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# Turnip yellow mosaic virus in Chinese cabbage in Spain: commercial seed transmission and molecular characterisation.

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| **Abstract:** | Seed transmission of Turnip yellow mosaic virus (TYMV, genus Tymovirus) was evaluated in the whole seeds and seedlings that emerged from three commercial Chinese cabbage (Brassica pekinensis) seed batches. Seedlings in the cotyledon stage and adult plants were assayed for TYMV by DAS-ELISA and confirmed by RT-PCR. The proportion of whole seeds infected with TYMV was at least 0.15%. The seeds of the three seed batches were grown in Petri dishes, and surveyed in the cotyledon stage in trays that contained a peat:sand mixture grown in greenhouses or growth chambers, which were analysed in the cotyledon and adult stages. The seed-to-seedling transmission rate ranged from 2.5% to 2.9% in two different seed batches (lot-08 and lot-09, respectively). Spanish isolates derived from turnip (Sp-03) and Chinese cabbage (Sp-09 and Sp-13), collected in 2003, 2009 and 2013 in two different Spanish regions, were molecularly characterised by analysing the partial nucleotide sequences of three TYMV genome regions: partial RNA-dependent RNA polymerase (RdRp), methyltransferase (MTR) and coat protein (CP) genes. Phylogenetic analyses showed that the CP gene represented two different groups: TYMV-1 and TYMV-2. The first was subdivided into three subclades: European, Australian and Japanese. Spanish isolate Sp-03 clustered together with European TYMV group, whereas Sp-09 and Sp-13 grouped with the Japanese TYMV group, and all differed from group TYMV-2. The sequences of the three different genomic regions examined clustered into the same groups. The results suggested that Spanish isolates grouped according to the original hosts from which they were isolated. The inoculation of the Spanish TYMV isolates to four crucifer plants species (turnip, broccoli, Brunswick cabbage and radish) |

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| | revealed that all the isolates infected turnip with typical symptoms, although differences were observed in other hosts. |
| **Response to Reviewers:** | Reviewer # 1:<br>Firstly, the reviewer pointed that there were no significant details about the seed origin. As he/she correctly stated the seeds of Chinese cabagge var. Sumiko are a F1 hybrid variety developed by Bejo Zaden, however no further information was available. They were probably produced in France, Italy, The Netherlands, Australia or the USA, and imported to Spain, but this information is not supplied.<br>Secondly, we have included more details in the seedling tests and corrected the Table 3, because the footnote (b) was incomplete. In the revised version, we have explained how was performed the Petri dishes sown and in the Table 3 clarified the groups of samples: 10 seedlings were considered a single sample in the Petri dishes assay and 4 in the trays.<br>Thirdly, we think that testing the plants individually probably gives a realistic data of TYMV seed transmission, however the assay was performed by grouped samples because it was designed in this way to analyse a higher number of plants faster and with a reduced price. Even so, we have rewritten that part in order not to exaggerate the level of infection, referring to the minimum transmission rate of 0.15%.<br>Fourth, we have rewritten the discussion part where the location of TYMV in the seed was explained to a better understanding of the text.<br>Later, we have referred to MNSV as internally-borne in the seed as the study of Campbell et al. (1996) reported.<br>All spelling, grammatical errors and word usage suggested by the reviewer have been addressed.<br>Reviewer # 2:<br>All spelling, grammatical errors and word usage suggested by the reviewer have been addressed. |

# *Turnip yellow mosaic virus* in Chinese cabbage in Spain: commercial seed transmission and molecular characterisation.

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## Abstract

Seed transmission of *Turnip yellow mosaic virus* (TYMV, genus *Tymovirus*) was evaluated in the whole seeds and seedlings that emerged from three commercial Chinese cabbage (*Brassica pekinensis*) seed batches. Seedlings in the cotyledon stage and adult plants were assayed for TYMV by DAS-ELISA and confirmed by RT-PCR. The proportion of whole seeds infected with TYMV was at least 0.15%. The seeds of the three seed batches were grown in Petri dishes, and surveyed in the cotyledon stage in trays that contained a peat:sand mixture grown in greenhouses or growth chambers, which were analysed in the cotyledon and adult stages. The seed-to-seedling transmission rate ranged from 2.5% to 2.9% in two different seed batches (lot-08 and lot-09, respectively). Spanish isolates derived from turnip (Sp-03) and Chinese cabbage (Sp-09 and Sp-13), collected in 2003, 2009 and 2013 in two different Spanish regions, were molecularly characterised by analysing the partial nucleotide sequences of three TYMV genome regions: partial RNA-dependent RNA polymerase (RdRp), methyltransferase (MTR) and coat protein (CP) genes. Phylogenetic analyses showed that the CP gene represented two different groups: TYMV-1 and TYMV-2. The first was subdivided into three subclades: European, Australian and Japanese. Spanish isolate Sp-03 clustered together with European TYMV group, whereas Sp-09 and Sp-13 grouped with the Japanese TYMV group, and all differed from group TYMV-2. The sequences of the three different genomic regions examined clustered into the same groups. The results suggested that Spanish isolates grouped according to the original hosts from which they were isolated. The inoculation of the Spanish TYMV isolates to four crucifer plants species (turnip, broccoli, Brunswick cabbage and radish) revealed that all the isolates infected turnip with typical symptoms, although differences were observed in other hosts.

