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The Potyviral Protein 6K2 from Turnip Mosaic Virus Increases Plant Resilience to Drought

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Virus infection can increase drought tolerance of infected plants compared with noninfected plants; however, the mechanisms mediating virus-induced drought tolerance remain unclear. In this study, we demonstrate turnip mosaic virus (TuMV) infection increases *Arabidopsis thaliana* survival under drought compared with uninfected plants. To determine if specific TuMV proteins mediate drought tolerance, we cloned the coding sequence for each of the major viral proteins and generated transgenic *A. thaliana* that constitutively express each protein. Three TuMV proteins, 6K1, 6K2, and NIa-Pro, enhanced drought tolerance of *A. thaliana* when expressed constitutively in plants compared with controls. While in the control plant, transcripts related to abscisic acid (ABA) biosynthesis and ABA levels were induced under drought, there were no changes in ABA or related transcripts in plants expressing 6K2 under drought compared with well-watered conditions. Expression of 6K2 also conveyed drought tolerance in another host plant, *Nicotiana benthamiana*, when expressed using a virus overexpression construct. In contrast to ABA, 6K2 expression enhanced salicylic acid (SA) accumulation in both *Arabidopsis* and *N. benthamiana*. These results suggest 6K2-induced drought tolerance is mediated through increased SA levels and SA-dependent induction of plant secondary metabolites, osmolytes, and antioxidants that convey drought tolerance.

Keywords: 6K2, abiotic stress, abscisic acid, *Arabidopsis thaliana*, drought, hormone, *Nicotiana benthamiana*, plant-virus interactions, potyvirus, salicylic acid, TuMV

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Crops are routinely subjected to biotic and abiotic stresses that adversely affect their productivity. Drought is one of the most important types of abiotic stress, impacting over three-fourths of global harvested area (approximately 454 million hectares) (Kim et al. 2019). Technological advances have led to improved drought management techniques (Lamaoui et al. 2018), however, these technologies often require significant infrastructure. Breeding and transgenic approaches have also led to the development of drought-resistant cultivars, but these plants often have lower yields compared with other cultivars under non-drought conditions (Kim et al. 2014; Martignago et al. 2020). Furthermore, climate change is predicted to intensify the severity and frequency of drought events in the future (Dai 2013); thus, identifying new sources of drought tolerance and drought management techniques is a high priority.

While plant viruses are best known as obligate parasites and pathogens of their hosts, recent evidence suggests viruses can enhance host survival during drought. For example, many plants have been shown to perform better under drought conditions when infected with viruses, such as brome mosaic virus, cucumber mosaic virus (CMV), cauliflower mosaic virus, tobacco mosaic virus, tomato yellow leaf curl virus (TYLCV), or tobacco rattle virus compared with uninfected control plants (Bergès et al. 2018, 2020; Corrales-Gutierrez et al. 2020; Shteinberg et al. 2021; Westwood et al. 2013; Xu et al. 2008). Virus-induced drought tolerance has been shown using diverse vegetative crops, including rice (*Oryza sativa*), wheat (*Triticum aestivum*), tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), watermelon (*Cucumis lanatus*), cucumber (*Cucumis sativus*), and zucchini squash (*Cucurbita pepo*) (Davis et al. 2015; Xu et al. 2008). More recently, it has been shown that grapevine fanleaf virus (GFLV)-infected grapevines, a long-lived perennial woody plant, also performs better in mild drought conditions compared with the healthy grapevine (Jež-Krebelj et al. 2022). These results suggest viruses may use conserved mechanisms to enhance drought tolerance, and thus, understanding the mechanisms of virus-induced drought may enhance our understanding of plant physiology more broadly.

Plants regulate appropriate responses to abiotic and biotic stress using phytohormone signaling pathways. Abscisic acid (ABA) is the primary hormone that regulates plant responses to drought (Wilkinson and Davies 2010), while salicylic acid (SA) and jasmonic acid (JA) are the primary hormones that regulate plant responses to pathogen infections and herbivore infestation, respectively (Prakash et al. 2017; Ryals et al. 1996). However, SA and JA have also been linked to increased plant resilience to abiotic stress in previous studies (Khan et al. 2015; Wang et al. 2020). Virus-induced changes in these phytohormones (or sensitivity) has been implicated in increased drought tolerance of infected

plants. For example, CMV-infected *Arabidopsis thaliana* are hypersensitive to ABA (Westwood et al. 2013) and host plants infected with plum pox virus (PPV*)* expressing the potato virus X RNA silencing suppressor molecule P25 (PPV-P25) had increased SA levels compared with controls and were also more tolerant of drought (Aguilar et al. 2017). Recently, it has been shown that differences in viral genomes can mediate the ability of viruses to enhance plant drought tolerance (Carr 2017, 2018; González et al. 2021; Hily et al. 2016; Westwood et al. 2013), suggesting individual viral proteins could be used as sources of drought resistance. To our knowledge however, there are only two drought tolerance–conveying viral proteins that have been identified so far (Corrales-Gutierrez et al. 2020; Westwood et al. 2013).

