Cauliflower mosaic virus (CaMV) Biology, Management, and Relevance to GM Plant Detection for Sustainable Organic Agriculture

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In today’s global market, some organic farmers must meet regulatory requirements to demonstrate that their plants and feedstocks are genetically modified organism (GMO)-free. Many GM plants are engineered to contain a promoter from the plant virus, *Cauliflower mosaic virus* (CaMV), in order to facilitate expression of an engineered target gene. The relative ubiquity of this CaMV 35S promoter (P35S) in GM constructs means that assays designed to detect GM plants often target the P35S DNA sequence, but these detection assays can yield false-positives from plants that are infected by naturally-occurring CaMV or its relatives within the *Caulimoviridae*. This review places CaMV infection and these ambiguous GM plant detection assays in context, serving as a resource for industry professionals, regulatory bodies, and researchers at the nexus of organic farming and global commerce. We first briefly introduce GM plants from a regulatory perspective, and then we describe CaMV biology, transmission, and management practices, highlighting the relatively widespread nature of CaMV infection in both GM and non-GM crops within the Brassicaceae and Solanaceae families. Finally, we discuss current knowledge of public food safety related to the consumption of CaMV-infected produce.

Keywords: *Cauliflower mosaic virus*, CaMV, organic agriculture, qPCR, GMO

INTRODUCTION

Organic agriculture, an ecological production management system that serves to promote and enhance biodiversity, biogeochemical cycles, and soil biological activity, has become increasingly popular, with global retail sales reaching more than $80 billion (Brantsæter et al., 2017; Mie et al., 2017). Organic agriculture relies on fertilizers of organic origin, such as compost, and encourages the use of biological pest control. It allows for the use of natural substances, such as pyrethrin and rotenone, while prohibiting most synthetic fertilizers and pesticides (some synthetic substances, such as copper sulfate and elemental sulfur, can be allowed). However, genetically modified organisms (GMOs) and plant growth regulators are prohibited in organic farming in regulated markets (Santhoshkumar et al., 2017).

A genetically modified organism (GMO) is any organism with genetic material that has been altered using genetic engineering techniques, and in the case of GM plants, a new trait is typically
introduced to the plant. GM plants can confer advantages such as herbicide resistance, disease resistance, stress (e.g., drought) resistance, and/or insect tolerance (Oliver, 2014), yet GMO use in commercial crops and feedstocks is becoming regulated in some countries (Sharples, 1982; Maghari and Ardekani, 2011; Zhang et al., 2016; Tsatsakis et al., 2017). For example, many countries in the European Union (EU) have banned or imposed legislation to regulate GMOs (Davison, 2010; Lau, 2015; Wong and Chan, 2016). GMO regulation has impacts on international trade (Krueger and Le Buanec, 2008; Ramessar et al., 2008), and the popularity of organic foods, along with the tight regulations surrounding GMOs in some markets, have made GMO detection technologies essential for product labeling and traceability. Indeed, many countries have instituted labeling laws stating that products must be labeled when they contain GMOs above a certain threshold concentration (Wong and Chan, 2016). These thresholds can vary—for example, the threshold is 0.9% in the EU and 5% in Japan, whereas the U.S. has voluntary labeling, and China has “yes-or-no” labeling (Fu et al., 2015). In the U.S., a new law has just passed with mandatory labeling for some GMO products that will be effective in 2020.\(^1\)

More than 80% of engineered genetic constructs in GM plants are built with the 35S promoter (P35S) from *Cauliflower mosaic virus* (CaMV) and/or the NOS terminator (TNOS) derived from the soil-borne bacterium, *Agrobacterium tumefaciens* (Figure 1A). Specifically, as of 2015, P35S and TNOS were used in 65.7 and 53.49%, respectively, of commercialized GMOs, with at least one of the two used in 81.4% of these constructs (Chaouachi et al., 2013; Wu et al., 2014; Fu et al., 2015). Thus, most GMO detection methods are based on marker sequences for P35S and TNOS detected through polymerase chain reaction (PCR) or quantitative PCR (qPCR) (Holden et al., 2010; Wu et al., 2014; Fraiture et al., 2015). However, it is known that CaMV infection of non-GM plants can yield false-positive results in some GMO detection assays, due to the presence of the P35S region in both the CaMV genome and many GMO constructs (Figures 1A,B; Wolf et al., 2000; Chaouachi et al., 2008; Becker and Ulrich, 2018). Although a detailed consideration of *A. tumefaciens* infections is beyond the scope of this mini-review, *A. tumefaciens* is naturally found in the soil and can infect plants, so assays designed to detect TNOS can yield false-positive GMO results too, as described elsewhere (Wolf et al., 2000; Escobar and Dandekar, 2003; Yang et al., 2013; Nabi et al., 2016; Becker and Ulrich, 2018).