**Keywords:** *Brassica pekinensis*, *Tymovirus*, RT-PCR, ELISA.

## Introduction

*Turnip yellow mosaic virus* (TYMV) is the type species of the genus *Tymovirus* and only infects species of the family *Brassicaceae*, and two species in two closely related families: *Capparidaceae* and *Resedaceae* (Brunt et al. 1996). This virus was first described in 1946 to infect turnip, swede and broccoli in the United Kingdom and Portugal (Markham and Smith 1949). Typical symptoms of TYMV infection in turnip (*Brassica rapa*) are a yellow clearing of veins and mosaic in younger leaves, followed by the appearance of small yellow patches on older leaves, which coalesce into large yellow areas (Markham and Smith 1949). TYMV-infected Chinese cabbage plants (*Brassica pekinensis* (Lour) Rupr.) present local chlorotic spots and vein clearing, followed by a distinct yellow mosaic. Severely affected plants of this species are stunted and develop small purple-brown necrotic spots on apical leaves (Kirino et al. 2008).

The occurrence of TYMV has been reported in different countries of Europe, and also in Australia, Japan, New Zealand and Canada (Brunt et al. 1996; Stobbs et al. 1998; Kirino et al. 2008). Although the virus is present in Spain, it has been reported only in a wild population of *Arabidopsis thaliana* plants surveyed in the spring of 2006, 2007 and 2008 in Central Spain, and was found in mixed infections with other viruses (Pagan et al. 2010).

The virus is transmitted mechanically by different flea-beetle species of the genus *Phyllotrecta*, and also via seeds (Markham and Smith 1949; Spak et al. 1993). Presence of TYMV in seeds collected from different crucifer species infected with the virus has been demonstrated, and infection percentages range from 0.2% to 19.7% (Rimmer et al. 2007). Seed embryo invasion is necessary for seed transmission in *A. thaliana* (Assis Filho and Sherwood 2000).

TYMV has isometric non-enveloped virions with a T=3 icosahedral symmetry, of approximately 30 nm in diameter. The most characteristic cytological effect of TYMV infection is the development of small vesicles near the periphery of the chloroplasts formed by the invagination of both chloroplast bilayer membranes (Mathews 1980). This virus has a single-stranded positive RNA genome of 6.3 kb that comprises three overlapping open-reading frames (ORF), which encode the replicase protein (ORF 1; 206 KDa), the movement protein (ORF 2; 69 KDa) and the virion capsid protein or coat protein (ORF3; 20 KDa). The capsid protein (CP) gene is transcribed from a subgenomic messenger RNA. To date, phylogenetic analyses have identified two distinct groups of isolates based on the studies of CP gene sequences of different isolates: TYMV-1, which encompasses the European and Australian subgroups; TYMV-2, which includes different UK isolates. The isolates of the European and Australian subgroups are more closely related than the UK isolates, which showed long genetic distances with the others (Blok et al. 1987; Hayden et al. 1998a; 1998b; Mitchell and Bond 2005). Two isolates of TYMV from Chinese cabbage collected in Japan have been recently identified, and their CP

sequences have revealed that they constitute a subgroup closely related to both the European and Australian isolates (Kirino et al. 2008).

All previous seed transmission studies of TYMV have been conducted with seeds obtained from infected plants. Therefore, the objectives of our study were to evaluate the transmission rate of TYMV from commercial seed batches to emerged seedlings. The characterisation of three TYMV isolates collected in different areas of Spain from turnip and Chinese cabbage is presented.

## Material and Methods

### Virus isolates and seed batches

The Chinese cabbage commercial seed batches used in this study were from the F1 hybrid variety Sumiko. Three seed batches were used for comparisons (lot-08, lot-09 and lot-10), from 2008, 2009 and 2010, respectively.

The TYMV isolates (namely Sp-09 and Sp-13) from Chinese cabbage (*B. pekinensis*) were collected from affected fields in October 2009 in Lleida (NE Spain) and January 2014 in Valencia (E Spain), respectively. Plants showed typical TYMV infection symptoms: vein clearing, stunting and yellow mosaic of leaves (Fig. 1). Another Spanish isolate (Sp-03) included in the assay was collected in 2003, from an infected turnip (*B. rapa*) from Lugo (NW Spain). Isolates Sp-03, Sp-09 and Sp-13 were frozen at -80ºC or leaf tissue was dehydrated, respectively, until used. The TYMV reference isolate DSMZ PV-0299 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) isolated from pak choi (*Brassica chinensis*) in Germany was also included in the characterisation assays.

