  S

1861  GACGCAGCCGAAGCGTACATTGTTATGAGAAATGCAAAGGAACCATACATGCCTAGGTAT
      D  A  A  E  A  Y  I  V  M  R  N  A  K  E  P  Y  M  P  R  Y

1921  GGAATTCGGCGAAATTTAACCGACAAGAGTTTGGCGAGGACAGCATTCGATTTTCACATT
      G  I  R  R  N  L  T  D  K  S  L  A  R  T  A  F  D  F  H  I

1981  AGTAAATCTGATACCTCGACACGCTCTCGAGAGGCTTCGATTCAAATGAAAGCAGCTGCA
      S  K  S  D  T  S  T  R  S  R  E  A  S  I  Q  M  K  A  A  A

2041  GTGAAAGGCAAATCAAGCAAGCTGTTTGGATTAGACGCGAACGTCGGAGCGAACGAAGAG
      V  K  G  K  S  S  K  L  F  G  L  D  A  N  V  G  A  N  E  E

2101  AACACAGAGAGACACACATCTGACGATGTGAACGCTGATATGCACAACTTAAATGGTGTG
      N  T  E  R  H  T  S  D  D  V  N  A  D  M  H  N  L  N  G  V

2161  CGATTTCATTAGTCTCTTATTTAAAATTACAAGCAATACAAATATATTATGTATGTTTTT
      R  F  H  *

2221  AAATAAAATATTGTGATCTCGAGTGAGCCTCTCACGCATGTTCACATTTAATTAAGTTAT

2281  TATTAAGTGTGGGCCTCCCACCTTCCTTTACGTCTTTAAATTAACGTTGCTTGTTGCTTT

2341  AGGTCGTCGTAGACTTCGTTCGGGAAGAGAATTGAGCTAAATGTGGTCTTACCATTGGGT

2401  TCAATTGGGCTTCCATTCACTCGCTTGTTTGCATGTTATTCGCAGCTAACTTAGTGTGGT

2461  TTTACCACGTGTGAATGGCGTCTGCATGTATGCAGGATGTTTGTGAGACA-poly(A)
```

Fig. 5.2. Nucleotide sequence of cDNA covering the 3'-terminal region of ISMV RNA. The sequence of the viral strand is given, numbered from the 5' terminus of the cDNA. The deduced amino acid sequence of the ORF is shown below the DNA sequence in single-letter code. The termination codon is indicated by an asterisk. The proposed site at which the coat protein is cleaved off from the polyprotein is indicated by //. Regions underlined were used for the design of primers.

The degenerate last nucleotide of the oligo(dT) primer was in all four clones recovered as a C residue, suggesting that this indeed is the last residue before the start of the poly(A) tail. The 2510 nucleotides long sequence of the 3' terminal

part of the ISMV genome is presented in Fig. 5.2, and has been filed in the EMBL, GenBank and DDJB Nucleotide Sequence Databases under the accession number X75939. It contains one open reading frame corresponding with the C terminal 723 amino acids of the viral polyprotein and a 3' non-coding sequence of 340 nucleotides before the start of the poly(A) tail. Comparison of the open reading frame in ISMV RNA with those of other potyviruses allowed the precise identification of the NIb and coat protein cistrons.

Coat protein cistron

The cleavage site between the NIb peptide and the coat protein was deduced from similarities found in other potyvirus sequences in this area of the genome. The conservation of a valine and a phenylalanine residue at positions P2 and P4 (Johansen *et al.*, 1991) suggests that the cleavage site in ISMV is between the glutamic acid and glycine residues at positions 471 and 472 respectively.

The conserved core of the coat protein starts 15 residues from the amino terminus showing the D-R/K/Q-D-V motif which is the proposed cleavage site of a trypsin-like proteinase (Shukla *et al.*, 1988). In comparison with other potyviruses this N-terminus is the shortest found so far (Table 5.1). The MW of the N-terminus is 1,953. The length of the C-terminus starting with TERH is 22 residues.

The overall similarity of the core with those of other potyviruses is 53-58% for nucleotides and 63-72% at the amino acid level, which values are usually found between distinct potyviruses (Shukla and Ward, 1988; 1989).

The predicted coat protein sequence is 252 amino acids long with a calculated MW of 29,822 which is in good accordance with the size determined on SDS-PAGE gels (Alper *et al.*, 1984). The finding of a 2 kD smaller product on SDS-PAGE gels (data not shown) corresponds exactly with a possible proteolytic degradation of the N-terminus part of the coat protein. A DAG-like motif normally found in the N-terminus of aphid borne potyviruses (Harrison and Robinson, 1988; Atreya, 1991) is not present.

Phylogenetic comparison of the CP of ISMV with other potyviral CP's

A phylogenetic analysis of the relatedness of ISMV and other potyviruses based on the core part of the coat protein sequences as described by Shukla and Ward (1989) and performed as described by Dekker *et al.* (1993) is shown in Fig. 5.3. The tree agrees in broad details with that of Dekker *et al.* (1993) and Burger *et al.* (1990). It reveals that ISMV is a taxonomically distinct potyvirus which has no specific relation with other bulb infecting potyviruses, such as lily mottle virus

(LMoV) or *Ornithogalum* mosaic virus (OrMV) or to other specifically monocotyledons infecting potyviruses, such as sugarcane mosaic virus strains (SCMV-MDB; SCMV-SC) or Johnson grass mosaic virus (JGMV).

Potyvirus	N-terminus of coat protein (amino acids)
ISMV	15
OrMV	20
TEV-NAT	27
TEV-HAT	27
SbMV N	28
TVMV	28
PVY-PepMo	30
PVY-N	30
PWV-S	32
ClYVV-N	35
BYMV-GDD	37
LMoV	38
LMV	40
ZYMV	42
WMV II	44
PRSV-W	51
TuMV	51
JGMV	67
SCMV-SC	75
SCMV-MDB	90
PPV-D	93

Table 5.1. Comparison of N-termini lengths of the coat protein (N-CP) of the listed potyviruses. Sequence data used were from : soybean mosaic virus (SbMV)-N (Eggenberger *et al.*, 1989); watermelon mosaic virus (WMV) II and ZYMV (Quemada *et al.*, 1990b); passionfruit woodiness virus (PWV)-S (Shukla and Ward, 1989); JGMV (Shukla *et al.*, 1987); pepper mottle virus (PVY-PepMo;Dougherty *et al.*, 1985); PVY-N (Robaglia *et al.*, 1989); lettuce mosaic virus (LMV;Dinant *et a.*, 1991); PRSV-W (Quemada *et al.*, 1990a); TEV-NAT (Allison *et al.*, 1985); TEV-HAT (Allison *et al.*, 1986); PPV-D (Ravelonandro *et al.*, 1988); turnip mosaic virus (TuMV; Tremblay *et al.*, 1990); LMoV (Langeveld *et al.*, 1991); OrMV (Burger *et al.*, 1990); bean yellow mosaic virus (BYMV-GDD; Hammond and Hammond, 1989); clover yellow vein virus (ClYVV)-N (Bryan *et al.*, 1992); SCMV-MDB and SCMV-SC (Frenkel *et al.*, 1991) and TVMV (Domier *et al.*, 1986).

The polymerase or NIb protein gene

The putative polymerase sequence as deduced from the ISMV RNA sequence was aligned with those of 4 dicot and 1 monocot infecting potyviruses (Fig. 5.4). The overall similarity of the NIb sequence of ISMV with other potyviruses is 71-77% for amino acids and 59-62% for nucleotides. Several specific, highly conserved sequence motifs found in the putative RNA-dependent RNA polymerases of positive-strand RNA viruses numbered I to VIII have been described by Koonin (1991) and are indicated in Fig. 5.4. All these motifs have been found in the NIb protein sequence. Region VI consists of the GDD motif, a diagnostic motif for RNA-dependent RNA polymerases. In the NIb protein of potyviruses additional regions of homology are apparent, which are also found in the ISMV protein : between motifs IV and V : 64% identity (Fig. 5.4, region 252-307) and a region of 37 amino acids downstream of motif VIII : 54% identity (Fig.

5.4, region 423-459). These conserved stretches may be specific to potyvirus RNA-dependent RNA polymerase.

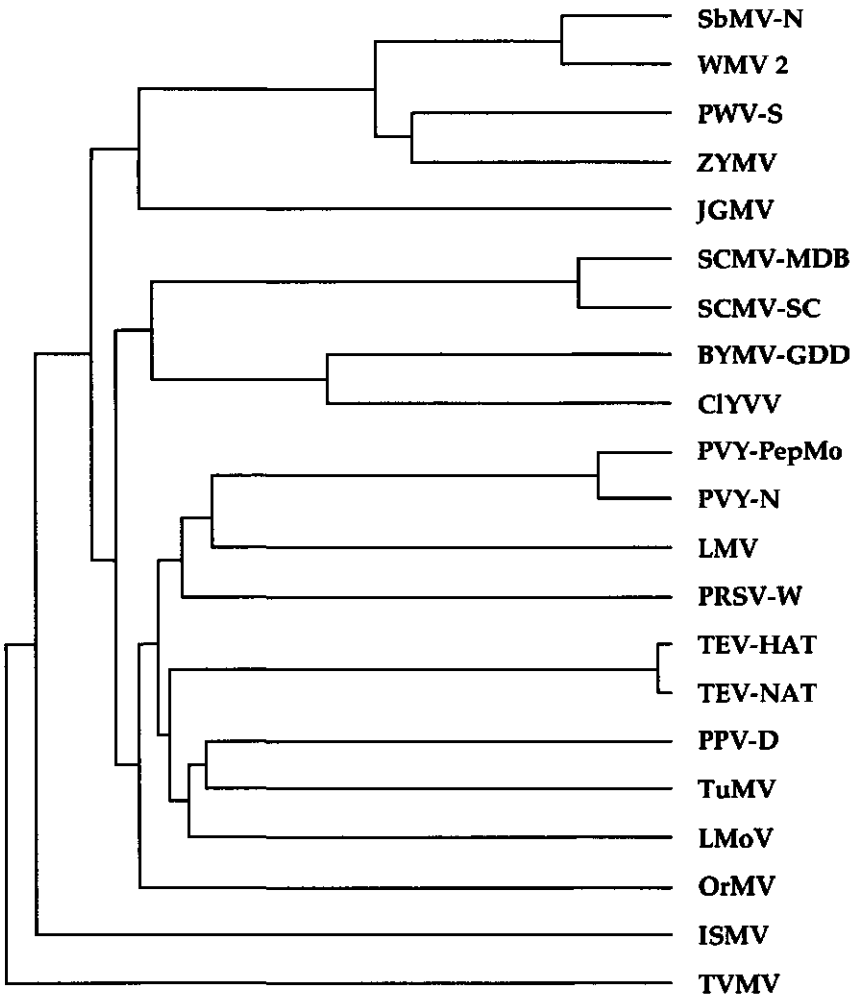

Fig. 5.3. Dendrogram based on the amino acids of the core region in the coat protein of 21 different potyviruses and computed according to the UPGMA clustering algorithm (Dekker *et al.*, 1993). Sequence data used as indicated in Table 5.1.

DISCUSSION

The sequence data obtained and described in this paper reveal that the ISMV isolate studied here is a distinct potyvirus with no special relationship to other

monocotyledon-infecting potyviruses.

The finding that an aphid-transmissibility motif (DAG, NAG or KAG, Atreya *et al.*, 1991) is missing in the CP-sequence would predict that no spread would occur in the field. The ISMV isolate used was maintained in crocus stocks for at least 10 years. Hence it is possible that this isolate has lost its aphid transmissibility. Such a loss of transmissibility by DAG-motif deletion was found for the PPV-NAT (Maiss *et al.*, 1989) and the PPV-AT (Laín *et al.*, 1989) which showed a difference in N-terminus length of 15 amino acids lacking in the PPV-NAT strain compared to the PPV-AT strain. Inspection of the N-terminus of ISMV-CP reveals a DAA sequence. It is tempting to assume that this sequence has been derived from a single point mutation. However ISMV can be spread by aphids, among crocus and iris stocks (C.J. Asjes, personal communication) though the spread from crocus can be minimal. Iris stocks on the field are infected with IMMV at high frequencies, a potyvirus which is much more contagious than ISMV. It might be possible that a non-aphid transmissible ISMV isolate from iris becomes aphid-transmissible by (partial) transencapsidation using IMMV CP subunits similar as found for the aphid transmissible papaya ringspot virus type W (PRSV-W) and a non-aphid transmissible strain of zucchini yellow mosaic virus (ZYMV; Bourdin and Lecoq, 1991). Indeed, antisera directed against ISMV or IMMV particles and only reacting with the homologous virus, showed partially decorated particles in material infected with both IMMV and ISMV using electron-microscopy (A.F.L.M. Derks, personal communication).

Based on homology in the surroundings of the CP-NIb proteolytic cleavage site (valine (V) at position P4, phenylalanine (F) at position P2) found for several potyviruses (Johansen *et al.*, 1991), a putative E/G cleavage site is proposed at this position in the ISMV polyprotein (Fig. 2). An E/G-cleavage site has not yet been reported for the NIb/CP junction, but was identified as such between the 6kD and the 49kD-Pro of pea seedborne mosaic virus (PSbMV) by Johansen *et al.* (1991) and as internal 49kD-Pro cleavage site. The glutamic acid (E) at position P1 was shown to occur in several picornavirus cleavage sites (Palmenberg, 1990). The most common sites for the picornaviral encoded 3C proteinase are Q/E-G/S/A. This supports the evidence that the potyvirus proteinase is also evolutionarily related with the picornaviruses, being part of the supergroup of picorna-like viruses (Goldbach, 1987; Domier *et al.*, 1987; Matthews, 1991).

Alignment of the coat protein sequences of 21 potyviruses reveals that except for the N-terminal part, the overall similarity is high, with a highly conserved LNGMVWCIENGTSP stretch in the core of the coat proteins. The host range of

```
                                1                                         32
TEV          fqpvkeatql  mn.elvysq//g  ekrkwvveal  sgnlrpvaec  psqlvtkhvv
PPV-NAT      fticklltdl  dg.efvytq//s  ktthwlrdrl  egnlkavgac  pgqlvtkhvv
TVMV         fmakktvaai  md.dlvrtq//g  ekrkwmleaa  htniqpvahl  qsqlvtkhiv
PVY-N        fkttklvqdl  idhdvvveq//a  khsawmfeal  tgnlqavatm  ksqlvtkhvv
OrMV         fqavkevsal  fs.davyeq//g  qevgwlfrel  kdnlkavavl  pnqlvtkhvv
ISMV         ..........  ..........//.  ..........  ..........  ..........
Consensus    ----------  ----------//-  ----------  ----------  ----------

             33                                                        82
TEV          kgkcplfely  l..qlnpEke  aYFkpMmgaY  kpSrLnreAF  lKDilKYase
PPV-NAT      kgkctlfety  l..lthpEeh  eFFrpLmgaY  qkSaLnkdAY  vKDlmKYskp
TVMV         kgrckmfaly  l..qenaDar  dFFksFmgaY  gpShLnkeAY  iKDimKYskq
PVY-N        kgecrhftef  ltvdaeaEae  aFFrpLmdaY  gkSlLnrdAY  iKDimKYskp
OrMV         kgpcqcfiqy  l..nespEas  aFFkpLmgqY  gkSiLskeAF  vKDimKYskp
ISMV         ..........  .......Esk  aFFepLcgfY  gkSnLnkaAC  vKDftKYase
Consensus    ----------  ----------  --F------Y  --S-L---A-  -KD--KY---

             83                                                       132
TEV          IeiGnVDcdl  Lelaismlvt  klkalGfptv  nYItDpeeIf  saLNMkAAmG
PPV-NAT      IvvGaVDcdq  Feravdvvis  mliskGfeec  nYVtDpddIf  saLNMkAAvG
TVMV         IvvGsVDcdt  Fesslkvlsr  kmkewGfenl  eYVtDeqtIk  naLNMdAAvG
PVY-N        IdvGvVDrmh  Lrkpsiglss  tcnvhGfkkc  aYVtDeqeIf  kaLNMkAAvG
OrMV         IvlGeVDfik  Feegynnvlr  mfhdiGfekc  eYVtDsmeVy  knLNLkAAvG
ISMV         IvvGkVDvdv  Fdrafqntyt  ilsraGikec  nYVnDtteIf  nsLNMkAAvG
Consensus    I--G-VD---  ----------  -----G----  -Y--D-----  --LN--AA-G

             133                                                      182
TEV          AlYkgkKkea  Lseltldeqe  amlkaSClRL  YtgklGIWNg  SLKAELRpiE
PPV-NAT      AlYsgkKrdy  Fenvsdqdke  sfvraSCkRL  FmgkkGVWNg  SLKAELRpkE
TVMV         AlYsgkKkqy  Fedlsddava  nlvqkSClRL  FknklGVWNg  SLKAELRpfE
PVY-N        AsYgckKkdy  Fehftdadke  eivmqSClRL  YkgllGIWNg  SLKAELRckE
OrMV         AmYtgkKqqy  Fegmsedeih  qlviaSCfRL  WsgkfGVWNg  SLKAELRplE
ISMV         AlYggqKkdy  Fahytdedke  niikeSCeRL  FlgklGIWNc  SLKAELRskE
Consensus    A-Y---K---  ----------  -----SC-RL  -----G-WN-  SLKAELR--E
                                                             ------I---

             183                                                      232
TEV          KvennKTRtF  TAAPiDTLLa  gKVCVDDFNn  qFYdlnikap  WtVGmtKFyq
PPV-NAT      KveanKTRsF  TAAPiDTLLg  gKVCVDDFNn  qFYslnlhcp  WsVGmtKFrg
TVMV         KlienKTRtF  TAAPiETLLg  gKVCVDDFNn  hFYskhiqcp  WsVGmtKFyg
PVY-N        KilanKTRtF  TAAPlDTLLg  gKVCVDDFNn  qFYskniecc  WtVGmtKFyg
OrMV         KvqacKTRtF  TAAPlDTLLg  aKVCVDDFNa  qFYdkhltap  WtVGicKYyk
ISMV         KieqnKTRtF  TAAPlETLLg  gKVCVDDFNn  qFYecnlrgp  WtVGisKFyg
Consensus    K----KTR-F  TAAP--TLL-  -KVCVDDFN-  -FY-------  W-VG--K---
             -     ----  -------II-  -------                   -----III-

