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Research

Virus infection improves drought tolerance

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Summary

• Viruses are obligate intracellular symbionts. Plant viruses are often discovered and studied as pathogenic parasites that cause diseases in agricultural plants. However, here it is shown that viruses can extend survival of their hosts under conditions of abiotic stress that could benefit hosts if they subsequently recover and reproduce.

• Various plant species were inoculated with four different RNA viruses, *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* and *Tobacco rattle virus*. The inoculated plants were stressed by withholding water. The onset of drought symptoms in virus-infected plants was compared with that in the plants that were inoculated with buffer (mock-inoculated plants). Metabolite profiling analysis was conducted and compared between mock-inoculated and virus-infected plants before and after being subjected to drought stress.

• In all cases, virus infection delayed the appearance of drought symptoms. Beet plants infected with CMV also exhibited significantly improved tolerance to freezing. Metabolite profiling analysis showed an increase in several osmoprotectants and antioxidants in BMV-infected rice and CMV-infected beet plants before and after drought stress.

• These results indicate that virus infection improves plant tolerance to abiotic stress, which correlates with increased osmoprotectant and antioxidant levels in infected plants.

Key words: beet (*Beta vulgaris*), infectious virus, metabolites, mutualistic symbiosis, rice (*Oryza sativa*), stress tolerance.

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Introduction

Viruses use host resources to support their own reproduction and dissemination, so it is widely believed that virus infections are harmful to the host. However, this paradigm represents an incomplete picture of virus–host relationships. Some fungal, bacterial and animal viruses are beneficial in the survival and reproduction of their hosts. For example, a virus in a mutualistic fungal endophyte is required for the thermal tolerance of the host plants, indicating a three-way mutualistic symbiosis (Márquez *et al.*, 2007). Several bacteriophages are required for the virulence of their host bacteria (Barksdale & Pappenheimer, 1954; Waldor & Mekalanos, 1996; Tinsley *et al.*, 2006). Some ascoviruses of wasps can be mutualistic, depending on the specific virus and wasp strains (Stasiak *et al.*, 2005). The polydnaviruses of parasitoid braconid wasps are required for the wasps to suppress the defense response and survive in their caterpillar hosts (Webb, 1998). Human endogenous retroviruses protect human tissue from infection by the exogenous retrovirus *Spleen necrosis virus*, and may also protect the developing fetus (Ryan, 2004). In many of these examples, the virus–host relationship has evolved to a stage of extreme mutualism where the two entities are no longer truly separate. However, all of these relationships originated with a simpler symbiosis.

Little is known about the biology of plant viruses and their hosts in natural systems. Plants support a large number of positive single-stranded RNA viruses that are less common in many other host kingdoms. The majority of recognized plant viruses are discovered and studied as pathogenic parasites

that cause diseases in cultivated agricultural plants (Zaitlin & Palukaitis, 2000). In natural, nonagricultural settings such as in a tropical forest, RNA viruses are present in *c.* 70% of the investigated nonsymptomatic or symptomatic plants (unpublished results), but their ecological roles remain unknown. Plants in nature are simultaneously exposed to combinations of biotic and abiotic stresses, so biotic and abiotic signaling pathways may share multiple nodes, and their output may have significant overlap for plants to survive under complex environmental conditions (Timmusk & Wagner, 1999; Xiong & Yang, 2003; Chini *et al.*, 2004). Here we report that infection of RNA viruses improves plant tolerance to abiotic stress, indicating that infectious RNA viruses may establish conditional mutualistic relationships with their plant hosts. For these studies, we used *Cucumber mosaic virus* (CMV), a virus with a very broad host range (Palukaitis *et al.*, 1992; Roossinck, 2001), *Tobacco mosaic virus* (TMV) and *Tobacco rattle virus* (TRV) viruses with intermediate host ranges, and *Brome mosaic virus* (BMV), a virus with a very narrow host range (Lane, 1981). All the viruses, generalist or specialist, provided tolerance to drought stress.

Abiotic stress such as drought and frost induces dehydration, resulting in osmotic stress and associated oxidative stress. One ubiquitous protective mechanism against drought and frost in plants is the accumulation of certain organic metabolites, the osmoprotectants and antioxidants (Bohnert *et al.*, 1995; Nuccio *et al.*, 1999; Rontein *et al.*, 2002). Here, the primary metabolic changes in the plants caused by virus infection and drought stress were investigated using metabolic profiling (Broeckling *et al.*, 2006).

Materials and Methods

Viruses and plant materials

The Russian strain of BMV and the U1 and mutant MIC-1,3 strains of TMV were kindly provided by Drs Xinshun Ding and Rick Nelson (Shintaku *et al.*, 1996). A cDNA clone derived from TRV and containing a portion of the GFP gene (TRV-GFP) was provided by Dr Kiran Mysore (Ryu *et al.*, 2004). The Fny strain of CMV was purified by the method previously described (Roossinck & White, 1998). Inoculum consisted of purified virus (CMV) or sap from infected plants (BMV, TMV and TRV-GFP).

Seedlings at the two- to three-leaf stage were used for inoculation of beet (*Beta vulgaris* cv. Detroit Dark Red), pepper (*Capsicum annum* cv. Marango), watermelon (*Cucumis lanatus* cv. Crimson Sweet), cucumber (*Cucumis sativus* cv. National Pickling), tomato (*Solanum lycopersicum* cv. Rutgers), *Solanum habrochaites*, rice (*Oryza sativa* cv. IR-8) and zucchini (*Cucurbita pepo* cv. Elite). One-month-old seedlings were used for inoculation of *Chenopodium amaranthicolor*, *Nicotiana benthamiana* and of tobacco (*N. tabacum* cv. Xanthi nc for CMV inoculation, and cv. Xanthi nn for TMV inoculation). For TMV infections, *N. benthamiana* was inoculated with the MIC-1.3 mutant of U1-TMV because U1-TMV is lethal, and tobacco was inoculated with the U1-strain of TMV.

Beet, pepper, watermelon, cucumber, tomato, zucchini, tobacco, *S. Habrochaites*, *C. amaranthicolor* and *N. benthamiana* were grown in metro-mix 350 soil (Sun Gro Horticulture, Bellevue, WA, USA) with one plant per 4.5-inch (11.4 cm) pot. Rice seedlings were grown in Redi-earth soil (Sun Gro Horticulture) with two seedlings per 4.5-inch pot. For each plant species, eight to fifteen individual plants were inoculated by virus or inoculation buffer (mock). All the plants except rice were inoculated with CMV. Rice plants were inoculated with the sap from BMV infected plants. In addition, the sap from TRV-GFP and TMV infected plants was also used to inoculate *N. benthamiana* and tobacco plants.