### TYMV detection in whole Chinese cabbage seeds.

In order to detect the level of virus in the three commercial seed batches, totals of 370, 540 and 640 seeds from lot-08, lot-09 and lot-10, respectively, were tested by the double-antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). Samples of 10 seeds were soaked for 60 min in 1 ml of sample extraction buffer, and were ground with a pestle to obtain the seed extract. DAS-ELISA was carried out in paired wells with TYMV-specific antisera supplied by Loewe Biochemica GmbH (Sauerlach, Germany) following the manufacturer's instructions, where 100 µl of the obtained extracts were used per well. The TYMV-infected turnip leaf samples were supplied by Loewe Biochemica GmbH (Sauerlach, Germany) and healthy Chinese cabbage seed extracts were included as the positive and negative controls, respectively. Absorbance values (*A*405nm) were measured in a Titertek Multiskan immunoplate reader (Flow Laboratories, Finland). Absorbance values of more than doubled those of the

healthy seed extract controls, were recorded as positive samples. Otherwise samples were considered to be non-infected.

**Seed-to-seedling transmission of TYMV**

To determine the transmission rates of the Chinese cabbage commercial seeds to seedlings, two different assays were performed. The first assay consisted in growing 100 seeds per batch on moist filter paper in Petri dishes (20 seeds per Petri dish) inside a greenhouse and then analysing the seedlings that emerged in the cotyledon stage by DAS-ELISA. In the second assay, 186 seeds from each seed batch (lot-08, lot-09 and lot-10) were sown in sterile 24-well trays that contained a sterilised substrate (2:1 peat:sand) with a single seed per well. One part of these sown trays (114 seedlings) was placed inside a growth chamber at day and night temperatures of 25ºC and 18°C, respectively, 16-hour daylight and 70% relative humidity. The rest of the seedlings (72 plants) were placed in a greenhouse (22-25°C). Stringent sanitary measures were used to prevent any spurious virus spread. The seedlings that emerged in the Petri dishes culture were analysed in the cotyledon stage (approximately 7 days after emerging) by considering groups of 10 seedlings as a single sample.

In the seedlings grown in trays and cultivated in a greenhouse or a growth chamber, sampling was performed at two different time points; 7 days after one cotyledon emerged from all four plants, which was pooled and homogenised in a plastic bag as described before; this screening procedure was repeated 3 weeks later when seedlings reached the four-leaf stage.

In both cases, grouped samples were homogenised in a plastic bag with 1:20 (wt/vol) ml of sample extraction buffer and 100-µl aliquots were assayed for TYMV by DAS-ELISA as described above. Healthy Chinese cabbage leaf extracts were included in each ELISA plate as the negative controls. The percentage of viral incidence from grouped samples was estimated with the formula of Gibbs and Gower (1960): $p = 1 - (1 - y/n)^{1/k}$, where $p$= probability of transmission by a single TYMV-infected seed, $y$ = number of positive samples, $n$ = total number of assayed samples, and $k$ = number of seedlings per sample ($k = 4$).

Positive results were confirmed by RT-PCR with TYMV-specific primers TYMV-D/R, detailed in Table 1 as described below.

**Characterisation of TYMV isolates**

The three Spanish isolates described above were molecularly characterised (Sp-03, Sp-09 and Sp-13), and the isolate supplied by DSMZ was named DSMZ PV-0299.

Total nucleic acid extraction was performed on 0.1 g of leaf tissue according to the silica capture extraction protocol (Rot and Jelkman 2001). Extracted nucleic acids were stored at −80°C until used.

Three different regions of the virus genome were studied in the molecular characterisation assay: partial RNA dependent RNA polymerase (RdRp), methyltransferase (MTR) and coat protein (CP) genes. The previously described primers were used to amplify these regions, plus one TYMV-specific primer pair (TYMV-D and TYMV-R), designed using version 4.0 of the OLIGO program (National Bioscience Inc.) on the basis of the complete TYMV genome sequences deposited in the GenBank database [TYMV-1 (Acc. No. X07441), TYMC (Acc. No. X16378), Blue Lake (Acc. No. AF035403), Club Lake (Acc. No. J04373)]. Primers and their characteristics are provided in Table 1.

RT-PCR was carried out using SuperScript™ II RT with the Platinum® Taq kit (Invitrogen Life Technologies, Barcelona, Spain) and the TYMV-specific primer pairs detailed in Table 1 at a final concentration of 0.4 pmol $\mu l^{-1}$. The PCR programme consisted of an initial incubation at 50°C for 30 min, followed by 2 min at 94°C, and 40 cycles of 94ºC for 15 s, and at an appropriate annealing temperature for specific primers for 30 s (Table 1) and 68°C for 1 min. A final incubation at 68°C, 10 min, was introduced to finish the incomplete PCR fragments. The amplified PCR products were analysed on 1.2% agarose gel in 1x TAE buffer (40 mM Tris-acetate, 20mM acetic acid and 1 mM EDTA, pH 8·0), stained with ethidium bromide and visualised under UV light. Fragment sizes were determined by comparing with 100 bp DNA Ladder Plus (MBI Fermentas). Amplified fragments were purified with the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) and directly sequenced.

