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Short Communication

A defect in carbohydrate metabolism ameliorates symptom severity in virus-infected *Arabidopsis thaliana*

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Altered starch accumulation is a characteristic biochemical symptom of virus infection in plants. To assess its biological importance, infection of *Arabidopsis thaliana* with *Turnip vein-clearing virus*, *Cucumber mosaic virus* or *Cauliflower mosaic virus* was investigated in plants grown under continuous illumination (under which there is no net breakdown of starch) and in *pgm1* mutant plants lacking chloroplastic phosphoglucomutase, an enzyme required for starch biosynthesis. Virus-infected wild-type plants grown under continuous light exhibited more severe leaf symptoms, but no reduction in growth compared with plants grown under diurnal illumination. Comparing lines grown in perpetual light, *pgm1* mutant plants displayed less severe symptoms than the wild-type controls. However, accumulation of all three viruses was similar in wild-type and mutant plants and was unaffected by the light regime. The results show that, although changes in starch accumulation during infection are not required for successful viral infection, carbohydrate metabolism does influence symptom development.

Virus multiplication in susceptible and resistant plants can profoundly affect host metabolism. Respiration, photosynthetic efficiency and the partitioning of carbon between starch, soluble sugars, organic and amino acids are frequently perturbed in both directly inoculated and systemically infected tissues (reviewed by Hull, 2002; Handford & Carr, 2006; Loebenstein & Akad, 2006). In directly inoculated leaves of susceptible plants, the alterations in carbohydrate metabolism frequently result in the occurrence of starch lesions or ringspots at viral infection sites and abnormal accumulation or depletion of this important storage compound in systemically infected tissues, which can be revealed by iodine staining (Holmes, 1931; Bawden, 1950; Cohen & Loebenstein, 1975; Roberts & Wood, 1982; Técsi *et al.*, 1994).

Starch lesions or ringspots on virus-inoculated leaves develop before the appearance of visible disease symptoms. They consist of rings of cells with chloroplasts containing enlarged starch grains surrounding a zone of starch-depleted cells (Cohen & Loebenstein, 1975). In marrow cotyledons inoculated with the cucumovirus *Cucumber mosaic virus* (CMV), starch accumulation occurs in cells that are behind the replication front of the expanding infection zone. These starch-accumulating cells display increased photosynthetic capacity relative to uninfected cells or cells in which virus replication is actively occurring (Doke & Hirai, 1970; Técsi *et al.*, 1996). The starch-depleted cells within the ringspot show enhanced respiratory activity and starch hydrolase activity, with decreased photosynthetic efficiency (Técsi *et al.*, 1996).

It is unclear if or how the complex biochemical changes reflected in the distribution of starch in the developing viral infection site influence the progress of viral infection. In this study we investigated the role of starch accumulation itself using *pgm1* mutant plants of *Arabidopsis thaliana* (L.) Heynh (ecotype Col-0). These plants cannot synthesize starch due to a defect in the gene encoding the plastidic phosphoglucomutase, an enzyme required for starch biosynthesis (EC 5.4.2.2; Caspar *et al.*, 1985). Three viruses that infect *A. thaliana* and induce recognizable disease symptoms were used for the study. These were the Tobamovirus *Turnip vein-clearing virus* (TVCV; Melcher, 2003) and the Fny strain of CMV (Roossinck & Palukaitis, 1990), which are both positive single-stranded RNA viruses, and the Cabb B-JI strain of the caulimovirus *Cauliflower mosaic virus* (CaMV), a double-stranded DNA pararetrovirus (Cecchini *et al.*, 1998).

To confirm that virus infection induces starch lesions in *A. thaliana*, wild-type plants at the six-leaf stage (17 days post-seeding) were mock-inoculated or inoculated on the two oldest true leaves with CMV at 10 μg ml^{-1} and grown at 22 °C in a 16 h light/8 h dark (diurnal) regime for various periods. Inoculated leaves were harvested 2 h into the light

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period, decolourized in boiling 80 % (v/v) ethanol and stained with iodine solution (5.7 mM I_2, 43 mM KI). By 2 days post-inoculation (p.i.) discrete regions of starch were detectable on leaves inoculated with CMV, but not on mock-inoculated leaves (Fig. 1a). The virus-induced starch lesions expanded into well defined rings of starch accumulation by 3 days p.i., but by 6 days p.i. distinct regions of starch accumulation were not apparent in stained, virus-infected leaves (Fig. 1a). TVCV is a very close relative of *Tobacco mosaic virus* (TMV), and the spatial and temporal development of starch lesions in wild-type *A. thaliana* inoculated with CMV are similar to these observed on leaves inoculated with TMV (Handford & Carr, 2006) and to those seen in CMV-infected marrow cotyledons (Técsi *et al.*, 1994).