Drought treatment

After inoculation, the plants were grown in four separate growth rooms. The temperature ranges of these growth rooms were 20°C (night) to 26°C (day) for TMV and CMV, 19°C (night) to 24°C (day) for BMV, and 18°C (night) to 23°C (day) for TRV-GFP with a 16-h daylength. The average daytime light levels in the growth rooms were 240 μmol m^{-2} s^{-1} for TMV, 300 μmol m^{-2} s^{-1} for CMV, 220 μmol m^{-2} s^{-1} for BMV and 290 μmol m^{-2} s^{-1} for TRV-GFP. For the survey of the responses of virus-infected plants to drought stress, plants grown in individual pots were bottom-watered for 2 d to saturate the soil at 8 d post inoculation (dpi) (watermelon, cucumber, tomato, pepper, *C. amaranthicolor* and *S. habrochaites* plants) or 14 dpi (*N. benthamiana* and tobacco), and then moved to dry flats where water was withheld. The rice plants were moved to dry flats at 28 dpi. The drought-treated plants were photographed with a Coolpix990 digital camera (Nikon, Melville, NY, USA) once every day from the onset of drought symptoms until the mock-inoculated plants showed wilted shoot tips or were entirely collapsed. Then the plants were rewatered for 1–2 wk. This comparative experiment was repeated once for pepper, zucchini, watermelon, tobacco and cucumber, and two or three times for the other plant species tested. The number of days after the water was withdrawn (daww) was recorded for each plant when the plants first showed drought symptoms and when the plants showed wilted shoot tips. For each plant–virus combination, means and 95% confidence limits of daww were calculated with bootstrapped data sets, which used variation present in the data. In this analysis, a normal distribution of daww data was not assumed. To do this, we generated 2000 data sets randomly resampled from the data with replacements: 1000 data sets from mock-inoculated plants and 1000 data sets from virus-inoculated plants. The number of samples from each experimental run was the same as in the original data set to preserve any potential differences among experimental runs. Thus, the bootstrapped data set for each treatment was

1000*N* data points, where *N* is the number of data points in the original data set for each treatment. The mean and 95% confidence limits for daww were then calculated for each of these two large data sets.

It is possible that differing soil water potential or the size of the pots in the original experiments effected the drought stress. Hence, to further test the improved drought tolerance of virus infected plants, four or five beet seedlings were grown in the same container (approx. 81 × 13 cm wallpaper water tray modified with holes at the bottom support; Lindar, Baxter, MN, USA) separated by 11–12 cm. When they reached the two- to three-leaf stage, half of the plants were mock-inoculated and the other half were inoculated with CMV. Plants were watered daily for 10 d, and then water was withheld until the shoot tips of mock-inoculated plants became wilted. The same planting and treatment regimes were done with rice and tobacco plants except that tobacco seedlings were inoculated with TMV and rice with BMV. The inoculated tobacco plants were watered for 7 d before withholding water and rice plants for 2 wk.

Cold treatment

Beet seedlings at the two- to three-leaf stage were mock-inoculated or inoculated with CMV. At 28 dpi the plants were moved into a 15°C growth chamber for 16 h for daytime growth and a −2°C chamber for 8 h in the dark. This treatment was repeated once, and then the plants were moved back to the 15°C chamber for 16 h followed by incubation in a −4°C chamber for 8°h. This analysis was repeated twice.

Identification of systemic infection of CMV

To test if CMV could systemically infect *C. amaranthicolor*, the inoculated leaves (positive control), the stem above the CMV-inoculated leaves and the noninoculated upper leaves of buffer- or CMV-inoculated *C. amaranthicolor* were harvested. The total RNA was extracted as described by Xu *et al*. (2004). Five micrograms of total RNA were used for reverse transcriptase polymerase chain reaction (RT-PCR) amplification of CMV RNA 3 (Xu *et al*., 2004). The amplified fragment was gel extracted and analysed by sequence analysis.

Water content and water loss analysis

All the leaves from individual beet plants inoculated with inoculation buffer or CMV were harvested at 16 dpi, weighed and incubated in a preheated oven at 105°C for 4.5 h. Twelve mock-inoculated and 12 CMV-infected plants were analysed. The leaves from each plant were loaded in an individual beaker and the beakers placed randomly in the oven. The dry leaf tissues were weighed again and the weight loss for each plant, which is equal to water weight, was calculated. The water content was calculated by dividing water weight with the fresh tissue weight for each sample. The averages of the water content between mock-inoculated and virus-infected plants were compared. The data was charted using Microsoft Excel and analysed with a Mann–Whitney *U* test. The same analysis was repeated once.

For water loss analysis, all the leaves from individual plants were detached and laid with adaxial side up in two sets of 150 × 155 mm Petri dish. The leaves were kept in growth chambers with light intensity at 300 μmol m^{-2} s^{-1} and temperature at 28°C. The weight of the leaves was measured at 30, 60, 90, 132, 192, 272, 392, 512 min after detachment. The ratio of water loss for each plant was calculated by dividing the weight loss at each time point with its total water weight. The water loss ratios at each time were averaged among six individual mock or CMV-inoculated plants that were analysed in the same growth chamber. The data were charted and analysed using Microsoft Excel. The analysis was repeated twice with slight differences in the time-points for weight measurement. Ten mock- and 10 CMV-inoculated plants were sampled at each time. The samples from 12 plants (six of each group) were analysed in the same growth chamber and the samples from the remaining eight plants were analysed in another growth chamber with the same settings.

Extraction of metabolites and metabolite profiling analysis

For metabolite analysis, the above-ground tissues were harvested from five to six of each, mock- and BMV-inoculated rice plants at 10 and at 20 dpi. The remaining plants were subjected to water deficit stress, and the tissues were harvested at 4 daww. The tissues were frozen in liquid nitrogen, ground to powder and lyophilized for 48–72 h until dry. The dried tissue (700 mg) was placed in a 4.0 ml glass vial. Two milliliters of chloroform–methanol (2 : 1, v : v) containing 100 μg ml^{-1} docosanol as an internal standard was added to dried tissue. The sample was thoroughly vortexed and extracted overnight at 25°C. Then, 0.75 ml of Milli-Q water (Millipore, Billerica, MA, USA) containing 25 μg ml^{-1} ribitol was added to the sample mixture. The sample was vortexed, and incubated for 4 h at room temperature. The biphasic solvent system was centrifuged at 2900 ***g*** for 30 min at 4°C to separate the layers, 100 μl of methanol–water layer (polar extracts) was collected and transferred to individual 2.0 ml autosampler vials and dried in a vacuum centrifuge at ambient temperature. The dried polar extracts were methoximated and trimethylsilylated and analysed using an Agilent 6890 GC coupled to a 5973 MSD according to the method of Broeckling *et al*. (Broeckling *et al*., 2005). For nonpolar extracts aliquots of the same plant samples were injected at a 1 : 1 split ratio, and the inlet and transfer line was held at 280°C. Separation was achieved with a temperature program of 80°C for 2 min, ramping at 5°C min^{-1} to 315°C and holding for 12 min, using a 60 m DB-5MS column (0.25 mm

