Effect of a water deficit on plant-virus interactions in increasing vectored-transmission

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SUBJECT:

EFFECT OF A WATER DEFICIT ON PLANT-VIRUS INTERACTIONS IN INCREASING VECTORED-TRANSMISSION

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ABSTRACT

La transmission est une étape cruciale du cycle viral pour persister dans un écosystème. La plupart des virus de plantes dépendent de vecteurs pour franchir la distance inter hôte. Il a été précédemment montré qu'un déficit hydrique (WD) appliqué à des plants de navets infectés par le virus de la mosaïque du chou-fleur (CaMV) augmentait significativement sa transmission par le puceron *Myzus persicae*. Deux hypothèses explicatives ont été proposées : 1) une altération de la relocalisation de la protéine virale P2, assistante de la transmission, sur les microtubules des cellules végétales et 2) une augmentation des transcrits viraux ou de P2 sous WD. La quantification des transcrits viraux par qRT-PCR et de P2 par western blots ont été réalisés. Grâce à des techniques d'imagerie nous avons étudié la localisation de P2 dans des plantes infectées WD vs. bien irriguées (WW). Les résultats préliminaires montrent que P2 semble présenter un phénotype différent dans les cellules infectées WD. Au niveau de la quantification de P2 dans les plantes WD, il ne semble pas y avoir de différences significatives comparé à des plantes WW. Ces résultats suggèrent que le déficit hydrique pourrait affecter la relocalisation de P2 mais pas la réplication virale.

Mots clés : CaMV, transmission, déficit hydrique, voies de signalisation, protéine P2, protéine auxiliaire, puceron.

Viral transmission is a key step in the virus cycle to maintain itself in an ecosystem. Most plant viruses use a vector, to span the distance between two hosts. It is known that environmental conditions influence vectored-transmission in many ways. It was previously shown that water deficit (WD) greatly enhanced the transmission by the aphid *Myzus persicae* of the *Cauliflower mosaic virus* (CaMV) in turnip plants. To explain this alteration of the transmission efficiency, two hypotheses have been suggested, (1) an alteration of the relocalization of the viral protein P2, the transmission helper factor, on plant cell microtubules, and (2) an increase of the viral transcripts and P2 in CaMV-infected plants under WD. qRT-PCR and western blots were performed to quantify CaMV transcripts and P2 protein, respectively. Localization of P2 in CaMV infected-plant cells in both WW and WD conditions was observed using confocal microscopy. Results show that P2 phenotype seems different in infected cells when plants are under WD. Concerning P2 quantification (transcription and protein level) we did not find clear difference in WD CaMV-infected plants compared to the WW condition. These results suggest a possible alteration in P2 relocalization rather than an alteration of P2 levels in plants under WD.

Key words: CaMV, Transmission, Water deficit, Signaling pathways, Protein P2, Helper protein, aphid.

ABBREVIATIONS:

ABA = Abscisic acid

AMV = Avian Myeloblastosis Virus

BSA = Sérum albumine bovine

CaMV = Cauliflower mosaic virus

CMV = Cucumber mosaic virus

DNA = Desoxyribonucleic Acid

DPI = Day Post Inoculation

ET = Ethylene

JA = Jasmonic Acid

kDa = kilo dalton

MAPK = mitogen-activated protein kinase

ORF = Open Reading Frame

P1 à P6 = Protéine 1 à 6

PFA = Paraformaldéhyde

PLRV = Potato leafroll virus

PVA = Potato virus A

PVY = Potato virus Y

qPCR = Quantitative Polymerase Chain Reaction

RNA = Ribonucleic Acid

ROS = Reactive Oxygen Species

SA = Salicilic acid

SAR = Systemic acquired resistance

SMV = Soybean mosaic virus

TB = Transmission bodies

TBS = Tris-buffered saline

TuMV = Turnip Mosaic Virus

VF = Viral factories

VOC = Volatile Organic Compound

WD = Water deficit

WW = Well-watered

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INTRODUCTION

Human-induced global climate change is predicted to become more frequent and severe in the coming years, resulting in increased temperatures, carbon dioxide levels, and extreme weather events. These changes will have far-reaching consequences for agriculture and food production, especially considering the growing global population and increasing food demand (ICPP, 2022). One of the consequences of environmental stresses like water scarcity is a reduction in plant yield. Record droughts this year have caused significant losses for farmers. Another factor affecting food security is the impact of pests and diseases on crops. Plant viruses, in particular, are a major cause of plant diseases and result in huge economic losses worldwide. Understanding the mechanisms of environmental stresses and their impact on crops is crucial to find ways to mitigate their negative effects.

Thus, studies are being conducted to understand viral epidemics under abiotic stresses in order to anticipate and prevent them. Changes in a plant's abiotic environment can affect its biotic interactions with insects and pathogens such as viruses. It is crucial to study how plant-virus interactions are likely to evolve in the context of climate change. Epidemiological data are often obtained under optimal conditions for infection success and do not consider the major changes in environmental conditions caused by climate change. These changes can alter plant-virus interactions and affect the epidemiological parameters involved in pathosystem functioning.

1. The Cauliflower mosaic virus

Viruses are obligated endocellular parasites as they depend on the host machinery to multiply. They are able to manipulate host metabolism to bypass its defenses in order to propagate in the host and to be transmitted to another host. The *Cauliflower mosaic virus* (CaMV) belongs to the Caulimoviridae family (https://viralzone.expasy.org/96) and infects mainly plant species of the Brassicaceae family and some Solanaceae provoking symptoms (Bouton et al., 2015) such as dwarfing of infected plants and yellowing of leaves (**Figure 1**). Leaves may also show a waffled appearance and vein clearing in the most virulent strains.

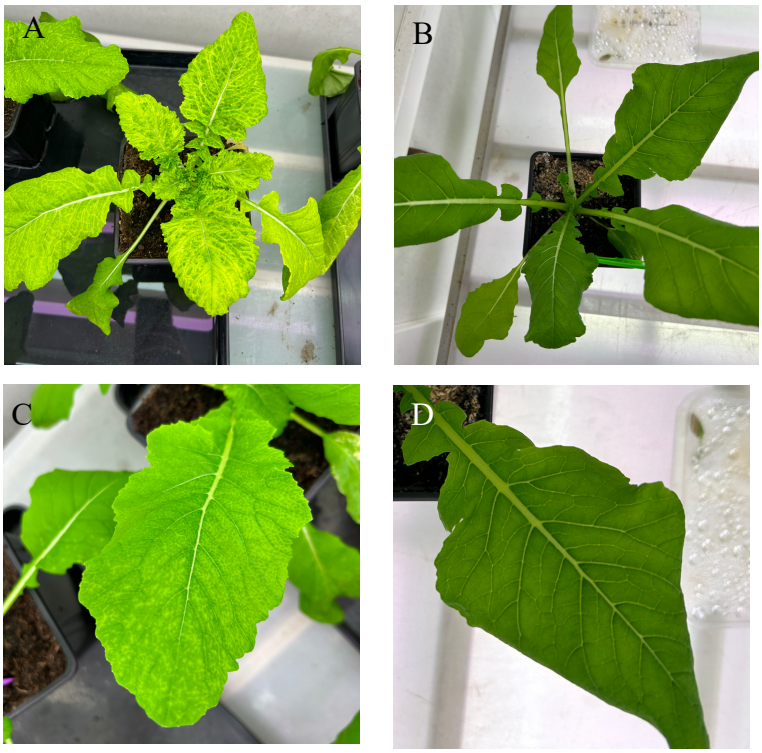


Figure 1: ***CaMV symptoms on turnip plants***. A and C: Dwarfing of the plant and infected leaves compared to B and D: healthy plants.

1.1. DNA structure

It is the first plant virus whose genome has been fully sequenced (Franck et al., 1980). Its genome consists of a double-stranded circular DNA molecule of 8000 bp long encapsidated in an icosahedral virus particle (**Figure 2A, 2C**). It has 7 open reading frames (ORFs) (located on

the - strand) coding for 6 proteins, from P1 to P6, each with a specific function (**Figure 2B**) (Haas et al., 2002)

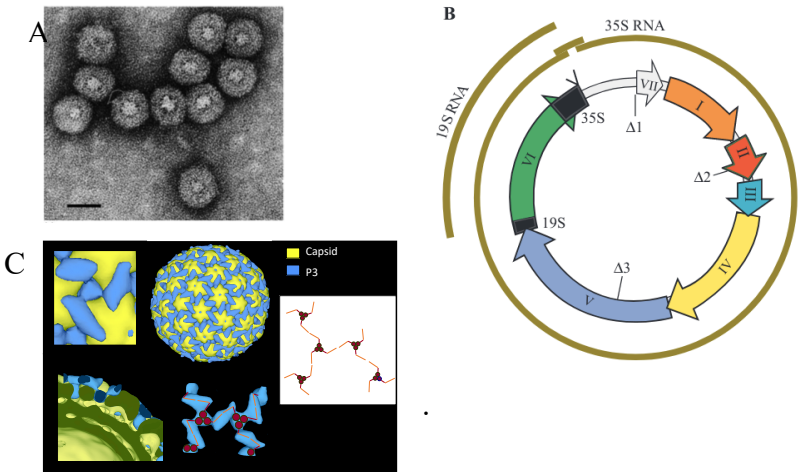


Figure 2 : ***Structure and genome of the Cauliflower mosaic virus.*** **A**: Electron micrographs of CaMV particles (bar, 50 nm). **B**: CaMV genome. The seven major ORFs are represented by colour coding and arrows. The circular viral genome is transcribed into 2 different RNAs, the 19s RNA and the 35s RNA. The 19s RNA translates the P6 protein which is essential for the translation of the 35s RNA which produces all other viral proteins. **C**: Surface representation of the CaMV (Plisson et al 2005).

1.2. Infection of the host cell - the CaMV life cycle

Once the CaMV particles entered the cell, viral particles migrate to the nucleus through nuclear pores where they are decapsidated (**Figure 3**). The replication of the CaMV genome has two phases: (**1**) transcription of the viral DNA in the nucleus with the cellular RNA polymerase II to give the various transcripts and (**2**) reverse transcription of the major transcripts in the cytoplasm (Bouton et al 2015).

It is at this stage that viral factories (UVs) are formed (**Figure 4**) within the host cell. Viral factories are non-membrane structures and contain mostly the P6 protein, virions, as well as most of P3 protein (Bak et al., 2013). Viral factories are the site of viral genome replication by reverse transcription, the newly transcribed genome is then encapsidated. They also have a protective role against host cell defenses (Bak et al., 2014). The newly formed virions are mostly stored in the UVs, others will go on to infect other host cells, and finally, some are integrated into specific structures: the Transmission Bodies (TB) implicated in host-to-host transmission (Bak et al .2014) (**Figure 4**). The virus infects host cells one after the other by passing through the plasmodesmata. Intra-host spread is also facilitated by the plant's vascular system, as the virus can enter the phloem vessels to infect other plant organs (long distance or systemy) (Hipper et al., 2013).