The obtained nucleotide sequences were compared with the sequences deposited in the NCBI database using the Blastn program, and an identity⁄similarity matrix of the nucleotide analysed sequences was calculated by the matrix global alignment tool software (Montclair, NJ, USA), version 2.02 (http://bitincka.com/ledion/matgat). Phylogenetic analyses were performed with MEGA (Molecular Evolutionary Genetics Analysis, Tempe, AZ, USA), version 5 (Tamura et al. 2011). The robustness of the inferred evolutionary relationships was assessed by 10,000 bootstrap pseudoreplicates.

Three isolates from different hosts in origin (Sp-03 from turnip, Sp-09 from Chinese cabbage and DSMZ PV-0299 from Pak-Choi) were inoculated to four different cruciferous species: turnip (*B. rapa* var. *rapa* subvar. *esculenta*), broccoli (*Brassica oleracea* var. *italica*), Brunswick cabbage (*Brassica oleracea* var. *viridis*) and radish (*Raphanus sativus*) var. De dix huit jours.

Seeds of these species were sown in sterile 24-well trays that contained sterilised substrate (2:1 peat: sand) with a single seed per well. Seedlings were analysed for TYMV by RT-PCR with the TYMV-specific primer pair, which amplified an RdRp gene fragment (Table 1), as described before when two true leaves were developed to ensure the health of plants. They were placed inside a greenhouse and strict sanitary measures were taken to ensure the hygiene of plants. Twelve plants per species were inoculated on the first true fully developed leaf when

they reached the four-leaf stage. The inoculum was prepared by grinding leaf material of the three TYMV isolates in inoculation buffer (0.01 m phosphate buffer, pH 7.2, that contained 0.2% sodium bisulphite and 0.2% sodium diethyldithiocarbamate) in 1:4 (wt /v), where Carborundum (600 mesh) was used as an abrasive. Six plants per species were mock-inoculated and served as a negative assay control.

Plants were monitored weekly to evaluate symptom development and were analysed by DAS-ELISA, as described above, on 15 and 30 days post-inoculation (dpi).

## Results

### TYMV infection of whole seeds

The results of the serological analyses performed on the different grouped seed samples for the three assayed batches (lot-08, lot-09 and lot-10) are shown in Table 2. The batch with the most TYMV-positive grouped samples was lot-08 (6 positive samples). In lot-09 and lot-10, TYMV was detected only in one group of seeds. If only one seed per group of 10 was infected in lot-08, the seed infection rate would be 1.62%. The worst situation would be if all the seeds of this TYMV-positive group were infected because it would imply an infection rate of 16.2%. Therefore, the infection rate was as minimum 0.15% (in lot-10, if only one seed in the TYMV-positive group was infected).

### Seed-to-seedling transmission of TYMV

Two different assays were carried out with the three seed batches as described before: (i) growing seeds in Petri dishes and analysing the emerging seedlings in the cotyledon stage; (ii) sowing seeds in trays with a peat:sand mixture and culturing seedlings in two different locations: inside the growing chamber and inside a greenhouse, and analysing them in the cotyledon and leaf stages. The results of the seed-to-seedling assays are detailed in Table 3. In the first assay, only two samples of the 100 seedlings of lot-08 grown in Petri dish (each comprised of cotyledon tissues from ten seedlings) tested positive for TYMV by DAS-ELISA. In lot-09, this assay was not performed because no seeds were left. In the second assay, none of the 186 seedlings grown under growth chamber conditions tested positive for TYMV. Under greenhouse conditions, only two samples of the 72 seedlings were positive for TYMV in both development stages (cotyledon and adult stage) in lot-09. All these data indicate that TYMV was seed-transmitted in these commercial seed batches at an infection rate that ranged from 2.5 to 2.9%, as calculated by the formula of Gibbs and Gower (1960). One plant of the ELISA-positive Chinese cabbage seedlings from lot-09 presented obvious TYMV infection symptoms

as its leaves presented yellow mosaic. All these positive results were confirmed by RT-PCR with TYMV-specific primers, which amplify an RdRp fragment (Table 1).

**Characterisation of TYMV isolates**

The nucleotide sequences of partial RdRp (857 nt), MTR (635nt) and CP (718 nt) were determined in the four studied TYMV isolates (Sp-03, Sp-09, Sp-13 and DSMZ PV-0299). These genomic regions were chosen to represent the viral genes that encode proteins with distinct functions. The range of the percentages of nucleotide identity of the RdRp, MTR and CP gene fragments among the Spanish isolates (Sp-13, Sp-09 and Sp-03) was 94-95%. If the comparison was made among the different subgroups of isolates retrieved from the GenBank database (European, Australian, Japanese or TYMV-2), the four TYMV isolates studied in this assay showed slight differences in identitying percentages (Table 4). Isolates Sp-09 and Sp-13 from Chinese cabbage were similar in the three studied regions. These isolates showed higher identity percentages with the Japanese cabbage isolates (97.8-98.6) in the CP gene. No sequences of the other studied regions were available in the GenBank database. Isolate Sp-03 showed more similar identities to European isolates in the three studied genes (96.2-97.8). Isolate DSMZ PV-0299 had similar percentages to the European and Australian subgroups of isolates. All the studied isolates showed clear differences with the TYMV-2 group (less than 74.7% of identity).