internal diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) and a constant flow of 1.0 ml min^{-1}. The metabolite profiling data generated by gas chromatography–mass spectrometry (GC-MS) was extracted by metabolomics ion-based data extraction algorithm (MET-IDEA) and then subjected to statistical analysis by *t*-test using the program JMP5. For anthocyanins, a portion of the methanol–water extracts was diluted with 0.5% HCl methanol and measured with a UV/visible spectrometer at an absorbance of 530 nm (Xie *et al.*, 2006).

Results

Infection with CMV improved drought tolerance of various host plants and enhanced freezing tolerance of beet plants

Cucumber mosaic virus was used to inoculate 10 different plant species. All of the plants were reported systemic hosts of CMV except for *C. amaranthicolor*, which was reported to be a local lesion host only (Francki *et al.*, 1979). However, a small amount of CMV was detected in the stem above the inoculated leaves in *C. amaranthicolor*. At the time for withholding water, infected pepper, tomato, watermelon, cucumber, zucchini, tobacco and *N. benthamiana* plants showed severe systemic disease symptoms, while infected beet, *S. habrochaites* and *C. amaranthicolor* showed mild symptoms or no systemic symptoms. After withholding water, drought symptoms first appeared as drooped, curled, or wilted leaves or dehydrated stems, depending on the plant species (Fig. 1a,c–e). The extended stress of water deficit eventually led to wilted shoot tips or plant collapse. In all plants except tobacco, all of the infected plants exhibited drought symptoms later than the control plants. In CMV-infected plants with or without systemic viral disease symptoms, the appearance of drought symptoms was delayed by 2–5 d when compared with the corresponding mock-inoculated plants, and responses varied among the tested plant species (Table 1). These results indicate that CMV infection can improve drought tolerance in many of the plant hosts of this virus.

Beet plants were used for a more in-depth study. Mock-inoculated beet plants collapsed after 4 daww while the young leaves of CMV-infected plants remained upright and turgid (Fig. 1a). After being subjected to an additional 4 d of water-deficit stress, the plants were rewatered for a week. All the CMV-infected plants recovered and resumed growth while only 30% of mock-inoculated plants grew new shoots. The water content in the leaves of mock- and CMV-inoculated plants growing under normal growth conditions was compared. The average water content was higher in the infected plants, and detached leaves from these plants lost water more slowly (Fig. 2). In addition to drought stress, tolerance to cold stress was compared between mock-inoculated and CMV-infected beet plants. Beet plants at 28°dpi were placed in a low-temperature (15°C) growth chamber for daytime growth and a freezing chamber at night for 3 d (−2°C for the first two nights and −4°C for one night) in order to simulate conditions that might occur in the field very early or late in the growing season. All the mock-inoculated plants died, but CMV-infected plants were tolerant of the frost stress (Fig. 1b).

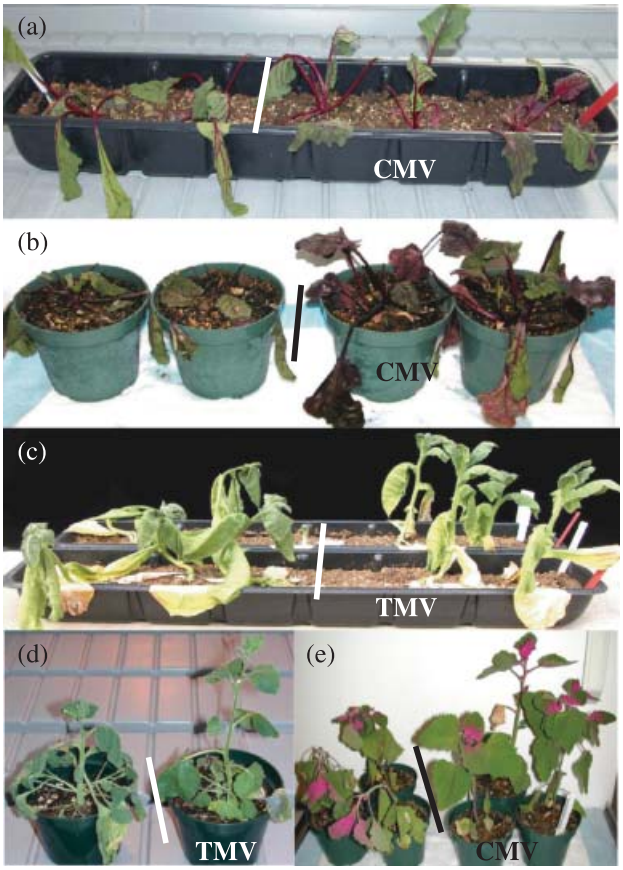

Fig. 1 Comparison of the symptoms in mock-inoculated and virus-infected plants caused by drought or frost stress. (a) Beet (*Beta vulgaris*) plants at 4 d after withholding water (daww). (b) Beet plants placed at 15°C for 16 h in the daytime and −2°C for 8 h at night for 2 d followed by another 15°C for 16 h and −4°C for 8 h. (c) Tobacco (*Nicotiana tabacum*) plants at 12 daww. (d) *Nicotiana benthamiana* plants at 8 daww. (e) *Chenopodium amaranthicolor* plants at 4 daww. Plants on the left side of the black or white lines are mock-inoculated plants and on the right are virus-infected plants.

Tolerance to water deficit stress improved in other plants infected with various viruses

Nicotiana benthamiana, a common host for many viruses, was inoculated with BMV, CMV, TMV or TRV-GFP to compare the effects of different viruses on drought tolerance in the same plant host. The appearance of drought symptoms in virus-infected plants was delayed by 2–5 d compared with mock-inoculated plants (Table 2). For example, at the time when watering was withdrawn, the TMV-infected and corresponding mock-inoculated plants were at a similar height.