Viruses belonging to the genus *Potyvirus* possess single-stranded positive-sense RNA genomes that are translated into a single polyprotein after host entry and are transmitted by aphids in a nonpersistent manner (Adams et al. 2012; Casteel and Falk 2016; Revers and García 2015). Recently it was proposed that the function of viral proteins can change in the presence of different ecological interactions and environments (Ray and Casteel 2022). For example, the viral protein NIa-Pro (Nuclear Inclusion a Protease), which is required for the cleavage of the polyprotein into individual mature proteins (Adams et al. 2005), also has a role in plant-aphid interactions when the aphid vector is present (Bak et al. 2017). Recent studies have demonstrated that specific lineages of the turnip mosaic virus (TuMV) increase plant tolerance to drought if they were evolved in the presence of drought-stressed plants (González et al. 2021). This suggests changes in the TuMV genome may convey new functions in drought tolerance under water-limited environments.

These previous findings led us to hypothesize that individual TuMV proteins may convey drought tolerance to host plants through changes in host physiology. Thus, in this study, we examined the ecological functions of viral proteins in conveying drought tolerance, using TuMV and *Arabidopsis thaliana* as a model. We determined at least three TuMV proteins (NIa-Pro, 6K1, and 6K2) increase drought tolerance of host plants (NIa-Pro, 6K1, and 6K2), with minimal negative impacts on host biomass. We investigated the underlying mechanisms by examining changes in phytohormones and related transcriptional responses. Expression of one potyviral protein, 6K2, increased drought tolerance and SA accumulation in *A. thaliana* and in another host species, *Nicotiana benthamiana*. Taken together, our study shows that the TuMV 6K2 protein may play additional ecological functions in host plants under drought conditions.

Results

TuMV-enhanced *A. thaliana* survival under drought is mediated by three viral proteins.

A greater number of *A. thaliana* plants infected with TuMV survived after drought compared with mock-inoculated plants (Fig. 1A) (66.21% compared with 25%, respectively). To determine if individual TuMV proteins may be mediating enhanced plant survival under drought, survival assays were conducted with *A. thaliana* expressing the 10 major TuMV proteins individually. Expression of 6K1, 6K2, and NIa-Pro increased plant survival after drought treatment compared with plants expressing the empty expression vector (Fig. 1B). In contrast, expression of cylindrical inclusion (CI), nuclear inclusion b (NIb), and coat protein (CP) decreased plant survival after drought treatment compared with the controls (Fig. 1B). Change in pot weight and plant phenotype during the drought treatment are shown in Supplementary Figure S1A and B. Plants with reduced size also have reduced water needs and are more tolerant to drought (Yang et al. 2021). TuMV-infected plants were visually smaller, thus, this may be the reason that these plants were more resilient to drought. To determine if this might also be the case for plants expressing 6K1, 6K2, and NIa-Pro, above ground biomass was recorded. While there was no impact of NIa-Pro expression on shoot biomass compared with controls, unexpectedly, plants expressing 6K1 or 6K2 had a greater biomass than control plants (Fig. 2A). As increases in root length have been associated with enhanced drought tolerance (Werner et al. 2010), we next measured root length of NIa-Pro-, 6K1-, and 6K2-overexpressing *A. thaliana* plants. There was no significant difference in root length of NIa-Pro or 6K2 plants compared with the EV control plants (Fig. 2B), however, root length was significantly increased in 6K1-expressing plants.

TuMV infection and the TuMV 6K2 protein do not alter ABA levels in *A. thaliana.*

Plants increase ABA levels in response to drought stress, which leads to physiological changes, such as regulating water status, activating of genes required for dehydration resistance, and elevating plant antioxidant defense system (Zhou et al. 2019). Thus, we hypothesized that ABA content may be elevated in plants infected with TuMV. To address this, we measured the ABA content in TuMV-infected plants. While ABA content increased slightly in infected plants, the increase was not significant compared with the control (Fig. 3A). Next, we wanted to check if drought affects ABA accumulation in *A. thaliana* plants individually expressing NIa-Pro, 6K1, and 6K2 relative to the control. We found that ABA content was increased in NIa-Pro- and 6K1-expressing plants under drought compared with

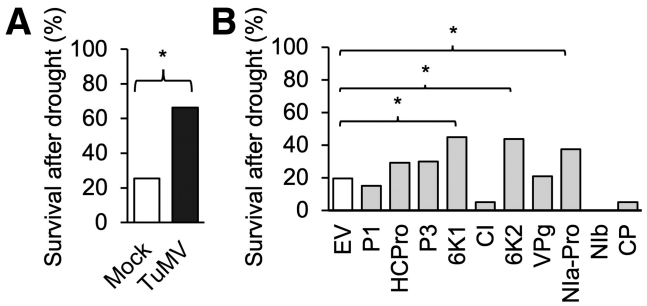

Fig. 1. A, Percent survival of turnip mosaic virus (TuMV)-infected and mock-infected *Arabidopsis thaliana* plants following 7 days of drought stress and 7 days of re-watering (Chi-square test = 78.0903, $P < 0.001$, $n = 51$ to 74). **B,** Percent survival of transgenic plants expressing the empty expression vector (EV) or different TuMV proteins following 14 days of drought stress and re-watering (Chi-square test, $P < 0.05$, $n = 18$ to 87).