             233                                                      282
TEV          GWneLMeaLP  sgWvYCDADG  SqFDSSLtPF  LINaVLkvRl  afMEeWDIGe
PPV-NAT      GWdkLLkaLP  egWiYCDADG  SqFDSSLsPY  LINaVLniRl  afMEeWDIGe
TVMV         GWneLLgkLP  dgWvYCDADG  SqFDSSLsPY  LINaVLrlRl  ssMEeWDVGq
PVY-N        GWdkLLrrLP  enWvYCDADG  SqFDSSLtPY  LINaVLtiRs  tyMEdWDVGl
OrMV         GWdtFMnkLP  egWlYCDADG  SqFDSSLtPF  LINsVLrlRl  efMEdWDIGa
ISMV         GWnrMLqqLP  dgWiYCDADG  SrFDSSLtPF  LINaVLrmRl  kfMEsWDIGe
Consensus    GW------LP  --W-YCDADG  S-FDSSL-P-  LIN-VL--R-  --ME-WD-G-
             ---------  --IV-----
```

```
            283                                                     332
TEV         qMLrNLYTEi  vYTPIlTPDG  tIIKKhkGNN  SGQPSTVVDN  tLMViiaMlY
PPV-NAT     qMLsNLYTEi  vYTPIaTPDG  tIVKKfkGNN  SGQPSTVVDN  tLMVilaMtY
TVMV        kMLqNLYTEi  vYTPIsTPDG  tIVKKfkGNN  SGQPSTVVDN  tLMVvlaMyY
PVY-N       qMLrNLYTEi  vYTPIsTPDG  tIVKKfrGNN  SGQPSTVVDN  sLMVvlaMhY
OrMV        rMLsNLYTEi  iYTPIaTPDG  tVVKKfrGNN  SGQPSTVVDN  tLMVvlaMnY
ISMV        iMLrNLYTEr  tYTPIaTPDG  sVVKKfkGNN  SGQPSTVVDN  tMMVmftMqY
Consensus   -ML-NLYTE-  -YTPI-TPDG  ---KK--GNN  SGQPSTVVDN  --MV---M-Y
                                          -----  ----V-----  ----------

            333                                                     382
TEV         tcekcginke  e....ivYYv  NGDDLliAIh  Pdk.aerlsr  FkesFgeLGL
PPV-NAT     sllklghhpd  thdcicrYFv  NGDDLvlAVh  Pay.esiyde  LqehFsqLGL
TVMV        alsklgvdin  sqedvckFFa  NGDDLiiAIs  Pel.ehvldg  FqqhFsdLGL
PVY-N       alikecvefe  eidstcvFFv  NGDDLliAVn  Pek.esildr  MsqhFsdLGL
OrMV        alaklsipye  emdsrirYFa  NGDDLlvAVe  Ptkggeilds  LqasFseLGL
ISMV        allrtgvsfe  dqsdsivYLa  NGDDLliAVr  Pdl.enildd  FqsiFdeLGL
Consensus   ----------  ----------  NGDDL--A--  P---------  ----F--LGL
                           ---  -VI----

            383                                                     432
TEV         kYeFdctTrd  KtqlwFMSHr  alerdgmYIP  KleeERIvsI  LeWDRskePs
PPV-NAT     nYtFttkTen  KeelwFMSHk  gvlyddmYIP  KlepERIvsI  LeWDRsnePi
TVMV        nYdFssrTrd  KkelwFMSHr  alskdgiLIP  KlepERIvsI  LeWDRsaePh
PVY-N       nYdFssrTrr  KeelwFMSHr  glliegmYVP  KleeERIvsI  LqWDRadlPe
OrMV        iYdFndrTfd  KtqlsFMSHq  alwdgdmFIP  KikqERVvsI  LeWDRstqPe
ISMV        nYdFtsrTrs  KeevwFMSHr  gklinnmYIP  KlepERIaaI  LeWDRstlPe
Consensus   -Y-F---T--  K----FMSH-  ---------P  K---ER---I  L-WDR---P-
                         ---VII--  --      ---  -VIII-----

            433                                                     482
TEV         hRlEAICAsM  IEaWGYdkLv  eeIRnFYaWv  leQaPYsqla  eeGkapYlae
PPV-NAT     hRlEAICAsM  VEaWGYkeLl  reIRkFYsWv  leQaPYnals  kdGkapYiae
TVMV        hRlEAICAsM  IEaWGYtdLl  qnIRrFYkWt  ieQePYrsla  eqGlapYlse
PVY-N       hRlEAICAaM  IEsWGYseLt  hqIRrFYsWl  lqQqPFatia  qeGkapYias
OrMV        hRiEAVCAaM  IEaWGYpeLl  qeIRkFYaFm  vtQePYsaih  aqGktrYise
ISMV        nRlEAICAaM  IEaWGYndLi  yhIRkFYkWv  leQhPYselv  aqGkapYlae
Consensus   -R-EA-CA-M  -E-WGY--L-  --IR-FY---  --Q-P-----  --G---Y---