Table 1 Time of the onset of drought symptoms after withholding water for mock-inoculated and *Cucumber mosaic virus* (CMV)-infected plants

		Appearance of symptoms (daww[a])		Occurrence of wilted shoot tip (daww[b])	
Plant species	Common name	Mock	CMV	Mock	CMV
Beta vulgaris	Beet	5.2 (5, 6)[c]	7.3 (7, 9)[c]	8.7 (8, 10)[c]	11.9 (11, 13)[c]
Capsicum annum	Pepper	2.2 (2, 3)[c]	5.9 (5, 7)[c]	7.4 (6, 9)[c]	12.4 (11, 14)[c]
Chenopodium amaranthicolor	–	3.2 (3, 4)[c]	6.1 (6, 7)[c]	6.2 (6, 7)[c]	8.1 (8, 9)[c]
Cucumis lanatus	Watermelon	5.2 (5, 6)[c]	9.6 (9, 11)[c]	9.2 (9, 10)[c]	11.8 (11, 13)[c]
Cucumis sativus	Cucumber	3.1 (3, 4)[c]	6.1 (6, 7)[c]	5.2 (5, 6)[c]	9.7 (9, 11)[c]
Cucurbita pepo	Zucchini	3.1 (3, 4)[c]	6.1 (6, 7)[c]	9.5 (9, 11)[c]	12.2 (12, 13)[c]
Solanum lycopersicum	Tomato	1.2 (1, 2)[c]	3 (3, 4)[c]	2.8 (2, 3)[c]	6.2 (5, 8)[c]
Solanum habrochaites	–	7.2 (7, 8)[c]	9.7 (9, 11)[c]	10.6 (10, 12)[d]	13 (12, 14)[d]
Nicotiana benthamiana	–	2.2 (2, 3)[c]	6.1 (6, 7)[c]	15.7 (15, 17)[c]	19.5 (18, 22)[c]
Nicotiana tabacum	Tobacco	2.4 (2, 4)[d]	4.7 (2, 6)[d]	8 (7, 11)[d]	10.1 (8, 11)[d]

[a]Mean days after water withdrawn (daww) when the tested plants first showed symptoms.
[b]Mean of daww when the tested plants had wilted shoot tips.
[c]Lower and upper 95% bootstrap confidence limits of daww. The confidence interval of daww is higher in infected plants than in mock-inoculated plants.
[d]Lower and upper 95% bootstrap confidence limits of daww. The means of daww are higher in infected plants but the 95% confidence intervals of both treatments overlap.

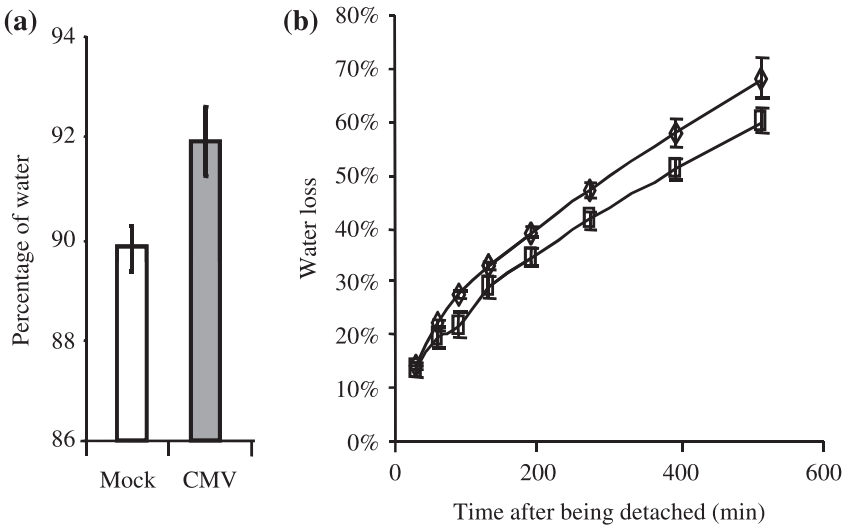

Fig. 2 Comparison of water content and water loss in the leaves of mock-inoculated and *Cucumber mosaic virus* (CMV)-infected beet (*Beta vulgaris*) plants. (a) Water content. Difference was deemed significant by Mann-Whitney U test with P value < 0.5%. (b) Water loss (mock-inoculated, diamonds; CMV-infected, rectangles). Calculations are described in the Materials and Methods section. Error bars, ± SD.

After 8 d without being watered, many leaves of the mock-inoculated plants were wilted while the leaves of infected plants just started to develop drought symptoms. Infected plants were also taller than mock-inoculated plants (Fig. 1d).

Rice seedlings were inoculated with BMV. This virus induces mild disease symptoms in rice, and the infected plants showed the first sign of drought stress (rolled leaves) at average 9.7 (9, 11) daww (mean and 95% confidence limits), when mock-inoculated plants were completely wilted (Fig. 3). The plants were rewatered for 2 wk. All the infected plants recovered while none of the mock-inoculated plants recovered. A similar phenomenon was seen in TMV-infected tobacco plants, which survived with turgid stems and green tips for 12 daww, when mock-inoculated plants had begun to collapse (Fig. 1c). Overall, these data demonstrate that drought tolerance in plants can be enhanced by the infection of several RNA viruses to varying degrees depending on the specific virus–plant combination.

Virus infection dramatically increased the accumulation of anti-oxidants and osmoprotectants in infected plants

The primary metabolic changes in rice and beet plants caused by virus infection and drought stress were investigated using metabolic profiling (Broeckling *et al.*, 2006). The above-ground tissues were harvested from mock- and BMV-inoculated rice

Table 2 Time of the onset of drought symptoms after withholding water for mock- and virus-infected *Nicotiana benthamiana* plants

	BMV (daww)		CMV (daww)		MIC-1,3-TMV (daww)		TRV-GFP (daww)	
	Mock	BMV	Mock	CMV	Mock	TMV	Mock	TRV-GFP
Appearance of drought symptoms	3.2 (3, 4)[a]	6.2 (6, 7)[a]	2.2 (2, 3)[a]	6.1 (6, 7)[a]	4.1 (4, 5)[a]	7.1 (7, 8)[a]	3.1 (3, 4)[a]	7.1 (7, 8)[a]
Occurrence of wilted shoot tip	21.9 (21, 23)[b]	23.5 (22, 25)[b]	15.7 (15, 17)[a]	19.5 (18, 22)[a]	18.9 (18, 20)[a]	21.7 (21, 23)[a]	16.8 (16,19)[b]	20.4 (19, 23)[b]

[a]Lower and upper 95% bootstrap confidence limits of days after water withdrawn (daww). The confidence interval of daww is higher in infected plants than in mock-inoculated plants.
[b]Lower and upper 95% bootstrap confidence limits of daww. The means of daww are higher in infected plants but the 95% confidence intervals of the two treatments overlap.
BMV, *Brome mosaic virus*; CMV, *Cucumber mosaic virus*; TMV, *Tobacco mosaic virus*; TRV-GFP, *Tobacco rattle virus* containing a portion of the GFP gene.