Here, we synthesize relevant information for navigating issues related to CaMV infection and false-positive GM plant detection due to CaMV infection, with particular relevance to sustainable organic farming and international trade of organic products in GMO-regulated markets. We outline CaMV biology and the impacts of CaMV infection on plant health and yield, recommended management practices for reducing CaMV infection of crop plants, available detection assays for GM plants with relevance to CaMV infection, and current knowledge of the safety of human consumption of CaMV in whole or in part.

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\(^1\)https://www.producer.com/2019/03/gmo-labelling-law-in-u-s-receives-mixed-reviews/

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**CAULIFLOWER MOSAIC VIRUS (CaMV) BIOLOGY, PATHOLOGY, AND TRANSMISSION**

*Cauliflower mosaic virus* (CaMV) belongs to the *Caulimoviridae* family of circular, double-stranded DNA viruses. It predominantly infects members of the Brassicaceae family, including radish, turnip, canola, mustard, cauliflower, broccoli, and cabbage. Some CaMV strains (D4 and W260) are also able to infect Solanaceae species, such as devil’s trumpets (genus *Datura*) and tobacco plants (genus *Nicotiana*) (Scholze and Shepherd, 1988). CaMV genetic variants have been described in different host species with different symptoms, virulence, and transmission rates (Covey et al., 1990; Al-Kaff and Covey, 1994; Doumayrou et al., 2013; Yasaka et al., 2014), and recent studies have identified a high diversity of CaMV genomic sequences (Farzadfar et al., 2014; Gong and Han, 2017; Becker and Ulrich, 2018; Sukal et al., 2018).

The CaMV genome consists of approximately 8,000 base-pairs of circular, double-stranded DNA. The genome encodes seven genes (gene I to gene VII), also called P1 to P7 for encoded proteins 1-7. CaMV replicates by reverse transcription (Haas et al., 2002), and its genes are transcribed from two promoters, the 19S and 35S promoters (Figure 1B), which are DNA sequences that define where transcription of a gene (or group of genes) begins. After entry into a plant host...
cell, the CaMV virus particle migrates to the nucleus, where the viral genome is separated from the viral particle (called decapsidation). The viral DNA is transcribed via the two promoters (P19S and P35S) into two long messenger RNAs (mRNAs), the 19S mRNA that encodes protein P6 and the 35S mRNA that encodes the other six proteins. Translation of the 19S mRNA results in the production of protein P6, which aggregates in numerous cytoplasmic virus factories (Harries et al., 2009; Angel et al., 2013; Rodriguez et al., 2014; Schoelz et al., 2016; Schoelz and Leisner, 2017), where the translation of other viral proteins will take place: the movement protein P1 (required for cell-to-cell movement), the helper component P2 (required for aphid transmission), the virus-associated protein P3, the coat protein P4, and the reverse transcriptase P5. Protein P7 has never been detected in planta and can be deleted by mutagenesis without any effect on virus infection or transmission (Dixon et al., 1986; Wurch et al., 1990).

CaMV is transmitted between host plants by more than 27 aphid species in a non-persistent and non-circulative manner (Kennedy et al., 1962), meaning that after an aphid acquires the virus from an infected plant, the virus does not circulate or replicate within the insect. The virus is retained for a short period (a few hours) in the aphid styllets (mouthparts), where CaMV receptor candidates have recently been identified (Bak et al., 2012, 2013a,b; Martinière et al., 2013; Webster et al., 2018). The virus can then be released to initiate a new infection during aphid feeding on healthy plants. There are no known cases of CaMV transmission via seeds.