            483
TEV         tALkfLYtsq  hgtnseieeY  lkvlydydip  ttenlyfq//
PPV-NAT     tALkkLYtdt  easeteierY  leafydnind  dgesnvvvhq//
TVMV        vALrrLYtsq  iatdneltdY  ykeilan ne  firetvrfq//
PVY-N       mALrkLYmdr  avdeeelraF  temmvaldde  feldsyevhh  q//
OrMV        rALvtLYkde  kvvlsdigpY  iqklaemslg  cvdevvmhq//
ISMV        tALtkLYtdv  daneqqieeY  vkafdnmhdf  npiidvhfe//
Consensus   -AL--LY---  ----------  ----------  ----------
```

Fig. 5.4. Protein sequence alignment of the NIb protein, the putative RNA-dependent RNA polymerase of 4 mainly dicotyledonous, tobacco vein mottling virus (TVMV), plum pox virus (PPV)-NAT, tobacco etch virus (TEV), potato virus Y (PVY)-N and 2 monocotyledonous, ISMV and OrMV, infecting potyviruses. Sequence data used were from TVMV (Domier *et al.*, 1986), TEV (Allison *et al.*, 1986), PPV-NAT (Maiss *et al.*, 1989), PVY-N (Robaglia *et al.*, 1989) and OrMV (Burger *et al.*, 1991). Gaps were introduced in the N-termini of the NIb for optimal alignment. Conserved amino acids are shown in upper case on the last row. // indicates the putative sites at which the NIb is cleaved off from the polyprotein. Conserved amino acids are shown in upper case and identical amino acids are shown in the consensus row. The eight conserved motif regions according to Koonin (1991) are indicated with Roman numbers.

ISMV comprises only the monocotyledonous Iridaceae, and it would be of interest to analyse whether this limited host range would be reflected by specific coat protein adaptations. The tree of Fig. 5.3 remarkably shows no specific relationship of ISMV with other bulbous crop or monocot-infecting potyviruses. It is concluded that the host-specificity of ISMV is not reflected in the specific core of its CP.

ACKNOWLEDGEMENTS

We thank Dr. A.F.L.M. Derks and Dr. P.M. Boonekamp for stimulating discussions, Dr. H.J.M. Linthorst and Dr. R.A.A. van der Vlugt for helpful advice.

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CHAPTER 6

Stress-induced metabolic activity is correlated with the multiplication of iris severe mosaic virus in the iris bulb*

SUMMARY

In earlier work it was found that after wounding or high-temperature stress, iris severe mosaic virus (ISMV) detection increased in iris bulbs. It was hypothesised that the multiplication was enhanced by increased metabolic activity after such stress. Metabolic activity, measured as oxygen uptake after wounding, high-temperature or ethylene treatment was studied to search for a possible correlation between metabolism and ISMV multiplication. An increased level of total oxygen uptake was found after all treatments. This correlated with the increase of ISMV detection. After wounding and ethylene treatment all measured respiratory pathways were enhanced, while after high-temperature treatment mainly an increase in residual respiration could be demonstrated.

These findings suggest that an increase in production of metabolic intermediates, rather than an increase in energy production, is important for the observed stress-induced multiplication of ISMV in iris bulbs.

* This chapter has been submitted as : Van der Vlugt, C.I.M., Gude, H., Goldbach, R.W., and Boonekamp, P.M. Stress-induced metabolic activity is correlated with the multiplication of iris severe mosaic virus in the iris bulb.

INTRODUCTION

The concentration of iris severe mosaic potyvirus (ISMV) in freshly-lifted iris bulbs usually remains below the detection limit of ELISA (Van der Vlugt *et al.*, 1988; 1993a; 1993b). Serological detection of the virus was improved during recovery after stress treatments such as wounding or high-temperature. Enhanced detection coincided with an increase of viral RNA levels. Thus it was shown that the improved virus detection was due to multiplication of the virus after stress treatment (Van der Vlugt *et al.*, 1993b). The phenomenon of an initially low concentration of virus in storage organs of bulbous crops and an improved detection after wounding was also found for bean yellow mosaic virus in gladioli corms (Stein *et al.*, 1986; Vunsh *et al.*, 1991).

To understand the relation between ISMV replication and stress, knowledge of the physiological changes in the iris bulb is required. It is hypothesised that the observed virus multiplication is enabled by an increased metabolic activity which is a consequence of stress. As a parameter for an activated metabolism, the oxygen uptake of the tissue can be measured. In iris three respiratory pathways occur, the energy yielding cytochrome pathway and the non-energy yielding alternative pathway in the mitochondria, and the so-called residual respiration in the cytoplasm. These pathways are related to each other as indicated in Fig. 6.1 (Marissen, 1989) and differ in production of energy.

Total respiration in plant tissue : $V_{tot} = V_{cyt} + \rho\, V_{alt} + V_{res}$
Mitochondrial respiration : $V_{tot'} = V_{tot} - V_{res} = V_{cyt} + \rho V_{alt}$

Fig. 6.1. V_{tot} is the total uninhibited respiration rate. V_{cyt} is the capacity of the cytochrome path. V_{alt} is the capacity of the alternative path, the factor ρ represents the fraction of the alternative pathway which is operative in uninhibited respiration ($0 \leq \rho \leq 1$). V_{res} represents the residual respiration outside the mitochondria (after Marissen, 1989).

It was investigated whether multiplication of the virus can be correlated to a specific pathway.

Ethylene is known to trigger wound or other stress responses (Yang and Pratt, 1978; Yang and Hoffman, 1984). One of the wound responses, an increase in respiration, can be evoked in iris bulbs by applying ethylene without actual wounding (Kamerbeek and Verlind, 1972). Moreover, ethylene is able to replace the high-temperature treatment used for induction of flower formation (Schipper, 1982; De Munk and Duineveld, 1986).

In this study we examined whether i) the enhanced detection by wounding and high-temperature stress is correlated with increased respiratory activity, ii) multiplication of the virus can be linked with a specific pathway of the increased respiration, iii) ethylene acts as an intermediate between stress on the one hand and respiration and virus multiplication on the other hand.

MATERIALS AND METHODS

ELISA

To detect ISMV in samples of iris bulbs ELISA was used according to Clark and Adams (1977) with some minor modifications as described by Van der Vlugt *et al.*, (1988, 1993a). Bulbs were scored reliably as infected, when A405 $\geq$ 0.15 (Van der Vlugt *et al.*, 1988). Samples were taken as indicated in Fig. 6.2.

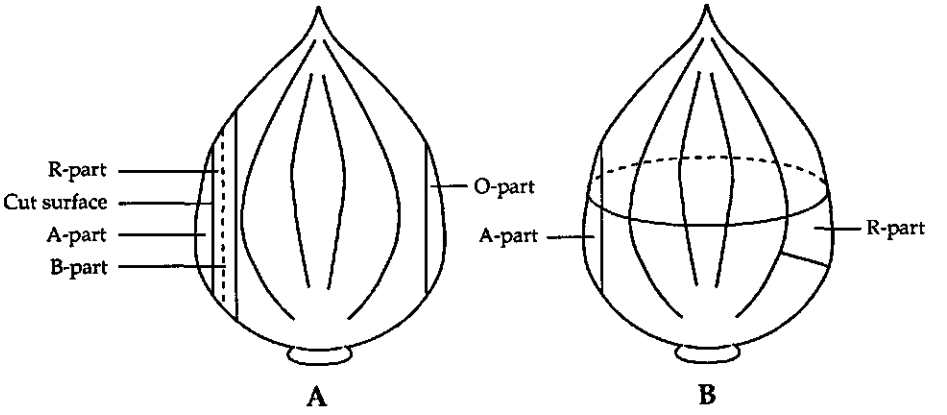

Fig. 6.2. Schematic view of an iris bulb with sampled areas, A, B, O or R in experiments with wounding (6.2A), 40°C or ethylene treatment (6.2B). Part R was used for respiration measurements, part A (40°C or ethylene treatment) or part B and O (wounding treatment) for testing in ELISA.

Bulb material

ISMV-infected and healthy stocks of *Iris x hollandica* cv. Professor Blaauw were acquired and grown in the field under a gauze and harvested at the end of August as described by Van der Vlugt *et al.*, (1988, 1993a).

Bulb treatments

Freshly harvested bulbs were stored in different lots at 30°C for circa 4 weeks.

At the end of the 30°C treatment, some lots were subjected to an additional treatment at 40°C or wounded by cutting a piece from the bulb as indicated in Fig. 6.2 and as described by Van der Vlugt *et al.*, (1988, 1993a). During treatment at 30°C, other lots obtained an additional treatment with 500 ppm ethylene at day 28 or 29 (for 24 or 48 hours) by placing the bulbs temporarily in a desiccator. At the end of the treatments all bulbs were transferred to 17°C and the respiration (O_2-uptake) was measured during this 17°C storage. The same bulbs were also tested for virus in ELISA .

Respiration measurements *in vivo*

Measurements were performed mainly as developed and described by Marissen (1989). For *in vivo* measurements parts of the outer scale of a bulb were used after removal of the tunica. To obtain a sample for respiration measurements from the wounded bulbs, dried tissue was removed and a 2-3 mm thick piece of tissue of about 1.0 g was sliced from the outer scale at the cut-surface area (R-part) and from the opposite (O)-part (Fig. 6.2A). From bulbs treated at 30°C and 40°C, a square piece of tissue of about 1.0 g (R-part, as indicated in Fig. 6.2B) was used for respiration measurements.

The bulb tissue was weighed, cut into circa 30 pieces and washed in a sieve under tap water for about 1 min, to remove loosened cell wall peroxidases, which may interfere with respiration measurements (Van der Plas *et al.*, 1987). Bulb pieces were dried with tissue paper and placed in 5 ml distilled water in a 25°C thermostated reaction vessel. The vessel was closed with a plug in which an YSI O_2-electrode, model 53, was fitted. Total uninhibited respiration (V_{tot}) was measured in distilled water. To distinguish between different respiration pathways (Fig. 6.1), inhibitors were used. To inhibit the cytochrome pathway, sodium azide (NaN_3, final concentration 5 mM) was added (Marissen, 1989). The remaining O_2 uptake consisted of the capacity of the alternative pathway (V_{alt}) and residual respiration (V_{res}). By subsequent addition of salicylhydroxamate (SHAM, final concentration 3 mM), inhibiting the alternative respiration (Marissen, 1989), the V_{res} was measured. The V_{alt} was calculated by subtraction of V_{res} from the NaN_3 inhibited respiration (Marissen, 1989; Fig. 6.1). Mitochondrial respiration (V_{tot}' or $V_{cyt} + \rho V_{alt}$) was calculated by subtraction of V_{res} from V_{tot} (Fig. 6.1). By this procedure only the *sum* (V_{tot}') of the *in vivo* cytochrome and the alternative pathway activities was determined, not the actual activities of the separate pathways. (Accurate determinations of V_{cyt} and ρV_{alt} in iris bulb tissue are impossible because of side effects of the alternative pathway inhibitors on the cytochrome pathway, Marissen, 1989).

At the start of each measurement and also immediately after an inhibitor had been added, the solution in which the bulb respiration was to be measured was aerated to achieve 100% air saturation (250 nmol $O_2.ml^{-1}$ at 25°C). Oxygen consumption of tissue pieces is expressed as nmol $O_2.min^{-1}.gFW^{-1}$.

RESULTS

Respiration and ISMV detection in wounded bulbs during recovery

Within 1 week after cutting and incubation at 17°C, total uninhibited respiration (V_{tot}), increased in tissue pieces from the wounded area of bulbs, and remained at a high level for several weeks (Fig. 6.3A). Detection of ISMV started to increase after the first week of incubation (Fig. 6.3B). All respiratory pathways contributed to the averaged six-fold rise in total respiration (Table 6.1). V_{tot}', the sum of the cytochrome and alternative path activities ($V_{cyt} + \rho V_{alt}$) increased 5-6 fold, while residual respiration increased 6-7 fold (Table 6.1).

Table 6.1. Mean (nmol $O_2.min^{-1}.gFW^{-1}$ ± S.E.) total uninhibited respiration (V_{tot}), capacity of the alternative pathway (V_{alt}), residual respiration (V_{res}) and mitochondrial respiration (V_{tot}') in the R-part of : 1. cut and control bulbs over the period from 5 days to 28 days after the wounding treatment and subsequent transfer to the 17°C, 2. 40°C treated and control bulbs over the period from 7 days to 28 days after the 40°C treatment and transfer to the 17°C, 3. ethylene (48 h) treated and control bulbs over the period from 7 days to 28 days after the 48 h ethylene treatment and transfer to the 17°C. Significance (Student's t-test) *1: $P \leq 0.003$; *2: $P \leq 0.590$; *3: $P \leq 0.003$; *4: $P \leq 0.974$; *5: $P \leq 0.073$; *6: $P \leq 0.002$; *7: $P \leq 0.046$; *8: $P \leq 0.166$.

Respiratory pathway	V_{tot}	V_{alt}	V_{res}	V_{tot}'
Cut	66.0 ± 2.9	14.7 ± 1.6	36.7 ± 2.3	29.3 ± 1.6
Control	11.1 ± 0.9	3.7 ± 0.7	5.5 ± 0.8	5.6 ± 0.5
40°C	11.3 ± 1.2*1	3.4 ± 0.6*2	7.9 ± 1.2*3	3.5 ± 0.5*4
Control	7.0 ± 0.6	3.0 ± 0.5	3.6 ± 0.5	3.5 ± 0.6
Ethylene	10.2 ± 1.6*5	4.1 ± 0.6*6	5.4 ± 0.9*7	5.0 ± 0.7*8
Control	6.9 ± 0.5	1.7 ± 0.2	3.1 ± 0.3	3.8 ± 0.2

The capacity of the alternative pathway was three times higher after wounding. Similar results were found in a second experiment in another year. From ELISA and RNA-hybridisation experiments it is known that ISMV titres are only enhanced in the wounded area and not in opposite parts of the bulbs (Van der Vlugt *et al.*, 1988; 1993b). It was found that after wounding, respiration

only increased in the wounded area but not in the opposite area of this bulb or in uncut bulbs (Table 6.2).

Respiratory pathway	V_{tot}
R-part of cut bulb	71.4 ± 4.5
O-part of cut bulb	15.4 ± 0.6
R-part of uncut bulb	15.4 ± 2.5

Table 6.2. Mean total uninhibited respiration (V_{tot}; nmol O_2 .min^{-1}.gFW^{-1} ± S.E.; n=2 to 5) in the cut surface area (R-part) of cut and uncut bulbs (see Fig. 6.2) and in the opposite area (O-part) of cut bulbs, 7 to 18 days after cutting and transfer to the 17°C.

Respiration and ISMV detection of 40°C treated bulbs during recovery

Respiration was measured after the stress treatment at 40°C and during the subsequent 17°C incubation. The V_{tot}, total uninhibited respiration, increased already during the stress treatment and remained at the same level during the next four weeks after transfer of the bulbs to the 17°C (Fig. 6.4A). The V_{tot} had increased almost two-fold (Table 6.1). The detection of ISMV by ELISA clearly improved after 2 weeks of incubation at 17°C (Fig. 6.4B). From Table 6.1 it can be concluded that the rise in residual respiration after the 40°C treatment completely accounted for the rise in total respiration. V_{tot}' nor V_{alt} were affected by this treatment.

Effect of exogenous ethylene on respiration and detection

The role of ethylene in the enhancement of virus detectability and respiration was studied by applying exogenous ethylene. Directly after ethylene application for 24 or 48 h the respiration was enhanced. Only when ethylene was applied for 48 h, this response continued during the first week of the 17°C incubation and then declined to the control level in the second week (Fig. 6.5A). After 4 weeks at 17°C, the total respiration in the treated bulbs showed a gradual increase. Both the residual respiration and the *sum* of the cytochrome and alternative pathway activities (V_{tot}') contributed to the averaged 1.5 fold increase in total respiration (Table 6.1). The capacity of the alternative pathway showed a 2.5 fold increase. Detection of ISMV by ELISA showed only a slight increase during recovery at 17°C in the ethylene treated bulbs after 4 to 6 weeks (Fig. 6.5B).

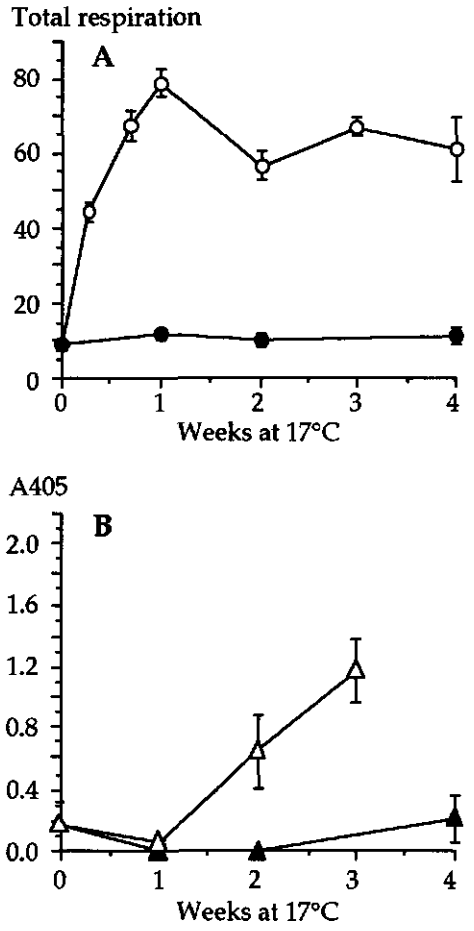

Fig. 6.3. A: Total respiration (nmol $O_2.min^{-1}.gFW^{-1}$) in the R-part of wounded (—○—) and control (—●—) iris bulbs during the 17°C recovery period after wounding (mean (n=2 to 5, usually 3) and S.E. are indicated).
B: Detection of ISMV by ELISA in the B-part of these wounded (—△—) and control (—▲—) iris bulbs (mean A405 (n=5) and S.E. are indicated). Bulbs were stored 30 days at 30°C prior to wounding.

Respiration correlates with ISMV detectability

Combining the respiration data and the virus-detection data of the wounding, the 40°C and ethylene experiments in one figure (Fig. 6.6), reveals that a correlation exists between metabolic activity (expressed as cumulative oxygen uptake) and virus detection.

DISCUSSION

Metabolic intermediates might play a main role in virus multiplication

It was expected that the increase in total oxygen uptake would consist of an enhancement of especially the mitochondrial respiration (V_{tot}') as this supplies

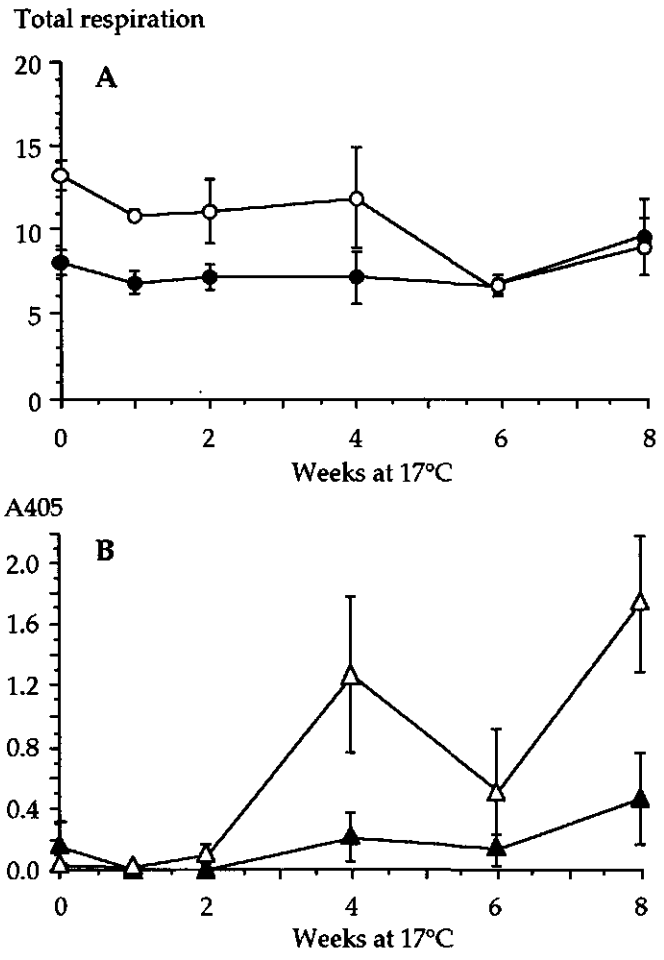

Fig. 6.4. A: Total respiration (nmol O_2 .min^{-1}.gFW^{-1}) in the R-part of iris bulbs stored for 24 days at 30°C and 6 days at 40°C (—○—) or 30 days at 30°C (—●—) (mean (n=2 to 5, usually 3) and S.E. are indicated). B: Detection of ISMV by ELISA, in the A-part of these 40°C (—△—) and 30°C control (—▲—) iris bulbs (mean A405 (n=5) and S.E. are indicated). After these temperature treatments, bulbs were transferred to 17°C and samples were tested during this recovery period.