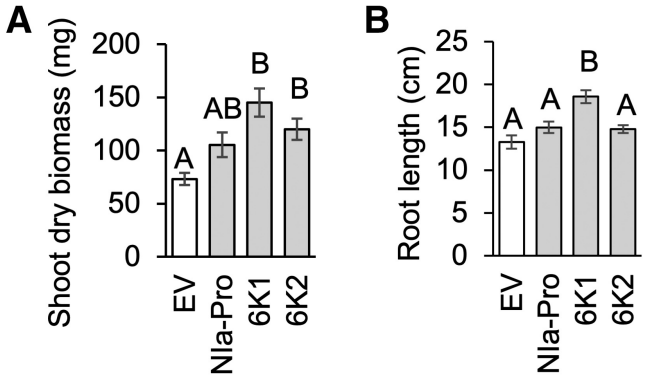

Fig. 2. A, Dry biomass of shoots and **B,** root length for 6-week-old *Arabidopsis thaliana* overexpressing the empty vector (EV), 6K1, 6K2, and NIa-Pro. The entire aerial portions of 6-week-old plants were harvested from the well-watered plants, were dried, and were weighed (one-way analysis of variance, Tukey's honest significant difference, $F = 8.15$, $P < 0.05$, $n = 10$). Significant differences are indicated using different upper-case letters.

the well-watered plants; however, surprisingly, there was no increase in ABA content in drought-stressed plants expressing 6K2 compared with well-watered controls (Fig. 3B).

SA is increased in 6K2-expressing *A. thaliana* under drought.

Reports suggest that enhanced SA provides drought tolerance in several plant species such as *Arabidopsis* (Miura et al. 2013), wheat (Kang et al. 2013; Munsif et al. 2022; Shemi et al. 2021), and rice (Munsif et al. 2022). As Casteel et al. (2015) already demonstrated that SA is significantly increased after TuMV infection in *Arabidopsis*, we hypothesized plants expressing individual TuMV proteins may have elevated SA levels under drought. To address this, we measured SA levels in NIa-Pro-, 6K1-, and 6K2-expressing *A. thaliana* under drought and well-watered conditions. We found plants expressing 6K2 had higher SA levels under drought conditions compared with the well-watered conditions (Fig. 4A), while there was no significant difference in EV, NIa-Pro, and 6K1 plants under drought and well-watered conditions (Fig. 4A). We also checked if JA was differentially accumulated in EV-, NIa-Pro-, 6K1-, and 6K2-expressing plants. We found that JA levels were drastically reduced in all plants under drought stress compared with well-watered controls (Fig. 4B).

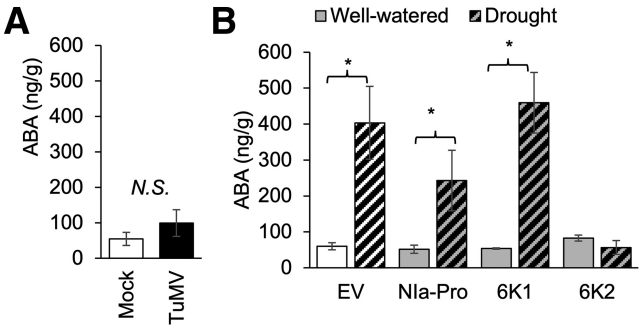

Fig. 3. **A,** Abscisic acid (ABA) levels in mock- and turnip mosaic virus (TuMV)-infected *Arabidopsis thaliana* and in **B,** plants expressing the empty vector (EV) or the TuMV proteins NIa-Pro, 6K1, or 6K2 under well-watered and drought conditions ($n = 5$ to 6, Mann-Whitney U test, $P < 0.05$). Stars indicate significant differences between well-watered and drought for B.

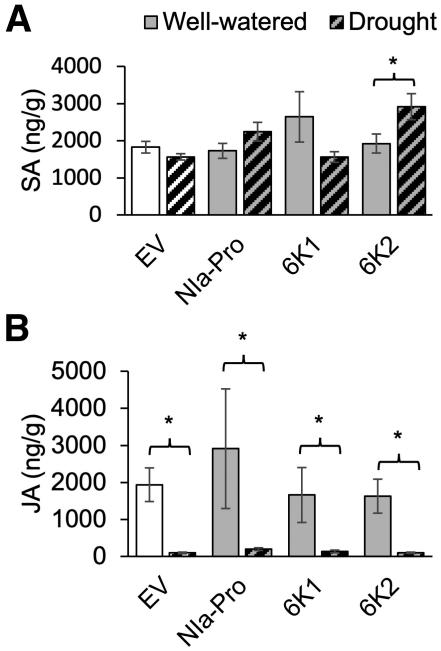

Fig. 4. **A,** Salicylic acid (SA) and **B,** jasmonic acid (JA) levels in *Arabidopsis thaliana* plants expressing the empty vector (EV) or the turnip mosaic virus proteins NIa-Pro, 6K1, or 6K2 under well-watered and drought conditions ($n = 5$, Mann-Whitney U test, $P < 0.05$). Stars indicate significant differences between well-watered and drought.

6K2 expression provides drought tolerance to *A. thaliana* independent of the ABA pathway.

