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A Virus in a Fungus in a Plant: Three-Way Symbiosis Required for Thermal Tolerance

Luis M. Márquez,[1] Regina S. Redman,[2,3] Russell J. Rodriguez,[2,4] Marilyn J. Roossinck[1]*

A mutualistic association between a fungal endophyte and a tropical panic grass allows both organisms to grow at high soil temperatures. We characterized a virus from this fungus that is involved in the mutualistic interaction. Fungal isolates cured of the virus are unable to confer heat tolerance, but heat tolerance is restored after the virus is reintroduced. The virus-infected fungus confers heat tolerance not only to its native monocot host but also to a eudicot host, which suggests that the underlying mechanism involves pathways conserved between these two groups of plants.

Endophytic fungi commonly grow within plant tissues and can be mutualistic in some cases, as they allow plant adaptation to extreme environments (*1*). A plant-fungal symbiosis between a tropical panic grass from geothermal soils, *Dichanthelium lanuginosum*, and the fungus *Curvularia protuberata* allows both organisms to grow at high soil temperatures in Yellowstone National Park (YNP) (*2*). Field and laboratory experiments have shown that when root zones are heated up to 65°C, nonsymbiotic plants either become shriveled and chlorotic or simply die, whereas symbiotic plants tolerate and survive the heat regime. When grown separately, neither the fungus nor the plant alone is able to grow at temperatures above 38°C, but symbiotically, they are able to tolerate elevated temperatures. In the absence of heat stress, symbiotic plants have enhanced growth rate compared with nonsymbiotic plants and also show significant drought tolerance (*3*).

Fungal viruses or mycoviruses can modulate plant-fungal symbioses. The best known example of this is the hypovirus that attenuates the virulence (hypovirulence) of the chestnut blight fungus, *Cryphonectria parasitica* (*4*). Virus regulation of hypovirulence has been demonstrated experimentally in several other pathogenic fungi (*5*–*8*). However, the effect of mycoviruses on mutualistic fungal endophytes is unknown. There is only one report of a mycovirus from the well-known mutualistic endophyte, *Epichloë festucae*, but no phenotype has been associated with this virus (*9*).

Fungal virus genomes are commonly composed of double-stranded RNA (dsRNA) (*10*). Large molecules of dsRNA do not normally occur in fungal cells and, therefore, their presence is a sign of a viral infection (*9*). Using a protocol for nucleic acid extraction with enrichment for dsRNA (*11*), we detected the presence of a virus in *C. protuberata*. The dsRNA banding pattern consists of two segments of about 2.2 and 1.8 kb. A smaller segment, less than 1 kb in length, was variable in presence and size in the isolates analyzed and, later, was confirmed to be a subgenomic element, most likely a defective RNA (fig. S1 and Fig. 1, A and B). Using tagged random hexamer primers, we transcribed the virus with reverse transcriptase (RT), followed by amplification and cloning. Sequence analysis revealed that each of the two RNA segments contains two open reading frames (ORFs) (fig. S2). The 2.2-kb fragment (RNA 1) is involved in virus replication, as both of its ORFs are similar to viral replicases. The first, ORF1a, has 29% amino acid sequence identity with a putative RNA-dependent RNA polymerase (RdRp) from the rabbit hemorrhagic disease virus. The amino acid sequence of the second, ORF1b, has 33% identity with the RdRp of a virus of the fungal pathogen *Discula destructiva*. These two ORFs overlap and could be expressed as a single protein by frameshifting, a common expression strategy of viral replicases. The two ORFs of RNA 2 have no similarity to any protein with known function. As in most dsRNA mycoviruses, the 5′ ends (21 bp) of both RNAs are conserved. Virus particles purified from *C. protuberata* are similar to those of other fungal viruses: spherical and ~27 nm in diameter (fig. S3). This virus is transmitted vertically in the conidiospores. We propose naming this virus *Curvularia* thermal tolerance virus (CThTV) to reflect its host of origin and its phenotype.