energy and metabolites. For the wounding treatment indeed such an increased mitochondrial respiration was found. However, after the 40°C treatment the increase in total respiration was caused by an increase in the residual respiration, instead of the expected increase in energy yielding mitochondrial respiration. Still, the virus detection was enhanced. Therefore it might be concluded that an

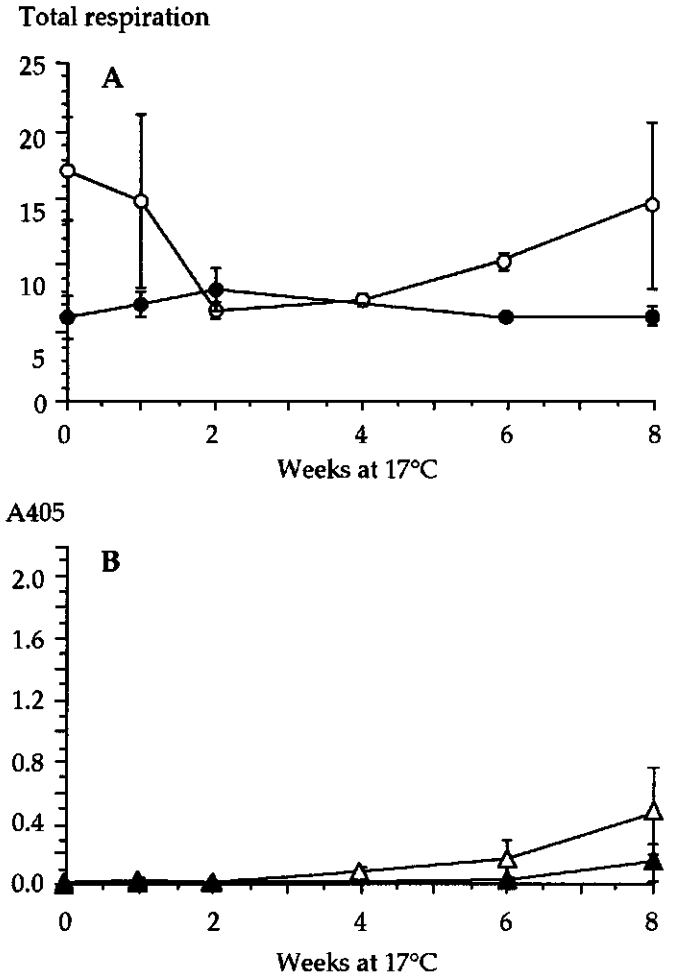

Fig. 6.5. A: Total respiration (nmol O_2 .min^{-1}.gFW^{-1}) in the R-part of bulbs stored for 30 days at 30°C with additional 500 ppm ethylene for 48 h during the last two days of this period (—○—) or without ethylene in a desiccator during these days as a control (—●—), (mean (n=2 to 5, usually 3) and S.E.). B: Detection of ISMV by ELISA in part A of bulbs treated with ethylene (—△—) or without (—▲—) as mentioned above (mean A405 (n=5) and S.E. are indicated). After ethylene treatments, bulbs were transferred to the 17°C and samples were tested during this recovery period.

increase in total respiration could account for an enhanced virus multiplication without an increase in the level of mitochondrial energy production.

The NAD(P)H used for all respiratory pathways (including residual respira-

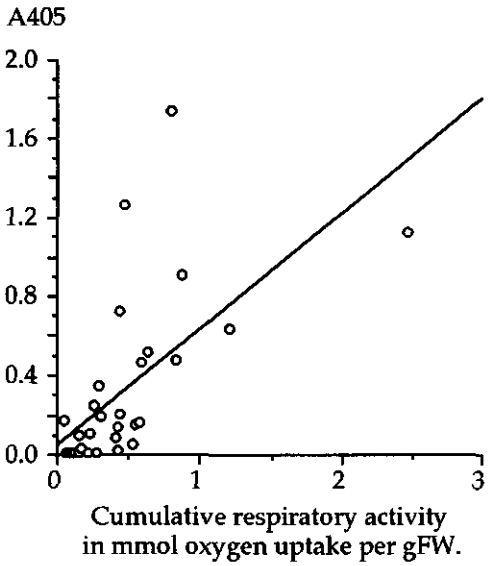

Fig. 6.6. Correlation between metabolic activity expressed as cumulative uninhibited oxygen uptake (in mmol per gFW) and the detection of ISMV by ELISA (A405) in bulbs transferred to 17°C, after wounding, high-temperature, ethylene and control treatments.

tion) originate from the sugar breakdown pathways the glycolysis, the Krebs cycle or the pentose phosphate pathway. This means that any increase in O_2-uptake coincides with an increase in NAD(P)H production, indicating an enhanced sugar breakdown. Apparently this enhanced sugar breakdown *per se* is sufficient to enable virus replication. This suggests that the production of metabolites is more important for viral replication in this system than the mitochondrial ATP production. The sugar breakdown pathway that theoretically would be beneficial for virus replication is the pentose phosphate pathway (PPP), since this pathway, besides NADPH, produces ribose intermediates, that can be used for viral RNA synthesis.

Increase of PPP enzymes as recently found in wounded iris bulbs (H. Gude and C.M. Brand, unpublished results), and increase of enzymes for glycolysis and PPP as observed during tobacco mosaic virus accumulation in tobacco (Makovcová and Sindelár, 1977) support the hypothesis that intermediates of the PPP are beneficial for virus multiplication.

Ethylene

Ethylene biosynthesis in iris increases after wounding and during the 40°C treatment (Marissen, 1989). As ethylene enhances respiration it may play a role in the stimulation of the metabolism which finally leads to virus-multiplication. In this study, ethylene resulted in a slight enhancement of the virus multiplication correlating with an increase in respiration.

The experiments described in this paper indicate that stress treatment results in induction of virus multiplication and leads to an increase in oxygen uptake, indicating an enhancement of sugar breakdown pathways. Metabolite production might be more important for virus multiplication than energy production.

ACKNOWLEDGEMENTS

We thank Dr. A.F.L.M. Derks and Dr. (Mrs.) J. Dijkstra for fruitful discussions and critically reading the manuscript.

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CHAPTER 7

Differences in iris severe mosaic virus distribution between primarily and secondarily infected iris plants*

SUMMARY

The distribution of iris severe mosaic virus (ISMV) in secondarily and primarily infected *Iris* cv. Professor Blaauw plants during the field and storage season was studied. In secondarily infected plants ISMV was distributed throughout the plant, but it was undetectable in the newly formed bulbs. In primarily infected plants, ISMV dispersed to various plant parts dependent on the time of inoculation. In case of an early infection, virus was spread first to the upper leaves and flowers and later on to the newly formed bulbs. However, towards the end of the field season, the detectable amount of ISMV in these newly formed bulbs decreased considerably. With late primary infections ISMV was hardly found in the leaves, but in high titres in the newly formed bulbs. At time of lifting the level of ISMV in primarily infected bulbs was therefore dependent on the date of inoculation: the later in the season infection took place, the better ISMV was detectable in the bulbs. Wounding of primarily infected bulbs resulted in an increase in virus titre in case of early-infected plants, but in a decrease in those of the late-primarily infected plants. Results are discussed in relation to metabolic activity. The consequences of these results for the development of routine tests are indicated.

* This chapter has been submitted as : Van der Vlugt, C.I.M., Derks, A.F.L.M., Boonekamp, P.M., Lohuis, H. and Goldbach, R.W. Differences in iris severe mosaic virus distribution between primarily and secondarily infected iris plants.

INTRODUCTION

During systemic infections of a host plant viruses are transported from cell to cell via plasmodesmata and over long distances via the plant vascular system (Matthews, 1991; Hull, 1989). A large number of viruses is known to be transported over long distances through the phloem to growing regions or to storage organs (Matthews, 1991; Samuel, 1934; Bennett, 1940). Therefore it is assumed that virus co-migrates with the nutrient flow (Fraser, 1987). Indications for this assumption were recently also obtained for cauliflower mosaic virus in young infected turnip plants in which the movement of viral particles in the leaves correlated with the sink-source transition of the photo assimilates (Leisner *et al.*, 1992). In plants with bulbs or tubers as underground storage organs, usually a carbohydrate flow starts from the storage organ towards the developing plant. Later on the new bulb becomes an increasing sink for photo assimilates while the leaves display a sink to source transition (Rees, 1992). Therefore it might be expected that when high titres of virus are found in the leaves, high amounts of virus will be transported to the new bulb as well.

For iris severe mosaic virus (ISMV), member of the Potyviridae, however, low virus titres were observed in the secondarily infected iris bulb just after lifting. (Van der Vlugt *et al.*, 1988; 1993b). ISMV became only detectable after specific stress treatments, which induce virus multiplication (Van der Vlugt *et al.*, 1993a; 1993b).

In the present study ISMV levels were determined in secondarily infected plants during the growing season in order to obtain insight in the translocation of ISMV in relation to the nutrient flow and to understand the reason for the low virus titre in bulbs of these plants. The results obtained were compared with ISMV localisation studies in primarily infected plants inoculated at various times during the field season. Differences and similarities found in ISMV presence between secondary and primary infections and the consequences for the development of an ISMV routine test are discussed.

MATERIALS AND METHODS

Bulb material

Virus-free and ISMV-infected stocks (*Iris x hollandica* cv. Professor Blaauw) were planted in late November and grown in the field under gauze, to prevent access of possible viruliferous aphids, until lifting at the end of August or beginning of September. Bulbs were harvested, wounded and tested as described

by Van der Vlugt *et al.* (1993a), unless otherwise stated.

Secondarily and primarily infected plant material

Secondarily infected iris plants were obtained by mechanical inoculation of virus-free iris plants in June and grown in successive years under gauze in the field as described by Van der Vlugt *et al.* (1993a). From the beginning of the fourth week of May until the beginning of the second week of August 1991, samples of five secondarily infected plants were harvested with intervals of two weeks. These plants were divided into, as far as when present: old bulb scales, basal plate with roots, new main bulb (A-bulb), new daughter bulbs (B-bulbs), sheath leaves, leaves, first developed flower (A-flower), second flower (B-flower), lower and upper stem part (Fig. 7.1). All parts were tested in ELISA.

Primarily infected plants and/or bulbs were obtained by mechanical inoculation of all leaves of virus-free plants on April 17, May 12, June 13, July 14 and August 5 in 1989 and on May 9, 23, June 11, 26 and July 13, 27 in 1990. The inoculum was obtained by grinding leaves of secondarily infected iris plants in 0.07 M phosphate buffer pH 7.0 containing 1% sodium sulphite (1:5 w/v). Infection percentage was usually 100%. In 1990 every two weeks, starting 14 days after inoculation, five plants were harvested and sampled as described above (Fig. 7.1)

ELISA

The ELISA procedure and ISMV coat protein antiserum used were as described earlier (Van der Vlugt *et al.*, 1988; 1993a). Plant parts were sampled and tested directly, or frozen in liquid nitrogen, stored at -70°C and tested directly after thawing. Plant parts were squeezed through a Pollähne roller press, 4 volumes of sample buffer were added to the obtained sap and then tested in ELISA. All A-bulbs of the same inoculation date (but from different sampling dates) were stored at -70°C and tested at the same time. Two grams of crushed, frozen bulbs were tested.

***In situ* blotting and immunogold-silver staining of tissue sections**

In situ blots and tissue sections were obtained and prepared as described earlier (Van der Vlugt *et al.*, 1993b). For *in situ* blots, freshly-cut surfaces of primarily infected iris bulbs were pressed onto NC membranes. These membranes were immuno labelled with antiserum against ISMV coat protein and this label was detected as described by Lin *et al.* (1990). Tissue sections of primarily infected iris bulbs were treated with ISMV antiserum and immunogold labelled and silver

stained mainly according to Van Lent and Verduin (1987) and Lohuis and Dijkstra (1992).

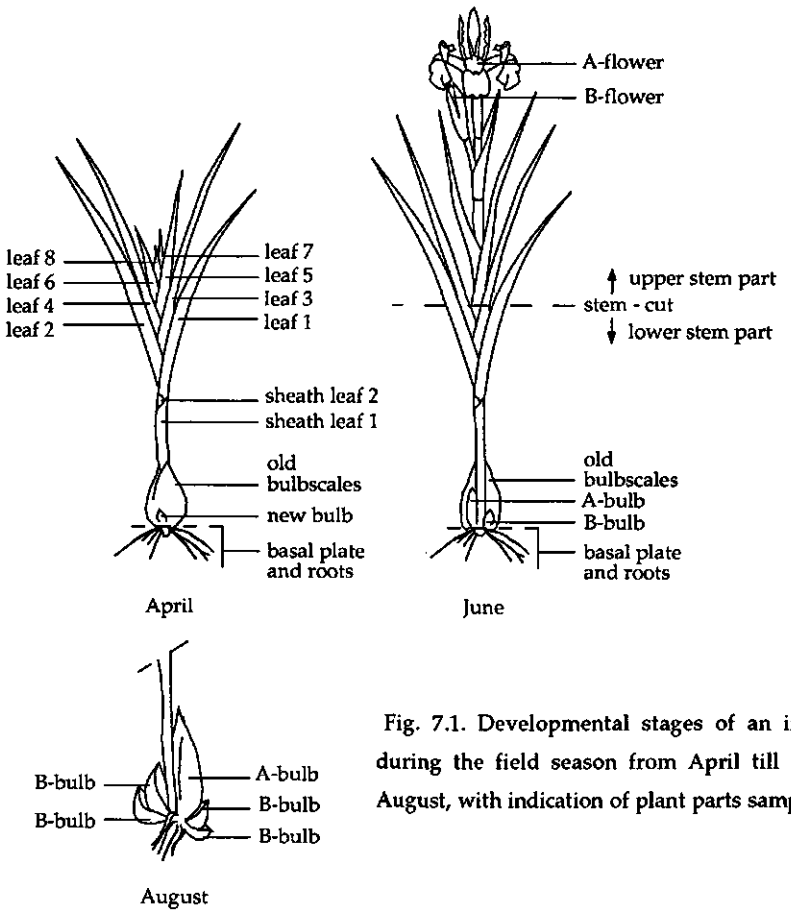

Fig. 7.1. Developmental stages of an iris plant during the field season from April till lifting in August, with indication of plant parts sampled.

RESULTS

Distribution of ISMV in secondarily infected plants during the growing season

In secondarily infected plants harvested on May 21, ISMV was present in the leaves and in the old bulb scales and to a lesser extend in the sheath leaves. Hardly any virus was detected in the newly formed bulbs or in the basal plate with roots, as shown in Fig. 7.2A. On June 6, the virus distribution was slightly changed; in only two out of five plants ISMV was detected in the old bulb scales which were rapidly deteriorating at this stage. In the stem, both in the upper and

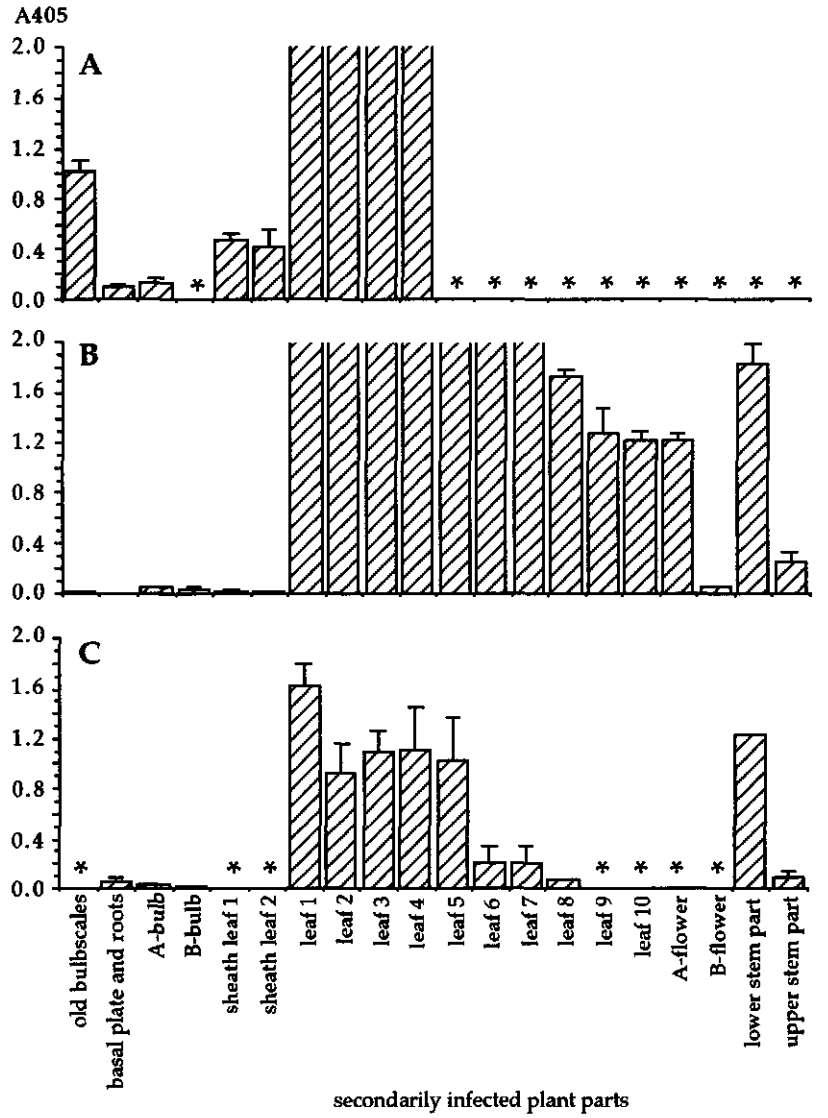

Fig. 7.2. Detection of ISMV by ELISA (mean A405 and S.E.) in secondarily infected plant parts of 4 non-flowering plants tested on May 21(A), 3 flowering plants tested on June 20 (B) and 5 plants tested on August 5 (C); (n=2 to 5 per plant part); *=plant parts not present.

in the lower part high amounts of virus were found, while hardly any virus was detected in the flower buds (data not shown). Two weeks later (June 20), when the plants started flowering, ISMV was found in the A-flower, the leaves and in the lower stem part. Furthermore, virus was present in low concentrations in the

upper stem part, while it was absent in the rest of the plant, namely the new bulbs, the (degenerating) bulb scales and sheath leaves (Fig. 7.2B). In July, the distribution of the virus in the plants was almost identical. As the plants finished flowering, and became senescent, the virus diminished in the flowers and sometimes in the upper leaves as well (data not shown). On August 5, the virus content of the leaves and upper stem part was decreased considerably, whereas the virus content of the lower stem part was still rather high and again no virus was detected in the newly formed bulbs (Fig. 7.2C).

Distribution of ISMV in primarily infected plants during the growing season

Virus-free plants were inoculated on various dates and every two weeks five plants were harvested and tested in ELISA. Results with respect to distribution of ISMV varied with the inoculation date. During the season ELISA values in the leaves of primarily infected plants never reached the high levels found for secondarily infected plants (Figs 7.2 to 7.5).

The data compiled in Figures 7.3 to 7.5 can be summarized as follows:
In early-inoculated plants tested at the end of May, ISMV was detected in the upper leaves, flowers and stem parts (Fig. 7.3A). Two weeks later the virus was spread through the whole plant, including the newly formed A and B-bulbs and the lower leaves, but with the exception of the sheath leaves and basal plate with roots (Fig. 7.3B). After flowering (June), virus concentration decreased in all plant parts including the newly formed A- and B-bulbs (Table 7.1), but the decrease was more pronounced in the upper than in the lower leaves (Fig. 7.3C and 7.3D). The ISMV content of the lower stem part remained highest until lifting (Fig. 7.3D). In contrast, in late-inoculated plants (July), virus was hardly present in the above-ground parts of the plants during the rest of the field season (Figs 7.4 and 7.5). However, ISMV level was high in the newly formed bulbs from four weeks after inoculation until time of lifting (Fig. 7.4B and 7.4C). In case of plants inoculated at the end of July, ISMV was present in high concentrations in the bulbs already two weeks after inoculation. The same was observed for the basal plate with roots (Fig. 7.5A and 7.5B).

Plants inoculated on intermediate dates showed results gradually in line with the above mentioned: In plants inoculated late May, virus was observed two weeks after inoculation in the flowers and stem only. Later on the virus was detected in low concentrations in some of the leaves (data not shown). ELISA values of the newly formed bulbs were comparable to those of the early-inoculated plants, except that the values in the A-bulbs four weeks after infection were

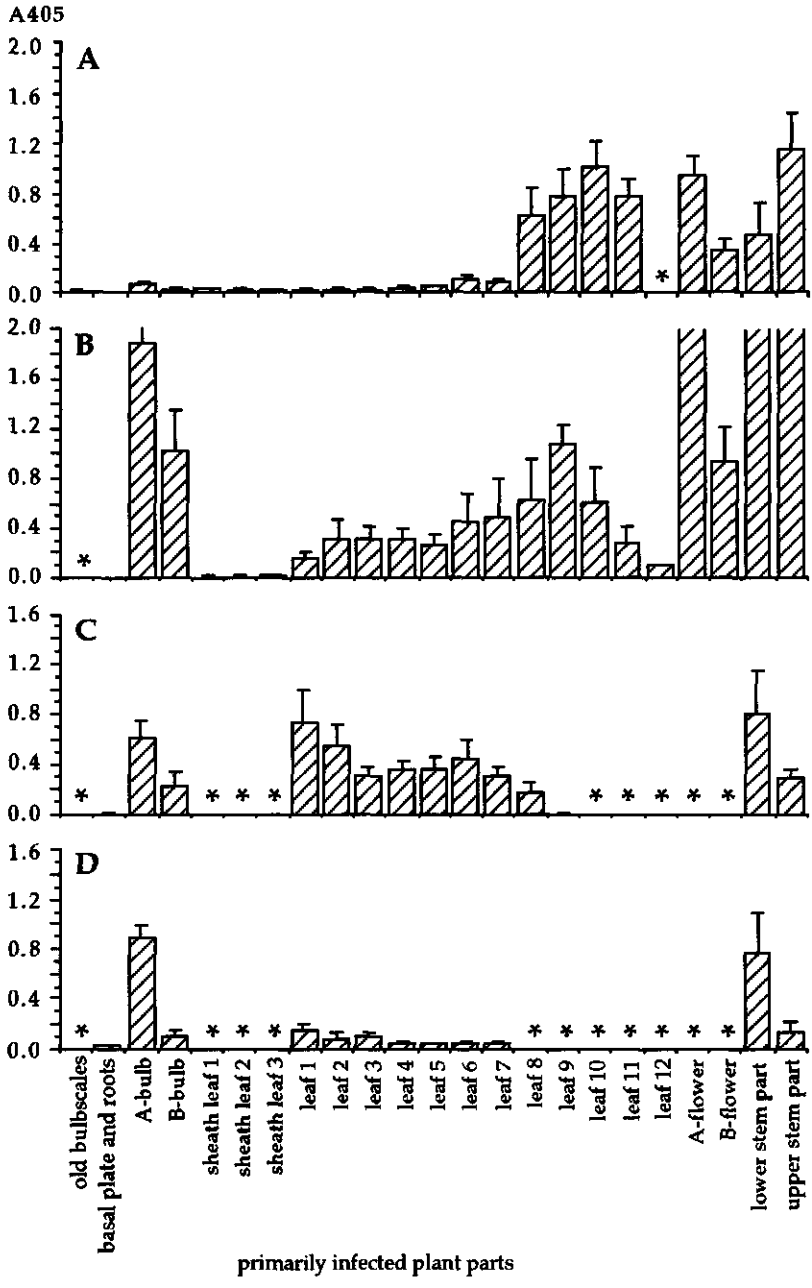

Fig. 7.3. Detection of ISMV by ELISA (mean A405 and S.E.) in plant parts, of 5 plants inoculated on *May 9* and tested on May 23 (A), June 11 (B), July 13 (C) and August 24 (D); (n=2 to 5 per plant part); *=plant parts not present.

lower (Table 7.1). In case of plants inoculated in June, ISMV was detected sometimes in the leaves from four weeks after inoculation until lifting, but was well detected in the newly formed bulbs.

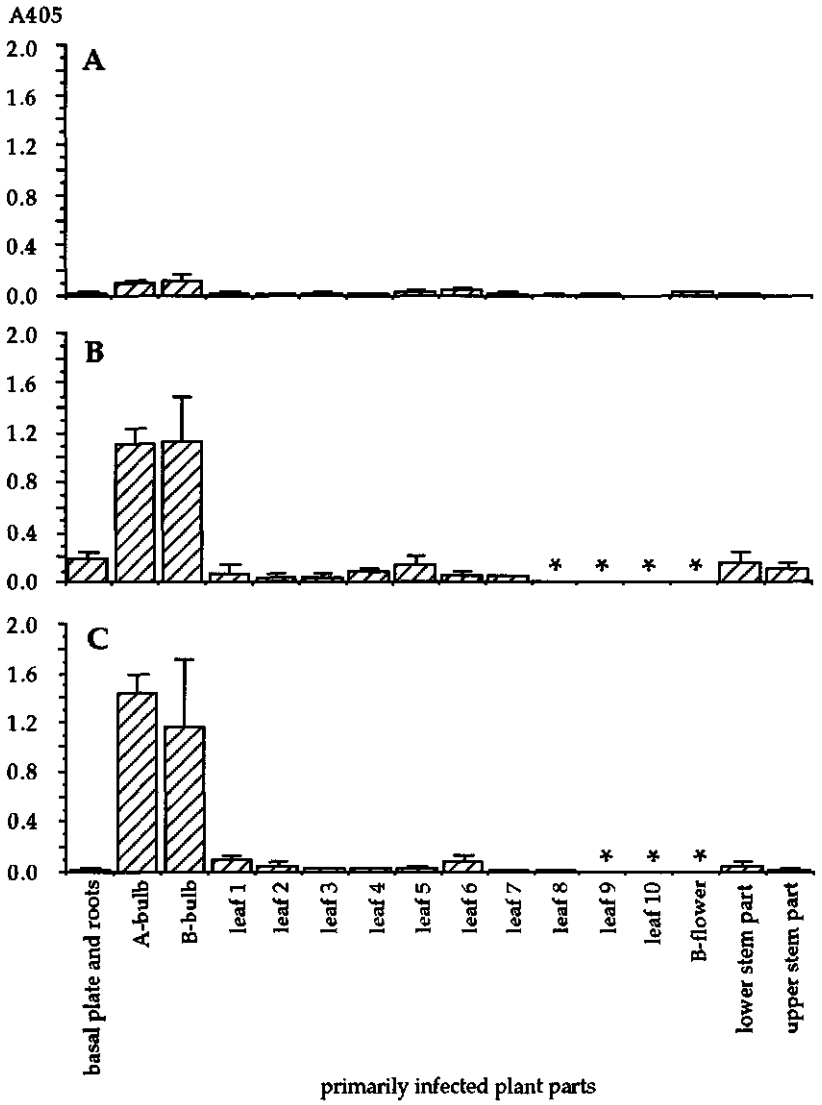

Fig. 7.4. Detection of ISMV by ELISA (mean A405 and S.E.) in plant parts, of 5 plants inoculated on *July 13* and tested on July 27 (A), August 13 (B) and August 24 (C); (n=2 to 5 per plant part); *=plant parts not present.

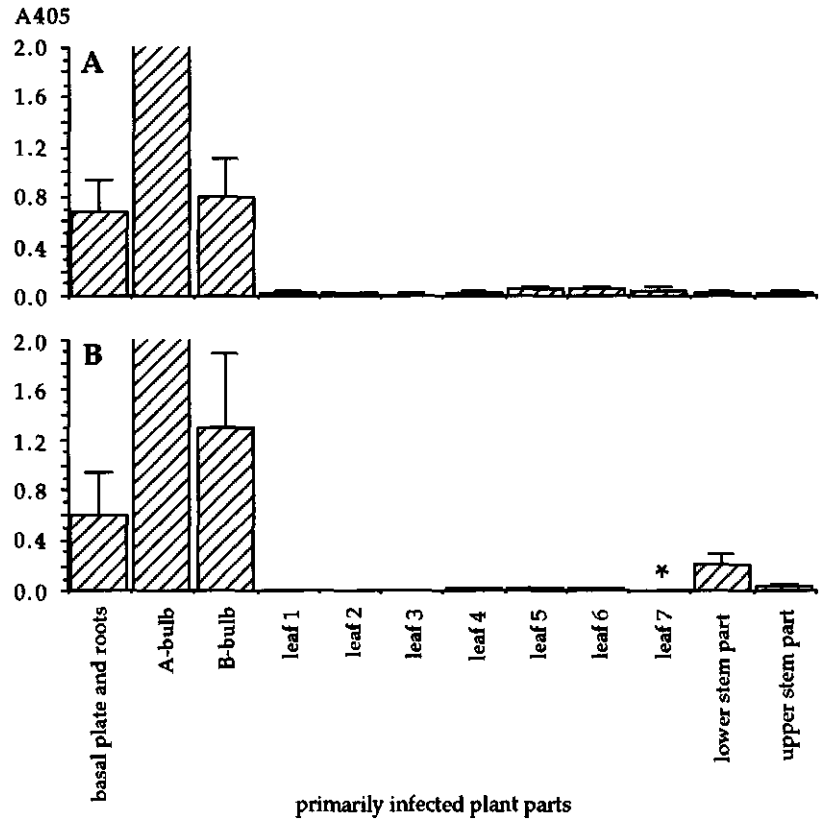

Fig. 7.5. Detection of ISMV by ELISA (mean A405 and S.E.) in plant parts, of 5 plants inoculated on *July* 27 and tested on August 13 (A) and August 24 (B); (n=2 to 5 per plant part); *=plant parts not present.

Detection of ISMV in bulbs of primarily infected plants

A comparison of the ISMV concentrations during the growing season in primarily infected A- and B-bulbs in relation to the inoculation date is shown in Table 7.1. The course of the virus titre is comparable in both types of bulbs, with the A-bulbs showing usually higher values, especially in early-inoculated plants (May 9). In general, ISMV accumulated to high ELISA values in the newly formed bulbs within four weeks after inoculation. However, in bulbs of early-inoculated plants these values decreased during the prolongation of the field season, resulting in rather low virus titres at the end of the growing season (time of lifting). In late-primarily infected plants however, ELISA values remained high during the field season, thus resulting in high virus titres of the bulbs at the

time of lifting.

