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Kyuri green mottle mosaic virus detected for the first time in Turkey

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Abstract
Turkey is among the top 10 producers of cucumber, melon, watermelon, and squash in the world. Lately, seed-borne viruses have become a major issue in greenhouse and field-grown cucurbits. In this study, the incidence of kyuri green mottle mosaic virus (KGMMV) was determined in seeds from various species (cucumber, melon, watermelon, summer squash, bottle gourd, winter squash) of *Cucurbitaceae*. KGMMV detection in a total of 20 seed lots of each cucurbit species was done by enzyme-linked immunosorbent assay (ELISA) and RT-PCR. The highest virus incidence was 45% in melon followed by 25% in cucumber and 10% in squash. To the best of our knowledge, this is the first report of KGMMV in cucurbit crops in Turkey and this highlights the potential risk of KGMMV to commercial cucurbit seed lots.

Keywords *Tobamovirus* · *Kyuri green mottle mosaic virus* · *Cucurbitaceae*

Introduction

The seed has strategic importance for the countries' agricultural sectors. Viruses have the utmost effect on seed health causing serious problems by decreasing yield and germination rate, and changing seed shape, colour, and biochemical composition (Inouye et al. 1967; Reingold et al. 2013). Until recently, twenty-eight virus species in cucurbit plants with serious damages have been reported in the Mediterranean basin (Lecoq and Katis 2014). Among many seed-borne viruses, *Tobamovirus* species are considered an important risk factor worldwide, especially for species in the *Solanaceae* and *Cucurbitaceae* families (Dombrovsky and Smith 2017). Seed transmission of tobamoviruses occurs at a high rate, which infects important vegetable crops (Davino et al. 2020). There are at least four different tobamoviruses described in cucurbit crops in the world thus far: cucumber green mottle mosaic virus (CGMMV), kyuri green mottle mosaic virus (KGMMV), cucumber fruit mottle mosaic virus (CFMMV), and zucchini green mottle mosaic virus (ZGMMV) (Kim et al. 2009). KGMMV was first described on cucumber in Japan in 1967 (Inouye et al. 1967). The virus is very stable, easily transmitted by foliage contact, soil contamination, and through infected seeds, and causes severe yield reduction in plants (Tan et al. 2000; Daryono et al. 2005). Contaminated cucurbit seeds used as scion or rootstock for grafting to seedling production may also be an important source of initial virus inoculum (Al-Tamimi et al. 2010). Therefore, seed transmission of viruses is considered an important threat to world agriculture. In modern agriculture, the introduction of new crops and/or varieties into a new region/country not only causes diseases but also poses a significant threat to endemic plants to which these varieties are related (Dombrovsky and Smith 2017). It is therefore critical to evaluate the seed-transmitted and/or seed-borne viruses in commercial cucurbit seeds to reduce the risk of the introduction of new diseases into a country.

Turkey is among the top 10 producers of cucumber, melon, watermelon, or squashes in the world (Faostat 2019) and severe output reductions caused by viral diseases have recently been reported in the greenhouse and field-grown cucurbits. Infected plants have been mostly tested after virus symptoms appear and there has been no record for the evaluation of the sanitary status of seeds used for cultivation. Therefore, this study was carried out to investigate the presence of kyuri green mottle mosaic virus (KGMMV) in seed lots of cucurbit crops.

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Materials and methods

Seed sources

Locally produced and imported commercial seed samples of cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* Thunb.), and summer squash (*Cucurbita pepo* L.) species were received from various seed companies. The imported seeds were from Peru, Chile, Latvia, India, and Morocco. Also, the seeds of local bottle gourd (*Lagenaria siceraria* (Molina) Standl.), and winter squash (*Cucurbita moschata* Pour) varieties were maintained by farmers in Turkey.

KGMMV detection in whole seeds

According to the International Seed Testing Association (ISTA) seed testing rules, 2000 seeds in 20 subsamples of 100 seeds were used for each type. KGMMV in seed lots was tested by the double-antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) according to the supplier's instructions (Agdia, USA). The subsamples were soaked in 10 vol. of 1x extraction buffer (Agdia, USA) overnight at 4 ^{0}C, and then grounded to obtain seed extracts. Following centrifugation of the homogenized seed extracts at low speed, hundred µl were used in duplicate wells per sample. The absorbance values (A405nm) were measured using an ELISA reader (Seac Sirio S). Samples were considered positive when the absorbance value was two times greater than the average absorbance value of healthy control.