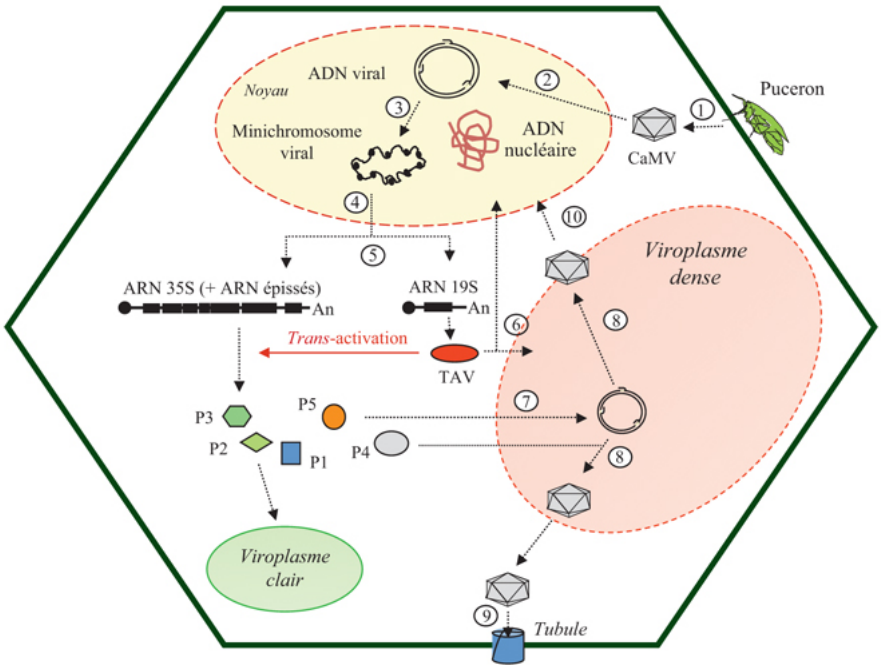


Figure 3 : ***The CaMV life cycle.*** **(Bouton et al. 2015)**

(1) CaMV particles enter the plant cell via the wounding triggered by the vector mouthparts (aphid stylets) that allows it to pass through the cell wall.
(2) Once inside the cell, viruses enter the nucleus through the nuclear pores where they are decapsidated.
(3) The virus genome associates with histones to form an episomal mini-chromosome
(4) which is transcribed by cellular RNA polymerase II to yield two RNAs, 19S RNA and 35S.
(5) The viral RNA is then translated in the cytoplasm, the 19S RNA giving a P6 protein while the 35S RNA gives the five other viral proteins (P1 to P5). The P6 protein is the first to be transcribed because it is essential for the translation of the other viral proteins.
(6) Viral factories formation and transport of P6 in the nuclear (necessary for its RNA silencing suppressor function).
(7) Viral genome replication inside viral factories by retro-transposition of the 35S RNA.
(8) Virions morphogenesis in viral factories.
(9) Virions intercellular movement through tubules formed in plasmodesmata.

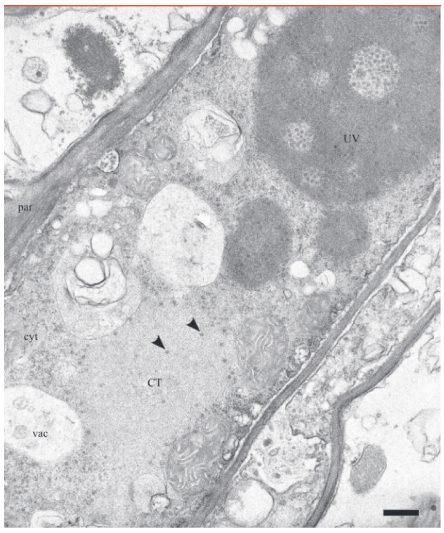


Figure 4 : ***Visualisation of a CaMV-infected cell by transmission electron microscopy.*** Longitudinal section of a companion cell showing viral factories (UV) and a transmission body (TB), characterised by its matrix being clearer than the cytoplasm, and the presence of some virions (arrowheads) (Bak et al. 2014).

1.3. Transmission is a crucial stage in the life cycle of a virus

1.3.1. Different transmission modes for plant viruses

Transmission is a very important stage in the virus life cycle because it allows its spread and survival in the environment by infecting new hosts. Through the course of evolution viruses, have developed different strategies to optimize their transmission. Unlike animal viruses, phytoviruses are confronted with the presence of a cell wall made of pectin and cellulose which prevents them from entering the cell. Moreover, viruses have to cope with the immobility of their host and find a way to move from one plant to another, and developed strategies to get around these obstacles. Several transmission processes exist across viruses' diversity, but the most widespread is transmission by a vector (e.g. nematodes, mites, fungi, arthropods …) (Bragard et al., 2013). Vectors are mostly arthropods with biting or sap-sucking feeding behavior (Bragard et al 2013). Among them, sap-sucking insects from the order Hemiptera (aphids, whiteflies, planthoppers, and leafhoppers) are by far the most widespread vectors of plant viruses (Bragard et al 2013). There are several types of vector-borne virus transmission according to the type of interactions between the two organisms (Brault et al., 2010). The first mode of transmission is called circulative transmission (**Figure 5**). In that case, the virus is taken up by the vector when it feeds on an infected plant (acquisition step). The virus is then internalized in the gut of the vector and eventually invades the salivary glands from where it will be inoculated to another host plant during long feeding (inoculation step). This route of transmission is quite slow, since the virus needs to transit through the vector's body before being released (latency step) (Brault et al 2010). The second mode of transmission by a vector is non-circulatory transmission (**Figure 5**). In general, before settling and feeding on a plant, vectors more specifically aphids make test probes to see if the plant is suitable. Thus, in this case, the virus is acquired by the vector during short probes on an infected plant. The virus binds to the mouthparts of the insect vector, such as the stylets in the case of aphids, directly or through the help of a viral protein acting as a bridge between the aphid stylets and the virus particle (Di Mattia et al. 2023) and is rapidly reinjected into a new host plant (inoculation phase) (**Figure 5**) (Uzest et al., 2010). In this case there is no internalization of the virus (Uzest et al. 2010).

The virus can in some cases manipulate its host or vector to increase its susceptibility of being transmitted (Blanc et Michalakis, 2016) (Dáder et al., 2017). Viruses can alter the phenotypes of their hosts and vectors in ways that influence the frequency and nature of

interactions between them, with significant implications for the transmission and spread of disease (Gutiérrez et al., 2013).

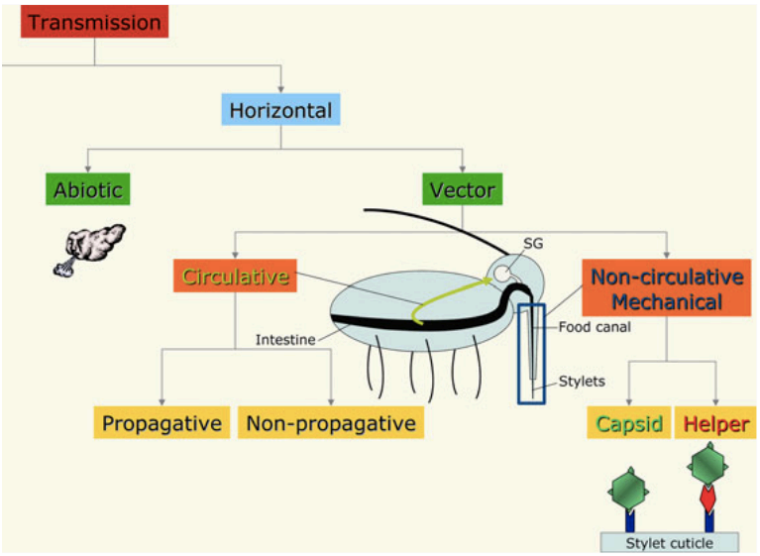


Figure 5: ***The different transmission modes of a virus.*** Abiotic transmission using the wind, or transmission via a vector organism. Two different modes of transmission with a vector:

- Circulatory transmission, which involves internalisation of the virus in the digestive system of the vector as well as in its salivary glands.
- Non-circulatory or mechanical transmission, the vector attaches to a cuticular receptor in the mouthparts of the vector. The virus can attach to the mouthparts of the vector directly or via a helper protein that acts as a bridge between the virus and the cuticular receptor (e.g. P2 protein in CaMV).

1.3.2. CaMV vectored- transmission

The CaMV is transmitted by several aphid species, such as *Myzus persicae* and *Acyrthosiphon pisum* in a non-circulating manner (Blanc et al. 2001). The attachment of CaMV particles to aphid stylets is achieved by the recruitment of the viral helper protein P2 protein (**Figure 6A**), recruited from the TBs and virus particles coming from the virus factories of infected cells. It has been shown that the P2 protein binds to a very specific organ of the aphid mouthparts: the acrostyle (Uzest et al., 2007) (**Figure 6B**). This specific organ at the tip of the stylets contains cuticular proteins, identified as CaMV receptors and named stylins (Webster et al., 2018) The transmissible CaMV complex (Virion-P3-P2) binds to these vector receptors in a reversible manner (Deshoux et al., 2018)

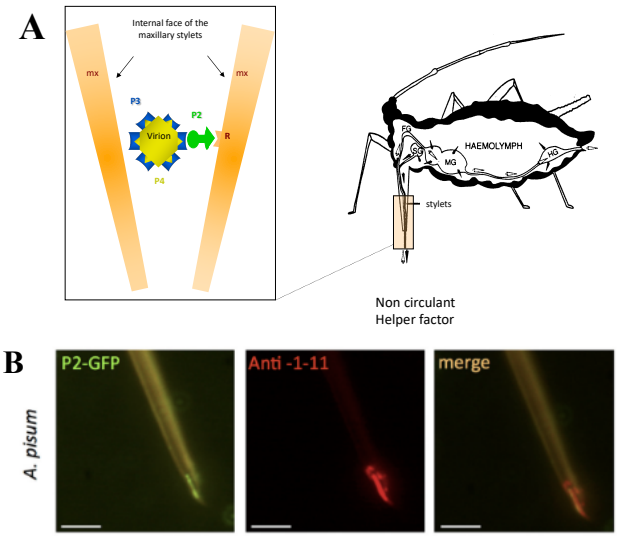


Figure 6 : ***The CaMV transmissible complex.***

A. CaMV binds to the acrostyle of aphid stylets on a cuticular receptor (Stylin-01) via the P2 protein. The transmissible complex of CaMV is composed of the virion to which the P3 protein is attached and the P2 protein.

B. CaMV protein P2 and anti-1-11 IgGs colocalize in and compete for the acrostyle. Co-incubation of P2-GFP and anti-1-11 antibody with *A. pisum* dissected stylets. P2-GFP (green fluorescence) and anti-1-11 antibody (red fluorescence) colocalize on the acrostyle (seen as orange labeling) (Webster et al 2018).

As natural transmission of the CaMV is vector-dependent, interactions between the plant and the vector are essential in the CaMV life cycle. The CaMV is also able to detect the presence of the aphid feeding on its host plant and react to optimize its transmission (Bak, Martinière, et al., 2013). At the cellular level, the CaMV intercepts stress signals emitted by the plant due to aphid probes. Indeed, it has been shown that the virus detects the Ca^{2+} flux induced by the aphid's mechanical stress and then actively prepares itself for its transmission by conformational changes visible under microscopy (Martinière et al., 2013). TBs react instantly (within seconds) to the presence of the vector (mechanical and chemical stimulation) (Bak, Martinière et al., 2013). The helper protein (or transmission factor) P2 is released from the TBs on cell microtubules and virions are recruited from viral factories (**Figure 7**) (Martinière et al., 2013). The homogeneous redistribution of P2 and virions on the microtubules increases the efficiency of the viral transmission, probably by increasing chances of stylets contact with transmissible complexes during aphid probing into cells. TB transformation is completely reversible. Soon after removal of the stress inducing the formation of the mixed network, both P2 and virions are released from microtubules and a standby TB reform that is ready for another round of transmission (**Figure 7**).