Fig. 3 Comparison of drought tolerance between mock-inoculated and *Brome mosaic virus* (BMV)-infected rice (*Oryza sativa*) plants. (a) Plants before withholding water. (b) Plants at 9 d after water withdrawn (daww). (c) Plants at 14 d after being re-watered. The plants on the left side were mock inoculated and those on the right side were BMV infected.

plants at 10 and 20 dpi, and the same portion of the tissues were harvested at 4 daww, 1 d before the mock-inoculated plants showed any drought symptoms. A GC-MS differentiated 174 metabolites in the polar phase of the extract, and the accumulation levels of 57 metabolites showed significant

Table 3 Number of metabolites showing significant change[a] after virus infection and water deficit stress in the leaves of mock- or virus-inoculated plants

Plant host	Comparison	Metabolites with increased levels	Metabolites with decreased levels	Metabolites with unchanged levels
Rice	Mock vs BMV (20 dpi)	51	6	117
	Mock vs drought	66	21	87
	BMV vs drought	31	12	131
	Mock vs BMV (4 daww)	49	48	77
Beet	Mock vs CMV (15 dpi)	18	3	115
	Mock vs drought	27	11	98
	CMV vs drought	24	9	103
	Mock vs CMV (3 daww)	42	11	83

[a]Changes were deemed significant by a standard *t*-test with $\alpha < 0.05$.
dpi, Days post inoculation; daww, days after water withdrawn; BMV, *Brome mosaic virus*; CMV, *Cucumber mosaic virus*.

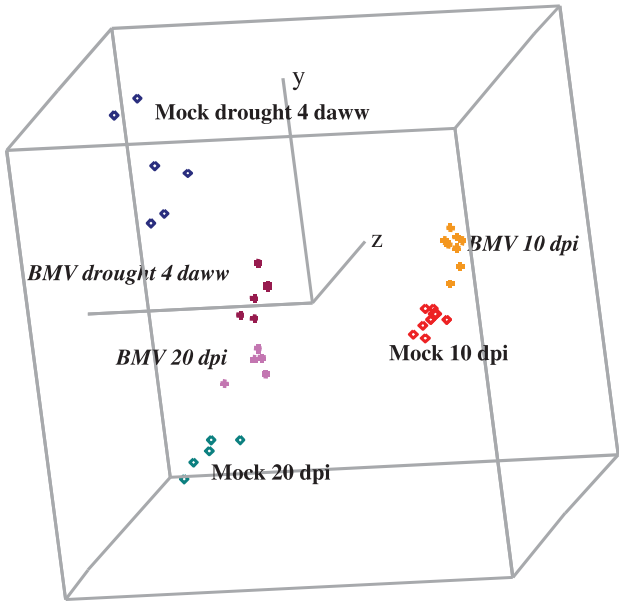

Fig. 4 Result from principal component analysis (PCA) with the metabolic profiling data from the extracts for rice (*Oryza sativa*) plants at polar phase. The samples from mock- or *Brome mosaic virus* (BMV)-infected plants were harvested at 10 d post infection (dpi) and 20 dpi and 4 d after water withdrawn (daww). Points for mock-inoculated plants are red, green and blue, respectively, while the corresponding points for BMV-infected plants are orange, pink and purple.

changes after BMV infection at 20 dpi (Table 3). Drought stress resulted in the altered accumulation of 87 metabolites in mock-inoculated plants and only 43 in BMV-infected plants (Table 3), which could indicate less sensitivity to drought in virus-infected plants. Principle component analysis showed that a larger difference in the metabolite composition occurred in mock-inoculated plants under water deficit stress, as compared to BMV-infected plants (Fig. 4). A similar assay was done with CMV-infected beet plants with tissues harvested at 12 and 15 dpi, and at 3 daww, 1 d before the mock-inoculated plants showed drought symptoms. A GC-MS analysis differentiated 136 metabolites in the polar phase. Similar to what occurred in BMV-infected rice plants, the number of metabolites changed significantly by drought stress was less in virus-infected plants than in mock-inoculated plants (Table 3).

Several metabolites with the known potential of improving plant stress tolerance were compared in detail after virus infection and drought treatment, including salicylic acid (SA) (Senaratna *et al.*, 2003; Chini *et al.*, 2004), proline (Kishor *et al.*, 1995), putrescine (Capell *et al.*, 2004), trehalose and other nonreducing sugars (Bohnert *et al.*, 1995; Garg *et al.*, 2002), tocopherols (Munné-Bosch, 2005), ascorbic acid (Foyer & Noctor, 2005) and anthocyanin (Gould, 2004). Both CMV and BMV infection increased the accumulation of trehalose, putrescine and SA, and their levels remained high under drought conditions (Fig. 5a). Infection with BMV also induced an increase in proline and α- and γ-tocopherol. The level of proline dropped under drought stress while α- and γ-tocopherols remained higher in drought-stressed infected plants than in mock-inoculated plants (Fig. 5b). In addition, drought stress enhanced the accumulation of ascorbic acid in BMV-infected plants but reduced its level in mock-inoculated plants (Fig. 5b). In CMV-infected beet plants, several sugars such as melezitose, maltose and galactose were increased (Fig. 5c). These sugars accumulated to levels 100–300% higher than mock-inoculated plants under water-deficit conditions. Infection with CMV also enhanced the accumulation of anthocyanins, and drought stress further increased the level to 300% above that in mock-inoculated plants. Thus, the increased accumulation of osmoprotectants in virus-infected plants, including trehalose, other sugars, putrescine, proline and increased antioxidants such as anthocyanins, tocopherols and ascorbic acid were associated with improved drought tolerance.