The samples resulting positive for KGMMV infection were submitted to RT-PCR. Total RNA was isolated from seed extracts according to Li and Trick (2005) and analyzed on 1% agarose gel. RNAs were reverse-transcribed with the specific reverse primer followed by cDNA synthesis using M-MLV reverse transcriptase (Promega). KGMMV detection was performed with the primer pair KGCP-F/KGCP-R (Kim et al. 2009) amplifying the coat protein (CP) genome. PCR amplicons were custom-sequenced by the Sanger method with both primers in two directions. Nucleotide maximum-likelihood tree (Yang 1994) was inferred with the program MEGA 7 (Kumar et al. 2016) with 1000 bootstrap replicates to assess the robustness of the nodes.

Determination of seed-to-seedling transmission rate of KGMMV

Two hundred seeds per KGMMV-infected seed lots were individually sowed in sterile plastic trays in potting soil and placed in a growth chamber with a 16 h photoperiod and constant temperature of 25 °C. Sampling was conducted at two different stages: the cotyledon stage (approximately 14 days after sowing), and seedlings at three leaf–stages. Leaf samples were taken from each plant, pooled together, and tested in groups of 4 seedlings as a single sample. All pooled samples homogenized in a plastic bag with 1:10 vol (w/v) of extraction buffer and hundred µl aliquots were assayed for KGMMV by DAS-ELISA and RT-PCR as described above. The percentage incidence from grouped samples was calculated with the formula $p = 1 - (1 - y/n)^{1/k}$, where p= probability of transmission by a single KGMMV-infected seed (0 to 1), y = number of positive samples, n = a total number of samples assayed, and k = number of seedlings per sample ($k = 4$) (Gibbs and Gower 1960).

Results

KGMMV detection and sequence analysis based on coat protein gene

Based on the DAS-ELISA results, the rate of KGMMV contamination in cucumber was 25% (5 seed lots out of 20), in melon was 45% (9 seed lots out of 20), and in summer squash was 10% (2 seed lots out of 20). The KGMMV was not detected in the bottle gourd and winter squash seeds. In addition, the presence of the KGMMV in DAS-ELISA positively-detected seeds was validated by RT-PCR. A total of sixteen KGMMV samples were sequenced but because of the low quality of DNA, only ten isolates were used for BLASTn and phylogenetic tree analyses. Sequence information for ten KGMMV isolates was deposited in the GenBank database with the accession numbers OQ915129.1-OQ915138.1 (see Supplementary Table 1). The amplified DNA sequences (484 bp) of the obtained RT-PCR shared nucleotide identity of 89.4 to 100% with each other, and the amplicons analyzed by the BLASTn program in NCBI showed an identity > 87% with sequences of KGMMV isolates reported in Japan on cucumber (LC649929.1, LC650021.1, LC650022.1, LC649923.1, LC649924.1, LC649927.1, AB015144.1, AB015145.1, NC_003610.1, AJ252162.1, AJ295948.1), in South Korea and Indonesia on melon (AB433895.1, MN593026.1, AB110084.1), in Indonesia on luffa (AB182577.1) (Fig. 1).

Seed-to-seedling transmission of KGMMV

In total, 1000 seedlings of cucumber, 1800 seedlings of melon, and 800 seedlings of summer squash were grouped consisting of 4 seedlings, and each was sampled in the cotyledon stage and three leaf–stages. The findings of the seed-to-seedling assays are shown in Table 1. As calculated by the formula of Gibbs and Gower (1960), in cucumber seeds, the seed transmission ratios in the cotyledon and the true leaf