**CaMV IN AGRICULTURE AND MANAGEMENT PRACTICES TO REDUCE CaMV INFECTION**

CaMV is a widespread virus in temperate regions and can cause significant loss in Brassicaceae crops, especially in cases of coinfection with other viruses (Shepherd, 1981; Sutic et al., 1999; Spence et al., 2007; Li et al., 2019). CaMV incidence can easily exceed 70%, and subsequent yields may be reduced up to 20–50% (Shepherd, 1981; Sutic et al., 1999). CaMV can affect plant development, especially in early infections, and the production of flowers can be blocked. Low seed yields have also been reported from plants with CaMV infection (Sutic et al., 1999). The virus can induce a range of systemic symptoms, such as chlorosis (loss of green leaf color), mosaic (patches of light and dark green on leaves), vein clearing (abnormal clear or translucent color of veins), and/or stunting (Figure 2). CaMV survives in Brassicaceae crop and weed hosts, including wild radish, turnip weed, canola, mustard, cauliflower, broccoli and cabbage, and weed hosts are known reservoirs for the virus outside the growing season (Farzadfar et al., 2005). Although global GM crop regulations vary widely (see discussion above), canola is a good example of a common GM crop that can also be infected by CaMV. It has recently been shown that water stress can influence CaMV virulence and transmission: under well-watered conditions, viral load, virulence, and transmission rate increased, whereas under water deficit, transmission rate, and virulence decreased (Bergès et al., 2018).

The best way to minimize CaMV infection is to inhibit aphid contact with seedlings, which are very susceptible to virus infection (Jenkinson and Glynne Jones, 1951; Farzadfar et al., 2005; Shah et al., 2015). Seedbeds can be isolated from aphids with a barrier of cereals or by growing the seedlings under insect-proof mesh (Jenkinson and Glynne Jones, 1951; Broadbent, 1957; Shah et al., 2015). A barrier of cereals may act as a sink for the viruses and/or as a physical barrier, such that aphids will be more likely to land on the tall cereals first and lose their virus contents while probing (Simons, 1957; Toba et al., 1977; Alegbejo and Uvah, 1986; Difonzo et al., 1996; Fereres, 2000). Still, non-persistently transmitted viruses are difficult to control, since the insects only need to feed on the plant briefly to release viruses (Fereres, 2000; Bak et al., 2019), and pesticides are usually not an effective solution because aphids can transmit viruses before the pesticide has an effect (Simons, 1957; Jayasena and Randles, 1985).

CaMV can also be transmitted mechanically by sap inoculation using contaminated hands and pruning tools (Yasaka et al., 2014). It has been shown that CaMV can stay for hours on surfaces such as doors, phones, and gloves, and can be exchanged by hand-shaking (Jiang et al., 1998; Dancer, 2014), so disinfecting tools, equipment, and anything that contacts plants will reduce infection. Though it did not include CaMV, a study that tested how to prevent propagation of plant viruses, such as Pepino mosaic virus (PepMV), Potato spindle tuber viroid (PSTVd), Tomato mosaic virus (ToMV), and Tobacco mosaic virus (TMV), examined sixteen commercially available disinfectants and found that 10% bleach is the most effective solution for preventing viral infection of plants in greenhouse facilities (Li et al., 2015).

**GM PLANT DETECTION METHODS**

Many GM plant detection assays target the 35S promoter (P35S) and/or the terminator NOS (TNOS). Polymerase chain reaction (PCR) was the first technique applied to GM plant detection, and it was mainly used as a fast (∼2 h) and low-cost method (Gachet et al., 1998; Iloh et al., 2018; Grohmann et al., 2019). Real-time PCR, also known as qPCR, was developed later and became the preferred technique, providing both qualitative and quantitative results by measuring both the presence and concentration of gene sequences in a given sample (Akiyama et al., 2009; Holden et al., 2010; Wu et al., 2014). Both PCR and qPCR have been reviewed extensively elsewhere (e.g., VanGuilder et al., 2008; Emerson et al., 2017). In the context of GM plant detection, both are used to identify specific genetic regions of interest, based on the hybridization of primers to conserved DNA (or, in some cases, RNA) regions flanking the genetic sequence to be identified, followed by the amplification of the sequence of interest by a polymerase enzyme. In the case of PCR, the result is typically binary (detection or non-detection) and can be visualized by gel electrophoresis. More quantitative results can be attained by qPCR, including the concentration and/or number of copies
of a specific genetic region, which can be useful if a threshold allowable concentration has been set by a regulatory agency.