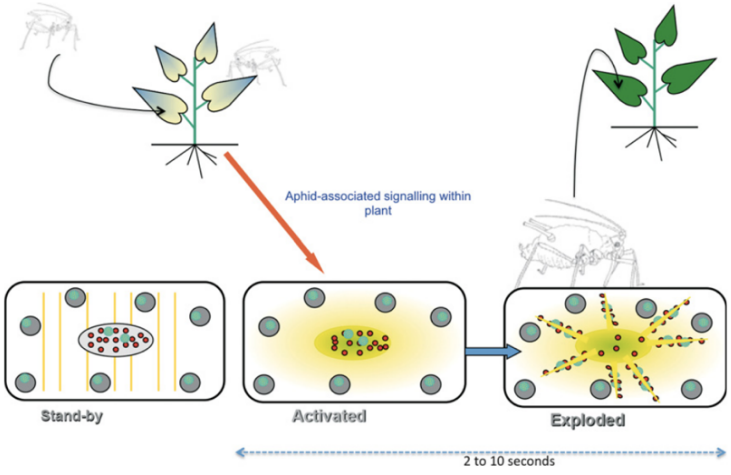


Figure 7 : ***During transmission CaMV recruts specialized structures.***
The whole infection cycle of CaMV is schematized chronologically from left to right. First an aphid vector inoculates the virus into a host plant, which becomes systemically infected after several days. In infected plant cells, the viral molecules required for vector transmission, the helper protein P2 (red dots) and the mature virus particles (yellow/bleu icosahedra) accumulate in a specific inclusion body designated the 'standby' transmission body (TB). When an aphid vector feeds on the infected plants, it triggers an immediate response of the TB, which is massively loaded with soluble tubulin (yellow) and then named the 'activated' TB. The activated TB subsequently disrupts and its components are released and distributed onto the cellular microtubule network (yellow), thus increasing their accessibility to the aphid vector, ensuring efficient acquisition and transmission. The time lapse between the aphid triggering signal and the virus acquisition by aphids from the 'disrupted' TB is in the order of few seconds (light blue dotted line) (from Gutierrez et al. 2013).

2. Plant signaling pathways triggered by biotic stresses and abiotic stresses are partly redundant

The interaction between biotic and abiotic factors in plants used to be analyzed taking the information from the plant's response to isolated stress. Some studies have shown that when the plant undergoes both biotic and abiotic stress, it could trigger a positive effect (tolerance) or a negative effect (sensibility) on plant performance (Bergès et al., 2020). A significant and growing body of evidence points to a close link between the physiological pathways of the plant involved in responses to different abiotic stresses and defense against pathogens and herbivores (**Figure 8**).

Plant susceptibility to stresses depends on several factors including the virus, the plant itself, or environmental factors. As a consequence, every single environmental factor which is able to modify the development, physiology, or reproduction of the plant could also be able to interact with the virus propagation, its transmission, or the plant's performance (Bergès et al. 2018, Bergès et al., 2021). Conversely, all the responses and signaling pathways put in place by the plant during a pathogen attack can interact with the responses that the plant settles to respond to environmental changes.

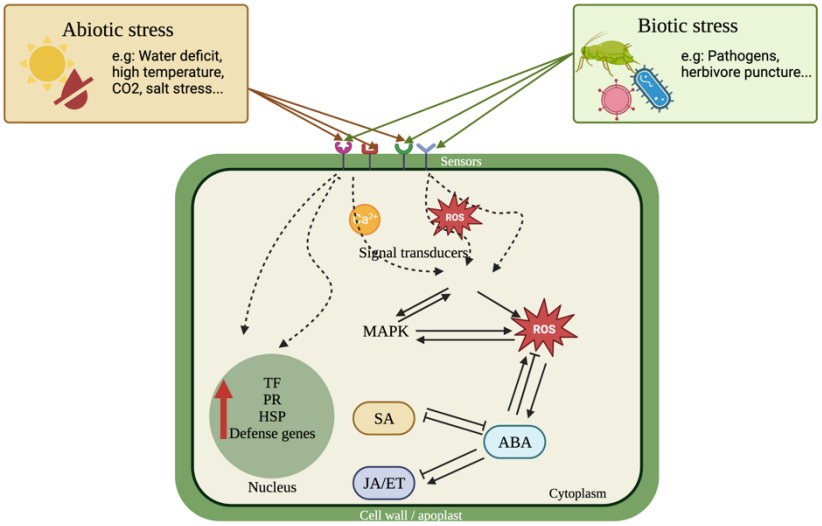


Figure 8: ***Model representing the interactions between abiotic stress and biotic stress (adapted from Rejeb et al., 2014).*** Biotic and abiotic stresses detected by the plant cell trigger rapid signaling (ROS, Ca2+) that activate common signaling pathways for different types of stress (MAPK and ROS production). MAPK plays a central role in the crosstalk between Ca2+ and ROS, as well as in the transmission of signals following specific stress. The significance of ROS signaling has been described for both abiotic and biotic stresses, suggesting it as a crucial element in cross-responses. Hormonal signaling plays a major role in stress adaptation. While ABA is primarily involved in abiotic stress adaptation, salicylic acid (SA), jasmonate, and ethylene (JA/ET) are more involved in responses to biotic stresses. These different hormonal signaling pathways can interact in a synergistic or antagonistic manner, creating a highly complex network of interactions.

2.1. Early responses: calcium signaling and reactive oxygen species (ROS) production

During biotic or abiotic stress, calcium signaling plays a key physiological role (Kudla et al., 2018). This constitutes a very rapid first level of signaling in the order of a millisecond (White et Broadley, 2003). In the presence of a pathogen or abiotic stress, Ca2+ ions are rapidly released into the cytosol (White et Broadley, 2003). This movement of Ca2+ ions creates calcium oscillations that trigger reactions in the cell concerned and which can be propagated to neighboring cells or to greater distances.

The detection of abiotic stress in plants is thought to cause an increase in cytoplasmic Ca2+ signaling, which can result in a higher virus load (Suntio et Mäkinen, 2012; Dorokhov et al 2012). Another intriguing idea is that mechanical inoculation or aphid transmission of viruses may cause a localized increase in Ca2+ levels in newly infected cells. When aphids use their stylets to probe phloem cells, they cause damage to the cell wall and plasma membrane of epidermal cells, which are the primary sites for viral infection initiation. Consequently, the first cells to receive viral particles from the stylets are already damaged.

Ca2+ oscillations are the precursors of the activation of other signal molecules such as ROS or hormonal signaling pathways (Mittler and Blumwald, 2015). ROS are generally considered to be highly reactive phytotoxic molecules able to oxidise proteins, lipids, carbohydrates, and nucleic acids (Sharma et al., 2021). ROS are used within plant cells as signal molecules to control many biological processes in relation to their biotic or abiotic environment.

ROS signaling during a biotic stress act as a secondary messenger locally and systemically to trigger immune responses (Baxter et al., 2014). Pathogen perception by plants leads to rapid, specific, and strong production of ROS molecules, mainly through the activation of particular proteins (Liu et al., 2017). The H_2O_2 produced following the activation of NADPH oxidase favors the deposition of callose and thus contributes to the occlusion of the sieve tubes of the phloem, which has an impact on the feeding of aphids (Louis and Shah, 2013).

ROS may also affect other signaling and defense mechanisms (Louis and Shah, 2013). Indeed, the production of ROS molecules can restrict the entry of pathogens by triggering processes such as stomatal closure or the strengthening of plant cell walls (Macho et al., 2012). ROS also activate the expression of essential genes such as a gene involved in the response to abiotic and biotic stresses (Atkinson et al., 2013). Finally, a recent study has again highlighted the importance of ROS in the response to environmental or biotic stresses (Choudhury et al., 2017).

2.2. MAP-Kinases

Phosphorylation or dephosphorylation of proteins significantly influences the expression of certain genes in response to environmental stresses, but also the physiology and morphology of the plant. Mitogen-activated protein kinase (MAPK)-activated signaling cascades are crucial in eukaryotes in the response to various environmental stimuli (Pitzschke et al., 2009). Whether for abiotic stresses (water deficit or heat) or following the detection of a pathogen, it is important for the plant to activate the synthesis of defense proteins (Taj et al., 2010).

2.3. Hormonal signaling

Phytohormones play a fundamental role in the different plant defense strategies. Indeed, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) are the main phytohormones that regulate the protective responses of plants against different biotic and abiotic stresses. They act synergistically or antagonistically depending on the environmental stress (Spoel and Dong, 2008). JA, SA, and ET are involved in biotic stress signaling upon pathogen infection while ABA is a phytohormone that plays a key role in responses to abiotic stress such as drought. ABA appears to be a negative regulator of the SA and JA/ethylene defense pathways normally activated during biotic stresses (Adie et al., 2007; Kissoudis et al., 2015; Lievens et al., 2017). SA plays an important role in activating plant defenses against pathogens including viruses (Bari and Jones, 2009). This hormone is known to induce the SAR (systemic acquired resistance) response, but according to some studies, it also causes an inhibition of CaMV long-distance movement without altering replication or cell-to-cell movement (Love et al., 2012). In addition, JA appears to be important in the response to abiotic stresses such as heat or salt stress (Kazan et al., 2015).

Since signaling pathways triggered by biotic and abiotic stresses are common and partly redundant it was suggested that abiotic stresses might influence virus epidemiological parameters, and thus modify its transmission efficiency in the same way as biotic stress (e.g. vector puncture) (Guttierez et al 2013).

3. Abiotic stress can modify plant virus transmission.

3.1. Abiotic stress can alter or enhance virus epidemiological traits.

Interactions between plants, viruses, and vectors are influenced by environmental conditions, which in turn affect the transmission efficiency of phytoviruses. In response to such drastic conditions, host plants undergo physiological changes that are accompanied by biochemical modifications in sap composition, as well as alterations in the levels of phytohormones and signaling pathways (Minocha et al., 2007; Podlešáková et al., 2019)). These physiological changes can have implications for various parameters related to the transmission of plant viruses (virus load, virulence), potentially impacting the efficiency and dynamics of transmission processes (Szczepaniec and Finke, 2019).

Several studies have examined the impact of various abiotic stresses on virus transmission. For instance, (Singh et al., 1988) found that high temperatures (25-30°C) and high relative humidity (80-90%) significantly increased the transmission of Potato virus Y (PVY) and Potato leafroll virus (PLRV) by 30-35%. In a study by Dader et al. (2016), it was observed that the transmission ability of Cucumber mosaic virus (CMV) by *Myzus persicae* decreased when pepper plants (*Capsicum annuum*) were grown under high concentrations of carbon dioxide (CO2). The researchers hypothesized that this decrease in transmission could be attributed to the activation of plant resistance mechanisms during CO2 treatment at the time of inoculation. Another study by (Nachappa et al., 2016) revealed that the transmission of Soybean mosaic virus (SMV), a potyvirus, was reduced in Soybean (Glycine max. L) plants under water deficit conditions, while it increased under water-saturated conditions. Furthermore, salt, osmotic, and wounding stress were found to increase the gene expression of Potato Virus A (PVA) in infected Nicotiana benthamiana leaves. Suntio et al. (2012) reported that an early response to these stresses is an elevation in cytosolic calcium ion (Ca2+) concentration. Interestingly, the application of salt stress or wounding enhanced virus production, suggesting a potential benefit for the virus. These findings highlight the possibility of greater crop losses in the field when plants are simultaneously subjected to abiotic stress and viral infection. Consequently, further investigation into the impact of abiotic stress on viral infections in plants is crucial (Suntio et al., 2012).