The *pgm1* mutant plants cannot synthesize starch from the products of photosynthesis and consequently these plants only grow and develop if propagated under continuous illumination (Caspar *et al.*, 1985; Caspar & Pickard, 1989). Iodine staining was used to confirm that under these growth conditions, leaves of wild-type *A. thaliana* plants accumulate high levels of starch, while leaves of the mutant plants do not react with the stain (Fig. 1b). Following viral infection, neither wild-type nor mutant plants maintained under continuous light developed starch lesions; at all time points, the decolourized leaves of these plants stained with iodine solution appeared like the 6 days p.i. samples shown in Fig. 1(a). Thus, starch lesion development can be blocked by altering the environment or by creating a defect in the biochemical pathway responsible for starch biosynthesis. We examined the effect of preventing the build-up or breakdown of starch on systemic infection and symptom induction by three highly distinct viruses.

Wild-type and *pgm1* mutant plants cultivated under continuous illumination at 22 °C grew and developed similarly (Fig. 2a, Table 1). Although wild-type plants developed faster under continuous illumination (reaching the six-leaf stage at 12 days post-seeding, rather than 17 days), they accumulated less biomass over that time, probably due to the disruption of the plant circadian system (Dodd *et al.*, 2005). Plants were inoculated at the six-leaf stage, grown under continuous light or diurnal conditions and analysed at 12 or 17 days p.i., respectively. The virus-induced symptoms were analysed by observing the physical appearance of the leaves (wrinkling, distortion, chlorosis, etc.) and then in terms of the effects of virus infection on plant growth.

In wild-type plants, the alteration of leaf appearance induced by all three viruses was exacerbated by growth in continuous light, yet those grown in these latter conditions did not suffer the loss of shoot biomass observed in plants propagated in a diurnal light regime. Plants grown under continuous illumination exhibited increased chlorosis and/or leaf distortion, compared with virus-infected plants maintained in the diurnal regime (Fig. 2a). TVCV caused mild stunting and perturbations in leaf morphology in wild-type plants grown in a day/night regime (Fig. 2a; Lartey

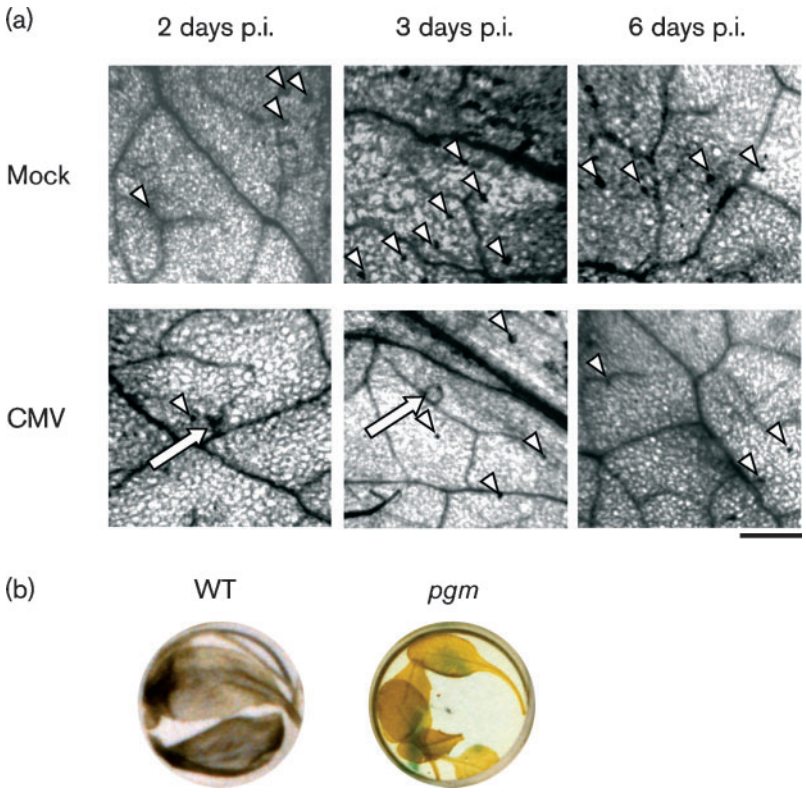

Fig. 1. (a) Virus-induced starch lesions in inoculated leaves of wild-type *A. thaliana* plants grown in a diurnal light regime. Wild-type plants at the six-leaf stage (17 days post-seeding) were lightly dusted with carborundum and two lower leaves were inoculated with either distilled water (mock-inoculated) or a 10 μg ml^{-1} suspension of CMV, using a roughened glass rod. Two, three and six days p.i., the inoculated leaves were removed after 2 h in the light period, decolourized in boiling ethanol, stained for starch in iodine solution and images taken with a digital camera attached to a microscope. Starch lesions (arrows) and carborundum particles (arrowheads) are indicated. Bar, 250 μm. (b) The leaves of wild-type and *pgm1* mutant plants grown under continuous light for 12 days were removed, decolourized in boiling ethanol and stained for starch in iodine solution. The wild-type leaves developed the characteristic blue/black stain for starch.