In another year, bulbs of plants inoculated throughout the season and tested just after lifting, showed an even greater difference : In bulbs of early-inoculated plants (April 17 and May 12), no ISMV could be detected at lifting (Fig. 7.6A). This was confirmed by immunogold labelling of tissue sections of these bulbs (data not shown). On the other hand, bulbs of late-inoculated plants (July 14 and August 15) showed high ELISA values at lifting (Fig. 7.6A). In tissue sections the virus was found evenly distributed over all the scales (data not shown).

inoculation date	May 9	May 23	June 11	June 26	July 13	July 27
date of testing						
A-BULBS						
May 23	0.08 ± 0.02					
June 11	1.90 ± 0.42	0.11 ± 0.05				
June 26	1.14 ± 0.19	0.59 ± 0.15	0.04 ± 0.01			
July 13	0.62 ± 0.11	0.33 ± 0.09	0.77 ± 0.04	0.10 ± 0.02		
July 27	0.92 ± 0.16	0.54 ± 0.05	0.71 ± 0.17	1.02 ± 0.22	0.09 ± 0.02	
August 13	0.68 ± 0.19	0.76 ± 0.11	0.95 ± 0.19	0.52 ± 0.15	1.11 ± 0.13	> 2.0
August 24	0.88 ± 0.10	0.38 ± 0.04	1.72 ± 0.29	0.85 ± 0.17	1.44 ± 0.14	> 2.0
September 8	0.35 ± 0.11	0.39 ± 0.16	1.27 ± 0.36	0.85 ± 0.10	1.17 ± 0.40	1.65 ± 0.41
B-BULBS						
May 23	0.03 ± 0.01					
June 11	1.03 ± 0.34	0.13 ± 0.03				
June 26	0.24 ± 0.10	0.10 ± 0.03	0.01 ± 0.01			
July 13	0.22 ± 0.13	1.40 ± 0.47	1.80 ± 0.20	0.23 ± 0.06		
July 27	0.12 ± 0.09	0.47 ± 0.20	0.24 ± 0.09	0.71 ± 0.36	0.12 ± 0.05	
August 13	0.06 ± 0.02	1.12 ± 0.39	0.92 ± 0.49	1.08 ± 0.39	1.13 ± 0.37	0.79 ± 0.31
August 24	0.10 ± 0.04	0.48 ± 0.24	0.98 ± 0.34	1.10 ± 0.33	1.16 ± 0.56	1.29 ± 0.60

Table 7.1. Detection of ISMV by ELISA (mean A405 and S.E., n=5) in primarily infected A- and B-bulbs of plants inoculated at various dates during the growing season. Bulbs were tested during the field season and, in case of the A-bulbs, also at lifting.

Influence of wounding on the detectability of ISMV in bulbs of primarily infected plants

For bulbs of secondarily infected plants it has been reported that the ISMV content greatly increased after stress treatments, such as wounding (Van der Vlugt *et al.*, 1993a). A similar treatment (followed by a 20°C incubation) was

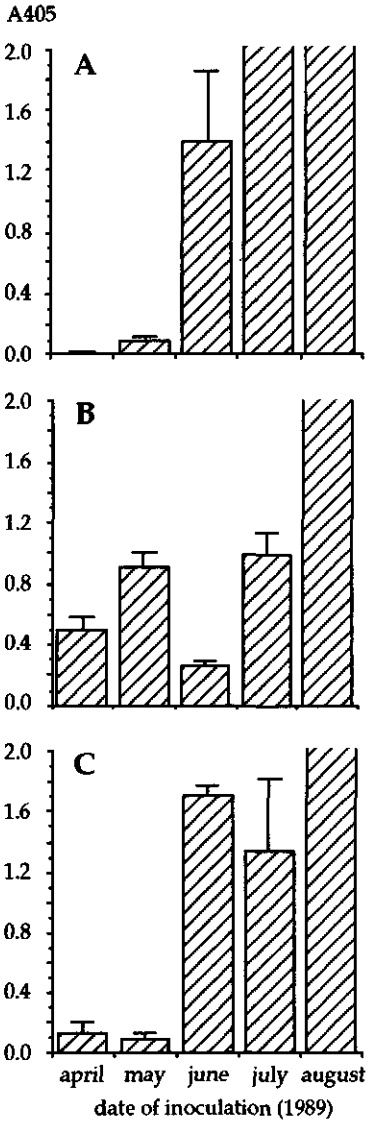

Fig. 7.6. Effect of inoculation date on the detection of ISMV (mean A405 and S.E. n=10 or 20) in just harvested bulbs at the beginning of September (A), after wounding treatment of these bulbs and subsequent storage at 20°C for 4-5 weeks (B) and in bulbs stored at 20°C only for 6 weeks after lifting (C).

applied to primarily infected bulbs just after lifting and resulted in increased ISMV detection in bulbs of early-inoculated plants (April and May), but in a decreased detection in bulbs of later inoculated plants (Fig. 7.6B). A 20°C incubation alone had hardly any effect on the detectability (Fig. 7.6C). More details on the distribution of the virus within the bulb were obtained by using *in situ* blots. After the wounding treatment the bulbs of early-inoculated plants showed ISMV located mainly in the basal plate and to a lesser degree along the

cut surface area (Fig. 7.7A). In the *in situ* blots of bulbs of late-inoculated plants ISMV was detected in all the scales after the wounding treatment (Fig. 7.7B).

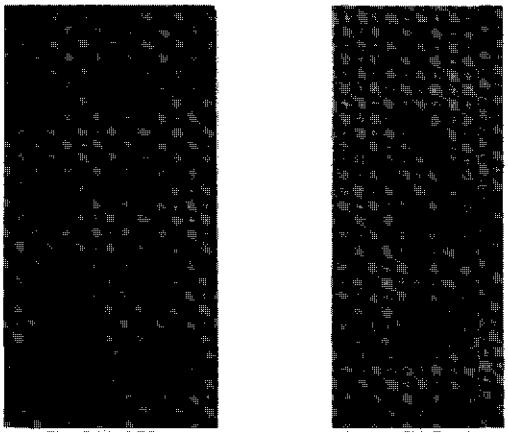

Fig. 7.7. An *in situ* immunoblot of wounded, early-(A) and late-(B) primarily infected bulbs 2 weeks after lifting, cutting and storage at 20°C. In bulbs of early-inoculated plants, positive staining is found mainly in the basal plate and to a lesser extent along the cut surface area of the wounded surface. The whole bulbs of late-inoculated plants reacted positively.

DISCUSSION

Relation between nutrient flow and virus translocation

As found earlier for other viruses, long distance movement mainly follows the nutrient flow in the phloem through the plant (Agrios, 1988; Bennett, 1940; Helms and Wardlaw, 1976; Matthews, 1991). It can be assumed therefore that ISMV moves also through the phloem. In case of secondary infections the bulb is already infected at the start of the growing season. Virus will presumably move first from the old bulb into the developing leaves, and then into the stem and flowers. At the end of the growing season during senescence, the amount of virus decreases in the leaves and upper stem. However, ISMV remains at high titre in the lower stem part, which is connected with the new bulbs via the basal plate. It is therefore remarkable, that hardly any ISMV can be observed in the newly formed bulbs at any time during the field season.

In case of early primarily infected plants, the spread of the virus is dependent on the developmental stage of the plant. As the nutrients first move from the bulb (source) into the leaves (sink), and probably for iris the development of the flower and the latter part of stem growth is more dependent on current photosynthesis (Ho and Rees, 1975; Rees, 1992) the virus inoculated on the leaves early in the season first moved to the upper leaves and flowers (Fig. 7.3A). Then the newly developing bulb acts as a sink for nutrients, and thus virus is presumably co-transported with the nutrients into the bulb as demonstrated by

the high initial ELISA values (Fig. 7.3B). However, long before lifting, a decrease in ELISA values is observed. As the bulbs keep on growing by nutrients transported from the leaves until the time of lifting, the virus was apparently no longer co-transported at this stage. The decreasing ELISA values in the new bulbs even suggest that the initial amount of ISMV was diluted with the increasing size of the bulbs. It is concluded that ISMV transport in early primarily infected plants initially follows the nutrient flow to the various organs. However, later on the virus is no longer co-transported to the new bulb.

In case of late-inoculated plants, when the sink-source transition has been completed, the transport of nutrients to upper leaves and flowers is shut down and is directed to the new bulbs only. It is plausible that the virus co-transports with the nutrient flow straight into the newly developing bulbs. Such has been found earlier for potato viruses (Agrios, 1988). It is concluded that ISMV transport in late-primarily infected plants follows the nutrient flow to the new bulbs.

Possible explanations for low virus titres in bulbs of early primarily infected and secondarily infected plants

Having compared the translocation of ISMV in secondarily and primarily infected plants, the question now arises why the virus is able to migrate into the bulb directly after infection, but is not able to accumulate in new bulbs of early primarily infected plants and secondarily infected bulbs. While the virus is not at all detectable in the newly formed bulbs of secondarily infected plants, it was present in rather high concentrations in most other parts of the infected plant prior to the onset of senescence, especially also in the lower part of the stem, suggesting that the loading of the virus into and transport through the phloem is not impeded.

Two alternative hypotheses may explain this phenomenon.

On the one hand, unloading of ISMV from the phloem may be limited because of a selective barrier in new bulbs of relatively long-term infected plants, preventing most of the ISMV to enter the bulb. Such a barrier might have been built as a defence mechanism of the plant against ISMV in e.g. the basal plate of a developing bulb. This phenomenon of selective passage has been found for other tissues as well, e.g. many viruses do not spread into seeds of a further totally infected plant (Matthews, 1991). It has recently been found that tomato spotted wilt virus is not able to move into iris bulbs either (A.F.L.M. Derks, unpublished results). The development of such a selective barrier might e.g. only be generated when the new bulb is still a metabolically-active, 'utilization sink' and has not

yet turned into a more passive 'storage sink' (Ho, 1988). On the other hand, virus in older infected plant parts might have become immobilized and thus is hardly able to migrate. Transport of virus may thus be possible during only a limited period after replication. Further research is needed to verify or disprove these hypotheses.

Implications for development of a routine test for ISMV in iris bulbs

In bulbs of secondarily infected iris, ISMV is not detectable immediately after lifting, due to extremely low amounts of virus present (Van der Vlugt *et al.*, 1993a,b). However, after specific stress and recovery treatments, ISMV detection could be improved due to induced virus multiplication. To test for the presence of ISMV in bulbs of early primarily infected plants, it would be advisable to give a wounding treatment, as for the secondarily infected bulbs. It must be mentioned though, that ELISA values of these primarily infected bulbs improved, but never at such a high rate as found for secondarily infected bulbs. In case of late-primarily infected bulbs however, ISMV was well detectable directly after lifting, and, instead, became less detectable after wounding and recovery treatment.

For a reliable routine test, it is necessary to detect secondarily as well as all primarily infected bulbs. Wounding in combination with a longer substrate incubation (more sensitive ELISA) seems to be reliable for all, primarily as well as secondarily, infected bulb material despite the decrease in values of late-primarily infected bulbs.

ACKNOWLEDGEMENTS

We thank Dr. A.R. van Schadewijk from the Bulb Inspection Service for providing laboratory assistance and Dr. (Mrs) J. Dijkstra for critically reading the manuscript.

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CHAPTER 8

Partial characterisation of iris severe mosaic potyvirus isolates in relation to an improved detection protocol*

SUMMARY

Seven iris severe mosaic virus (ISMV) isolates from different origins, including an isolate of bearded iris mosaic virus (BIMV), were transferred to iris cv. Professor Blaauw. Based on disease symptoms, these isolates could be divided into three groups. All isolates in leaf samples of this iris were detected in a hybridisation assay using part of the NIb gene of one ISMV strain as a probe and in immunoblotting using either anti BIMV or ISMV antisera. Using the latter antiserum, all isolates were also detected in DAS-ELISA. The previously developed wounding treatment to enhance virus detection in bulbs resulted in improved detection of all isolates tested. These data suggest that all isolates are strains of one virus.

* This chapter has been submitted as : Van der Vlugt, C.I.M., Derks, A.F.L.M. and Muñoz Labiano, D.M. Partial characterisation of iris severe mosaic potyvirus isolates in relation to an improved detection protocol.

INTRODUCTION

Iris severe mosaic potyvirus (ISMV), is known to occur in bulbous and rhizomatous iris and in crocus. The ISMV isolates studied so far have been divided into three strains based on symptoms in *Belamcanda chinensis* (Brunt, Derks and Barnett, 1988) and into mild and severe isolates referring to symptoms in *Iris x hollandica* cv. Professor Blaauw (Van der Vlugt *et al.*, 1992). Barnett (personal communication; Brunt *et al.*, 1988) found no sequence homology in reciprocal molecular hybridisation assays between ISMV and bearded iris mosaic virus (BIMV) using random-primed cDNA probes, while on basis of serology and host range BIMV is considered to be a strain of ISMV (Brunt *et al.*, 1988; Derks and Hollinger, 1986).

In this paper various isolates of ISMV from different origins, including the bearded iris isolate from Barnett (Barnett *et al.*, 1971), have been compared to establish whether the isolates are strains of one virus on basis of symptoms in iris cvs. Professor Blaauw and Apollo, by serology including immunoblotting and by hybridisation assay. Iris bulbs infected with these isolates have been subjected to the wounding treatment as described earlier (Van der Vlugt *et al.*, 1988; 1993a) to verify whether the assay protocol developed for ISMV is generally applicable in virus indexing of iris stocks.

MATERIALS AND METHODS

Virus isolates

The ISMV isolate obtained from *Crocus vernus* cv. Remembrance was used in earlier studies (Van der Vlugt *et al.*, 1988; 1993a; 1993b). Symptoms caused by this isolate were described by Derks, Hollinger and Vink-Van den Abeele (1985).
Three other ISMV isolates were obtained from *Iris oncocyclus*, *I. gatesii* and *I. germanica*. Symptoms caused by ISMV in these plants were described by Derks and Hollinger (1986). Other virus isolates originated from *I. bucharica* showing light-green to yellow stripes in the leaves and from *C. flavus* with a light-green mosaic pattern mainly at the leaf bases. The latter symptoms were indiscernible from those caused by bean yellow mosaic potyvirus in this crocus. The ISMV isolate described as bearded iris mosaic virus (BIMV, Barnett *et al.*, 1971) was kindly provided by Dr. O.W. Barnett (United States of America).

Other potyviruses used in the experiments were potato virus Y (PVY) kindly provided by Dr. R.A.A. van der Vlugt (The Netherlands) and iris mild mosaic virus (IMMV, Derks *et al.*, 1985).

Plant and bulb material

All 7 isolates were mechanically inoculated onto virus-free plants of *Iris* cvs. Professor Blaauw and Apollo. Moreover the isolate from *C. vernus* in part of the iris stock was passed through iris cv. Professor Blaauw once more by mechanical inoculation. Bulbs from all plants, except those with the BIMV isolate, had been propagated in the field under gauze for 2 to 7 years until actual use for detection. Harvesting and wounding treatment were performed as described earlier (Van der Vlugt *et al.*, 1988; 1993a).

Serology

DAS-ELISA and immunoblotting were essentially performed as described by Clark and Adams (1977) and Van der Vlugt *et al.*, (1988; 1993a,b). Tests were performed with ISMV antiserum prepared against the isolate from *C. vernus* (Van der Vlugt *et al.*, 1988), and BIMV antiserum provided by Dr. O.W. Barnett (Barnett *et al.*, 1971). Leaf material was squeezed through a Pollähne roller press: the undiluted sap was used for the hybridisation assay, while for ELISA this sap was 1:5 (v/v) diluted in phosphate buffer (Van der Vlugt *et al.*, 1988).

Hybridisation assay

A nitrocellulose sheet (pretreated with 3 M NaCl and 0.3 M sodiumcitrate (20xSSC)), Genescreen or Hybond-N was used to spot 2 μl samples of undiluted leaf sap. These blots were baked during 2 h at 80°C, prehybridised for 30 min to 2 h at 65°C and hybridised overnight in a 0.25 M phosphate buffer pH 7.2 containing 1 mM EDTA, 1% BSA and 7 % SDS (Church and Gilbert, 1984) with a random primed (Prime-a-gene, Promega) ^{32}P-CTP-labeled *Hind*III 838 bp DNA fragment (Feinberg and Vogelstein, 1983) coding for a part of the polymerase gene ranging from bp 349 to bp 1187 (Van der Vlugt *et al.*, 1994). Blots were washed with 2xSSC and 0.1% SDS at 65°C. An X-ray film was exposed to the blot. A five-fold dilution series starting with 0.3-0.7 μg of purified *C. vernus* ISMV isolate was used as a reference for measuring the intensity of the blackening.

RESULTS

Symptom expression

All isolates, except the BIMV, easily infected field-grown plants of iris cv. Professor Blaauw upon mechanical inoculation. Several attempts were made in the greenhouse before the plants became infected with the BIMV isolate.

Dependent on the time of inoculation, symptoms did or did not appear in the

leaves of iris cvs. Professor Blaauw and Apollo in the year of infection. All isolates, except the one from *Iris bucharica* induced a mosaic of light-green and yellow stripes on the inner leaves (Fig. 8.1A). The later the inoculation during the growing season, the more the symptoms were restricted to the top of the plant. Inoculation after flowering generally did not result in leaf symptoms in the first growing season. The isolate from *I. bucharica* deviated by evoking a complete yellowing of the inner leaves and of the lower parts of the outer leaves (Fig. 8.1B). Most plants died prematurely; a majority of the surviving bulbs showed black and brown spots, which was also observed in some bulbs with the isolate of *Crocus flavus*, but not in bulbs infected with the other isolates. Symptoms in the leaves caused by ISMV from *C. flavus* were of intermediate severity between those of most isolates and those with the *I. bucharica* isolate.