The 6K2 protein localizes to the endoplasmic reticulum (ER) (Cotton et al. 2009) and chloroplasts of plant cells (Cheng et al. 2021; Wei et al. 2010) and several chloroplast proteins have been linked to increased drought resistance (Lim et al. 2014; Nawaz et al. 2018); therefore, we decided to investigate plants expressing 6K2 in more detail.

We measured the expression of genes that are known to be differentially expressed under drought stress. *AtNCED3*, a transcript related to ABA biosynthesis, is induced in plants under drought (Bhaskara et al. 2012; Sato et al. 2018). *AtNCED3* was drastically increased in the drought-treated EV control plants compared with EV plants under the well-watered conditions, which was expected (Fig. 5A). However, there was no significant change in *AtNCED3* transcripts or ABA content of the 6K2 plants in well-watered or drought conditions compared with controls (Fig. 5A), which is consistent with the ABA levels in 6K2 plants under drought and well-watered conditions (Fig. 3B).

Previous studies show that the expression of *AtRD29* is induced by drought and ABA (Yamaguchi-Shinozaki and Shinozaki 1993; Yamaguchi-Shinozaki et al. 1992), and overexpression of *AtRAP2.6L* and *AtDREB19* enhances the performance of the plants under drought (Krishnaswamy et al. 2011). Although all three transcripts increased in EV plants under drought conditions compared with well-watered conditions, there were only significant differences for *AtRD29* (Fig. 5B to D).

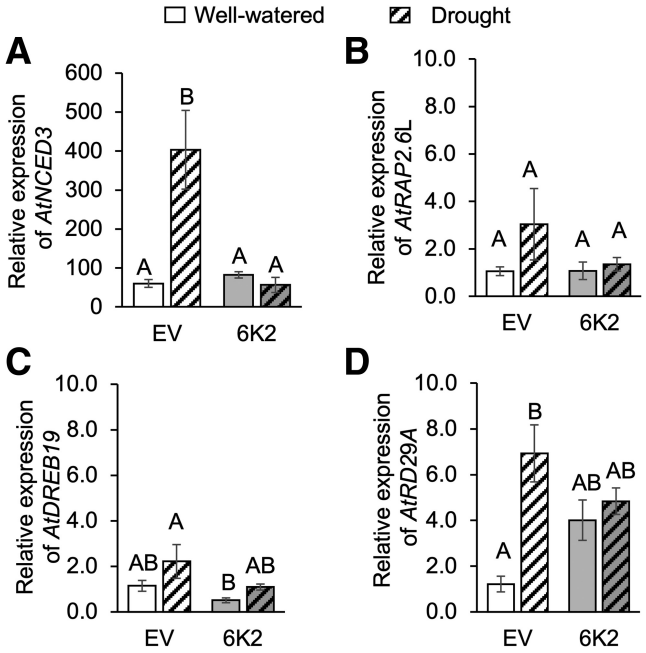

Fig. 5. Relative abundance of four drought-responsive genes, **A,** *AtNCED3*, **B,** *AtRAP2.6L*, **C,** *AtDREB19*, and **D,** *AtRD29A*, in *Arabidopsis thaliana* expressing the empty vector (EV) or the turnip mosaic virus protein 6K2 under well-watered and drought conditions. *AtUbiquitin* was used as an internal control and each replicate had leaf samples pooled from two individual plants. For transcript abundance: $n = 5$, two-way analysis of variance, Tukey's honest significant difference (for *AtNCED3*, $F = 17.2120$, $P < 0.05$; for *AtRAP2.6L*, $F = 0.3477$, $P > 0.05$; for *AtDREB19*, $F = 3.0379$, $P > 0.05$; for *AtRD29*, $F = 8.0594$, $P < 0.05$). Significant differences are indicated using different upper-case letters.

Similar to *AtNCED3* and ABA, we did not observe significant difference in the accumulation of any of the three transcripts in well-watered or drought conditions for plants expressing 6K2 compared with controls (Fig. 5B, C, and D).

TuMV 6K2 provides drought tolerance to *N. benthamiana.*

A foxtail mosaic virus plasmid expression system (pFoMV) was constructed previously to express *cspB2*, a bacterial cold-shock protein known to increase plant tolerance to drought stress (Castiglioni et al. 2008; Guddimalli et al. 2021; Nemali et al. 2015). To evaluate if 6K2 expression enhances *cspB2* drought tolerance and also can be used in other host plants and stacked with other genes, we modified this pFoMV:cspB2 construct to also express 6K2 (i.e., pFoMV:cspB2:6K2) and assessed survival of plants infected with the constructs following a drought treatment (Fig. 6A). We found that *N. benthamiana* plants infected with pFoMV:cspB2:6K2 had greater survival than plants infected with pFoMV and pFoMV:cspB2 (Fig. 6A). However, there was no difference in survival between plants infected with pFoMV and pFoMV:cspB2 (Fig. 6A). Changes in pot weight and plant phenotype during the drought treatment are shown in Supplementary Figure S2A and B. We also checked the accumulation of ABA, JA, and SA in these plants following 5 days of drought. Consistent with the *Arabidopsis* data, SA accumulation was increased in *N. benthamiana* infected with pFoMV:cspB2:6K2 compared with the pFoMV control after 5 days of drought (Fig. 6D) and there was no significant difference in the ABA and JA level (Fig. 6B and C).