3.2. A severe water deficit of the host plant infected by the CaMV triggers an increase in aphid transmission

It has been shown in the hosting lab that a severe drought applied to CaMV-infected turnip (*Brassica rapa*) plants increases dramatically viral transmission (about 34%) by aphids (van Munster et al. 2017). This increased transmission could not be attributed to increased viral accumulation since viral load was similar between control plants control and plants under water deficit (van Munster et al. 2017). Similar results using several ecotypes of the host plant *Arabidopsis thaliana* were obtained (Bergès et al. 2018; Bergès et al. 2021).

INTERNSHIP OBJECTIVES

Here we hypothesize that in a similar way that the CaMV reacts to an aphid probe, water stress 'stimulates' virus responses. Indeed, as signaling pathways are redundant between biotic stress (aphid probes) and abiotic stress (water deficit), a water deficit applied to a CaMV-infected plant could trigger the same reaction of the virus in the presence of the aphid (i.e. the relocalization of P2 on the microtubules in infected cells) or an increase of the viral transmission factor P2 explaining the described transmission increase (van Munster et al. 2017).

This internship project will study plant-virus interactions at the cell level in a water deficit context to identify the mechanisms responsible for increased transmission by vector. The study will focus on two factors: (1) the amount of the helper viral protein P2 available (at the mRNA level and protein level), and (2) the accessibility/relocalization of viral partners in cells of water-deficient plants compared to control plants. Techniques of molecular biology, biochemistry, and cellular imaging will be used to study virus load and viral protein P2 under different hydric conditions. The first step of the study will involve quantifying CaMV viral load, P2 transcripts, and protein P2 directly using specific primers and antibodies in water-deficient vs. control-infected turnip plants. In the second step, we will study the accessibility of the virus and the viral protein P2 in infected cells. Microscopy approaches will be used to evaluate the dynamics of the formation of activated CaMV viral morphs in cells of infected turnip plants under water deficit conditions, which can be observed by fluorescence and confocal microscopy.

MATERIAL AND METHODS

1. Plant material and growth conditions.

Turnip seeds (*Brassica rapa* L. var. "Just Right") were individually grown in 250 g of soil substrate (Neuhaus Huminsubstrat N2; Klasmann Delmann, Geeste, Allemagne) (pH 6) in square pots (9 cm x 9 cm), and cultivated at 8 h dark/ 16 h light photoperiod and 24 ± 1℃/19 ± 1°C Day/night. Soil water content was maintained to 320g until the application of the water deficit treatment.

2. Virus isolate and plant inoculation

The CaMV isolates Cabb B-JI (Delseny and Hull, 1983) was used in this study. Plants were mechanically inoculated at the first true leaf stage (9-day-old) with a mix containing CaMV-infected turnip extract. CaMV-infected turnip extract was prepared from 1 g of infected leaf material (leaves presenting systemic symptoms collected at 21 days post inoculation (dpi)) ground in 1 ml of milliQ water and carborundum. Mock-inoculation was performed with a mix containing non-infected plant extract. When plants developed clear symptoms (10 ± 2 dpi), the water deficit treatment was applied for 9 days. Then leaf 9 of each plant was collected and stored at -80°C for further nucleic extraction (RNA and DNA), quantification of virus accumulation and protein analysis. Four biological replicates were done with 12 technical replicates for each condition (**Figure 9**).

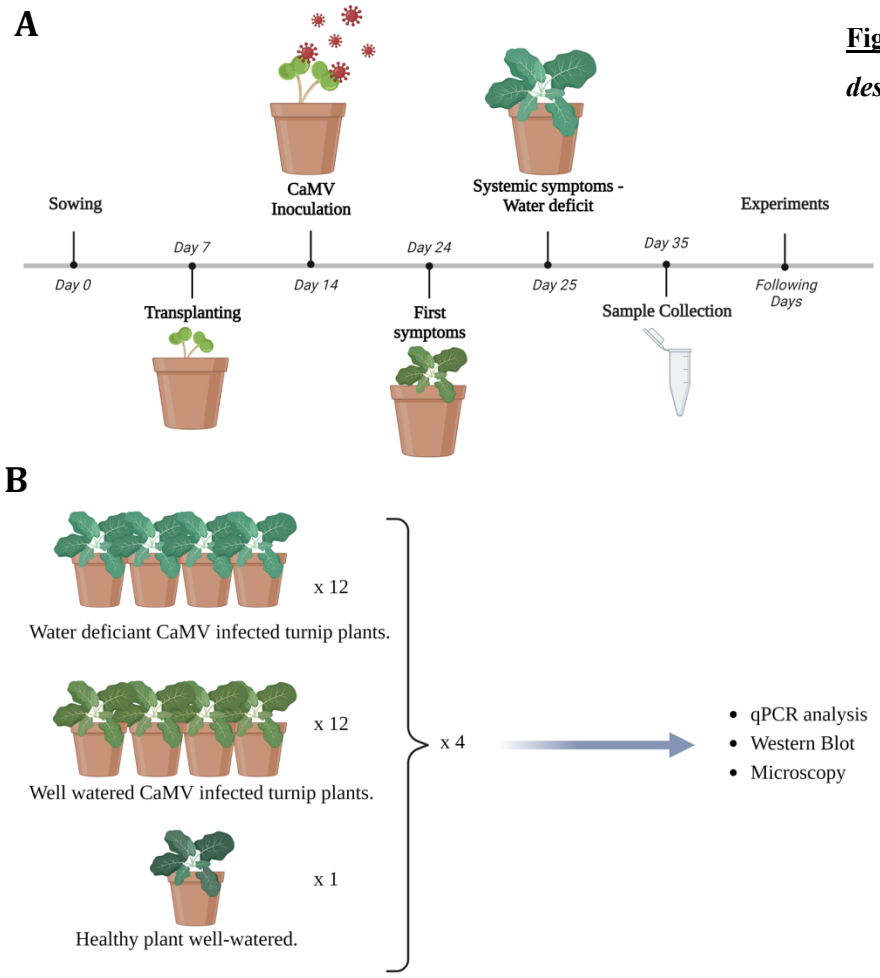


Figure 9: ***Experimental design***

A. Chronology of turnip development from sowing to plant experiments.

B. 4 biological replicates of 12 plants were grown in every condition (Well-watered and Water deficit), and then experiments were carried out (qPCR, Western Blot, Microscopy).

3. Irrigation protocol

For turnip plants, water treatment protocols (control, and deficit) had already been established before the course. It requires a calculation of the quantity of water to be provided per plant each day, as explained below.

For each experiment, the initial relative humidity of the soil is determined. To this purpose the dry weight of a defined quantity of potting soil (dried in a Poupinel oven at 80 °C, 24 hours) is determined, and the initial relative humidity of the potting soil is calculated using formula (1). The dry weight of the potting soil in the pots is then calculated using formula (2), and will be used to determine the amount of daily water to be supplied to each plant according to the water treatment.

Control plants are kept in potting soil with a moisture content of 3.3 g water/ g dry soil, while the water-deficient plants are kept in potting soil with a moisture content of 0.8 g water/ g dry soil. In order to calculate the quantity of water to be supplied to maintain these humidity levels, the formulas (3) and (4) are used.

The pots were weighed approximately every day from the first day of water stress to control the loss of water every day. After collecting samples from all the turnips, the fresh weight of the aboveground part of the turnips was measured with a precision scale, and the leaves were counted. Subsequently, the aboveground parts were placed in an oven at 90°C for 48 hours to measure the dry weights of each turnip.

$$(1)\ \text{Initial soil humidity (water g / dry soil g)} = \frac{\text{Fresh soil mass}}{\text{Dry soil mass}} - 1$$

$$(2)\ \text{Dry moisture} = \frac{\text{Fresh soil mass}}{1 + \text{Soil initial humidity}}$$

$$(3)\ \text{Expected soil weight (g)} = \text{Dry soil mass} \times (\text{Humidity to maintain} + 1)$$

$$(4)\ \text{quantity of water to bring (g)} = \text{Expected soil weight} - \text{Measures soil weight}$$

4. DNA and RNA quantification

4.2. Plant DNA and RNA Extraction

Total RNA from CaMV-infected leaf-disc samples (pools of 2 leaf discs collected on the sixth leaf per plant) was extracted using the Monarch kit (Qiagen) following the manufacturer's instructions prior to cDNA synthesis. Total RNA was resuspended in 60µL of water. Finally, samples were diluted in milliQ water to in order to have a final concentration of 40 ng/µl per tube and stored at -80°C.

Total DNA from CaMV-infected leaf disc samples (pools of the 2 leaf discs collected per plant) was extracted according to a modified Edwards protocol with an additional washing step with 70% ethanol. DNA was resuspended with 200 µl of water, and 10-fold diluted samples were used for Q-PCR. Total DNA was stored at -20°C. The quality and quantity of nucleic acid extraction were assessed by spectroscopic measurements at 230, 260, and 280 nm (NanoDrop 2000 Spectrophotometer).

4.3. Single-stranded cDNA synthesis.

Four micrograms of total RNA extracted from CaMV-infected leaf-discs samples were used for single-strand cDNA synthesis with 1 µg of three reverse primers with a 1/10 dilution (P2_418-R, UBC21_Bn_R, R-ActBra; **Table 1**) using Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega kit) according to the manufacturer's instructions. Finally, the reverse transcriptase product was diluted to 1/10 prior to the qPCR analysis.

Table 1: Primers used for RT-qPCR (P2, Actin and Ubiquitin).

Primers name	Forward or Reverse	Sequence (5' – 3')
P2_198F	F	CTTCGGACTAAGCAAAGACC
P2_418R	R	CTTCTTCTTTAGTTAAGGGCT-CTG
R-ActBra	R	GATCTCTTTGCTCATACGGTCTG
F-Act2	F	GACYTBTAYGGTAACATTGTGCTC
UBC21_Bn_F	F	CCTCTGCAGCCTCCTCAAGT
UBC21_Bn_R	R	CATATCTCCCCTGTCTTGAAATGC

4.4. DNA and RNA quantification by qPCR

DNA and RNA quantification was performed in triplicates by real-time quantitative PCR (qPCR) in 384-well optical plates using the LightCycler FastStart DNA Master Plus SYBRgreen I kit (Roche) in a LightCycler 480 thermocycler (Roche), following the manufacturer's instructions on the DNA extraction (viral load) and on the cDNA (viral transcripts).

Specific primers designed for quantification of the viral gene P2 (P2_198F, and P2_418R; **Table 1**) were used at a final concentration of 0.4 µM. All qPCR reactions were carried out with 40 cycles (95°C for 15 s, 62°C for 15 s, and 72°C for 15 s) after an initial step at 95°C for 10 min. Two host plant genes: Ubiquitin and Actin were used as housekeeping genes and specific primers (R-ActBra/F-Act2 and UBC21 _Bn_F/ UBC21 _Bn_R) also used at a final concentration of 0.4 µM.

The qPCR data were analyzed with the LinReg PCR program to account for the efficiency of every single PCR reaction. The absolute initial viral concentration in *B*. *rapa* plants, expressed in arbitrary fluorescence units: N0 CaMV, was divided by that of a host plant gene (N0 actin; *B*. *napus* actin gene, primers R-ActBra and F-Act2, and NO ubiquitin, primers

UBC21_Bn_F, UBC21_Bn_R; **Table 1**), in order to normalize the amount of plant material analyzed in all samples. Actin and Ubiquitin are commonly used house-keeping gene and were previously used for quantification of gene expression in plants under water stress (van Munster 2017, Berges et al. 2018).