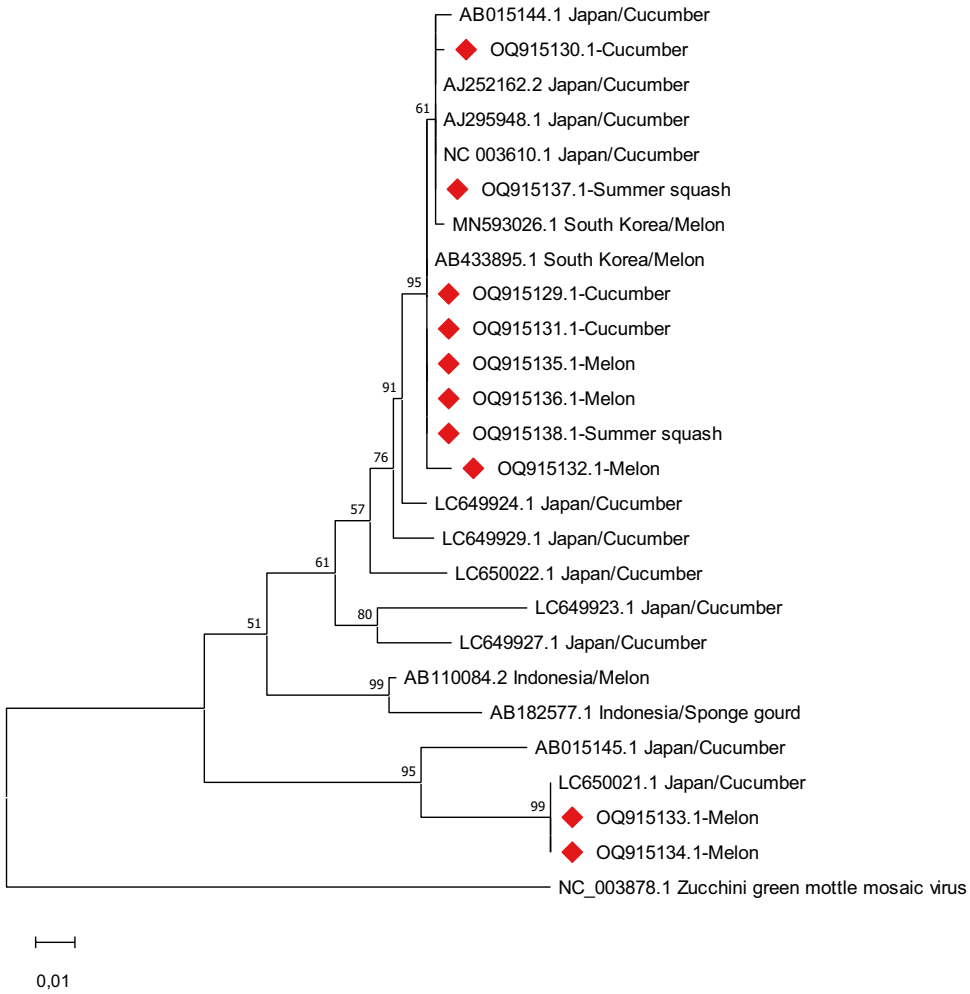

Fig. 1 Maximum likelihood phylogenetic tree constructed using an alignment of 25 sequences from the coat protein region of KGMMV isolates. The host and origin of isolates are shown in the tree. Zucchini green mottle mosaic virus (accession number: NC_003878) was used as an outgroup. KGMMV isolates obtained in this study are labelled with the symbol (). This Maximum likelihood phylogenetic tree was constructed in MEGA7 software with a bootstrap test (1000 replicates). Percentage bootstrap values (greater than 50) are shown on each branch

stage were found 2.3% and 0.5%, respectively. Also, seed transmission ratios in melon seeds were found 1.1% in the cotyledon stage, and 0.5% in the true leaf stage (Table 1). None of the germinating seeds developed virus infection in a grow-out assay of summer squash seeds. As a consequence of assays, it was discovered that KGMMV seed contamination ratios in cucumber and melon seeds were extremely high proportions, although seed transmission rates were quite low. The contact between the virus-infected seed coat and the growing seedling is most likely what causes cotyledon and true leaf infections. Nonetheless, these data do not entirely explain the mechanism of the seed transmission of KGMMV.

Discussion

Seeds have played an important role as primary inoculum sources of several important plant viruses and viroids (Sastry 2013). Multinational companies' expansion of the global

Table 1 Results of the seed-to-seedling transmission of KGMMV in the cucurbit seed lots

Types	Total number of seedlings	Assay	Number of positive subsamples	Probability of infection
Cucumber	1000	Cotyledon	22	0.023
		Three leaf–stage	5	0.005
Melon	1800	Cotyledon	20	0.011
		Three leaf–stage	10	0.005
Summer squash	400	Cotyledon	0	0
		Three leaf–stage	0	0

seed trade is introducing damaging virus diseases to parts of the world where they were previously absent. Among the main cucurbit-infecting tobamoviruses, KGMMV has been reported to cause severe reduction in cucurbit plants in several countries (Daryono et al. 2005; Tan et al. 2000; Fukuta et al. 2012). Similar to other tobamoviruses. KGMMV has a highly stable structure that is easily transmitted by foliage contact, soil contamination, and seed (Daryono et al. 2005; Fukuta et al. 2012). We demonstrated that local and imported seed lots have a KGMMV infection. Especially, the virus was found in samples of seed lots identified as originating from North Africa and South America. As a result of the grow-out assay, KGMMV could be transmitted mechanically from seed to seedling. It can be concluded that the use of KGMMV-contaminated seed lots has a risk to cucurbit growers. It is important to prevent its spread, during cultivation by human activities, contaminated trays and tools. The effectiveness and duration of virus seed transmission are greatly influenced by factors such as host cultivar, virus isolate, and environmental conditions (Simmons and Munkvold 2014). The narrow host range could be an advantage in applying control measures to the spread of the virus. Using seeds free of viruses is the key to controlling initial infections with tobamoviruses. Meanwhile, efficient disinfection protocols should be discovered, and KGMMV-seed lots should be eliminated to avoid the dissemination of the virus.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13314-023-00504-3.

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Declarations

Ethical approval This study did not involve human or animal participants.

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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