Discussion

In this study, we demonstrate TuMV can positively impact host plants by increasing plant survival under drought conditions (Figs. 1A and 6A). We go further and show at least three TuMV proteins, 6K1, 6K2, and NIa-Pro, are responsible for enhanced drought tolerance in TuMV-infected plants (Fig. 1B). Due to the small size of most viral genomes, many viral proteins have evolved multifunctionality (Callaway et al. 2001; Deng et al. 2015; Valli et al. 2018). The ability of 6K1, 6K2, and NIa-Pro to enhance drought tolerance may be related to the proteins primary function, or, alternatively, it may be an additional ecological function (Ray and Casteel 2022). Recently it was determined that the potyviral NIa-Pro has evolved the ability to inhibit plant defenses only when insect vectors are present (Bak et al. 2017), which should benefit the virus through enhanced transmission (Ray and Casteel 2022). It is tempting to speculate that drought tolerance–inducing viral proteins will benefit the virus as well as the host, as greater host survival under drought conditions would increase the time viruses can replicate and spread in the environment before the host dies.

Virus infection causes molecular, physiological, and biochemical changes in plants that can induce a state of drought tolerance (Carr et al. 2018; González et al. 2021; Hily et al. 2016; Xu et al. 2008). In our study, expression of 6K1 and 6K2 increased shoot dry biomass relative to controls (Fig. 2A); however, there was no impact of 6K2 expression on root length of *A. thaliana* (Fig. 2B). While the soil was carefully washed from roots, loss of some root material was unavoidable. It is possible that small differences in root length or biomass could be detected among treatments if the experiment was repeated using plates and drought mimicking chemicals, such as mannitol. Virus infection can trigger the induction of ABA (Alazem and Lin 2015, 2017; Alazem et al. 2014) and related genes, such as *PP2C*, that regulate extreme resistance in soybean against soybean mosaic virus (Seo et al. 2014). In contrast, we did not observe significant increases in ABA levels in TuMV infected plants (Fig. 3A), potentially due to the expression of the TuMV proteins P1 and helper component-protease (HC-Pro), which negatively impact ABA accumulation in *Arabidopsis* (Chiu et al. 2021). ABA regulates stomatal conductance and expression of various genes, such as *AtRD29*, *AtDREB19*, and *AtRAP2.6L*, which improves plant performance under drought (Iuchi et al. 2001; Jia et al. 2012; Krishnaswamy et al. 2011; Munemasa et al. 2015). To our surprise, 6K2-overexpressing plants failed to increase the expression of *AtNCED3* and ABA content under drought stress as compared with well-watered conditions (Figs. 3B and 5A). Negative impacts on ABA content were also shown for transgenic *Arabidopsis* expressing the TuMV proteins P1/HcPro (Chiu et al. 2021). Although expression of *AtRD29*, *AtRAP2.6L*, and *AtDREB19* increased in control plants under drought, this was only significant for *AtRD29* (Fig. 5B, C, and D) and none of these transcripts increased in *6K2*-overexpressing *A. thaliana* leaves under drought compared with well-watered conditions (Fig. 5B, C, and D). Taken together, these results suggest that TuMV 6K2 transgenic plants are either insensitive to drought or inhibiting drought induction of ABA and that enhanced drought tolerance of 6K2 plants is independent of the ABA pathway.

A similar study has shown that C4 protein of TYLCV provides drought tolerance in *A. thaliana* via an ABA-independent pathway (Corrales-Gutierrez et al. 2020). It has been suggested that the ability of TuMV to enhance drought tolerance in particular *A. thaliana* accessions (Oy-1) involves the downregulation of ABA-independent gene expression pathways (González et al. 2021); however, the same differential gene expression patterns were not observed for other TuMV-infected *A. thaliana* accessions under drought. These results suggest the mechanisms used by TuMV to enhance drought tolerance may be dependent on plant genotype (González et al. 2021). Despite this, 6K2 expression enhanced survival of two different plant species under drought conditions (*A. thaliana* [Fig. 1A] and *N. benthamiana*