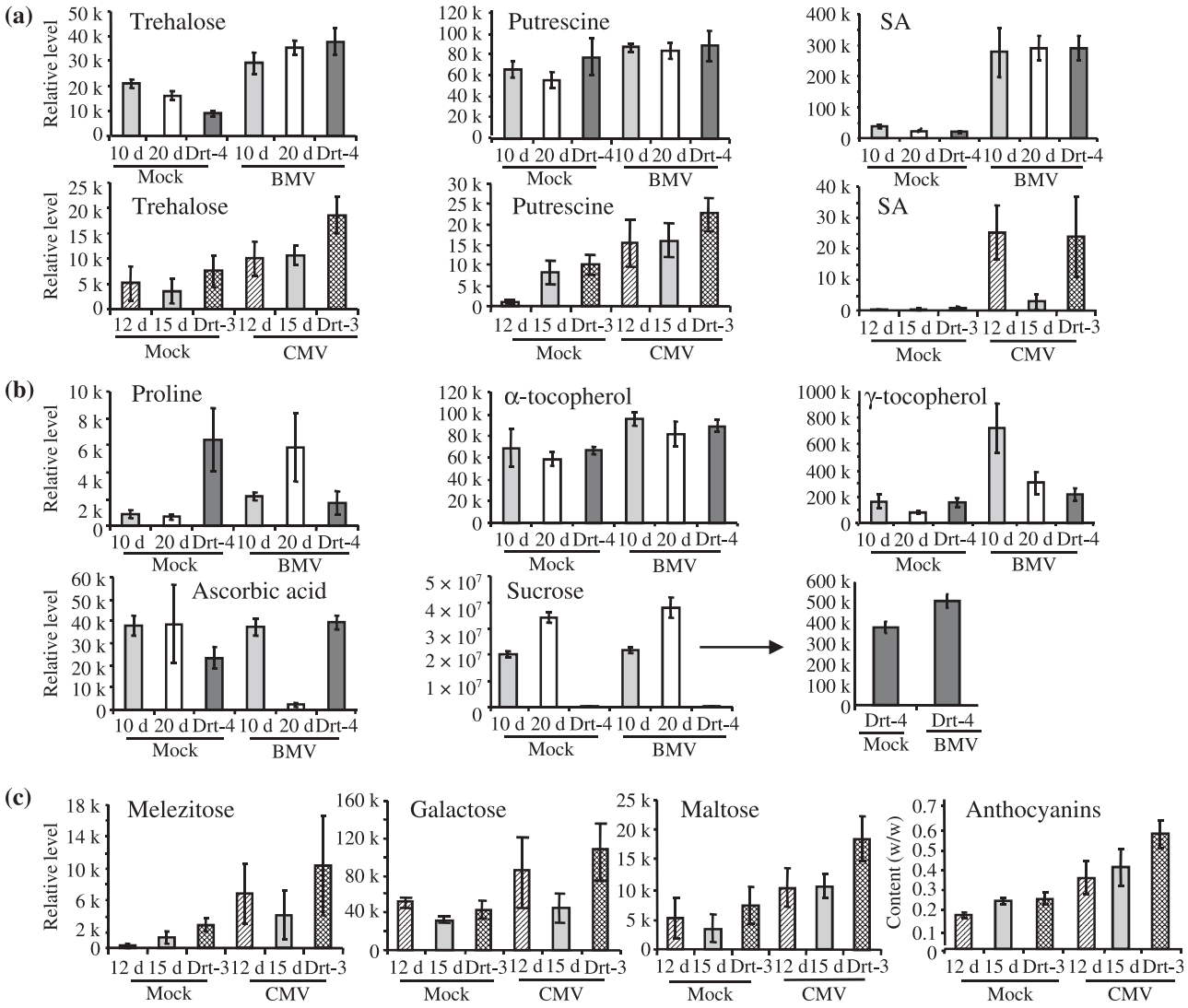

Fig. 5 Change of several metabolites with potential contribution to improved drought tolerance in virus-infected plants under water deficit conditions. (a) Metabolites that were increased in both *Brome mosaic virus* (BMV)-infected rice (*Oryza sativa*) and *Cucumber mosaic virus* (CMV)-infected beet (*Beta vulgaris*) plants under drought conditions. (b) Metabolites that were increased or stayed at high concentrations in BMV infected rice plants. (c) Metabolites that were increased in CMV infected beet plants. d, days post inoculation; Drt-3, samples harvested at 3 d after water withdrawn (daww); Drt-4, samples harvested at 4 daww; SA, salicylic acid.

Discussion

Viruses are parasitic symbionts that use host resources and systems for their own reproduction, so are normally considered to be harmful to the hosts. However, here we found that infection of pathogenic viruses improved the survival of plant hosts under some extreme conditions, indicating a potential mutualistic relationship between viruses and their hosts. Conditional mutualistic symbioses have been reported in other symbiotic systems (Bronstein, 1994; Redman *et al.*, 2001; Clay & Shardl, 2002; Schardl *et al.*, 2004). Some pathogenic fungi such as *Colletotrichum* spp. are able to express a mutualistic lifestyle based on the host genotype (Redman *et al.*, 2001). Endophyte-infected tall fescue exhibits improved recovery after drought (Schardl *et al.*, 2004). The DpAV4 ascovirus is a mutualist in certain *Diadromus* wasps but is pathogenic when infecting other species of this genus (Stasiak *et al.*, 2005). Mycorrhizal associations are generally considered mutualistic fungus–plant relationship, but they can be antagonistic depending on environmental conditions and plant physiology (Johnson *et al.*, 1997). Thus, symbiotic interactions are dynamic particularly under complicated natural settings. Symbiosis often involves exchange of either benefits or costs among the partners. In mutualistic interactions, benefits outweigh costs for both partners. In parasitism, there are more costs to the host. However, the relative benefits and costs

depend on host species and environmental and ecological contexts. At the evolutionary level, true mutualism is a reciprocal increase in fitness for both partners. A virus always benefits if it can prevent the death of its host. While we did not measure reproductive fitness of the plant host *pe se*, the death of mock-inoculated plants versus survival of infected plants under extreme drought stress represents a conditional difference in fitness provided that the surviving virus-infected plants can subsequently produce offspring. Virus-infected beet and rice plants recovered after being rewatered following drought stress, demonstrating this possibility.

Plants in nature and crop fields are simultaneously exposed to numerous environmental stresses. Although in-depth stress research has often focused on plant responses to a single environmental stress, cross-kingdom resistance to biotic attack and cross tolerance to multiple abiotic stresses have been described. However, the responses of plants to simultaneous abiotic and biotic stresses are complicated (Garrett *et al.*, 2006). In some cases, abiotic and biotic stress are synergistic. For example, drought causes more severe charcoal rot symptoms in common bean and sorghum plants infected with the fungal pathogen *Macrophomina phaseolina*, and increased drought stress effects also occur in the infected plants (Diourte *et al.*, 1995; Mayek-Pérez *et al.*, 2002). Similar phenomena were reported in grape plants infected by the bacterial pathogen *Xyllela fastidiosa* (McElrone *et al.*, 2001). By contrast, inoculation of rhizobacteria, which is a mild biotic stress for plant hosts, enhances drought tolerance and confers partial resistance to the pathogenic bacteria *Erwinia carotovora* (Timmusk & Wagner, 1999). Results from some field studies also suggest complicated consequences of viral infection and drought stress in crop loss, with varying additive effects, but other environmental variables in the field may contribute to this complication (Olson *et al.*, 1990; McLaughlin & Windham, 1996; Clover *et al.*, 1999). Results from the present study show that plants infected with several RNA viruses exhibited better tolerance and survival to drought or cold stress. These interactions between plants and microbes may be part of the elaborate mechanisms plants use to survive various environmental changes.