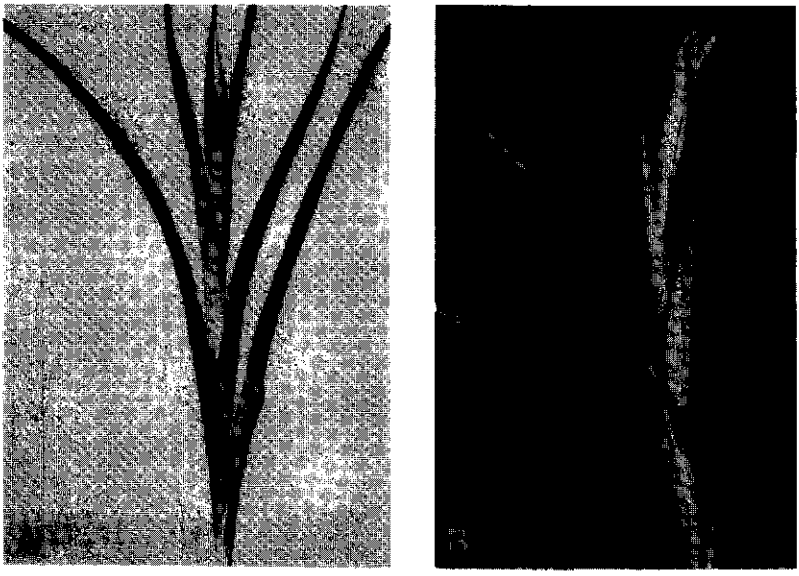

Fig. 8.1. Light-green and yellow striping on leaves of iris cv. Professor Blaauw caused by an ISMV isolate from *Iris germanica* (A) and leaf yellowing in the same cultivar caused by an ISMV isolate from *I. bucharica* (B);

In the second and following years, all isolates caused light-green and yellow stripes in the leaves extending below soil level as described earlier (Asjes, 1979; Derks and Hollinger, 1986) irrespective of the inoculation date. Later on, usually gray-green stripes and spots developed in the leaves. Dark spots or stripes were visible in the flowers.

Again, the *I. bucharica* isolate deviated, viz. by showing additional necrotic

stripes in the leaves soon after emergence of the irises leading to premature death of all plants, including the bulbs. In general, the symptoms in cv. Apollo were comparable with those in cv. Professor Blaauw but less pronounced both in the year of infection and in later years. On basis of symptoms in these bulbous irises a division can be made into three groups. The first group consists of 5 isolates, including the isolate from bearded iris (BIMV) with slight mutual differences. The second group contains the isolate from *I. bucharica* causing very severe symptoms and the third group contains the *C. flavus* isolate showing symptoms of intermediate severity.

ELISA and immunoblotting

All 7 ISMV-isolates could be serologically detected both in iris cv. Professor Blaauw (Table 8.1) and in the original hosts using the ISMV-coat protein antiserum in ELISA. Highest ELISA values in leaves of iris cv. Professor Blaauw were obtained with the homologous isolate from *Crocus vernus*. In immunoblotting, all isolates reacted with both ISMV and BIMV antisera (Fig. 8.2). The only obvious difference was the double banding of several ISMV isolates. The apparent MWs found on a 12% SDS-PAGE gel (Fig. 8.2) were circa 31 and 33 kD. However, the occurrence of double banding was not consistent for the ISMV isolates in different immunoblots.

Hybridisation assay

All isolates in leaf material of iris cv. Professor Blaauw hybridised with the 838 cDNA-probe (Table 8.1), indicating a high degree of homology for the NIb gene. Other potyviruses, viz. IMMV and PVY, did not hybridise with this probe. High hybridisation signals did not always coincide with high ELISA values. In comparison to the *Crocus vernus* isolate, the isolates from *Iris germanica*. *I. gatesii* and *C. flavus* seemed to be more, while those of *I. oncocyclus*. and *I. bucharica* seemed to be less reactive in a hybridisation assay than in ELISA. With BIMV both the hybridisation signal and the ELISA values were low (Table 8.1), corresponding with low virus concentration in electron-microscopical preparations.

Detectability after stress treatment

Previously a sensitive ELISA has been described based on the multiplication of ISMV in iris bulbs upon wounding (Van der Vlugt *et al.*, 1988; 1993a,b). For routine use it is necessary that such an ELISA can be generally applied.

Table 8.1. Detection of ISMV isolates from *Iris germanica, I. oncocyclus, I. bucharica, I. gatesii, Crocus flavus,* and *C. vernus,* after inoculation onto iris cv. Professor Blaauw and one extra passage through this iris (*C. vernus/I*) and BIMV in leaf material of iris cv. Professor Blaauw by ELISA (A405 and S.E.) and hybridisation assay (intensity of blackening; label 5 corresponds with 0.3-0.7 μg ISMV. Label 4 is a five-fold dilution of label 5 etc.). *: test number; three series of hybridisation and ELISA assays were performed, indicated as A, B and C. **: n is number of samples tested.

Iris cv. Professor Blaauw with ISMV isolates from:		A405	blackening	n**
I.bucharica	C*	0.19 ± 0.15	3.0 ± 0.0	2
I.bucharica	B	0.43 ± 0.08	3.1 ± 0.4	4
bearded iris (BIMV)	C	0.54 ± 0.01	2.0 ± 0.0	3
I.germanica	B	0.48	5.0	1
I.germanica	A	0.66 ± 0.17	4.0 ± 0.0	2
I.gatesii	A	0.65 ± 0.39	4.5 ± 0.5	2
I.gatesii	B	0.90	5.0	1
I.oncocyclus	B	0.66 ± 0.50	2.8 ± 1.2	2
I.oncocyclus	A	0.91 ± 0.41	2.8 ± 1.2	2
C.flavus	B	0.68 ± 0.13	4.5 ± 0.5	2
C.vernus/I	B	1.32 ± 0.23	4.3 ± 0.3	3
C.vernus/I	C	1.36 ± 0.04	4.0 ± 0.0	2
C.vernus/I	A	> 2.0	4.7 ± 0.1	4

Therefore, six ISMV isolates in bulbs of iris cv. Professor Blaauw were tested in ELISA one to two weeks after lifting and for a second time 4 weeks after cutting with consecutive storage at 20°C (Table 8.2). Already 1 week after lifting several bulbs infected with some ISMV isolates showed rather high levels of virus. With all isolates reliability of detection improved after wounding and storage of the bulbs, although the average ELISA values did not always increase.

Although values decreased after wounding in iris cv. Apollo, detection of ISMV in this cultivar did not pose any problem.

DISCUSSION

Our division of strains based on symptom severity in bulbous iris does not coincide with that based on symptoms in *Belamcanda chinensis* (Brunt *et al.*,

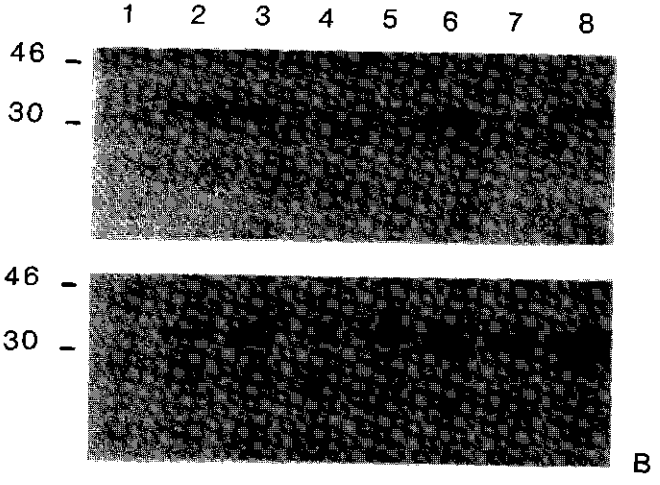

Fig. 8.2. Electroblot immunoassay of leaf sample preparations from a virus-free plant (lane 1) and from plants infected with ISMV isolates from *Crocus vernus, C. flavus, Iris gatesii, I. germanica, I. bucharica, I. oncocyclus,* and bearded iris (BIMV) (lanes 2 to 8 respectively). The coat protein bands reacted with antiserum to ISMV in panel A and with antiserum to BIMV in panel B. Numbers to the left are MWs of marker proteins.

1988). This host dependency makes these divisions of limited value, not denying their agricultural importance.

The minor differences in reaction pattern with ISMV and BIMV antisera in immunoblotting are probably due to the use of respectively more or less degraded virus particles for the preparation of these antisera. The finding of two bands of 31 and 33 kD in some lanes (Fig. 8.1) with the ISMV-coat protein (CP) instead of one is probably due to proteolytic degradation of the coat protein by splicing off the N-terminus of the CP, as is often found for potyviruses (Hollings and Brunt, 1981). Alper, Salomon and Loebenstein (1984) also found a MW of about 33 kD whereas the calculated MW was 30 kD (Van der Vlugt *et al.*, 1994). Thus the experimentally determined and calculated MW of the coat protein and its degradation product are in good accordance.

Barnett (personal communication; Brunt *et al.*, 1988) found no cross-hybridisation using BIMV and ISMV derived random-primed cDNA probes suggesting a difference between ISMV and BIMV. Using a NIb ISMV-derived probe, however, all tested ISMV-isolates, including BIMV, hybridised, whereas IMMV and PVY did not. Together with the ELISA results (CP homology) this suggests that all isolates are strains of one virus. For definitive conclusions it is better to sequence (at least part of) the CP genes of all isolates and to compare these on nucleotide

and amino-acid level as is done e.g. for the colour breaking viruses of tulip (Dekker *et al.*, 1993).

It is remarkable that at least in the year of testing, the detectability of most ISMV isolates in bulbs differed from that of the crocus isolate used standardly. However, the wounding treatment in combination with a sensitive ELISA seems to be a reliable method for the detection of ISMV in primarily as well as in secondarily infected iris bulbs (Van der Vlugt *et al.*, 1993a,b ; submitted) since this method is also suitable to detect other ISMV-isolates in iris cv. Professor Blaauw as shown here. Moreover, the wounding treatment did not lead to any problem in the detection of ISMV in another iris cultivar. So far, we succeeded to develop a test protocol for the detection of ISMV in iris bulbs.

Table 8.2. Detection of ISMV isolates from *Iris germanica , I.oncocyclus, I. bucharica, I. gatesii, Crocus flavus, C. vernus,* all in bulbs of iris cv. Professor Blaauw and ISMV from *C. vernus* after inoculation onto iris cv. Professor Blaauw and one extra passage through iris cv. Professor Blaauw (*C. vernus* /I) and of ISMV from *C. vernus* in bulbs of iris. cv. Apollo by ELISA (Mean A405 and S.E.). *: n is number of bulbs tested. ** : pos(n) indicates number of bulbs scored positive in ELISA (A405>0.15).

		t=0, after lifting		t=4 weeks, after lifting, and wounding	
	n*	A405	pos(n)**	A405	pos(n)**
Iris cv. Professor Blaauw with ISMV from					
I.germanica	10	0.30 ± 0.06	8	0.32 ± 0.06	10
I.oncocyclus	9	0.28 ± 0.13	5	0.72 ± 0.08	9
I.bucharica	5	0.28 ± 0.15	2	too dry for testing	
I.gatesii	10	0.24 ± 0.06	6	0.39 ± 0.07	10
C.flavus	10	0.58 ± 0.06	10	0.42 ± 0.03	10
C.vernus	10	0.67 ± 0.13	8	0.53 ± 0.07	9
C.vernus/I	10	0.08 ± 0.04	2	0.82 ± 0.02	10
Iris cv.Apollo with ISMV from					
C.vernus	10	1.00 ± 0.16	10	0.54 ± 0.09	10

ACKNOWLEDGEMENTS

We thank Dr. P.M. Boonekamp, Dr. (Mrs). J. Dijkstra and Prof. dr. R. W. Goldbach for critically reading the manuscript.

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CHAPTER 9

General Discussion

A model for transport and multiplication of ISMV in bulbous iris during the growing season in the field

The studies presented in this thesis deal with the detection and distribution of iris severe mosaic virus (ISMV) in iris bulbs. It is shown that upon changing the physiological conditions within the bulb by stress treatment, the virus becomes detectable, due to active replication and spread. Furthermore it was investigated why, in case of secondary infection, virus apparently did not move from highly infected leaf tissue into the bulb.

The process of virus translocation within infected plant tissue has been extensively described in various reviews (e.g. Atabekov and Taliansky, 1990; Deom *et al.*, 1992; Hull, 1989; Maule, 1991; Wolf and Lucas, 1994). In case of systemic infections these processes can be divided into cell-to-cell transport, supposedly through plasmodesmata, and long-distance transport, involving the vascular system.

When leaves become infected either by mechanical inoculation or by a biological vector, virus particles usually first enter the epidermal cells (Matthews, 1991). After entry and replication, progeny virus spreads to adjacent healthy cells if the virus is capable of movement.

To move to adjacent cells, a virus has to pass through the protoplasmic connections, the plasmodesmata. However, the size exclusion limit of the plasmodesmata under normal physiological conditions range from 800-1000 Da (Wolf and Lucas, 1994) and therefore do not accommodate transport of virus particles. Up to now at least two types of cell-to-cell transport have been proposed.

1. Transport can take place in a non-virion form e.g. as a viral nucleoprotein complex as exemplified by tobacco mosaic virus (TMV; Atabekov and Taliansky, 1990; Dawson, 1992). Due to the availability of transgenic plants expressing the TMV-30 kDa movement protein (MP), the understanding of TMV transport through plasmodesmata is progressing. These studies demonstrated that the gating capacity of the plasmodesmata was enlarged by the MP of TMV, which is also found inside the plasmodesmata (Deom *et al.*, 1992; Maule, 1991; Wolf and

Lucas, 1994). Still the viral nucleoprotein complex has to be unfolded to be able to move through these channels. The MP is postulated to play a central role in this process as well (Citovsky *et al.*, 1992; Deom *et al.*, 1992; Wolf and Lucas, 1994).

2. Another transport mechanism allows the movement of whole virus particles through tubular structures of heavily modified plasmodesmata, as is exemplified by cowpea mosaic virus and cauliflower mosaic virus. Movement proteins are involved in this process as well (Van Lent *et al.*, 1990, 1991; Maule, 1991).

Virus-coded movement proteins have also been proposed or identified for other plant viruses, e.g. on basis of homology (Koonin *et al.*, 1991). For potyviruses the P1 protein might carry such a function (Domier *et al.*, 1987). Alternatively, it has also been suggested that the cylindrical inclusion (CI) protein plays a role in cell-to-cell movement based on observations that these proteins are closely associated with plasmodesmata (Atabekov and Taliansky, 1990).

The first experiments concerning long-distance virus transport were reported by Samuel (1934) who proposed that vascular bundles are involved. Bennett (1940), Helms and Wardlaw (1976), and Leisner *et al.*, (1992) gained more evidence for transport through the phloem, and nowadays it is assumed that during systemic infection viruses co-migrate with the nutrient flow (Fraser, 1987; Leisner and Turgeon, 1993). Recently the interest in long distance is reviving (Leisner and Turgeon, 1993) and studies are carried out in more detail.

TMV mutants defective in coat protein spread within inoculated leaves but usually do not move into upper and lower uninoculated leaves suggesting that coat protein is involved in long-distance movement (Atabekov and Taliansky, 1990; Dawson, 1992; Meshi and Okada, 1986). TRV-RNA-1 infections alone (no particles) are also unable to spread rapidly to more distal tissues of the plant, although some systemic movement occurs (Cadman, 1962; Maule, 1991). These studies indicate that coat protein might be essential at some stage of long-distance spread of viruses (Hull, 1989). Therefore, long distance is more than a transport just through the plasmodesmata into the phloem and through the plasmodesmata out of this phloem because it shows other requirements than short distance transport.

Nutrients may be apoplastically or symplastically transported through the phloem (Robards and Lucas, 1990; Van Bel, 1993). Envisioning that virus is co-transported with the nutrients through the phloem, and that the size of virus or its nucleic acid pose large problems for apoplastic movement, viruses presumably move symplastically through plasmodesmata or sieve pores of the phloem

(Helms and Wardlaw, 1976; Leisner and Turgeon, 1993). The direction of sugar/assimilate movement, and the supposedly co-transported viruses, is dependent on the strength of the different metabolic sinks present in the plant. In this respect flower bulbs are complicated because they contain underground storage organs with extra sink/source capacities during one growing season: a bulb, shortly after planting, acts as a source for the developing plantlet (1), when the plant is large enough, assimilation products are transported from the older leaves towards the younger leaves (2), and finally from all the leaves to the new bulb which becomes a sink at this stage (3). Virus apparently moves likewise, as virus infected bulbs give rise to infected plants (1), virus is detected earlier in upper younger leaves, then, if at all, in older lower leaves in a systemic primary infection (Derks and Vink-Van den Abeele, 1980; Derks, 1988; Samuel, 1934; Leisner *et al.*, 1992) (2). In addition, at the end of the growing season, when carbohydrates are transported to the new bulbs, in general the progeny of virus-infected bulbous crops are infected too (Derks, 1988) (3).

ISMV in iris infects its progeny also for 100% and therefore seems to be translocated probably according to the nutrient flows. Nonetheless, upon lifting at the end of the growing season, the virus is usually hardly detectable in the bulb.

In order to gain insight into the cause of the failure to detect virus in the bulbs, investigations have been carried out on the spread of the virus in the plant and its bulb.

In this thesis the distribution of ISMV was determined both in plants with a first-year-infection (primary infection) and in plants infected in a preceding year (older or secondary infections) (Chapter 7). The results are summarized in Fig. 9.1.

In early primarily infected plants virus could be found first in the upper leaves according to the assimilate flow, and later also in the lower leaves. With these early primary infections, the virus initially reached high titres in the bulb, but virus levels decreased drastically towards the end of the season. With late primary infections, when inoculation took place after flowering, virus could not be detected in above-ground parts anymore as expected on basis of assimilate flow which should then be directed to the newly developing bulb. Only in these latter cases, the virus appeared to move freely into the new bulbs as well.