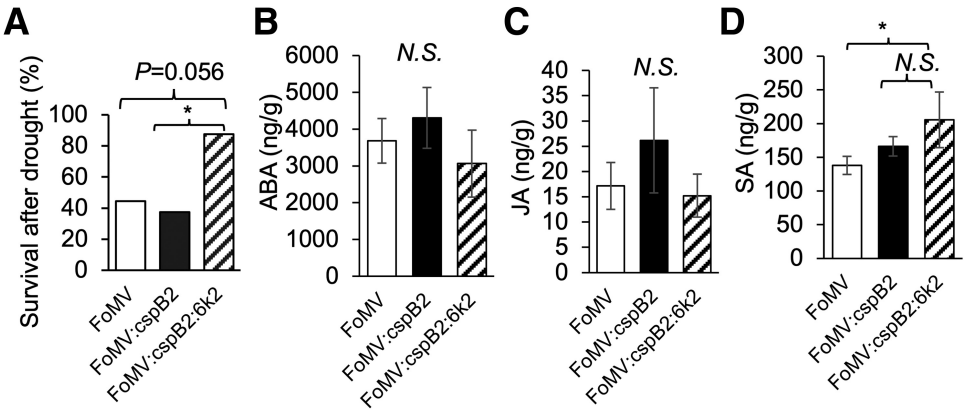

Fig. 6. **A,** *Nicotiana benthamiana* plants were infected with pFoMV, pFoMV:cspB2, or pFoMV:cspB2:6K2 and percent survival was recorded following 7 days of drought stress and re-watering (Chi-square test, one asterisk (*) indicates $P < 0.05$, $n = 8$). **B,** abscisic acid (ABA), **C,** jasmonic acid (JA), and **D,** salicylic acid (SA) levels in plants after 5 days of drought treatment (Mann-Whitney U test, one asterisk (*) indicates $P < 0.05$, $n = 7$ to 8).

[Fig. 6A]), suggesting the mechanisms used by 6K2 to enhance drought tolerance may be somewhat conserved.

Our data shows SA levels are increased in *A. thaliana* plants expressing TuMV 6K2 under drought compared with well-watered conditions (Fig. 4A). Consistently, SA levels are also increased in *N. benthamiana* infected with pFoMV:cspB2:6K2 compared with pFoMV- and pFoMV:cspB2-infected under drought conditions (although not significantly different compared with the latter) (Fig. 6D). Increased SA levels in 6K2-expressing plants may have contributed to improved drought resilience by blocking stomata opening, as was shown previously using SA-accumulating and SA-deficient *Arabidopsis* mutants (Okuma et al. 2014). Elevated SA levels in 6K2-expressing plants may also be contributing to increased drought resilience by blocking ABA induction (Moeder et al. 2010), which is consistent with the lack of ABA induction in 6K2-expressing plants under drought conditions (Fig. 3). Antagonistic relationships between SA and ABA have been reported during several host-microbe interactions (De Torres Zabala et al. 2009; Jiang et al. 2010; Meguro and Sato 2014; Moeder et al. 2010). A variant of the PPV protein P1 has been shown to enhance early symptoms and increase transcript accumulation of the SA-inducible pathogenesis-related (PR) protein. This suggests SA levels may also be increased in plants expressing P1 (Pasin et al. 2014). Despite this, we did not observe increased survival of P1-expressing plants under drought compared with controls (Fig. 1B), potentially due to differences in activation downstream of SA signaling. On the other hand, plants expressing CI, NIb, or CP were all more susceptible to drought stress compared with controls (Fig. 1B); however, SA levels were not measured in these plants. Previously it was shown that expression of the PR1 transcript is reduced in transgenic *Arabidopsis* plants expressing NIb, suggesting SA levels are reduced and potentially mediating increased drought susceptibility (Cheng et al. 2017).

SA is known to provide resistance against virus infection (Prakash et al. 2017; White 1979) by inducing the expression of the antiviral gene *RDR1* (*RNA dependent RNA polymerase 1*) (Xie et al. 2001), which in turn induces various defense related proteins (Prakash et al. 2020). Elevated levels of SA or SA application to plants has also been shown to activate the unfolded protein response (UPR), a signaling pathway that protects cells from ER stress. NPR1 (NONEXPRESSOR OF PR1), a master regulator of the SA signaling pathway, was recently shown to also function in the UPR response. NPR1 translocates to the nucleus to negatively regulate bZIP60 and bZIP28, two transcription factors that are required to activate the UPR (Gayral et al. 2020; Lai et al. 2018). Furthermore, TuMV accumulates to higher levels in leaves of *bZIP28* and *bzip60* mutants compared with controls, suggesting the UPR plays a defensive role against TuMV (Gayral et al. 2020). As 6K2 expression increases ER stress and the expression of *bZIP60* and *bZIP28* (Gayral et al. 2020), increased SA accumulation may be due to NPRI levels increasing in order to regulate the ER stress and UPR pathway.

TuMV has also been reported to have the opposite impact on host plants, decreasing drought tolerance (Manacorda et al. 2021). In this work, water was withheld from *A. thaliana* plants immediately after the plants were infected with TuMV; thus, infection was not established in host plants before drought. In our study, plants were not used in drought experiments until approximately two weeks after infection. Manacorda et al. (2021) found that plants that were infected with TuMV immediately before drought had higher mortality compared with uninfected control plants. The inability of TuMV to increase host drought tolerance at early stages in the infection process may be due to crucial 6K2 and 6K1 roles in viral replication and infection establishment at this time (Cui and Wang 2016; Laliberté and Sanfaçon 2010; Wei and Wang 2008). NIa-Pro is required for the cleavage of the viral polyprotein into individual mature proteins, another essential function in establishing infections (Adams et al. 2005). These primary functions of 6K1, 6K2, and NIa-Pro may have to be prioritized over enhancing drought tolerance to ensure a successful infection, although additional experiments would be needed to test this.