Multiplex qPCR can allow for amplification of several DNA targets at the same time, e.g., for the simultaneous detection of multiple GMO markers in a single reaction (Akiyama et al., 2009; Singh et al., 2016; Cottenet et al., 2019). For example, in Cottenet et al. (2019), the authors developed a new GMO screening method based on two multiplex real-time PCR reactions, targeting six major GM markers in one reaction and six other GM events in another reaction. The method showed a broad screening capacity, due to the large number of targets, and the limit of detection ranged from 0.005 and 0.02% (Cottenet et al., 2019). However, the same fluorophore was used for all markers, so detection was based on the presence or absence of signal with no ability to distinguish among the markers. To distinguish among markers in the same reaction, a high level of multiplexing can be achieved using qPCR with different fluorophores for each target; depending on the instrument, up to five distinguishable targets have been successfully amplified in a single multiplexed qPCR reaction (Reller et al., 2013; Datukishvili et al., 2015). For example, we recently developed a multiplex qPCR assay that can distinguish CaMV infection from GM plants containing P35S in a single reaction, based on detection of four different targets: P35S, CaMV gene III, TNOS, and actin (a universal plant gene used as a positive control for the assay) (Bak and Emerson, 2019).

Given the increasing number and diversity of GMOs developed and the number of molecular biology technologies available in addition to PCR and qPCR, many different GMO detection methods have been developed (Broeders et al., 2012; Fraiture et al., 2015; Demeke and Dobnik, 2018) (for more information, see Supplementary Table 1 and references therein). Databases of GMO sequences and detection assays have also been compiled, facilitating the identification of an appropriate detection method or the development of a new technique for a given need (Dong et al., 2008; Petriello et al., 2015; Wilkes et al., 2017). To date, there is no international standardized GMO or CaMV detection method, but PCR and qPCR (most commonly targeting P35S and/or TNOS) are still the most commonly used (Grohmann et al., 2019). From a regulatory standpoint, detection thresholds are typically evoked for various requirements. For example, in the European Union, detection of >0.9% GMO content requires a GMO label, but for specific GMOs that are not allowed in the EU under any circumstances, GMO contents must be 0%, which, practically speaking, means below the limit of detection of the assay (Davison, 2010). Thus, for regulatory purposes, the chosen GMO detection method must be sensitive enough to detect a GMO at the required threshold concentration(s), and qPCR is the most widely used method that provides this quantitative information.

**CaMV AND FOOD SAFETY**

Public concerns exist regarding whether plant viruses can infect humans, and a number of studies have attempted to answer this question (Bawa and Anilakumar, 2012; Rastogi Verma, 2013; Wunderlich and Gatto, 2015). Although a definitive, universal answer is not possible, here we point to existing literature for insights. Numerous viruses infect plants and are consumed through various types of fresh food and food products (Balique

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**FIGURE 2** | CaMV symptoms on leaves. For each plant type, the left panel shows an uninfected leaf and the right panel shows a leaf from a CaMV-infected plant with typical symptoms, such as mosaic (mottling, e.g., lighter and/or darker green patches, puckered or curled leaves) or vein clearing (yellow or white veins). Top left: turnip (Brassica rapa); bottom left: watercress (Nasturtium officinale); right: canola (Brassica napus).
et al., 2015), and two widespread plant viruses (Pepper mild mottle virus, PMMoV, and tobacco mosaic virus, TMV) are the most comprehensively studied, in terms of their effects on human consumption. To the extent that human consumption of CaMV has been studied, we will first present results specific to CaMV, and then we will consider human consumption of PMMoV and TMV.

CaMV commonly and widely infects crucifers, also known as cole crops (cabbage, kale, cauliflower, broccoli, and mustard), and it has been reported that 10% of the cabbages and cauliflower on sale in supermarkets are infected with CaMV (Raafat El-Gewely, 2001). In an epidemiological study of cauliflower in England, it was shown that 50% of the plants were infected with CaMV (Gong and Han, 2017), yet no ill effects or evidence of human pathogenicity have been found resulting from CaMV-infected plants (Hull et al., 2000). In terms of consumption of parts of CaMV, e.g., the P3SS promoter in GM plants, some concerns have been raised, but to our knowledge, there are no scientific publications that have demonstrated a safety risk of eating CaMV, P3SS, or any CaMV genetic sequences. In fact, many plants already contain sequences from members of the Caulimoviridae in their genomes as endogenous viral elements (EVEs), including commonly consumed crops, such as Vitis vinifera (grape), Oryza sativa (rice), and a variety of Citrus species (e.g., Citrus clementina, clementines) (Bertsch et al., 2009; Geering et al., 2014), meaning that we have been consuming parts of viruses in our food for centuries.