The phylogenetic trees of the three studied TYMV genome regions are depicted in Fig. 2. These analyses showed that Spanish isolate Sp-03 from the turnip collected in 2003 clustered together with the European subgroup, and that DSMZ PV-0299 grouped with the Australian isolates in the three studied genome regions. Isolates Sp-09 and Sp-13, collected respectively in 2009 in Lleida (Spain) and in 2013 from Valencia, clustered with the Japanese isolates collected from Chinese cabbage A-1 and B-3 (Kirino et al., 2008) in the CP gene. In the other two genes, RdRp and MTR, they branched separately because only the nucleotide sequences of the European and Australian isolates are published in the GenBank database, and no sequence of these genes of the Japanese or UK isolates was found. Therefore, the phylogenetic tree of the CP gene of TYMV presented two big clades: the TYMV-2 group, which included different UK isolates (collected from cauliflower and other wild cabbage species from *B. oleracea*, and the TYMV-1 group, which presented three subclades: European (isolated mainly from turnip); Australian (collected from *Cardamine lilaciana*); and Japanese TYMV isolates (from Chinese cabbage) (Paul et al. 1980; Dreher and Bransom, 1982; Blok et al. 1987; Mitchell and Bond 2005; Kirino et al. 2008). The phylogenetic analyses revealed that the sequences of the three different studied regions maintained the same groups. Apparently, Spanish isolates grouped separately according to the original host where they were isolated, but more isolates should be studied to reinforce this observation.

The inoculation of the three TYMV isolates to the four species of crucifer plants revealed that all the isolates infected turnip and presented the typical TYMV symptoms, such as mosaic, vein clearing, and yellowing. Only the DSMZ PV-0299 TYMV isolate was able to infect all the crucifer species and distinguishable symptoms were observed in all the plants. However, Spanish isolates Sp-03 (collected from turnip) and Sp-09 (from Chinese cabbage) failed to infect some species. Specifically, isolate Sp-03 was able to infect turnip and Brunswick cabbage, and showed clear infection symptoms, whereas isolate Sp-09 infected only inoculated turnip.

## Discussion

Seed transmission is one of the most important mechanisms of viral transmission. It is effective because not only is tissue infection produced in an early plant development stage, but low seed transmission rates, in conjunction with secondary spread by vectors, can also lead to introduction of viruses into new areas that can produce viral disease epidemics (Johansen et al. 1994; Hull 2002). As viruses may persist in seeds for long periods, this mechanism is the most effective form of long- distance viral transmission (Hull 2002). Plant virus perpetuation by infected seeds is a survival strategy as it acts as a protective link between growing seasons. A virus in seeds can be located on the seed surface. Seedling infection occurs primarily by mechanical transmission and virus infection in the embryo, and is probably produced indirectly by the infection of reproductive tissues before embryogenesis, or directly by the invasion of the embryo in some embryogenesis stage (Johansen et al. 1994). TYMV has been reported to be transmitted through seeds in several hosts with variable transmission rates. TYMV seed transmission was first described in 1983, when naturally- and experimentally-infected broccoli presented 2.2% and 9.5% of seed-to-seedling transmission, respectively (Benneti and Kaswalder 1983). Further studies have determined TYMV seed transmission in artificially inoculated plants of *A. thaliana*, Chinese cabbage, *Crambe hispanica*, *Camelina sativa* and garlic mustard (*Alliara petiolata*) (Benneti and Kaswalder 1983; Hein 1984; Pelikanova 1990; Assis Filho and Sherwood 2000; Kirino 2008). Natural infection has also been reported in winter turnip rape (*B. napus* var. *silvestris*) with transmission rates ranging from 1.6% to 8.3% in three different localities of the Czech Republic and Slovakia (Spak et al. 1993). In this assay, the main difference with the previously reported seed-to-seedling transmission studies is that the three seed batches used were commercial. So although seed transmission was detected only in two seed lots (lot-08 and lot-09), the transmission rate we obtained (2.5-2.9%) was very high. Moreover, when seeds come from commercial suppliers, there may be a risk of long-distance virus spread. To our knowledge, our study is the first to determine the presence and infectivity of TYMV in commercial seeds (in a range over 0.15%). The location of TYMV in infected seeds is determined in either the embryo or the seed coat, but the higher concentration was

found in the seed coat, although the embryo invasion is necessary for seed transmission in *A. thaliana* (Assis Filho and Sherwood 2000).