In secondarily infected plants early in spring the virus was readily detectable in the leaves and at this stage the virus was also present, in detectable amounts, in the old bulb scales from which the nutrient flow started for plant-development. Obviously, the virus was co-transported with this nutrient flow. The virus was ubiquitous in the above-ground parts for the rest of the season and levels of

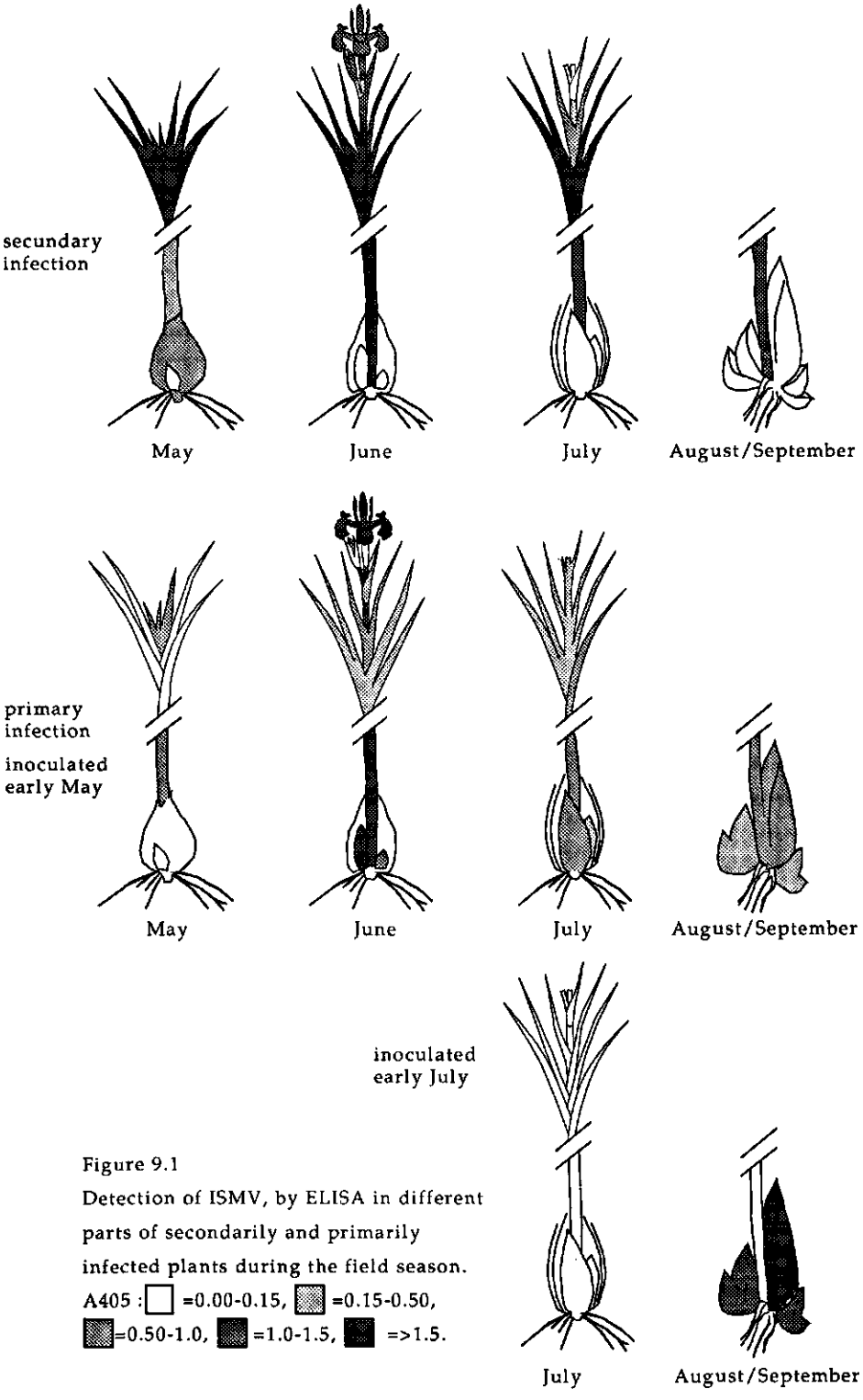

Figure 9.1
Detection of ISMV, by ELISA in different parts of secondarily and primarily infected plants during the field season.
A405 : □ =0.00-0.15, ▨ =0.15-0.50, ▨ =0.50-1.0, ■ =1.0-1.5, ■ =>1.5.

detection in ELISA in these parts were among the highest found in iris plant parts and bulbs of primarily and secondarily infected origins tested so far. Most remarkable therefore was the almost complete absence of the virus in the newly developing bulblet, being fed with a nutrient flow which originated from highly infected tissue. In secondarily infected plants, hardly any virus was transported into the new bulb at any time during the growing season in the field at all. However, ISMV was shown to have invaded the bulb (lifted and tested in August, Chapter 4) in hardly detectable amounts in the basal plate and in small spots in the bulb scale tissue, leading to infection of all daughter bulbs. Also the viral RNA could hardly be detected, suggesting that the virus had not gone 'undercover', thus escaping serological detection (Chapters 4 and 5).

Assuming that the virus can only be translocated due to co-transport, two alternative hypotheses may explain this situation.

Firstly, ISMV is impeded to enter the newly formed bulb in secondarily infected plants, suggesting a barrier for the virus. However, such a barrier could not have been absolute. In early primarily infected plants (May) the virus co-migrated initially with the nutrients into the new bulb. After this initial invasion of the bulb by the virus, the bulb apparently reacts by 'closing the gate'. With late infections, the bulb is not able to block virus import. Apparently the bulb is only able to block virus entrance into the bulb when infection had taken place the year before or early in the growing season. In correlation with the possibility of barrier forming only during the start of bulb formation, is the supposition that the bulb initially is metabolically more active, as a 'utilization' sink, and that later on the metabolic activity is limited to just filling the bulb with nutrients, the bulb being a passive 'storage' sink (Ho, 1988), and is then no longer able to defend the gate.

Selective barriers are known in seeds (Matthews, 1991; Carroll, 1981), where invasion of the embryo can occur dependent on the virus strain, host genotypes or time of infection (Wang and Maule, 1992; Carroll, 1981). A limited period of access of virus into the pea seed, called a 'window of opportunity', was also found by Wang and Maule (1992) in case of pea seedborne mosaic virus (PSbMV) in a cultivar of pea susceptible to seed transmission of PSbMV. Also, tobacco rattle virus is not equally distributed in tulip bulbs and the virus does not infect all daughter bulbs (Van der Vlugt *et al.*, 1988). The phenomenon of 'green islands' in infected tissue also points into the direction of a barrier. The occurrence of tobacco necrosis virus in tulip bulbs and in the above-ground parts is also dependent on the growth conditions of the host (planting time and soil type) in the year after infection (Asjes, 1989). The fact that tomato spotted wilt virus (TSWV) is not transported into the iris bulb (Derks, in press) also suggests a kind of virus import

barrier.

A second possibility is that the virus is not always available for transport. It might be that virus is only able to move during a particular, for instance replicative, stage immediately after infection or in reactivated tissue. Virus in older infected plant parts might have become less movable due to partial degradation of the virus itself or due to decreased availability of movement proteins. In case of bulbous crops, secondary infections and early primarily infections are relatively old towards the end of the growing season and it is suggested that e.g. the CI proteins which may aid in the virus transport only early after infection (Calder and Ingerfeld, 1990) become trapped in inclusion bodies in case of older infections. Virus transport may therefore only be possible during a limited period after replication, e.g. in the front zone of infection. Such a front zone of replicative activity was found in case of PSbMV in pea tissue by Wang and Maule (A.J. Maule, personal communication).

Model for distribution and multiplication of ISMV in the iris bulb during different storage conditions.

With respect to the behaviour of the virus in secondarily infected bulbs during storage, it should be first mentioned that temperature is an environmental factor which greatly influences the state of activity and development of the bulb. Long exposure to a low temperature of e.g. 2°C after lifting results in bulbing or pupation, while exposure to 30°C apparently imposes dormancy on the iris bulb. Temperatures around 17°C induce leaf and/or root formation.

Detection of virus in secondarily infected bulbs, especially after stress-treatment, is particularly enhanced in bulbs stored around 17°C, thus coinciding with metabolic activity needed for active growth (Chapter 2). The imposed dormancy during 30°C apparently does not reduce developmental activity to zero, as bulbs transferred to metabolically more favourable temperatures after 'dormancy' start to develop in a normal fashion but at high(er) speed. High temperature treatment can also induce flower formation in, for flowering, critically sized bulbs. Multiplication of virus in such treated bulbs is also speeded up, indicating a correlation between the metabolic activity or developmental stage of the bulb and virus increase (Chapter 3).

Removal of the tunic from an iris bulb is a form of stress, and is followed by enhanced respiration (Kamerbeek, 1962). A stress treatment consisting of wounding (by cutting a piece of scale from the side of the bulb) and subsequent recovery resulted not only in enhanced detection of the virus, but also in

increased respiration (Chapters 2, 3 and 6). Within a broad temperature range (5 to 30°C), again metabolically favourable temperatures of 17-20°C proved to be optimal for enhanced detection (Chapter 3). The virus had become detectable due to multiplication of an initial low amount of virus at the site of wounding (Chapters 3 and 5) and not due to e.g. serological alteration. High-temperature treatment (30/40°C) as an alternative stress, followed by a 17°C period to allow recovery, also showed an increased level of ISMV. After either stress treatment the total respiratory activity increased (Chapter 6) and it was found that especially the residual respiration as part of the total respiration was enhanced in both cases. As the pentose phosphate pathway (PPP) also yields pentose intermediates for nucleotide synthesis, beneficial for virus multiplication, it is tempting to assume that especially the pentose phosphate pathway increases. Increase of PPP enzymes was recently found in wounded iris bulbs (H. Gude and C.M. Brand, unpublished results), and increase of enzymes involved in glycolysis and PPP was observed during tobacco mosaic virus accumulation in tobacco (Makovcová and Sindelár, 1977). Thus it might be concluded that the increase in respiration led to an increase in production of metabolic intermediates, rather than in an increase in energy.

More detailed localisation studies of ISMV in secondarily infected iris bulbs revealed its presence in the basal plate and sometimes in small spots within the scales immediately after lifting in August (Chapter 4). After application of local stress (i.e. wounding) the virus multiplied mainly along the cut surface area which presumably is the activated tissue. The increase of virus was not correlated to specific spots, suggesting that the virus must have been present in many cells in the tissue adjacent to the wounded area.

However, after total stress (high-temperature treatment) virus was located in spots in the vascular tissue in the scales and in the basal plate, the latter containing a junction of vascular tissue. Thus the tissue activated by total stress probably comprised in first instance the vascular tissue. Subsequently the virus may have been transported from the vascular tissue in the basal plate upwards to other tissues. However, the observation that top halves of bulbs cut during 'dormancy' also gained higher virus titres, indicates that the virus was presumably present scattered in places in the scales too. Whether translocation was also stimulated still remains a question.

With respect to the behaviour of ISMV in primarily infected bulbs, early infections may be regarded as comparable to secondary infections, as ISMV detection was also enhanced after wounding. However the situation in late

primarily infected bulbs is different, as ISMV was readily detectable in the freshly harvested bulbs. When these bulbs were wounded the virus titre even decreased instead of increased. It is assumed that these bulbs are also metabolically activated, enhancing virus multiplication, but there seemed to be less viral epitopes available for serological detection by ELISA. This might be explained as follows: the virus might aggregate into clusters, resulting in a decrease of available epitopes, or the virus could be broken down due to proteolytic degradation, resulting in degradation products which became undetectable either. In the latter case degradation should be faster than *de novo* synthesis of the virus.

It would be of interest to know whether, and if so to what extent, virus is degraded, suggesting a means of defense. This phenomenon may be studied in late primarily infected bulbs, as these can be kept as explants on tissue culture medium under controlled conditions. Furthermore, in case of bulbs, one is able to work with established infections thus ruling out artifacts occurring when a virus is introduced into a plant. This makes flower bulbs excellent objects to study plant-virus interactions.

Implications for development of a routine test for ISMV in iris bulbs

With respect to the detectability in primarily and secondarily infected iris bulbs, it can be concluded that in secondarily infected iris bulbs ISMV can be detected satisfactorily after a stress treatment. For primarily infected bulbs the situation is slightly more complicated as virus titres vary enormously after lifting. Nevertheless, after a wounding treatment ISMV detection is always enhanced or maintained, and reliable monitoring is possible when a sensitive ELISA is used (e.g. prolonged substrate incubation, Chapter 7).

Other ISMV-isolates in iris cv. Professor Blaauw were also detected using this wounding method, indicating that no escapes due to virological variation occurred so far (Chapter 8). Thus one simple test procedure, wounding immediately after lifting followed by a storage at 20°C for approximately three weeks, will cover all types of infections.

However, before putting the test into practice some other aspects should be considered, e.g. the interference of other viruses in iris has to be ruled out, such as for instance IMMV, another important potyvirus occurring in iris in the Netherlands. This virus is easily detectable in the bulb already some weeks after storage without any stress treatment. The wounding treatment had no effect on the reliability of detection of IMMV (A.F.L.M. Derks, personal communication). This was confirmed in an assay of doubly (ISMV and IMMV) infected bulbs

which were cut in top and bottom halves (Chapter 5). The level of IMMV in both parts was hardly changed while that of ISMV was highly enhanced (Chapters 5, 8; unpublished data).

In summary it can be concluded that the investigation described in this thesis demonstrated the multiplication and distribution of ISMV in iris in relation to the host metabolism during normal growth and after specific treatments of the bulbs. This not only resulted in a greater knowledge of the interaction between ISMV and bulbous iris but also led to the development of a reliable test procedure to detect ISMV in bulbous iris.

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Summary

During cultivation of iris, several viruses may cause severe damage like yield reduction and discoloration of the plant. In commercial stocks in the Netherlands virtually all plants are infected with iris mild mosaic virus (IMMV) while iris severe mosaic virus (ISMV) and narcissus latent virus (NLV) can also be present at high incidence. ISMV and IMMV both belong to the genus *Potyvirus* of the largest plant virus family, the Potyviridae.

As the quality of virus-free iris is superior and methods are available now to produce and grow virus-free iris for commercial practice, it is of great importance to control the spread of iris viruses. Therefore rapid and reliable assays for the detection of these viruses are needed. In the Netherlands such tests are being developed mainly at the Bulb Research Centre in Lisse. At the start of the research described in this thesis, detection of ISMV in the iris bulb was problematic, in contrast to that of IMMV. It has been difficult to detect ISMV reliably in iris bulbs from lifting in late August to planting in October by means of ELISA or electron microscopy.

The aim of the research was to develop a reliable detection protocol suitable for monitoring ISMV infection, and, to understand the behaviour of this virus in the iris plant, especially the bulb, with respect to the multiplication and movement of the virus in relation to the metabolic activity of the plant. Preliminary results concerning the development of a test protocol are presented in Chapter 2, and further elaborated in Chapter 3, 7 and 8. In Chapters 4, 6 and 7 the behaviour of ISMV in relation to the metabolic activity of the plant is elaborated. In Chapters 5 and 8 a further characterisation of ISMV is presented.

In freshly-lifted bulbs secondarily infected with ISMV, the virus was not always detected in the basal plate and rarely in bulb scale tissue (Chapter 4), but it gradually became better detectable in the bulb scale tissue when bulbs were incubated during several months at a temperature of circa 17°C (Chapter 2). When a wounding method was applied on the iris bulb by cutting a slice of bulb scale tissue from a side of the bulb, ISMV became readily detectable in all bulbs, though only in tissue adjacent to the cut surface, if the cut bulbs were incubated for three weeks at an optimal temperature of 17-20°C (tested within a range of 5 to 30°C; Chapters 2 and 3). It was concluded that stress followed by a recovery period is favourable for an enhanced detection of the virus. Indeed, high-

temperature treatment, applied as an alternative stress, also gave rise to improved detection of ISMV (Chapter 3).

To investigate whether the virus became better detectable by multiplication rather than by modification of the antigenicity of the coat protein, the levels of the viral antigen as well as those of the viral RNA were followed after wounding. From this analysis it was concluded that the increase of the virus titre was due to multiplication (Chapter 4). For the detection of the viral RNA, a cDNA clone corresponding to a part of the 3'-terminal region of the ISMV genome was used. The availability of this clone led to the determination of the nucleotide sequence of the ISMV coat protein (CP) gene, thus allowing a definitive classification of the virus. Phylogenetic comparisons of potyviral CP sequences revealed that ISMV is a taxonomically distinct potyvirus not closer related to other bulbous or monocotyledonous infecting potyviruses than to other potyviruses. The sequence data also allowed to conclude that the CP is probably cleaved off from the NIb protein at an unusual glutamine acid-glycine (E/G) dipeptide cleavage site. Furthermore the N-terminus of the CP appeared to be only 15 amino acids long, being the shortest found among potyvirus CPs studied so far.