The ability of the fungus to confer heat tolerance to its host plant is related to the presence of CThTV. Wild-type isolates of *C. protuberata* contained the virus in high titers, as evidenced by their high concentration of dsRNA (~2 μg/g of lyophilized mycelium). However, an isolate obtained from sectoring (change in morphology) of a wild-type colony contained a very low titer of the virus, as indicated by a low concentration of dsRNA (~0.02 μg/g of lyophilized mycelium). These two isolates were identical by simple sequence repeat (SSR) analysis with two single-primer polymerase chain reaction (PCR) reactions and by sequence analysis of the rDNA ITS1-5.8*S*-ITS2 region (figs. S4 and S5). Desiccation and freezing-thawing cycles are known to disrupt virus particles (*12*); thus, mycelium of the isolate obtained by sectoring was

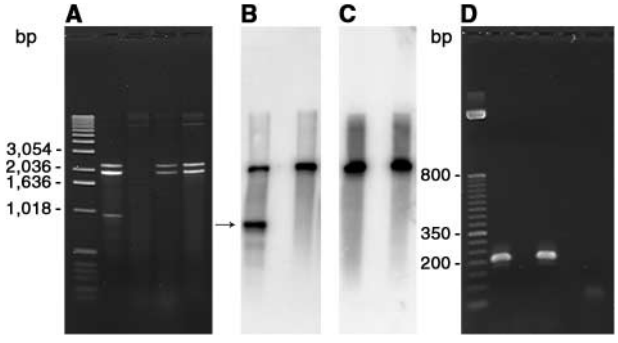

Fig. 1. Presence or absence of CThTV in different strains of *C. protuberata*, detected by ethidium bromide staining (**A**), Northern blot using RNA 1 (**B**) and RNA 2 (**C**) transcripts of the virus as probes, and RT-PCR using primers specific for a section of the RNA 2 (**D**). The isolate of the fungus obtained by sectoring was made virus-free (VF) by freezing-thawing. The virus was reintroduced into the virus-free isolate through hyphal anastomosis (An) with the wild type (Wt). The wild-type isolate of the fungus sometimes contains a subgenomic fragment of the virus that hybridizes to the RNA 1 probe (arrow).

[1]Plant Biology Division, Samuel Roberts Noble Foundation, Post Office Box 2180, Ardmore, OK 73402, USA. [2]Department of Botany, University of Washington, Seattle, WA 98195, USA. [3]Department of Microbiology, Montana State University, Bozeman, MT 59717, USA. [4]U.S. Geological Survey, Seattle, WA 98115, USA.

*To whom correspondence should be addressed. E-mail: mroossinck@noble.org

lyophilized, frozen at –80°C, and subcultured to cure it completely of the virus. The complete absence of CThTV in this isolate was confirmed by dsRNA extraction, Northern blotting, RT-PCR (Fig. 1), and electron microscopy (no particles were observed in four grids). We assessed experimentally the ability of the wild-type and virus-free isolates to confer heat tolerance by using thermal soil simulators (*2*, *11*). Plants inoculated with the virus-infected wild-type isolate of the fungus tolerated intermittent soil temperatures as high as 65°C for 2 weeks (10 hours of heat per day), whereas both nonsymbiotic plants and plants inoculated with the virus-free isolate of the fungus became shriveled and chlorotic and died (Fig. 2).