Further studies into effective seed treatments should be carried out to ensure that commercial seeds are TYMV-free. As this virus is carried in the embryo, chemical treatments might not be effective enough to eliminate the virus. Consequently, different thermal treatments have to be evaluated; e.g. for MNSV, which is internally-borne (Campbell et al. 1996) , a thermal treatment (144 h at 70ºC) was used to eradicate the virus from the seed without hindering germination (Herrera-Vasquez et al. 2009).

The CP gene of TYMV has been widely studied because it plays an important role in the pathogenicity and virulence of the virus. Variations in the nucleotide sequence of the CP and the resulting amino acid structure of the CP of individual isolates both indicate differences in pathology and ecology (Mitchel and Bond 2005). Based on the sequences of the CP gene of TYMV, two different groups were originally defined, one formed by European and Australian isolates named TYMV-1, which included the type strain; a second group named the TYMV-2 isolates, which included the cauliflower strain (Blok et al. 1987). Further studies revealed that TYMV-1 and TYMV-2 also included Japanese Chinese cabbage and UK isolates collected from wild cabbage, respectively (Mitchell and Bond 2005; Kirino et al. 2008). Three Spanish isolates collected from different hosts, locations and in different years were analysed herein. These isolates clustered within TYMV-1; isolate Sp-03 collected in 2003 from turnip and grouped with the European isolates, and Sp-09 and Sp-13 collected in 2009 and 2013 from Chinese cabbage clustered in the Japanese group formed by isolates from Chinese cabbage. Although the exact geographical origin and original host of many sequences of the isolates retrieved from the GenBank database are unclear, a correlation was found between the hosts of the Spanish isolates and the group they matched. Moreover, the geographical proximity of isolate source should not be presumed to mean lack of variance between TYMV isolates, which has occurred with different TYMV-2 isolates (Mitchell and Bond 2005). These molecular differences in the Spanish isolates translated into different phylogenetic clusters were maintained in the other studied genome regions (MTR and RdRp). However, their genomic regions have been studied less and few sequences are available in the GenBank database to compare them with.

The inoculation of Spanish isolates Sp-03 and Sp-09 resulted in a different response among the four tested cruciferous species. While Sp-03 was able to infect turnip and Brunswick cabbage, Sp-09 only infected turnip. Different strains among the isolates collected in one area have been defined according to serology and symptom development in test plants (Matthews 1980). Thus, molecular differences could also induce a difference in host response, which has been described

for other viruses (Procházková 1980; Špak et al. 2000; Fakhro et al. 2011). However, no correlation between the sequence differences in the CP gene and symptoms caused by different TYMV isolates has been established. So the molecular basis of symptom and host response differences between TYMV is probably encoded by virus sequences other than the virion protein, as shown for many other viruses (Hayden et al. 1998a).

The hypothesis of a progenitor TYMV has given rise to group TYMV-1 and TYMV-2 populations during the diversification of brassicas. More recently, a TYMV-1 isolate has been reported to migrate with the newly established brassicas to other areas, and has established new populations which have lately diverged, as has been suggested for Australian isolates (Blok et al. 1987). Furthermore, this hypothesis could also be applied to the Japanese group of isolates and is reinforced by the fact that TYMV is often introduced into new areas by seed transmission. Seed transmission has to be taken into account and effective seed treatments need to be evaluated to avoid introducing TYMV via infected seeds also from commercial batches into new areas as new population of the virus could be established, which could develop differences in the host range or viral detection, and even in vector transmission.

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## Figure captions

**Fig. 1:** Symptoms of TYMV in Chinese cabbage: (**A**) TYMV affected field in Lleida (Spain) in October 2009. (**B**) General appearance of infected plants. (**C**) Typical symptoms of TYMV infection such as vein clearing, stunting and yellow mosaic of the leaves. (**D**) When TYMV infection occurs on young plants, the symptoms are more severe and the affected plants remain small and stunted (left) when compared with healthy plants (right).

**Fig. 2:** Neighbour-joining phylogenetic trees obtained from distance matrix (Kimura 2-parameters) with MEGA version 5 (Tamura et al. 2011) from nucleotide sequences coding for the RdRp (a), MTR (b) and CP (c) genes of Spanish (Sp-03, Sp-09 and Sp-13) and a reference TYMV (DSMZ PV-0299) isolates with other isolates retrieved from the Genbank database: TYMV-1 (Acc. No. X07441), TYMC (Acc. No. X16378), 62226 (Acc. No. V01418), 332241 (Acc. No. K00602), Blue Lake (Acc. No. AF035403), Club Lake (Acc. No. J04373), D5 (Acc. No. U88845), P1 (Acc. No. U88849), F41 (Acc. No. U88847), Q18 (Acc. No.U88850), F39 (Acc. No. U88846), N37 (Acc. No. U88848), A-2 (Acc. No. AB358971), B-3 (Acc. No. AB358972), Rothamstead (Acc. No. AF035635), Cauliflower (Acc. No. AF035636), Dorset 18 (Acc. No. AY673644), Dorset 17 (Acc. No. AY673642), and Dorset 50 (Acc. No. AY673645). Sequence of *Kennedya yellow mosaic virus* (KYMV, Acc. No. D00637) was used as outgroup. The statistical reliability of the constructed trees was assessed by the bootstrap method based on 10,000 pseudoreplicates. Numbers above nodes indicate percentages of bootstrap replicates which supported branching. Scale bars represent genetic distance of 0.05.