In the field, crops often experience a combination of abiotic stresses that cause reduced yield and enormous economic loss. While our study demonstrates that TuMV and specific TuMV proteins increase plant survival under drought, other aspects of plant-virus interactions and environmental conditions may influence these impacts. For example, when *Arabidopsis* was subjected to drought and heat stress, plants became more susceptible to TuMV infection (Prasch and Sonnewald 2013). Under drought conditions, TuMV accumulation may be limited to a certain threshold to ensure host survival and the combined impact of heat and drought stress may break this threshold with unknown impact on host drought tolerance. Clearly, additional work is still needed, and our study and others help set the stage for advancing our understanding of the plant-microbe interactions in complex environments.

Materials and Methods

Plants and growth conditions.

A. thaliana and *N. benthamiana* plants were grown in Lambert LM-111 All Purpose Mix in nursery flats at 25°C and a 16-h-light and 8-h-dark cycle. The same growth conditions were used in all subsequent experiments. Plants used for all experiments were 4 weeks old, unless otherwise noted. *A. thaliana* plants were used in experiments with TuMV and individual TuMV protiens, and *N. benthamiana* plants were used in experiments with the pFoMV overexpression system (Bouton et al. 2018; Liu et al. 2016; Mei and Whitham 2018).

TuMV infection of *A. thaliana* and *N. benthamiana.*

To determine the impact of TuMV on *A. thaliana* drought tolerance, TuMV-GFP (green fluorescent protein) was propagated from infectious clone p35TuMVGFP (Lellis et al. 2002) and was used to inoculate 4-week-old *A. thaliana* plants, as described by Casteel et al. (2015). Briefly, one leaf from each plant was dusted with Carborundum and, using a cotton stick applicator, rub-inoculated with sap from a TuMV-GFP-infected *N. benthamiana* plant suspended in 20 mM phosphate buffer. A corresponding set of control plants was dusted with Carborundum and mock-inoculated with a cotton stick applicator that was soaked in uninfected sap in 20 mM phosphate buffer. Ten days after inoculation, a Blak Ray B 100AP UV lamp (UV Products) was used to identify fully infected leaves. Six-week-old plants were used in drought survival assays, unless otherwise noted.

Stable expression of TuMV proteins in *A. thaliana.*

To determine the effect of individual TuMV proteins on *A. thaliana* drought tolerance, we transformed *A. thaliana* individually with one of the ten proteins from TuMV. Individual TuMV proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, viral genome-linked protein, NIa-Pro, NIb, and CP) were previously cloned into the pMDC32 expression vector and recombinant *Agrobacterium tumefaciens* GV3101 was generated for each TuMV protein (Casteel et al. 2014; Curtis and Grossniklaus 2003). Wild-type *A. thaliana* were transformed, with the pMDC32 TuMV protein constructs or the pMDC32 empty vector construct, using floral dip transformation (Clough and Bent 1998). Successful transformation was previously confirmed using kanamycin-containing Murashige and Skoog agar plates (Murashige and Skoog 1962) and, then, confirmed by reverse-transcription PCR (RT-PCR).

Ectopic expression of *N. benthamiana* with pFoMV.

To further examine the effect of the TuMV protein 6K2 on plant drought tolerance, we cloned the entire coding sequence of *cspB2*, a gene that encodes a bacterial cold-shock protein, which was previously shown to increase plant tolerance to drought, heat, and salt stress (Castiglioni et al. 2008; Guddimalli et al. 2021; Nemali et al. 2015), and of cspB2:6K2 into a pFoMV-based expression system (Bouton et al. 2018; Liu et al. 2016; Mei and Whitham 2018). Sequences were cloned into the multiple cloning site I of pFoMV using *Xba*I and *Xho*I restriction enzymes. For the cspB2:6K2 construct, a self-cleaving peptide sequence was included to ensure cspB2 and 6K2 are cleaved after translation (Ryan et al. 1991). *Agrobacterium tumefaciens* GV3101 was transformed with pCambiaFoMV, pFoMV:cspB2, or pFoMV:cspB2:6K2 and was grown individually in Luria-Bertani with 50 μM kanamycin and 10 μM rifampicin. The culture was pelleted at 1,500 rpm and re-suspended in 10 mM of morpholineethane sulfonic acid buffer containing 10 mM $MgSO_4$ and 150 μM acetosyringone to a final optical density at 600 nm. The bacterial suspension was kept in the dark for 3 h and was then infiltrated into the underside of the first fully expanded leaf of 4-week-old *N. benthamiana* with a needleless syringe. Infiltrated *N. benthamiana* plants were kept in the dark for 24 h in a growth chamber at 25°C and were then transferred to a growth room with a photoperiod of 16 h of light and 8 h of dark at 25°C. After 1 week, infection was verified by RT-PCR.

Drought-stress treatments and survival assays.

A. thaliana drought treatment assays were performed on six-week-old plants. Plants were subjected either to well-watered or drought treatment. For the well-watered treatment, plants remained in trays saturated with water for 14 days. For the drought treatment, water was withheld from plants for 7 days. Weight of the well-watered as well as drought-treated pots was recorded at days 1, 3, 5, and 7, to control for variation in the soil water content (Supplementary Fig. S1). After the drought treatment, plants were re-watered and survival was assessed 7 days later. The number of plants that recovered was recorded and the percentage of plants surviving was calculated. *Nicotiana benthamiana* drought treatment assays were performed on five-week-old plants. For this experiment, all plants were subjected to a drought treatment. Prior to the start of the drought treatment, potted plants were watered until run-off and were weighed 24 h later to obtain a baseline mass. Plants were then subjected to a 7-day drought period in which soil water content was reduced and was maintained at 40% of the baseline pot mass (Supplementary Fig. S2). To maintain the 40% baseline mass, potted plants were weighed each day during the drought period and water was added if the pots fell below the 40% threshold. At the end of the 7-day drought period, plants were re-watered, and survival was assessed 4 days later. The number of plants that recovered was recorded and the percentage of plants surviving was calculated.