5. Western Blot

Total leaf extracts were prepared as follows: sixth leaf samples were flash-frozen in liquid N2 and ground in a mortar with a pistil to a fine powder. 400 mg of the powder was transferred to a 1.5 ml reaction tube and 2× Laemmli buffer (Laemmli, 1970) was added at a ratio of 1:1 (wt/vol). The samples were heated for 5 min at 95 °C and centrifuged for 10 min at 16,000×g. Aliquots 15 µl of the supernatant were loaded on 13.5% discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels which allow small molecules separation (P2 is around 18kDA). Gels migrate in a running buffer at 40mA and 100V for 10 minutes and at 200V for 50 minutes.

Proteins were transferred after SDS polyacrylamide gel electrophoresis onto nitrocellulose membranes using the wet blotting technique (Towbin et al., 1979). The transfer efficiency was controlled by Ponceau red staining. Membranes were blocked for 30 min with 5% low-fat milk powder in Tris-buffered saline (TBS) and incubated overnight at 4 °C with the anti P2 primary antibodies (Blanc et al., 1993; dilution 1/1000) at 4°C. After three washes with TBS, membranes were incubated with the secondary antibodies (goat anti-rabbit peroxidase, dilution 1/5000) for 3–4 hr at 4°C. After another three washes with TBS, protein bands were visualized by enhanced chemiluminescence using a G-Box.

To control the presence of P2, positive control was used P2-GFP, obtained in *Spodoptera frugiperda* 9 cells (sf9).

The following 1:1,000 dilutions of primary antibodies were used: anti-P2 (Blanc et al., 1993). Secondary antibodies were goat anti-rabbit peroxidase conjugates used at a 1:5000 dilution.

6. Microscopy

Three rectangles of the sixth leaf of each plant were harvested and put in a paraformaldehyde (PFA) 4% solution to stop all enzymatic reactions overnight at 4°C. Samples were then included in agarose low melting 8%, before making 100µm thick sections with the vibratome. Slides were faded with Carnoy 6 :3 :1 (chloroform, ethanol, acetic acid). After 2 rinses with PBS 1X + Tween 0,05%, samples were incubated in a solution of PBS 1X + BSA

5% to saturate aspecific sites for one hour and a half. Slides were incubated with primary antibodies (anti-P2 (Blanc et al. 1993) and mouse anti-α-tubulin DM1A (http://www.sigmaaldrich.com; Blose et al., 1984) diluted 1:250 in a 5% BSA buffer) overnight at 4 °C. After three rinses with PBS 1X, slides were incubated for at least 1 h at 37°C with secondary antibodies (Alexa 488 and Alexa 594 conjugates (http://www.lifetechnologies.com; Bak and Martinière, 2013) diluted 1:250 in BSA. After three rinses with PBS 1X, slides were mounted in an antifading medium, which included DAPI (50 ng/ml). DAPI is a fluorochrome that complexes with double-stranded DNA marking nuclei. Some slides were incubated only with BSA without primary antibodies and then with secondary antibodies to verify their specificity.

Slides were observed with Zeiss LSM700 (http://www.zeiss.com) confocal microscope at different magnificent (x10, x20, x40, x63). Images were processed using ZEN software and final figures were prepared using ImageJ and PowerPoint.

7. Statistical analysis

Statistical analyses of the various tests were carried out using R software version 4.2.2 (R Core Team 2023) with a critical threshold of 5%. The repetition effect was verified for each type of data. In the absence of a repeat effect, the data were grouped together or processed separately if the repeat effect was too strong. For the analysis of fresh weights, dry weights, viral loads and transcript levels obtained, the hypotheses of normality and homoscedasticity were verified using the Shapiro-Wilk and Bartlett tests respectively. If these hypotheses were not valid, a Kruskall-Wallis test was performed. A non-parametric analysis of variance (ANOVA) was also performed on the qPCR data to verify the presence of a possible repeat effect. A post hoc test was then performed. Leaf count data were analyzed using a fish-type generalized linear model (GLM).

RESULTS

1. Water deficit effect on turnip physiology.

In the first step, physiological parameters of turnip plants were measured to confirm the effect of water treatments (WW and WD), such as plants' aboveground fresh and dry mass (g) after sampling, as well as the number of leaves. These parameters have been measured individually on each plant at 21 dpi (day post inoculation) on 4 biological replicates.

The fresh mass indicates the water content in plants under the two different water conditions is a good indicator of the effectiveness of the water deficit treatment. The WW turnip's fresh weight is approximately three times higher than that of turnips under water deficit (**Figure 10A**) with a mean of 11,10 g/plant for WW (n=12/biological replicate) and 3,20 g/plant for WD (n=12/biological replicate). This difference in fresh weight between WW and WD conditions is significant with a p-value of 9.144e-09 according to the Kruskal-Wallis test (**Table 2**).

The number of leaves (**Figure 10B**) and the dry mass are used to assess the water deficit effect on turnip development. WW plants dry mass is twice higher than WD plants'one with a mean of 2,21 g/plant for the WW (n=12/biological replicate) and 1,06 g/plant for the WD (n=12/biological replicate) (p-value of 0.003604). The leaf number per turnip plant is around 8,72 for WD (n=12/biological replicate) plants and around 10,04 for WW plants (n=12/biological replicate). This difference is significant with a p-value of 9.167e-05, again based on the Kruskal-Wallis test (**Table 2**).

Quantification by qPCR and RT-qPCR was realized on leaf 6 of each turnip plant for 3 biological replicates (virus quantification) and for 4 biological replicates (RNA transcripts quantification) respectively. A non-parametric ANOVA was carried out on qPCR and RT-qPCR data to test the effect of water treatment and biological replicates on the variability of the results obtained. In both cases, the non-parametric ANOVA results indicated that a significant proportion of the variability was explained by the water treatment, but also by biological replicates with significant p.value (**Table 3, Table 4**). These results indicate that, even though differences in qPCR data are significantly explained by the water deficit treatment, the repetition effect remains more important. In consequence we chose to analyze each repetition independently.

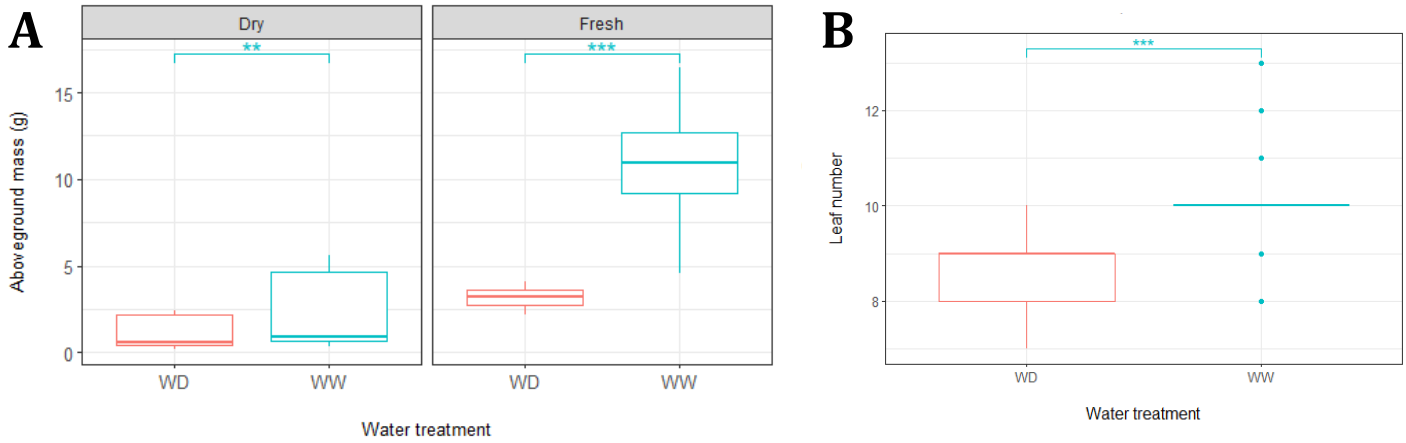


Figure 10: ***Impact of water deficit on plant physiology.***

A. Comparison of the effect of water deficit on the aboveground dry and fresh mass (g) of individual turnip plant at 21 dpi. A Kruskal-Wallis test was performed on the data.

B. Leaf number of 35-day-aged turnip plant (21 dpi). A Kruskal-Wallis test was performed on the data. *** : p.value < 0,001 ; ** : p.value < 0,01

Table 2: ***Water deficit effect on plant physiology used for the*** *experiments (qPCR, Western Blot, and microscopy).* ***: p.value < 0,001; **: p.value < 0,01

Parameter	WW	WD	p.value
Fresh mass (g)	11,10268	3,205	9.144e-09 ***
Dry mass (g)	2,21514	1,06588	0.003604 **
Leaf number	10,0476	8,7272	9.167e-05 ***

Table 3: ***No parametric ANOVA results on qPCR data.***

*** : p.value < 0,001; ** : p.value < 0,01

	Df	F value	Pr(>F)
Water treatment	1	13,4338	0,00053743 ***
Repetition	2	11,3342	6,9984 e-05 ***
Water treatment:Repetition	2	3,7535	0,02931338

Table 4: ***Non parametric ANOVA results on RT-qPCR data).***

***: p.value < 0,001; ** : p.value < 0,01

	Df	F value	Pr(>F)
Water treatment	1	12,9906	0,00065138 ***
Repetition	2	49,9666	2,4193 e-13 ***
Water treatment:Repetition	2	3,2942	0,04415111

2. Water deficit effect on viral load

The amount of viral DNA was quantified in WW and WD plants in order to detect a change in viral accumulation depending of the plant status. **Figure 11** shows the results of viral accumulation in three biological replicates (n=12/treatment/biological replicate). A similar trend can be observed in all replicates, with a higher viral load in WW plants. However, this difference is only significative for the third replicate (p.value <0.0001; **Table 5**).

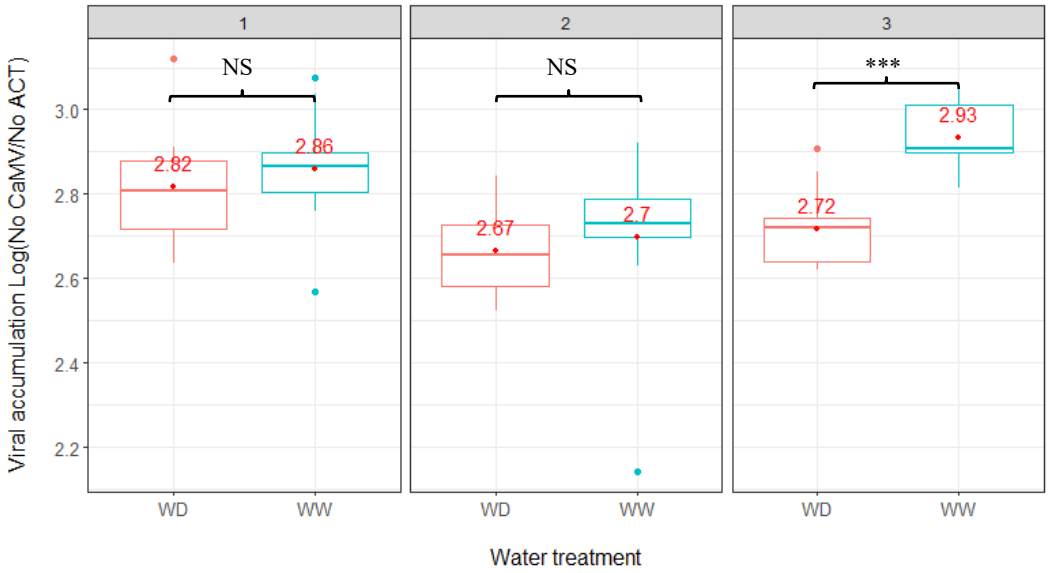


Figure 11: ***Effect of water deficit on the viral accumulation by repetition in turnip leaves.***

Amount of viral DNA in infected turnip leaves in well-watered plants (WW in blue) compared to plants in water deficit (WD in red). Viral accumulation in the infected plants was estimated in leaf 6 by qPCR. The DNA amount is normalized to a housekeeping gene (Actin).