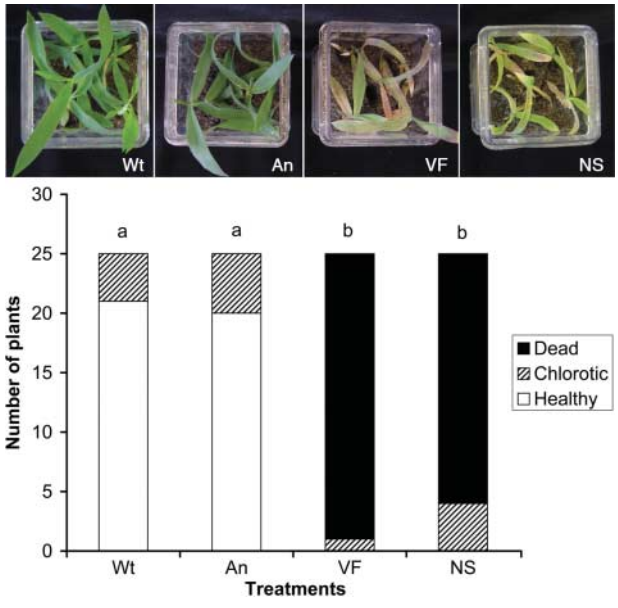

Fig. 2. (**Top**) Representative *D. lanuginosum* plants after the heat-stress experiment with thermal soil simulators. Rhizosphere temperature was maintained at 65°C for 10 hours and 37°C for 14 hours/day for 14 days under greenhouse conditions. Plants were nonsymbiotic (NS) and symbiotic with the wild-type virus-infected isolate of *C. protuberata* (Wt), the hygromycin-resistant isolate newly infected with the virus through hyphal anastomosis (An), or the virus-free hygromycin-resistant isolate (VF). (**Bottom**) The histogram presents the number of plants chlorotic, dead, and alive at the end of the experiment. The small letters on top of the bars indicate statistical differences or similarities (chi-square test, $P < 0.01$).

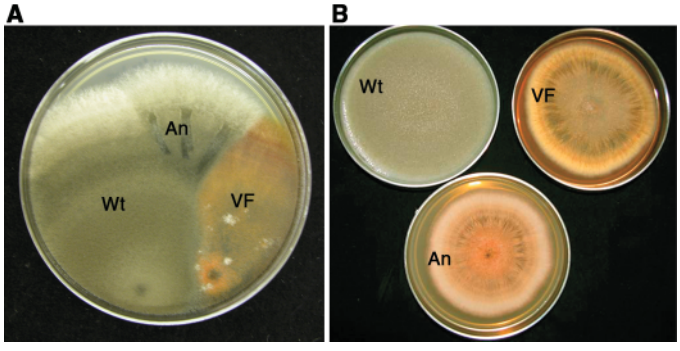

Fig. 3. (**A**) Anastomosis of the wild-type virus-infected isolate of *C. protuberata* (Wt) and the virus-free hygromycin-resistant isolate (VF) to produce a virus-infected hygromycin-resistant isolate (An). (**B**) After single-spore isolation to produce pure cultures, the isolate newly infected with the virus (An) retained the hygromycin-resistance and the morphology of the VF isolate.

To confirm that CThTV was involved in heat tolerance in the plant-fungal symbiosis, we reintroduced the virus into the virus-free fungal isolate and tested its ability to confer heat tolerance. To provide a selectable marker, the virus-free isolate was transformed with a pCT74 vector containing a hygromycin-resistance gene (*13*) by restriction enzyme–mediated integration (REMI) transformation (*14*). Virus-containing wild-type hygromycin-sensitive (Wt) and virus-free hygromycin-resistant (VF) isolates of *C. protuberata* were cultured on single Petri dishes and allowed to undergo hyphal fusion or anastomosis (Fig. 3A). The mycelium from the area of anastomosis was subcultured twice with single conidiospores grown on hygromycin-containing plates. Thirty-five hygromycin-resistant isolates obtained in this way were screened for their dsRNA profiles, but only one was found to have acquired the virus (Figs. 1 and 3B). This fungal isolate, newly infected by hyphal anastomosis with CThTV (An), was tested for its ability to confer heat tolerance by the same experimental approach indicated above. The heat-stress experiment confirmed that the isolate newly infected with CThTV confers the same level of heat tolerance as that conferred by the wild-type isolate (Fig. 2).