Virus infection sometimes reduces the size of plant hosts (Hull, 2002). This in itself could reduce water requirements and hence improve plant survival during extreme drought stress. Virus infection can also cause comprehensive physiological changes in some plant hosts, such as altered water content of tissues and the synthesis and translocation of metabolites (Hull, 2002). Here, CMV infection increased the water content of the tissues above the ground. The detached leaves from infected plants lost water more slowly than those from mock-inoculated plants, indicating better water retention in infected plants. Water retention can be correlated with a reduction of stomatal opening and lowered transpiration rate in virus infected plants (Hall & Loomis, 1972; Lindsey & Gudauskas, 1975; Keller *et al.*, 1989). It is quite common for levels of glucose, fructose and sucrose to increase in virus-infected plants (Hull, 2002). More complete investigation through metabolite profiling analysis in the present study showed both similar changes of primary metabolites caused by the infection of CMV and BMV, and changes specific to the individual viruses. Salicylic acid, a defense mediator, and some osmoprotectants and antioxidants accumulated to high concentrations in virus-infected plants before and after drought. Salicylic acid increases plant tolerance to abiotic stress (Singh & Usha, 2003). Metabolic acclimation via the accumulation of protective metabolites is regarded as a basic strategy for protection and survival of plants in extreme environments (Bohnert *et al.*, 1995). These metabolites may mediate osmotic adjustment, stabilize membranes, and protect proteins and metabolic machinery against oxidative damage caused by drought or frost stress (Bohnert *et al.*, 1995; Hare *et al.*, 1998). The increase in these protective metabolites in virus-infected plants reflects a stressed physiological status, and may make the plants more acclimated to further stress. Under water stress, some metabolites were maintained at high levels or showed increased accumulation, which further correlates with improved stress tolerance in the infected plants.

Adaptation to abiotic stress is often regulated by the combined activity of interconnected ABA-dependent and ABA-independent signaling pathways. TMV infection dramatically increases ABA concentration in tobacco plants (Whenham *et al.*, 1986), but it is unclear if this is a general response to viral infection. Transcriptome changes induced by different viruses demonstrate a common induction of stress or defense responses in various host plants including the expression of some genes involved in osmotic stress response and regulation (Irian *et al.*, 2007). The pathways involved in stress sensing, defense, and acclimatization are usually complex. There are some overlapping networks that control abiotic stress tolerance and biotic disease susceptibility or resistance (Timmusk & Wagner, 1999; Xiong & Yang, 2003; Chini *et al.*, 2004). Further understanding of the underlying mechanisms of virus infection and improved tolerance to abiotic stress provides insight into the important role of viruses in the evolution and ecology of their hosts. In the field, a delay of drought-stress symptoms of even a few days can be very significant. Thus, understanding of the associated mechanisms also provides potential for agricultural applications, which are of prime importance during climate change, as drought becomes one of the most limiting factors to crop production worldwide (Wollenweber *et al.*, 2005).

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References

Barksdale WL, Pappenheimer AM Jr. 1954. Phage–host relationships in nontoxigenic and toxigenic diphtheria bacilli. *Journal of Bacteriology* **67**: 220–232.

Bohnert HJ, Nelson DE, Jensen RG. 1995. Adaptations to environmental stresses. *The Plant Cell* **7**: 1099–1111.

Broeckling CD, Huhman DV, Farag MA, Smith JT, May GD, Mendes P, Dixon RA, Sumner LW. 2005. Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. *Journal of Experimental Botany* **56**: 323–326.

Broeckling CD, Reddy IR, Duran AL, Zhao X, Sumner LW. 2006. MET-IDEA: data extraction tool for mass spectrometry-based metabolomics. *Analytical Chemistry* **78**: 4334–4341.

Bronstein JL. 1994. Conditional outcomes in mutualistic interactions. *TREE* **9**: 214–217.

Capell T, Bassie L, Christou P. 2004. Modulation of the polyamine biosynthetic pathway in transgenic rice confers tolerance to drought stress. *Proceedings of the National Academy of Sciences, USA* **101**: 9909–9914.

Chini A, Grant JJ, Seki M, Shinozaki K, Loake GJ. 2004. Drought tolerance established by enhanced expression of the *CC-NBS-LRR* gene, *ADR1*, requires salicylic acid, EDS1 and ABI1. *Plant Journal* **38**: 810–822.

Clay K, Shardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *The American Naturalist* **160**: S99–S127.

Clover GRG, Smith HG, Azam-Ali SN, Jaggard KW. 1999. The effects of drought on sugar beet growth in isolation and in combination with beet yellows virus infection. *Journal of Agricultural Science Cambridge* **133**: 251–261.

Diourte M, Starr JL, Jeger MJ, Stack JP, Rosenow DT. 1995. Charcoal rot (*Macrophomina phaseolina*) resistance and the effects of water stress on disease development in sorghum. *Plant Pathology* **44**: 196–202.

Foyer CH, Noctor G. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* **17**: 1866–1875.

Francki RIB, Mossop DW, Hatta T. 1979. Cucumber mosaic virus. *CMI/AAB Description of Plant Viruses*, Set 13, Article 213.

Garg AK, Kim J-K, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ. 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proceedings of the National Academy of Sciences, USA* **99**: 15898–15903.

Garrett KA, Dendy SP, Frank EE, Rouse MN, Travers SE. 2006. Climate change effects of plant disease: genomes to ecosystems. *Annual Review of Phytopathology* **44**: 489–509.

Gould KS. 2004. Nature's Swiss army knife: the diverse protective roles of anthocyanins in leaves. *Journal of Biomedicine & Biotechnology* **5**: 314–320.

Hall AE, Loomis RS. 1972. An explanation for the difference in photosynthetic capabilities of healthy and beet yellows virus-infected sugar beets (*Beta vulgaris* L.). *Plant Physiology* **50**: 576–580.

Hare PD, Cress WA, VanStaden J. 1998. Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell & Environment* **21**: 535–553.

Hull R. 2002. *Matthews' plant virology*. San Diego, CA, USA: Academic Press.

Irian S, Xu P, Dai X, Zhao PX, Roossinck MJ. 2007. Regulation of a virus-induced lethal disease in tomato revealed by LongSAGE analysis. *Molecular Plant–Microbe Interactions* **20**: 1477–1488.