Further research on the localisation of the virus after high-temperature treatment showed that the virus was well detectable in the bulb base and usually also in the vascular bundles and surrounding tissue. This suggested that the virus did spread from the basal plate towards the bulb scales. However when the wounded (cut) apical parts of infected, but in ELISA negative reacting, bulbs were incubated at an optimal recovery temperature, the virus became detectable in these upper parts of the bulbs (Chapter 4). Thus, virus must have been originally present in the scales, albeit at a very low and at non-detectable concentration. This provides another indication that multiplication is likely to be the main factor involved in the improved sensitivity of viral detection. It is, therefore, hypothesised that the multiplication is enhanced by increased metabolic activity after stress. A possible correlation between metabolism and ISMV multiplication was further investigated in Chapter 6, with oxygen uptake as a measure for the metabolic activity after application of wounding, high-temperature stress or ethylene treatment.

An increased level of total oxygen uptake was found after wounding as well as high-temperature treatment, thus positively correlating with the enhancement of ISMV detection. Application of ethylene, an important plant hormone in relation to stress, caused a limited increase in respiration and a slight improvement of ISMV detectability. After wounding, the mitochondrial respiration, the residual respiration and the capacity of the alternative pathway had increased,

while after high-temperature treatment there was mainly an increase in residual respiration measured. These findings suggest that increased production of metabolic intermediates, possibly by the pentose phosphate pathway, rather then an increase in energy is important for the observed stress-induced multiplication of ISMV in iris bulbs.

For the development of a satisfactory test method, it is imperative that virus is reliably detected not only in secondarily infected bulbs but also in primarily-infected bulbs. To obtain primarily infected bulbs, virus-free plants were mechanically inoculated with ISMV at different times during the growing season. At lifting the level of ISMV in primarily infected bulbs appeared to be dependent on the date of inoculation. Surprisingly, it was found that early infections were scarcely detectable in the bulbs in contrast to late infections. The later in the season infection took place, the better ISMV was detectable in (untreated) bulbs (Chapter 7). Wounding of these primarily infected bulbs generally resulted in an increased detection in bulbs of the early infected plants, but the virus titre in bulbs of late infections decreased. However, these infections were still reliably detectable. Another potential problem for implementation of the developed test for routine use could be the existence of differently reacting isolates of ISMV. In spite of causing slightly different symptoms and serological reactions, all could be detected by the wounding method (Chapter 8).

The reason why ISMV is so difficult to detect in secondarily infected and in early primarily infected bulbs was investigated further in Chapter 7. The virus titre was monitored in the whole plant, including the bulb, during the growing season for both secondarily and primarily infected plants. The distribution of ISMV in the above-ground parts of secondarily infected as well as primarily infected plants correlated with the nutrient flow via the vascular system. This implied that above-ground parts of secondarily infected plants were totally infected while in primary infections the presence of virus was dependent on the time of infection: during an early infection virus still spread to the upper leaves of the plant and only later to the new bulb, while in late infected plants the virus was found mainly in the new bulb. However, in secondarily infected plants hardly any virus could be detected in the new bulbs at any time during the whole growing season. Besides, the detectability of ISMV in bulbs of early infected plants decreased considerably towards the end of the growing season. This might be explained by assuming that the plant develops a barrier at some time after infection blocking virus avenue to the bulb, or that the virus in secondarily and early primarily infected plants is no longer available for transport anymore (Chapters 7 and 9). It must be concluded that detection of ISMV in these

secondarily and early primarily infected bulbs immediately after lifting is unreliable due to impeded import of virus into the bulb.

Samenvatting

Bij de teelt van iris kunnen verschillende virussen grote schade veroorzaken. Hierbij treedt oogstderving op en een verminderde kwaliteit van het produkt. In de praktijk-partijen in Nederland zijn in feite alle planten met het irismozaiekvirus (iris mild mosaic virus; IMMV) geïnfecteerd, terwijl het irisgrijsvirus (iris severe mosaic virus; ISMV) en het irisbontvirus (narcissus latent virus; NLV) ook in belangrijke mate kunnen voorkomen. Zowel ISMV als IMMV behoren tot het geslacht *Potyvirus,* dat deel uitmaakt van de grootste plantevirusfamilie, de Potyviridae.

Omdat virus-vrije irissen van betere kwaliteit zijn en er methoden beschikbaar zijn om virus-vrije irissen in de praktijk te telen, is het van groot belang de verspreiding van iris-virussen onder contrôle te houden. Daarom zijn snelle en betrouwbare toetsen nodig. In Nederland worden zulke toetsen voor virussen in bloembollen voornamelijk ontwikkeld op het Laboratorium voor Bloembollenonderzoek te Lisse. Voor de aanvang van het in dit proefschrift beschreven onderzoek was het ISMV, in tegenstelling tot het IMMV, slecht aantoonbaar in de irisbol. Vanaf het rooien eind augustus tot het planten in oktober was het moeilijk om het ISMV betrouwbaar in de irisbollen met behulp van ELISA of elektronenmicroscopie aan te tonen.

Doel van het onderzoek was een betrouwbare toetsmethode te ontwikkelen voor het ISMV en inzicht te verkrijgen in het gedrag van dit virus in de waardplant m.b.t. de vermeerdering en verspreiding. De eerste resultaten betreffende de ontwikkeling van een toets zijn vermeld in Hoofdstuk 2. De toets is verder uitgewerkt in de Hoofdstukken 3, 7 en 8. In de Hoofdstukken 4, 6 en 7 is het gedrag van het virus, in relatie tot de metabole activiteit van de irisbol, verder uitgewerkt. In de Hoofdstukken 5 en 8 is een nadere karakterisering van het ISMV gegeven.

In vers gerooide, secundair (in het voorgaande groeiseizoen) met ISMV geïnfecteerde bollen kon het ISMV niet altijd betrouwbaar aangetoond worden in de bolbodem (of basale plaat) van de bol en zelden in bolrokweefsel (Hoofdstuk 4). Het virus werd echter geleidelijk beter aantoonbaar in de bolrokken wanneer de bollen gedurende enige maanden bewaard werden bij een temperatuur van ongeveer 17°C (Hoofdstuk 2). Wanneer de irisbol werd verwond door een stukje

bolrok aan de zijkant van de bol af te snijden, bleek dat het ISMV in alle bollen betrouwbaar aantoonbaar was geworden, maar alleen in het aan het snijvlak grenzende bolrokdeel en na drie weken incubatie bij een temperatuur van 17 of 20°C (Hoofdstukken 2 en 3). Hieruit werd geconcludeerd dat stress gevolgd door een herstelperiode gunstig is voor het verbeteren van de aantoonbaarheid van het virus. Inderdaad leidde een hoge-temperatuur behandeling, toegepast als een alternatieve vorm van stress, eveneens tot een verbetering van de aantoonbaarheid (Hoofdstuk 3).

Om vast te stellen of het virus beter aantoonbaar werd als gevolg van vermeerdering danwel verandering van het manteleiwit waarop met ELISA getoetst wordt, werden op verschillende tijdstippen na verwonding de niveau's van zowel het virale antigen, het manteleiwit, als die van het virale RNA bepaald. Hieruit volgde dat de verbetering van de aantoonbaarheid verklaard kon worden door vermeerdering van het virus. Voor het aantonen van het virale RNA werd een cDNA-kloon gebruikt corresponderend met een deel van het 3'-terminale gedeelte van het ISMV-genoom. Door de beschikbaarheid van deze kloon kon tevens de nucleotidenvolgorde van het ISMV manteleiwitgen bepaald worden, waardoor een definitieve taxonomische klassificering van het virus kon worden gemaakt. Vergelijking met aminozuurvolgorden van het manteleiwit van andere potyvirussen suggereerde dat het ISMV taxonomisch een aparte potyvirussoort is die niet meer verwant is met bol- of monocotyl-infecterende potyvirussen dan met andere potyvirussen. Uit de opheldering van de aminozuurvolgorde werd ook geconcludeerd dat het manteleiwit waarschijnlijk van het grote nucleaire inclusie eiwit (NIb) wordt afgesplitst op een ongebruikelijke glutaminezuur-glycine (E/G) dipeptide klievingsplaats. Tevens bleek dat het N-terminale gedeelte van het manteleiwit slechts 15 aminozuren lang is, waardoor het ISMV-manteleiwit het kleinste van de tot nu toe bestudeerde potyvirus-manteleiwitten is.

Nader onderzoek naar de *in situ* lokalisatie van het virus na een hoge-temperatuur behandeling liet zien dat het virus in hoge concentratie aanwezig was in de bolbodem en meestal ook in de vaatbundels en omringend weefsel. Dit wijst erop dat het virus zich vanuit de bolbodem via de vaatbundels naar de bolrokken verspreidt. Wanneer echter het apicale (top) deel van geïnfecteerde, maar negatief reagerende bollen werd afgesneden (zodat hiernaar geen transport mogelijk is) en dit deel vervolgens bij een optimale herstel-temperatuur werd geïncubeerd, dan werd ook in dit boldeel de aantoonbaarheid van het virus verhoogd (Hoofdstuk 4). Dit betekent dat transport niet de enige verklaring is,

maar dat het virus oorspronkelijk ook in deze bolrokdelen aanwezig heeft moeten zijn, zij het in een zeer lage, niet detecteerbare hoeveelheid. Verder is dit opnieuw een aanwijzing dat virusvermeerdering de belangrijkste factor is bij de verbeterde detectie.

Een mogelijke correlatie tussen het metabolisme van het (verwonde) bolweefsel en virusvermeerdering wordt besproken in Hoofdstuk 6. Bij dit onderzoek werd zuurstofopname door de bol als maat genomen voor metabolische activiteit.

Er werd een verhoogd totaal-niveau van de zuurstofopname gevonden zowel na de verwonding als na de hoge-temperatuurbehandeling. Na verwonding namen zowel de mitochondriële ademhaling als de rest-ademhaling en de capaciteit van de alternatieve ademhaling toe, terwijl na de hoge-temperatuur behandeling voornamelijk een verhoging van de rest-ademhaling gemeten werd. Deze bevindingen kunnen erop wijzen dat een toename in de productie van bouwstenen, mogelijkerwijs via de pentose-fosfaatweg, meer dan een toename in energieproductie, belangrijk is voor de waargenomen stress-geïnduceerde virusvermeerdering.

Voor een goede praktijk-toets is het noodzakelijk dat niet alleen in secundair geïnfecteerde bollen maar ook in primair (gedurende het lopende seizoen) geïnfecteerde bollen, het virus betrouwbaar aantoonbaar is. Voor het verkrijgen van primair geïnfecteerde bollen werden virus-vrije irisplanten op het veld gedurende het groeiseizoen mechanisch geïnfecteerd met het ISMV. Vlak na het rooien bleek dat de aantoonbaarheid van ISMV afhankelijk was van het tijdstip van infectie. Verrassend genoeg bleek dat vroege infecties slecht en late infecties juist heel goed aantoonbaar waren in de bollen. Hoe later in het seizoen de primaire infectie plaats gevonden had, des te beter werd het ISMV aantoonbaar in de (onbehandelde) bol (Hoofdstuk 7; Figuur 9.1). Verwonding (gevolgd door herstel) van deze primair geïnfecteerde bollen resulteerde over het algemeen in een verbeterde aantoonbaarheid van het ISMV in de vroege, maar in een verminderde aantoonbaarheid in late infecties. Deze late infecties bleven nog wel betrouwbaar aan te tonen. Een andere potentiële complicatie voor routinegebruik van de ontwikkelde test zou het bestaan van verschillend reagerende isolaten van het ISMV kunnen zijn. Hoewel verschillende geteste isolaten wel enigszins verschilden in symptoomexpressie en serologische reacties, konden zij allen goed aangetoond worden met behulp van de verwondingsmethode.

Onderzoek naar de oorzaak van de slechte aantoonbaarheid van het virus in secundair geïnfecteerde en in vroeg primair geïnfecteerde bollen wordt beschreven in Hoofdstuk 7. De virustiter in de hele plant, inclusief de bol, werd

gedurende het groeiseizoen gevolgd in zowel secundair geïnfecteerde planten als in primair geïnfecteerde planten (Hoofdstuk 7; Figuur 9.1). De verdeling van het ISMV in de bovengrondse delen van zowel de primair als secundair geïnfecteerde plant correleerde met de nutriëntenstroom via de vaatbundels. Dit betekende dat de bovengrondse delen van de secundair geïnfecteerde planten volledig geïnfecteerd waren terwijl dit in primair geïnfecteerde planten afhankelijk was van het tijdstip van inoculatie: na een vroege infectie verspreidde het virus zich eerst nog naar de bovenste bladeren en later naar de nieuwe bol, terwijl bij late infecties het virus voornamelijk in de nieuw gevormde bollen gevonden werd. Echter, in de nieuwe bollen van secundair geïnfecteerde planten werd gedurende het hele groeiseizoen nauwelijks virus aangetroffen. Tevens nam de aantoonbaarheid van het ISMV in bollen van vroeg geïnfecteerde planten drastisch af tegen het einde van het groeiseizoen (Hoofdstuk 7). Dit kan verklaard worden door aan te nemen dat de plant op een bepaald tijdstip na infectie een barrière heeft opgeworpen zodat de toegang van het virus naar de bol wordt geblokkeerd, of dat het virus in de secundaire en vroeg primair geïnfecteerde planten niet meer beschikbaar is voor transport (Hoofdstukken 7 en 9). Zodoende moet geconcludeerd worden dat ISMV in deze secundair en primair geïnfecteerde bollen direct na het rooien onbetrouwbaar is aan te tonen, doordat het transport van het virus naar de bol belemmerd wordt.

Het hier gepresenteerde onderzoek beschrijft de relatie tussen de vermeerdering en verdeling van het ISMV en het metabolisme van de waardplant. Het onderzoek resulteerde niet alleen in meer kennis over de interactie tussen ISMV en de iris, maar heeft ook geleid tot de ontwikkeling van een betrouwbare toets voor het aantonen van dit virus in de irisbol.

Curriculum vitae

Cornelia Ida Maria van der Vlugt (Lia) werd geboren op 6 februari 1961 te Leiden. Zij bezocht College Leeuwenhorst te Noordwijkerhout en deed in 1979 eindexamen VWO-B met Latijn. In 1979 startte zij de studie Biologie aan de Rijksuniversiteit te Leiden, waar in october 1982 het kandidaatsexamen B4 (Biologie met als tweede hoofdvak Scheikunde) werd behaald. Tijdens de doctoraalstudie volgde zij het hoofdvak Microbiologie en de bijvakken Plantevirologie en Milieukunde. Tevens nam zij deel aan een uitwisselingsproject met de Kent State University (Ohio, USA). In februari 1986 werd het doctoraalexamen Biologie behaald.

Van april 1986 tot en met november 1987 was zij werkzaam bij de Bloembollenkeuringsdienst te Lisse in een samenwerkingsverband met de werkgroep Plantevirussen van de Rijksuniversiteit Leiden en het Laboratorium voor Bloembollenonderzoek te Lisse, waarbij de mogelijkheid van toepassing van een cDNA-hybridisatietoets voor het routinematig aantonen van het tabaksratelvirus werd onderzocht.

Vanaf 1 december 1987 tot 1 februari 1992 had zij een aanstelling als onderzoeksassistent en daarna als gastmedewerker, bij de vakgroep Virologie van de Landbouwuniversiteit Wageningen en was zij gestationeerd op het Laboratorium voor Bloembollenonderzoek te Lisse. Tijdens deze periode werd onderzoek gedaan naar het irisgrijsvirus in irisbollen. Het onderzoek was vooral gericht op het verbeteren van de aantoonbaarheid van dit virus in de bol en het verkrijgen van inzicht in de verplaatsing en de vermeerdering van het virus mede in relatie tot de metabole activiteit van de irisplant. In dit proefschrift wordt hiervan verslag gedaan.

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