Previously, we found that some beneficial endophytes isolated from monocots could be transferred to eudicots and still function as mutualists (*3*). Thus, we tested the ability of the *C. protuberata* isolates to confer heat tolerance to tomato (*Solanum lycopersicon*). Using a slightly modified protocol for the heat-stress experiment (*11*), we obtained similar results to those obtained with *D. lanuginosum* (Fig. 4). However, it was not possible to attain 100% fungal colonization of tomato plants (*11*), and this may explain the higher proportion of dead plants colonized with the Wt or An fungus, compared with the experiment using *D. lanuginosum*. Given that *C. protuberata*, when infected with CThTV, provides similar mutualistic benefits to both a monocot and a eudicot, it is possible that the underlying mechanism is conserved between these two groups of plants.

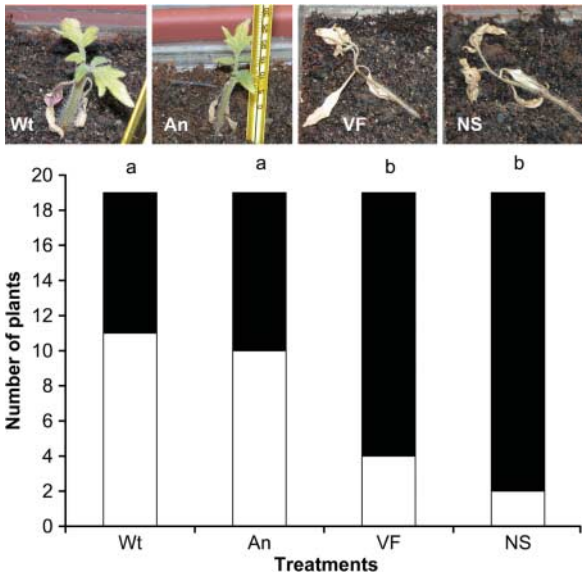

Fig. 4. (**Top**) Representative tomato (*Solanum lycopersicon*, var. Rutgers) plants after the heat-stress experiment. Plants were nonsymbiotic (NS) and symbiotic with the wild-type virus-infected isolate of *C. protuberata* (Wt), the hygromycin-resistant isolate newly infected with the virus through hyphal anastomosis (An) or the virus-free hygromycin-resistant isolate (VF). Rhizosphere temperature was maintained at 65°C for 10 hours and ambient temperature (26°C) for 14 hours/day for 14 days under greenhouse conditions. (**Bottom**) The histogram presents the number of plants dead (white) and alive (black) at the end of the experiment. The small letters on top of the bars indicate statistical differences or similarities (Fisher's exact test, $P < 0.05$).

Plants inoculated with *C. protuberata* infected with CThTV do not activate their stress-response system in the usual way. For example, the osmolyte concentration in these plants does not increase as a response to heat stress, although the levels are constitutively higher than in plants colonized with the virus-free isolate or the nonsymbiotic plants (fig. S6). It has been hypoth-

esized that endophytes may protect their host plants by scavenging the damaging reactive oxygen species (ROS) generated by the plant defense mechanisms in response to environmental stress (*15*). The leaves of nonsymbiotic plants generated detectable ROS when stressed with heat, whereas those of symbiotically colonized plants did not (table S1). However, there was no difference in the ROS response to heat between plants inoculated with the virus-free and the CThTV-infected isolates of *C. protuberata*.

Complex tripartite symbioses have been found among arthropods, bacteria, and mutualistic bacteriophages (*16*, *17*). This study reports a three-way mutualistic symbiosis involving a virus, a fungal endophyte, and either a monocot or eudicot plant.