*** : p.value < 0,001; NS : Non-significative

Table 5: ***Post Hoc test results on qPCR data.***

*** : p.value < 0,001; ** : p.value < 0,01

Compared condition	p.value
WD-1 / WW-1	0,3268
WD-2 / WW-2	0,1038
WD-3 / WW-3	<0,0001 ***

3. Water deficit effect on transcription of the RNA35S of the CaMV

We then looked at CaMV major transcript RNA35S amount in order to potentially detect a change in transcription levels depending of the watering status. Noteworthy, the 35S RNA is coding for all CaMV proteins but P6. Indeed, CaMV 35s RNA is polycistronic (**Figure 3**), enabling the synthesis of P1 to P5 viral proteins from the same RNA strand. Thus, total amount of transcripts was quantified, including transcripts corresponding to the P2 protein.

Figure 12 shows the results of viral transcripts accumulation as a function of water treatment for each replicate. A same trend, i.e., a higher transcripts accumulation in WD plants (n=12/biological replicate) compared to WW plants (n=12/biological replicate), was observed in all replicates but was only significative for the second replicate (p.value <0.0001; **Table 6**).

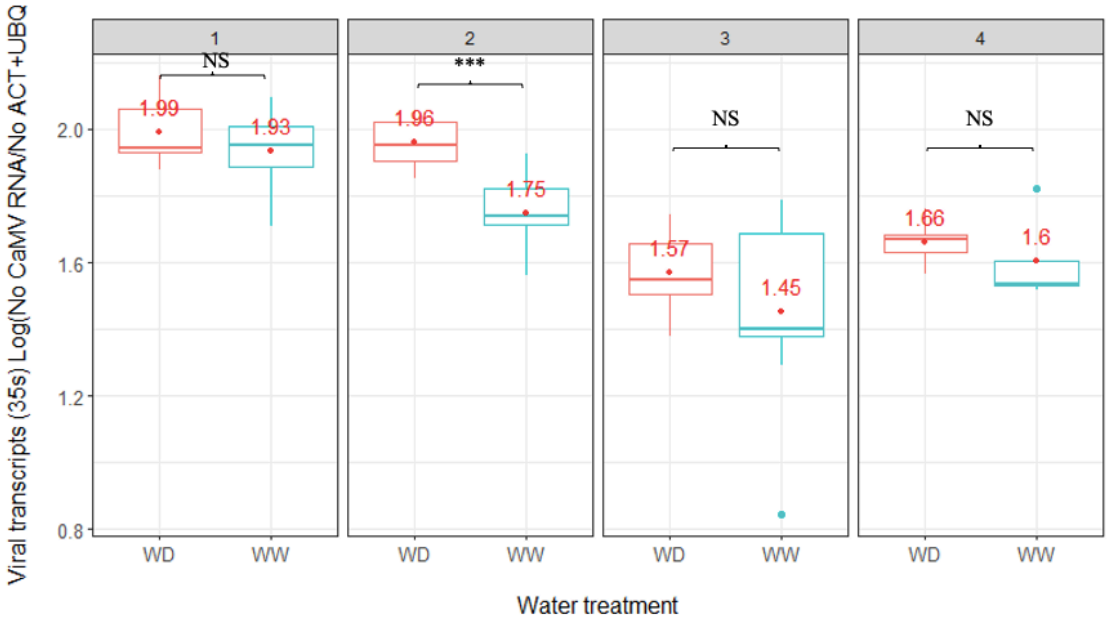


Figure 12: ***Effect of water deficit on the viral transcripts amount in turnip leave isn 4 biological replicates.***

Amount of viral RNA in infected turnip leaves for well-watered plants (WW in blue) compared to plants in water deficit (WD in red). Viral transcript accumulation in infected plants was estimated in leaf 6 by RT-qPCR. The RNA amount is normalized to 2 housekeeping genes: Actin and Ubiquitin. *** : p.value < 0,001; NS : Non-significative

Table 6: ***Post Hoc test results on RT-qPCR data.***

*** : p.value < 0,001; ** : p.value < 0,01

Compared condition	p.value
WD-1 / WW-1	0,3241
WD-2 / WW-2	<0,0001
WD-3 / WW-3	0,6376
WD-4 / WW-4	0,4676

4. Water deficit effect on P2 protein amount

Since having a clear correlation between RNA transcripts (including P2 transcripts) levels and P2 protein levels, we tried to quantify P2 protein in WW and WD plants by Western blot. The same leaf used for DNA and RNA quantification was used in this assay. **Figure 13** shows two examples of protein gels performed on a representative plant subset. In both WW and WD plants a band (approx. 18kDa) corresponding to the size of P2 viral protein was detected using specific anti-P2 antibodies (**Figure 13**). The P2-GFP positive control validates that this is indeed P2. Noteworthy, other bands can also be observed higher up on the gel for P2-GFP, corresponding to higher molecular weights. This is due to the polymerization of P2, which is mostly found in the form of aggregates in cells. Bands of the same type are less intense in infected plant samples. No visually discernible difference in brightness was observed between WW and WD plants, suggesting a similar amount of P2 between the two conditions. Staining with Ponceau red verifies that the same amount of material was deposited in each well allowing comparison of the different samples.

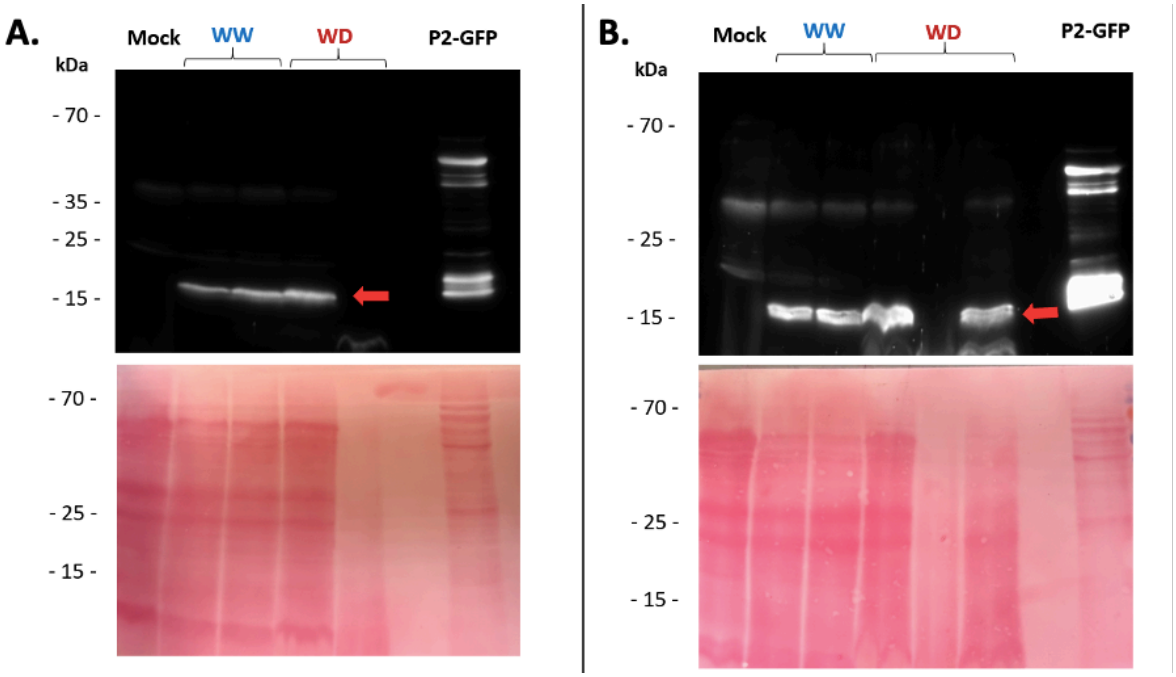


Figure 13: ***Effect of water deficit on the amount of P2 protein in CaMV-infected turnip leaves.*** Nitrocellulose membrane on the upper part and the corresponding ponceau red coloration on the lower part. WW (blue): CaMV-infected well-watered plant samples, and WD (red) : CaMV-infected water deficit plant samples. In both examples, the first well contains a Mock inoculated plant (healthy), and the last well is a positive control (P2-GFP). The red arrow indicates the presence of P2 protein.

A. Western blot analysis of the accumulation of P2 (18 kDa), on CaMV-infected turnip at 21 dpi from first and second biological repetition.

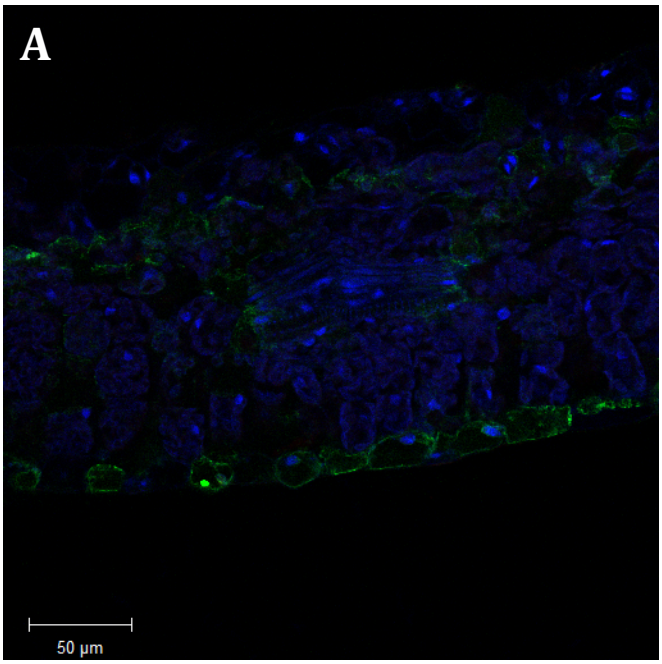
B. Another example of western blot analysis of the accumulation of P2 (18 kDa), on CaMV-infected turnip at 21 dpi from the first, second, and third biological repetition.