Johnson NC, Graham JG, Smith FA. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* **135**: 575–585.

Keller P, Lüttge U, Wang X-C, Büttner G. 1989. Influence of rhizomania disease on gas exchange and water relations of a susceptible and a tolerant sugar beet variety. *Physiological and Molecular Plant Pathology* **34**: 379–392.

Kishor PBK, Hong Z, Miao G-H, Hu C-A, Verma DPS. 1995. Overexpression of Δ^1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* **108**: 1387–1394.

Lane LC. 1981. Bromoviruses. In: Kurstack E, ed. *Handbook of plant virus infections and comparative diagnosis*, North Holland: Elsevier Biomedical Press, 333–376.

Lindsey DW, Gudauskas RT. 1975. Effects of maize dwarf virus on water relations of corn. *Phytopathology* **65**: 434–440.

Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ. 2007. A virus in a fungus in a plant – three way symbiosis required for thermal tolerance. *Science* **315**: 513–515.

Mayek-Pérez N, García-Espinosa R, López-Casteñeda C, Acosta-Gallegos JA, Simpson J. 2002. Water relations, histopathology and growth of common bean (*Phaseolus vulgaris* L.) during pathogenesis of *Macrophomina phaseolina* under drought stress. *Physiological and Molecular Plant Pathology* **60**: 185–195.

McElrone AJ, Sherald JL, Forseth IN. 2001. Effects of water stress on symptomatology and growth of *Parthenocissus quinquefolia* infected by *Xylella fastidiosa*. *Plant Disease* **85**: 1160–1164.

McLaughlin MR, Windham GL. 1996. Effects of peanut stunt virus, *Meloidogyne incognita*, and drought on growth and persistence of white clover. *Phytopathology* **86**: 1105–1111.

Munné-Bosch S. 2005. The role of α-tocopherol in plant stress tolerance. *Journal of Plant Physiology* **162**: 743–748.

Nuccio ML, Rhodes D, McNeil SD, Hanson AD. 1999. Metobolic engineering of plants for osmotic stress resistance. *Current Opinion in Plant Biology* **2**: 128–134.

Olson AJ, Pataky JK, D'Arcy CJ, Ford RE. 1990. Effects of drought stress and infection by maize dwarf mosaic virus on sweet corn. *Plant Disease* **74**: 147–151.

Palukaitis P, Roossinck MJ, Dietzgen RG, Francki RIB. 1992. Cucumber mosaic virus. In: Maramorosch K, Murphy FA, Shatkin AJ, eds. *Advances in virus research*. San Diego, CA, USA: Academic Press, 281–348.

Redman RS, Dunigan DD, Rodriguez RJ. 2001. Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytologist* **151**: 705–716.

Rontein D, Basset G, Hanson AD. 2002. Metabolic engineering of osmoprotectant accumulation in plants. *Metabolic Engineering* **4**: 49–56.

Roossinck MJ. 2001. *Cucumber mosaic virus*, a model for RNA virus evolution. *Molecular Plant Pathology* **2**: 59–63.

Roossinck MJ, White PS. 1998. Cucumovirus isolation and RNA extraction. In: Foster GD, Taylor SC, eds. *Plant virology protocols*. Totowa, NJ, USA: Humana Press, 189–196.

Ryan FP. 2004. Human endogenous retroviruses in health and disease: a symbiotic perspective. *Journal of the Royal Society of Medicine* **97**: 560–565.

Ryu C-M, Anand A, Kang L, Mysore KS. 2004. Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse solanaceous species. *Plant Journal* **40**: 322–331.

Schardl CL, Leuchtmann A, Spiering MJ. 2004. Symbioses of grasses with seedborne fungal endophytes. *Annual Review of Plant Biology* **55**: 315–340.

Senaratna T, Merritt D, Dixon K, Bunn E, Touchell D, Sivasithamparam K. 2003. Benzoic acid may act as the functional group in salicylic acid and derivatives in the induction of multiple stress tolerance in plants. *Plant Growth Regulation* **39**: 77–81.

Shintaku MH, Carter SA, Bao Y, Nelson RS. 1996. Mapping nucleotides in the 126-kDa protein gene that controls the differential symptoms induced by two strains of tobacco mosiac virus. *Virology* **221**: 218–225.

Singh B, Usha K. 2003. Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. *Plant Growth Regulation* **39**: 137–141.

Stasiak K, Renault S, Federici BA, Bigot Y. 2005. Characteristics of pathogenic and mutualistic relationships of ascoviruses in field populations of parasitoid wasps. *Journal of Insect Physiology* **51**: 103–115.

Timmusk S, Wagner EGH. 1999. The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. *Molecular Plant–Microbe Interactions* **12**: 951–959.

Tinsley CR, Bille E, Nassif X. 2006. Bacteriophages and pathogenicity: more than just providing a toxin? *Microbes and Infection* **8**: 1365–1371.

Waldor MK, Mekalanos JJ. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science* **272**: 1910–1914.

Webb BA. 1998. Polydnavirus biology, genome structure, and evolution. In: Miller LK, Ball LA, eds. *The insect viruses*. New York, NY, USA: Plenum Publishing Corporation, 105–139.

Whenham RJ, Fraser RSS, Brown LP, Payne JA. 1986. Tobacco-mosaic-virus-induced increase in absicisic acid concentration in tobacco leaves: intracellular location in light and dark-green areas, and relationship to symptom development. *Planta* **168**: 592–598.

Wollenweber B, Porter JR, Lubberstedt T. 2005. Need for multidisiplinary research towards a second green revolution. *Current Opinion in Plant Biology* **8**: 337–341.

Xie D-Y, Sharma SB, Wright E, Wang Z-Y, Dixon RA. 2006. Metabolic engineering of proanthocyanidins through co-expression of anthocyanidin reductase and the PAP1 MYB transcription factor. *Plant Journal* **45**: 895–907.

Xiong L, Yang Y. 2003. Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. *The Plant Cell* **15**: 745–759.

Xu P, Rogers SJ, Roossinck MJ. 2004. Expression of antiapoptotic genes *bcl-xL* and *ced-9* in tomato enhances tolerance to virus-induced necrosis and abiotic stress. *Proceedings of the National Academy of Sciences, USA* **101**: 15805–15810.

Zaitlin M, Palukaitis P. 2000. Advances in understanding plant viruses and virus diseases. In: Webster RK, Shaner G, VanAlfen NK, eds. *Annual Review of Phytopathology*. Palo Alto, CA, USA: Annual Reviews, 117–143.