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The Neural Basis of Loss Aversion in Decision-Making Under Risk

Sabrina M. Tom,[1] Craig R. Fox,[1,2] Christopher Trepel,[2] Russell A. Poldrack[1,3,4]*

People typically exhibit greater sensitivity to losses than to equivalent gains when making decisions. We investigated neural correlates of loss aversion while individuals decided whether to accept or reject gambles that offered a 50/50 chance of gaining or losing money. A broad set of areas (including midbrain dopaminergic regions and their targets) showed increasing activity as potential gains increased. Potential losses were represented by decreasing activity in several of these same gain-sensitive areas. Finally, individual differences in behavioral loss aversion were predicted by a measure of neural loss aversion in several regions, including the ventral striatum and prefrontal cortex.

Many decisions, such as whether to invest in the stock market or to accept a new job, involve the possibility of gaining or losing relative to the status quo. When faced with such decisions, most people are markedly risk averse. For instance, people typically reject gambles that offer a 50/50 chance of gaining or losing money, unless the amount that could be gained is at least twice the amount that could be lost (e.g., a 50/50 chance to either gain $100 or lose $50) (*1*). Prospect theory, the most successful behavioral model of decision-making under risk and uncertainty (*1*, *2*), explains risk aversion for "mixed" (gain/loss) gambles using the concept of loss aversion: People are more sensitive to the possibility of losing objects or money than they are to the possibility of gaining the same objects or amounts of money (*1*, *3*–*5*). Thus, people typically require a potential gain of at least $100 to make up for exposure to a potential loss of $50 because the subjective impact of losses is roughly twice that of gains. Similarly, people demand substantially more money to part with objects that they have been given than what they would have been willing to pay to acquire those objects in the first place (*6*). Loss aversion also has been used to explain a wide range of economic behaviors outside the laboratory (*7*, *8*). Further, loss aversion is seen in trading behavior of both children as young as age five (*9*) and capuchin monkeys (*10*), which suggests that it may reflect a fundamental feature of how potential outcomes are assessed by the primate brain.

Previous neuroimaging studies of responses to monetary gains or losses have focused on activity associated with the anticipation of immediate outcomes ("anticipated" utility) (*11*, *12*) or the actual experience of gaining or losing money ("experienced" utility) (*11*, *13*, *14*) rather than specifically investigating which brain systems represent potential losses versus gains when a decision is being made ("decision" utility). Behavioral researchers have shown that anticipated, experienced, and decision utilities often diverge in dramatic ways, which raises the possibility that the corresponding brain systems involved may also differ (*15*). In the current study, we aimed to isolate activity associated with the evaluation of a gamble when choosing whether or not to accept it (i.e., decision utility) without the expectation that the gamble would be immediately resolved. This allowed us to test whether neural responses during the evaluation of potential outcomes are similar to patterns previously reported in studies of anticipated and experienced outcomes.

One fundamental question for the study of decision-making is whether loss aversion reflects the engagement of distinct emotional processes when potential losses are considered. It has been suggested that enhanced sensitivity to losses is driven by negative emotions, such as fear or anxiety (*16*). This notion predicts that exposure to increasing potential losses should be associated with increased activity in brain structures thought to mediate negative emotions in decision-making [such as the amygdala or anterior insula; compare with (*17*, *18*)]. Alternatively, loss aversion could reflect an asymmetric response to losses versus gains within a single system that codes for the subjective value of the potential gamble, such as ventromedial prefrontal cortex (VMPFC)/orbitofrontal cortex (OFC) and ventral striatum (*11*, *19*, *20*).

To examine the neural systems that process decision utility, we collected functional magnetic resonance imaging (fMRI) data while partici-

[1]Department of Psychology, University of California Los Angeles (UCLA), Franz Hall, Box 951563, Los Angeles, CA 90095–1563, USA. [2]Anderson School of Management, UCLA, 110 Westwood Plaza, Los Angeles, CA 90095–1481, USA. [3]Brain Research Institute, UCLA, Los Angeles, CA 90095, USA. [4]Department of Psychiatry and Biobehavioral Sciences, UCLA, Los Angeles, CA 90095, USA.

*To whom correspondence should be addressed. E-mail: poldrack@ucla.edu