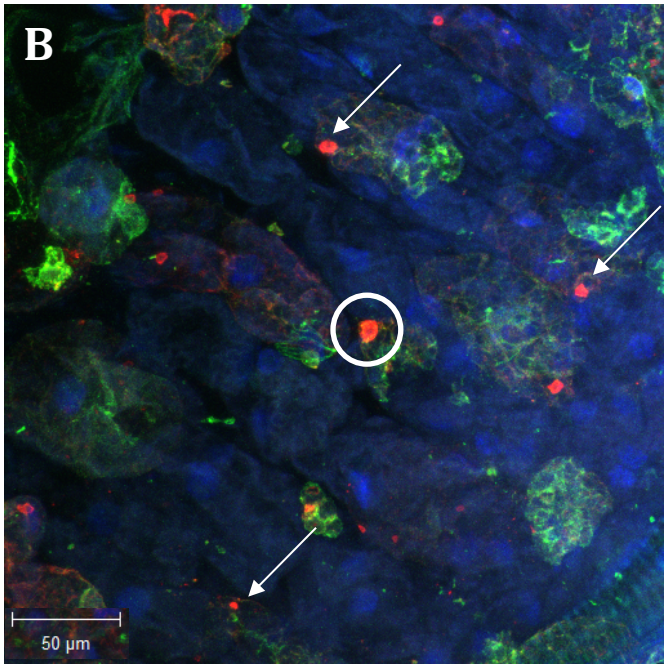
5. Water deficit triggers different phenotypes of TBs

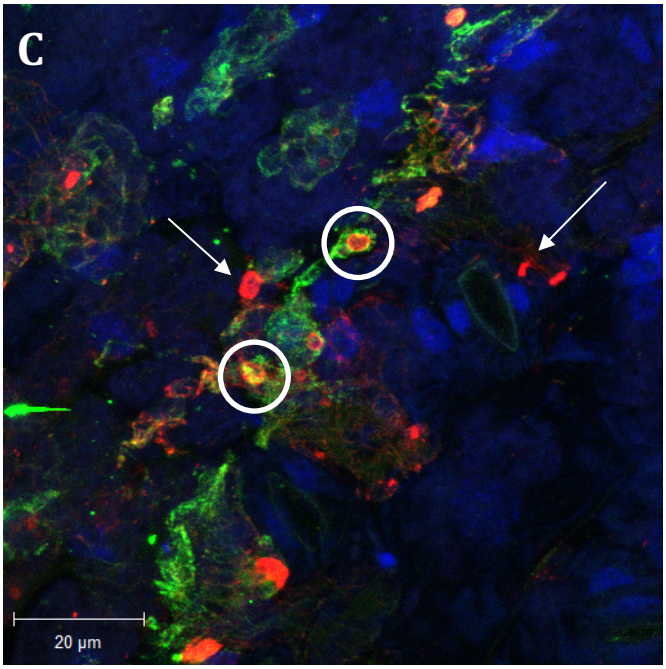
As explained in the introduction, we hypothesize that, just as aphid puncture induces P2 relocalization on plant cell microtubules, water deficit could trigger activation of the same signaling pathways, leading to the same relocalization of P2. This hypothesis would potentially explain the increase in CaMV transmission in WD condition. To test this hypothesis, confocal microscope observations were made on leaf sections from CaMV-infected turnip plants grown under controlled or water-deficit conditions, as well as on healthy plants (negative control). CaMV-infected plant cells phenotype were observed using specific antibodies against P2 (in red) and alpha-tubulin (in green) (double-labeling experiment).

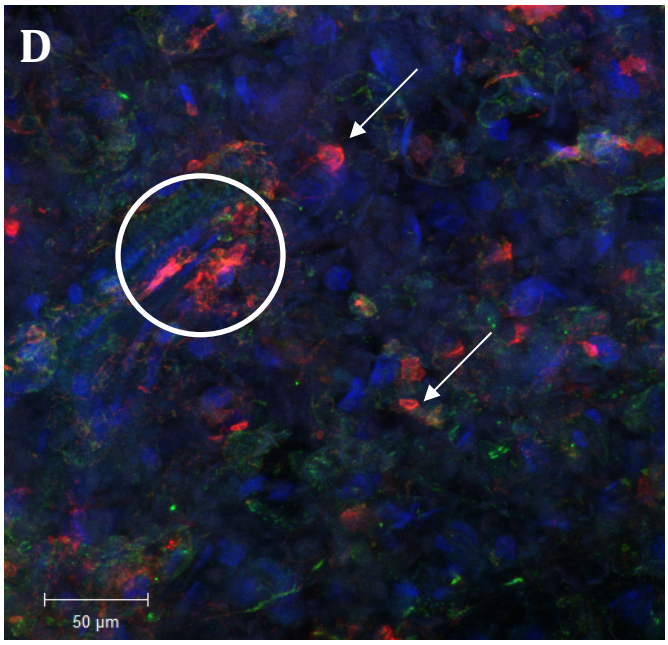
An example of observation under microscope of plant cells for each condition (mock, WW and WD) is shown on **Figure 14** using 3 magnifications (x20, x40 and x63). In the mock-inoculated condition, that serves as negative control, P2, as expected, is not detected while microtubule networks (in green) are visible. In the WW condition classic P2 aggregates forming vesicules (TBs) could be observed. Other common phenotype could be seen with TBs partly labelled in yellow (resulting of the merge of the red and green labelling) corresponding to tubulin relocalizing with P2. At x63 magnification, P2 aggregates are often located at the level of microtubule networks (green).

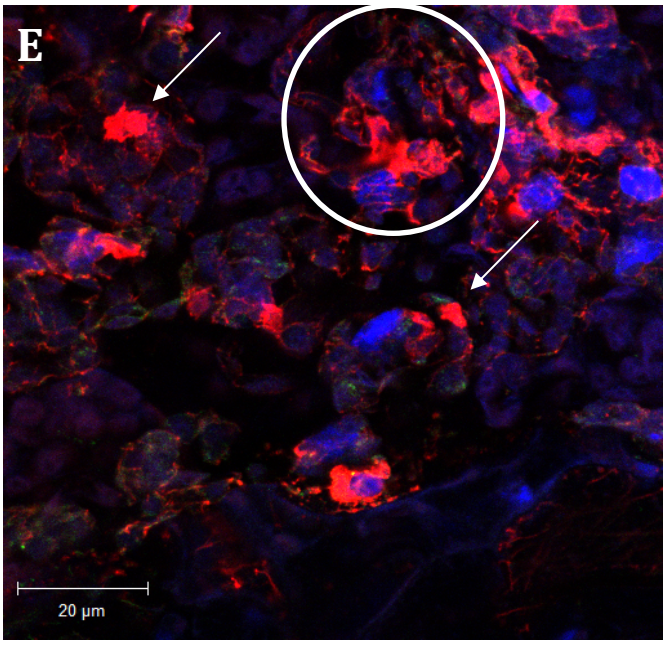
It seems that in WD CaMV-infected cells we observe a different phenotype (**Figure 14**). P2 aggregates seem to be more important. Moreover, P2 is not confined in TBs but appears to be more widespread in within the cell, showing a different localization of P2 in WD plants. The microtubule network is barely visible on WD plants, making it impossible to determine whether P2 is localized along them. These observations are not significant, as they were made on too few individuals (8 slides of each condition), and more observations need to be done before concluding whether P2 is located differently in WW and WD CaMV-infected plants.











<u>Figure 14:</u> ***<u>Effect</u> of a water deficit on P2 localization in CaMV-infected turnip cells.***

A. Immunofluorescence of MOCK-inoculated (healthy plants) leaves against alpha-tubulin (green). DAPI coloration for nuclei (blue). **B-E.** Immunofluorescence of infected leaves against P2 (red) and alpha-tubulin (green) with co-labeling appearing as yellow/ orange. DAPI coloration for nuclei (blue). **B-C.** CaMV-infected plant well-watered. Arrows show the tubulin-less TB, and circle a Tub+/TB**. D-E.** CaMV-infected plant in water deficit. Arrows show TB formation, and circles show a zone where P2 is more diffuse in the cell and not retained in aggregates.

DISCUSSION

It was previously shown in the hosting lab that viral transmission by aphid vectors (*M. persicae*) significantly increased in turnip plants (*B. rapa*) infected by the CaMV-isolate BJI and submitted to a severe water deficit compared to CaMV-infected plants well-watered (van Munster et al. 2017). One of the potential explanations to this result would be an alteration of the plant-virus interactions in this abiotic stress condition. In this study we investigated turnip-CaMV-BJI interactions when plants were submitted to WD in terms of virus accumulation, increase of viral transcription and virus "reactivity". Thus, we tested the hypothesis that perturbating effects of the WD on plant physiology would lead to changes 1) in viral load, 2) viral transcripts (35s) and/or P2 amount and 3) P2 localization in infected plant cells leading ultimately to an increase of the transmission rate.

1. Plant physiology is altered under water deficit

Plant physiological parameters were measured to ensure that the water deficit was properly established. Turnip fresh mass, dry mass, and the number of leaves were measured for each plant of both conditions (CaMV:WW and CaMV:WD) of the four biological replicates. As expected, and already observed in various studies (van Munster et al. 2017; Bergès and al. 2018), WD had a significant impact on plant development and physiology (decrease in fresh and dry mass, as well as in the number of leaves) in CaMV-infected plants (CaMV:WD), compared with control CaMV-infected plants (CaMV:WW).

2. Water deficit might alter viral load

Abiotic stresses on host physiological status might also influence the life cycle of viruses and the relationships between viral traits such as within-host accumulation that may have an impact on virus transmission itself (Suntio and Makinen, 2012; Szczepaniec et al.,2019). Van Munster et al. (2017) showed that in the case of CaMV there was no difference in viral accumulation due to water deficit applied to turnip plants that could potentially explain increase of the viral transmission. Here we reconducted quantification of virus accumulation in CaMV:WW plants using other primer sets to confirm previous results. Interestingly, we could observe a different trend that previously reported with a potentially higher accumulation in WW CaMV plants. However, this was significantly different with the WD condition in only one of the three replicates. Thus, at this stage we cannot conclude on a clear difference of virus accumulation triggered by a water treatment. One explanation that could explain this higher viral load, in WW CaMV-infected plants would be the following: experiments were conducted

at 21 dpi, corresponding to the plateau accumulation for CaMV in standard conditions. However, it is possible that this plateau is not reached by WD plants, due to slower transcription, which would explain the smaller difference in viral load for WD plants. It would be interesting to repeat this experiment kinetically to check whether water deficit influences viral load, with a plateau arriving later than in WW plants.

3. WD might impact viral transcription including P2 transcription

One of the hypotheses that would explain the increase in transmission in WD plants was an increase in the transcription and/or quantity of the P2 protein, the viral helper of the transmission. A higher amount of P2 could induce a higher chance of encounter between the aphid and the P2-virion complex in infected plant cells.

From a transcriptional point of view, water deficit did not induce a clear and significant difference of CaMV RNA 35S transcription levels that includes transcripts of P2 when pooling all together all replicates. However, there was a trend with a higher CaMV RNA 35S transcripts levels in WD conditions which was significative in one of the four replicates. To gain in robustness, number of samples should be increased per biological replicate in order to see potential statistical differences.

It would be interesting to normalize RNA transcripts levels by amount of viral DNA to have a ratio of transcription/viral load. However, to have exploitable results, RNA transcription levels and DNA levels should have been done on the same samples. It is impossible to be sure that the viral load is identical at all points on the same leaf. It is known that virus accumulation can vary from one leaf to another one and even on the same leaf (Gutierrez et al., 2012). To verify this, the correlation between viral load and the position of the sample on the leaf could be measured.

The qPCR data obtained showed a strong and significant effect of the biological replicates that explain qPCR results. It is likely that this variation simply stems from the biological variability of the replicates, which is why it would be necessary to carry out additional replicates to confirm the results. Our hypothesis has not yet fully revealed all its answers. Further RT-qPCR replicates would be necessary to reinforce our conclusions and verify accuracy of the results.

4. Impact of WD on P2 amount

As P2 transcript is localized on the CaMV 35s RNA , a long transcript polycistronic (Bouton et al. 2015) comprising several segments translating five of the 6 viral proteins, it is not possible to quantify only P2 transcripts. To analyze specifically P2, it was important to quantify P2 at the protein level under the two water conditions. For this purpose, we did semi-quantification by western blot using specific anti-P2 antibodies and normalization with the RUBISCO protein, a major constitutive protein of plant cells involved in photosynthesis. However, the Western blot results did not distinguish a difference in the amount of P2 protein depending of the plant water status suggesting that water deficit does not induce an increase in P2 protein. The primary aim of this experiment was to do the quantification using ImageJ software, normalizing the data with Rubisco (approx. 55kDa for large subunits) thanks to Coomassie blue or Ponceau red staining. However, it was difficult to obtain clear results for Rubisco, preventing us from normalizing the data. A more precise protein quantification method would be needed to verify the western blot results. One possibility would be a DAS-ELISA for spectrophotometric protein quantification. Combining RT-qPCR and Western Blot results, it suggests that water deficit had no strong effect at the transcriptional and translational level of the P2 protein. The higher CaMV transmission rate observable in water-deficient turnip plants seems not to be due to an effect on viral transcripts quantity or P2 amount. However, further experiments are needed to dismiss the first hypothesis.