Biomass and root length measurements.

To measure the aboveground dry biomass in experiments done with *A. thaliana*, the entire aboveground portion of the plant was cut, was dried at 37°C for 7 days, and dry weight was determined. To measure root length, roots of each plant were carefully removed from the soil by rinsing in water. Root length was then measured using a ruler.

Hormone content measurement.

To quantify ABA, JA, and SA in *A. thaliana* following drought, leaves were collected on the final day of the drought treatment, were lyophilized, and were weighed. Extractions were performed as described previously (Casteel et al. 2015). For *N. benthamiana*, leaves were collected on the fifth day of drought treatment. Briefly, leaves were ground into a fine powder and 1 ml of extraction buffer (2:1:0.005 of iso-propanol, high-pressure liquid chromatography–grade H_2O, and hydrochloric acid) was added to each tube with 1,000 ng of D_6-ABA and D_4-SA, and 10 ng of D_5-JA as an internal control. Samples were shaken in a Harbil paint shaker for 1 min for homogenization. Samples were then centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was added to a tube containing 1 ml of dichloromethane, was vortexed for 30 min at 750 rpm, and was centrifuged in an Eppendorf 5424 for 3 min at 12,000 rpm at room temperature. The bottom layer of the solution was transferred to a new tube, was dried, and was resuspended in 125 ml of methanol. Samples were injected into a Dionex UHPLC system (Thermo Scientific) and ion masses were detected using an Orbitrap-Q Exactive mass spectrometer (Thermo Scientific). ABA, SA, JA, and the internal standards were identified by the signature ion masses and retention, using the Xcalibur 3.0 program (Thermo Fisher Scientific). Concentrations of ABA, SA, and JA were quantified by comparing the peak area of the endogenous compound with the internal standard and are expressed relative to weight in milligrams.

Total RNA isolation and transcripts abundance analysis.

For quantification of ABA- and drought-related gene transcript abundances in *A. thaliana*, leaf samples were collected on the final day of drought treatment, were flash frozen in liquid nitrogen, and were stored at −80°C until analysis. Each replicate represented a pool of leaf tissue from two individual plants. Total RNA was isolated using a Quick-RNA miniprep kit (ZymoResearch), and 1 μg of RNA was used to generate complementary DNA, using SMART MMLV reverse transcriptase (Takara Bio USA, Inc.). Transcript abundance of an ABA biosynthesis gene, *AtNCED3*, and several drought-inducible genes (*AtRD29A*, *AtDREB19*, and *AtRAP2.6L*) were measured, using real-time quantitative RT-PCR (qRT-PCR). Expression of *AtNCED3* and *AtRD29* was previously shown to be induced by drought stress (Sato et al. 2018; Yamaguchi-Shinozaki and Shinozaki 1993; Yamaguchi-Shinozaki et al. 1992), while overexpression of *AtDREB19* and *AtRAP2.6L* increases the performance of plants during drought (Krishnaswamy et al. 2011). qRT-PCR was conducted using SYBR Green PCR master mix (Applied Biosystems) and a CFX384 Optics Module real-time system (BioRad Laboratories, Inc.). The qRT-PCR program had an initial denaturation for 2 min at 94°C, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Primers were designed using primer3 for *AtNCED3*-AT3G14440.1 (forward: TCCGGTGGTTTACGACAAGA and reverse: TTCCCAAGCGTTCCAGAGAT), *AtRD29A*-AT5G52310.1 (forward: TGAGGAGACGAGAGATGAGAAA and reverse: CCGGAGTAACCTAGCATTGAAG), *AtDREB19*-AT2G38340.1 (forward: CTCCTCTGCTTCTGTTGTATCC and reverse: CAATCCTTCTTCCTCCCATCTC) and *AtRAP2.6L*-AT5G13330.1 (forward: GAAATCCGCGATCCAAAGAAAG and reverse: CAGCTCGGTCATAGGCTAAAG). Ubiquitin was used as internal control (AT4G05320 forward: GGCCTTGTATAATCCCTGATGAATAA and reverse: AAAGAGATAACAGGAACGGAAACATA).

Statistical analysis.

Data transformations were performed to meet the assumptions of each statistical test when necessary. Chi-square analysis was used for survival experiments. One-way analysis of variance (ANOVA) was used to analyze the effect on biomass and root length. Mann-Whitney U tests were used to test for differences in shoot hormone content with and without drought. Two-way ANOVA was performed to evaluate changes in the

relative abundance of ABA- and drought-related gene transcripts (i.e., *AtNCED3*, *AtRAP2.6L*, *AtDREB19*, and *AtRD29*). The R statistical software (R Core Team 2018) and SAS JMP software (version 16.2.0) were used to perform all statistical analyses.

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