Still, we review some of the CaMV- and 35S promoter-specific concerns here for context. Sequence overlap exists between P3SS and the coding sequences of one CaMV gene, gene VI, also known as P6 (Figure 1), indicating the potential for virus protein expression in humans (Podevin and Du Jardin, 2012). Another concern is the horizontal gene transfer risk with DNA recombination that can potentially occur between P3SS and human genes, along with the potential for P3SS to facilitate expression of human genes (Chiter et al., 2000; Morel and Tepfer, 2000; Nielsen and Townsend, 2004; Paparini and Romano-Spica, 2006; Bawa and Anilakumar, 2012). In two studies that have attempted to address these concerns, no evidence was observed for the activity of the P3SS promoter in mammalian cells (Vlasák et al., 2003; Paparini and Romano-Spica, 2006). In terms of the potential for viruses to recombine with and acquire genes from transgenic plants (i.e., horizontal gene transfer from transgenic plants to a virus), earlier studies of CaMV have demonstrated that this is possible (Schoelz and Wintermantel, 1993; Wintermantel and Schoelz, 1996), but only under very specific conditions, such as strong selection pressure in the laboratory (Wintermantel and Schoelz, 1996).

PMMoV is a widespread plant virus that infects pepper and is found in numerous products containing chilis and peppers (sauce, spicy powder, etc.). For example, PMMoV was found in 57% of 28 pepper-based foods found in supermarkets (Colson et al., 2010). Colson et al. found that humans who consumed PMMoV presented a specific immune response to the virus, including fever and abdominal pain symptoms (Colson et al., 2010). This observation may not be the infection of human cells by the virus itself, but rather viral RNA interfering with the function of human RNA. Tobacco mosaic virus (TMV) is often present in smoked tobacco and, therefore, it is resistant to manufacturing processes (Smith, 1957; Wetter, 1975; Wahyuni et al., 2008). A study found that TMV was viable in 53% of the cigarettes of six different brands (Balique et al., 2012). In addition, 45% of the saliva from 12 smokers, compared to 0% of the saliva from 15 non-smokers, tested positive for TMV RNA (Liu et al., 2013). Another study found that exposure to TMV-infected tobacco products can induce an immune response to TMV in humans; specifically, using an ELISA assay, the authors found anti-TMV antibodies in tobacco smokers (Kamthan et al., 2016). Nevertheless, there was no direct evidence of viral infection in any of these studies, and the symptoms described seem to be an indirect response to the viruses. The extent to which these results for PMMoV and TMV are relevant to CaMV is largely unknown (for example, both PMMoV and TMV have RNA genomes, whereas CaMV is a DNA virus), but these cases demonstrate the potential for some minor symptoms associated with an autoimmune response to plant viruses in some cases. In general, plant viruses are not considered to present pathogenicity to humans (Balique et al., 2015).

CONCLUSIONS

In parallel with the development of new GMOs in agriculture, organic farming is increasing (Shi-ming and Sauerborn, 2006; Kamthan et al., 2016). As a result of these farming trends and, in some countries, regulation of GMOs, international trade can necessitate quantification of the GMO content of a given product. Thus, numerous GMO detection methods are emerging (Kamble et al., 2017; Salisu et al., 2017; Umesh and Manukumar, 2018), but the potential for crop infection with CaMV can lead to false-positive results in the most commonly used GMO detection assays, due to the use of a CaMV-derived P3SS promoter in many GM constructs (Lipp et al., 1999; Becker and Ulrich, 2018; Lübeck, 2019). This potential for false-positive GMO detection is particularly relevant for organic farmers trying to meet regulatory requirements for non-GM plants in a sustainable international market. Here we have provided information about GMO detection assays and how to disambiguate GMO detection from CaMV infection (e.g., by considering detection targets in the CaMV genome outside the P3SS region, such as gene III), management practices to minimize CaMV infection (e.g., planting tall cereals around Brassicaceae crops and adding protective aphid-proof netting around seedlings), and our current understanding of the food safety risk associated with CaMV infection (CaMV is not currently known to pose a risk to humans, but specific studies related to CaMV impacts on human health are limited).

AUTHOR CONTRIBUTIONS

JE conceived of the article. AB performed the literature research and prepared the figures. AB and JE wrote and edited the article. All authors have approved of the final version of the manuscript.
FUNDING

We gratefully acknowledge Amway for funding this work in the form of salary support for AB from a grant awarded to JE.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2020.00021/full#supplementary-material

Frontiers in Sustainable Food Systems | www.frontiersin.org 6 March 2020 | Volume 4 | Article 21
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