5. WD induce different transmission bodies phenotypes

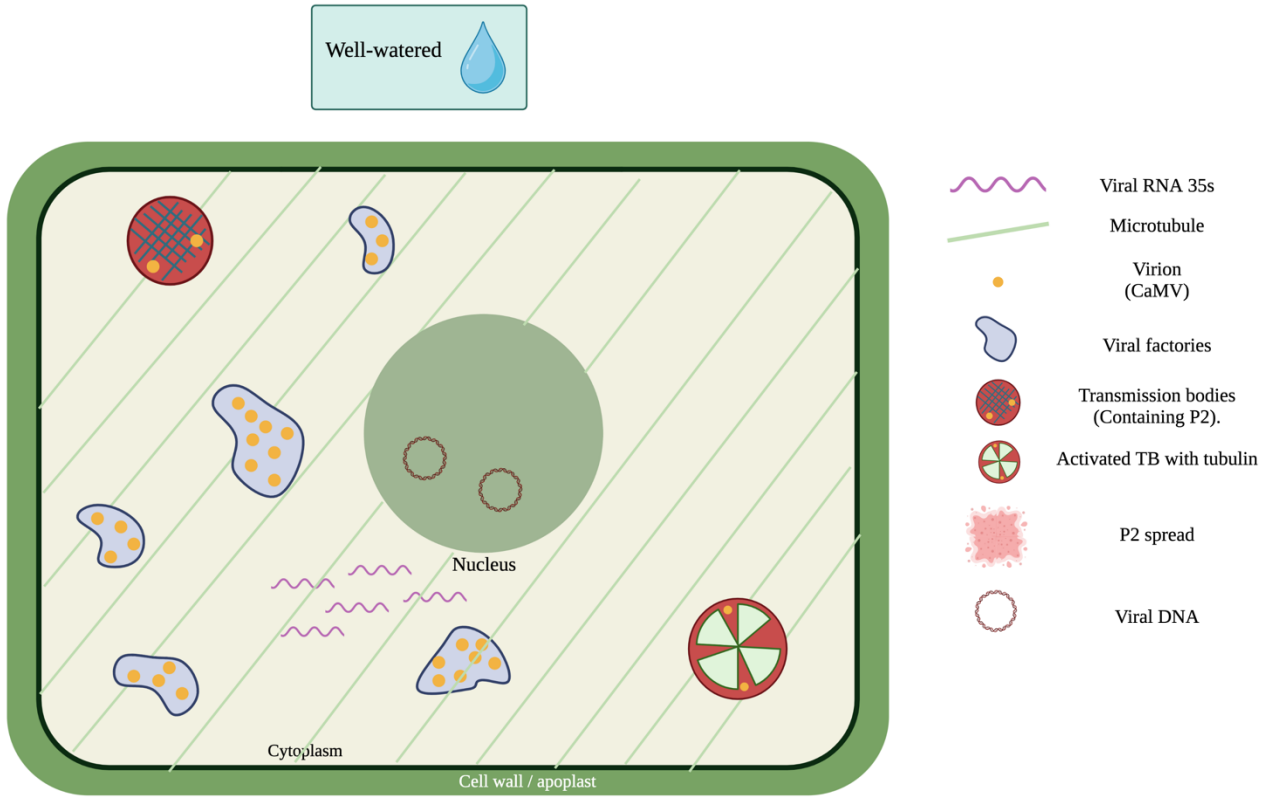
The second hypothesis explored was a possible increase of P2 relocalization of P2 protein within cells on microtubules similarly to what was observed after aphid puncture (Martiniere, Bak et al., 2013). Water deficit would likely induce stress signals in the plant that would activate common signaling pathways (Rejeb et al., 2014) , inducing a higher P2 relocalization on microtubules. To test this hypothesis, we did microscopic observation on plant cell sections CaMV-infected and under WW and WD conditions. Even if the number of observations is too low (8 slides per conditions) to conclude a clear and specific phenotype was seen in CaMV-plant cells under WD. In CaMV-infected cells WW, we observed the classic phenotype with 1 or 2 TBs par cell containing all the P2. Another phenotype of TB described in Martinière, Bak et al. (2013) et corresponding to P2 and tubulin which colocalized on a mixed network was not identified here. In WD plants, TBs are highly irregular and spread throughout the cells. Water deficit seems to lead to a phenotypic difference in P2 arrangement and TB

formation. It is possible that this phenotype of much more dispersed and diffuse TBs in water-deficient plants makes P2 more accessible to the vector. However, microtubules are difficult to observe on WD plants, suggesting either a problem with microtubule labelling or a physiological alteration due to water deficit. This hypothesis could easily be verified by observing plant sections to see the effect of water deficit alone on microtubule networks. The influence of dehydration on microtubules has already been demonstrated (Chen et al., 2003). In this paper, they showed that after dehydration, the microtubule organization was remarkably altered and the fine microtubule structure disappeared whereas some thicker cables formed. Cell counting would also be necessary to verify the significance of the results.

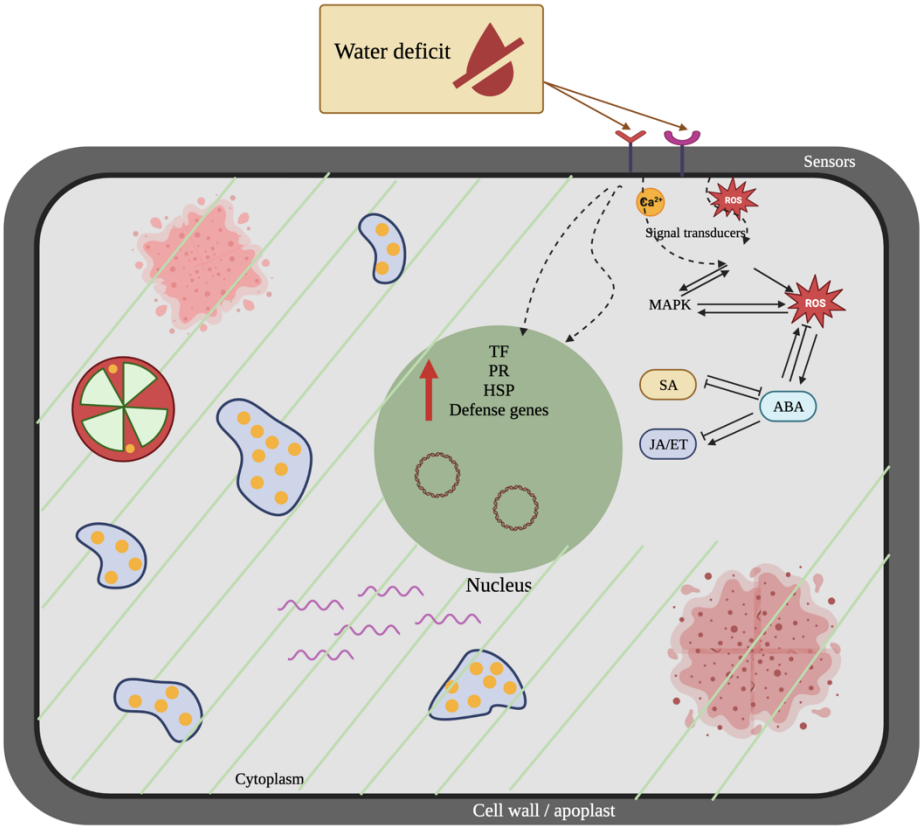
6. Perspectives

As a reminder, to identify the mechanisms responsible for increased transmission by vector two hypotheses were mentioned (1) the amount of the helper viral protein P2 available (at the mRNA level and protein level), and (2) the accessibility/relocalization of viral partners in cells of water-deficient plants compared to WW plants. However, another student studied another hypothesis in the team: (3) the vector feeding behavior could be responsible for the higher transmission rate. Vector feeding behavior was monitored using ElectroPenetroGraphy (EPG), a device that monitored in real-time stylest pathway using electric signals. Results showed an increase in intracellular punctures in both healthy plants in water deficit and infected plants in water deficit during the acquisition phase. These results suggest an influence of the alteration of the feeding behavior of the vector *M. persicae* on CaMV transmission when infected plants are under WD conditions. The higher transmission rate due to the water deficit is probably caused by an interaction of the different hypotheses, they seem to be complementary (**Figure 15**). Indeed, changes in the P2 and TB phenotype in water deficit conditions could modify the vector feeding behavior leading to a difference in the transmission rate. There are probably also some mechanisms still unknown to explain the influence of the environment on virus vectored-transmission. As it has also been observed that the transmission rate of TuMV increases when the host plant is under water stress, it would be interesting to study the mechanisms involved in this increase in transmission. This study should be linked to the one on CaMV to determine whether similar mechanisms emerge. Finally, it would be possible to extend this research to other environmental stresses (CO2, temperature, water stress, salt stress, etc.) and other viruses with non-circulating transmission with capsid strategy (work in progress) or circulating transmission, in order to determine whether there are any common mechanisms.

A



B



<u>Figure 15:</u> ***Summary diagramm: Effect of water deficit on a CaMV-infected-plant cell.***

A. A CaMV-infected-plant cell in well-watered conditions. Different TB morphs are observable, and viral factories containing CaMV virions. **B.** A CaMV-infected- plant cell in water deficit. The stress induced by water deficit triggers a cascade of signaling reactions, involving ROS, hormone, MAPK… In response to this signal, TB morphs are reacting, P2 is spread in the cell, but there is no difference concerning the amounts of transcript or P2 protein.

CONCLUSION

The economic and ecological impact of viral epidemics and their consequences for food safety are major problems for our society. To make matters worse, climate change and the scenarios expected for the coming years will have an impact on these epidemics by modifying plant/virus/vector interactions and the associated epidemiological parameters. One of the consequences of climate change is an increase in the frequency and intensity of climatic hazards, particularly drought and rainfall, leading to problems of water deficit and saturation in agricultural soils. In this context, the aim of this study is to assess the effects of water deficit on plant/virus/vector interactions and the associated mechanisms.

The higher CaMV transmission rate observed in water deficit condition for the host plant seems to be due to changes in P2 relocalization in plant infected cells and of the specific vesicules involved in virus transmission (the transmission bodies) that seems to have a different phenotype than in infected cells from well-watered plants. No clear variation in the viral load, viral transcripts or of the P2 viral protein, the helper component of the transmission, could be detected. Further research must be carried out to a better understanding of the mechanisms involved in the alteration of the transmission efficiency under WD and to confirm our conclusions. Moreover, the feeding behavior of the aphid vector may also be involved in the higher transmission rate observed. As a matter of fact, preliminary results obtained during another internship showed an alteration of the aphid vector feeding on turnip plants CaMV-infected and under water deficit compared to plants well irrigated that could partly explain this increase of transmission. Looking at the interactions between transmission bodies morphs kinetics and feeding behavior of the vector in water deficit conditions should be the next step to follow this research.

Subsequently, other pathosystems should be studied in order to determine the common mechanisms to several virus species in response to different abiotic stresses (CO_2, salt stress, temperature, etc.). In addition, we need to see whether these results can be transposed to the field, on entire crops. Therefore, further work is needed to understand the effects of climate change on plant-virus-vector interactions and their consequences on virus epidemiology.

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