**Figure 1.**

Figure 2

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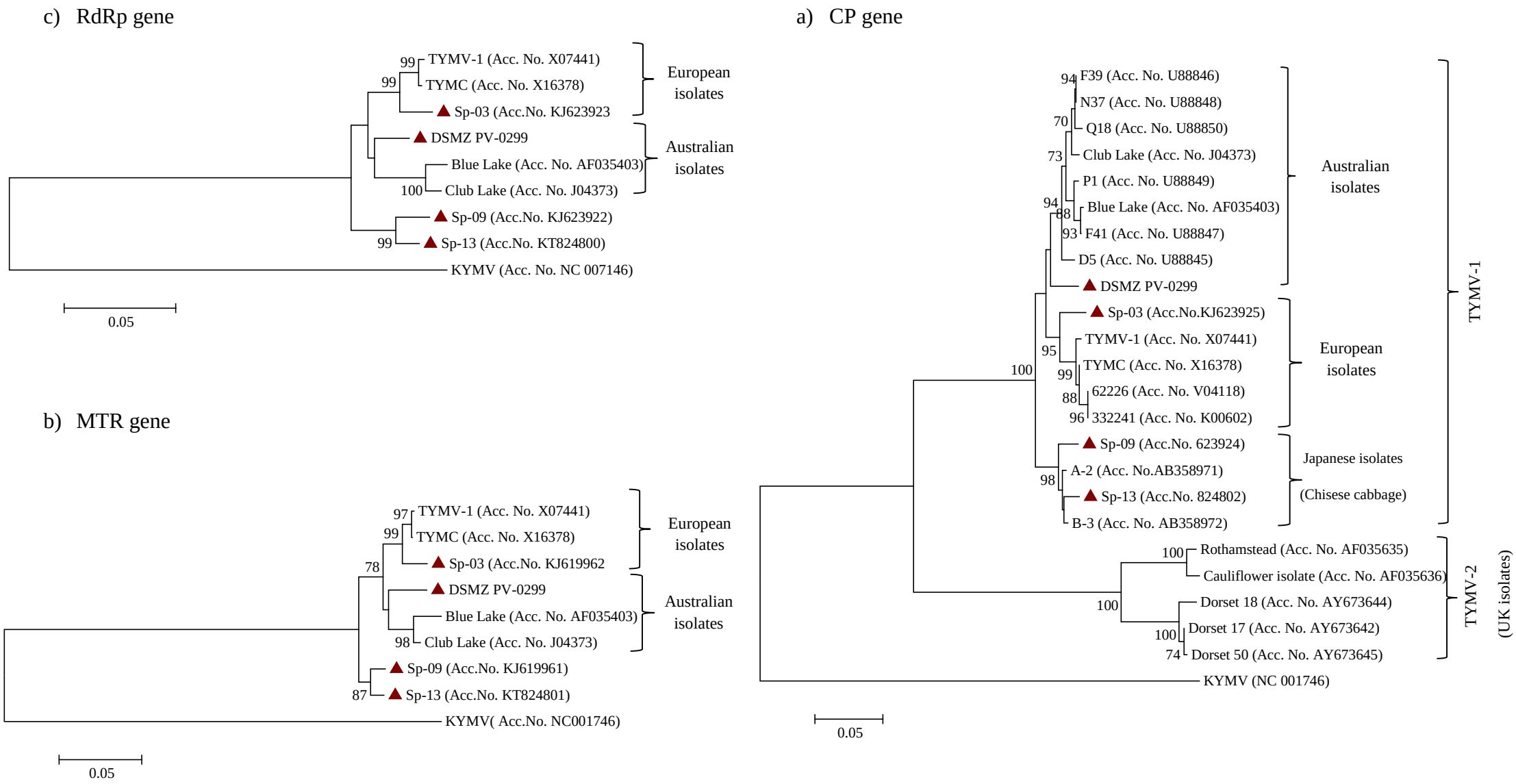


**Figure 2.**

**Table 1:** Sequences of the *Turnip yellow mosaic virus* (TYMV)-specific primers used in the assay.

| Primer name | Sense | Sequence (5´-3´)[a] | Ta (ºC) | Fragment (bp) | Amplified Region | Reference[b] |
|---|---|---|---|---|---|---|
| TYMV-D | F[c] | CTCCACAAAGATCAATCTAGCAACC | 58 | 857 | RdRp | In this study |
| TYMV-R | R[d] | GATGGGGCAGGAACCGACGTCATA | | | | |
| MTR1 | F[c] | TTCATGCAYGAYGCYMTSATGT | 55 | 635 | MTR | Sabanadzovic *et al.*, 2000 |
| MTR2 | R[d] | TCCCAVGCNBHBGVRGTGACCCA | | | | |
| TALL-COMP | F[c] | CCCTCGAGTYTGAATTGCTTC | 50 | 718 | CP | Hayden *et al.*, 1998a |
| CL-3 | R[d] | GGTCTAGACATATGGTTCCGATGACCCTCGG | | | | |

[a] Y= C or T; M=A or C ; V=A or C or G; N= A or C or G or T; R= A o G; B=C or G or T; H=A or C or T; S=G or C.

[b]In which primer was designed

[c]Specific forward primers.

[d]Specific reverse primers

**Table 2:** Results of the analyses by DAS-ELISA performed on the whole seed samples of the three seed batches analysed (lot-08, lot-09 and lot-10).

| **Batch number** | **Tested[a]** | **Positive samples[b]** | **Range of infection (%)** | **Emergence (%)** |
|---|---|---|---|---|
| Lot-08 | 370 | 6 | 1.62-16.2 | 100 |
| Lot-09 | 540 | 1 | 0.18-1.85 | 91.67 |
| Lot-10 | 640 | 1 | 0.15-1.56 | 100 |

[a] Number of whole seed analysed from each seed batch.

[b] Number of TYMV-positive grouped seed samples. A sample corresponds to a group of ten whole seeds.

**Table 3:** Results of the analyses of seed to seedling transmission performed to plants emerged from the three seed batches analysed (lot-08, lot-09 and lot-10).

| Batch number | Assay | Tested[a] | Positive samples[b] | Probability of infection[c] |
|---|---|---|---|---|
| Lot-08 | Petri dish | 100 | 2 | 0.0259 |
| | Growing chamber culture | 186 | 0 | 0 |
| | Greenhouse culture | 72 | 0 | 0 |
| Lot-09 | Petri dish | - | - | - |
| | Growing chamber culture | 186 | 0 | 0 |
| | Greenhouse culture | 72 | 2 | 0.0290 |
| Lot-10 | Petri dish | 100 | 0 | 0 |
| | Growing chamber culture | 186 | 0 | 0 |
| | Greenhouse culture | 72 | 0 | 0 |

[a] Number of seedlings tested by enzyme-linked immunosorbent assay (DAS-ELISA).
[b] Number of TYMV-positive grouped leaf samples. A sample corresponds to a group of ten leaves from ten seedlings grown in Petri dishes or four leaves from four seedlings grown in pots cultivated growing chamber or greenhouse.
[c] Probability of infected seedlings grown from infected seeds calculated using the formula of Gibbs and Gower (1960) to estimate proportions from group samples.

**Table 4:** Percentages of identity of the sequences of RdRp, MTR and CP partial genes of the three TYMV isolates studied compared with the sequences of isolates belonging to different groups retrieved from the GenBank database.

| Gene Fragment | Group of isolates[a] | Sp-03 | Sp-09 | Sp-13 | DSMZ PV-0299 |
|---|---|---|---|---|---|
| RdRp | European group | 97.3-97.8 | 94.0-94.5 | 94.1-94.5 | 95.8-95.9 |
| | Australian group | 94.0 | 92.5 | 93.5-93.8 | 95.3-95.4 |
| MTR | European group | 97.3-97.8 | 94.9-95.5 | 94.9-95.5 | 95.1-95.5 |
| | Australian group | 94.2-95.5 | 93.7-94.9 | 93.3-94.9 | 94.9-95.1 |
| CP | European group | 96.2-96.8 | 93.7-94.5 | 93.7-94.3 | 94.9-94.7 |
| | Australian group | 94.5-95.6 | 93.1-94.1 | 93.2-94.9 | 95.2-96.0 |
| | TYMV-2 group | 72.5-73.9 | 73.9-75.0 | 72.8-74.2 | 73.7-74.7 |
| | Japanese group (Chinese cabbage) | 94.7-34.9 | 97.8-98.2 | 98.6 | 93.5-93.3 |

[a]The groups of isolates encompassed the following isolates: European subgroup =TYMV-1 (Acc. No. X07441), TYMC (Acc. No. X16378), and in the CP gene also isolates 62226 (Acc. No. V01418) and 332241 (Acc. No. K00602); Australian group = Blue Lake (Acc. No. AF035403), Club Lake (Acc. No. J04373), and in the CP gene also isolates D5 (Acc. No. U88845), P1 (Acc. No. U88849), F41(Acc. No. U88847), Q18 (Acc. No.U88850), F39 (Acc. No. U88846), N37 (Acc. No. U88848) and Japanese group (Chinese cabbage)= A-2 (Acc. No. AB358971), B-3 (Acc. No. AB358972); TYMV-2 group = Rothamstead (Acc. No. AF035635), Cauliflower (Acc. No. AF035696), Dorset 18 (Acc. No. AY673644), Dorset 17 (Acc. No. AY673642), Dorset 50 (Acc. No. AY673645).