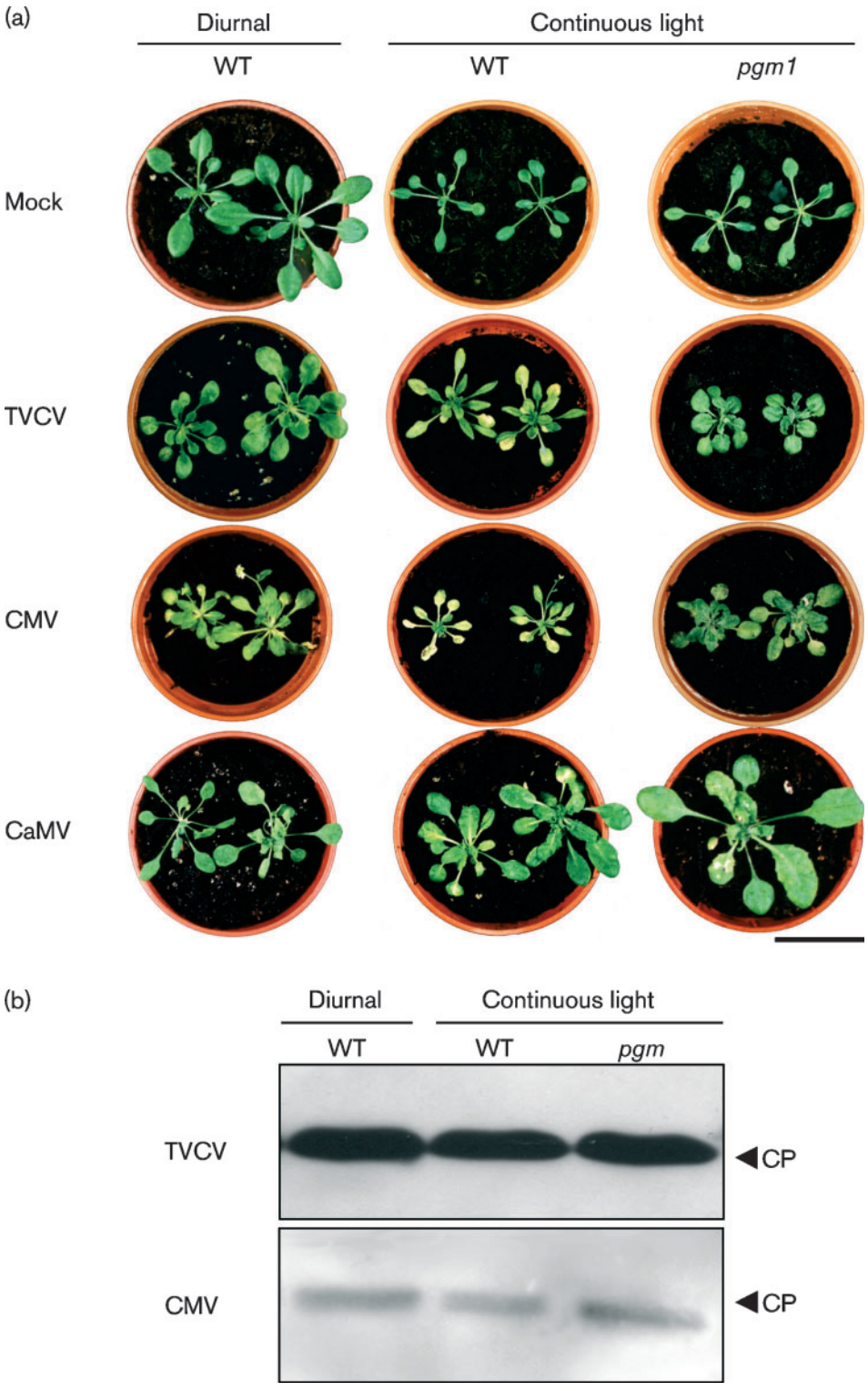

Fig. 2. Virus-induced symptoms and viral coat protein accumulation in wild-type or *pgm1* mutant *A. thaliana* plants. Wild-type plants were grown in a diurnal light regime or under continuous light in parallel with *pgm1* mutant plants before mock-inoculation or inoculation with CMV, TVCV or CaMV. Plants grown under diurnal conditions were inoculated at 17 days post-seeding and those grown under continuous light at 12 days post-seeding. (a) Plants were photographed after a further 17 days (diurnally grown plants) or 12 days (continuous light) and harvested. Bar, 5 cm. (b) Total soluble protein was extracted from the pooled aerial parts of the harvested plants and equal amounts of protein from each pool were analysed by Western immunoblotting using appropriate antisera to detect viral coat protein (CP) accumulation. The results of analysis for CMV and TVCV are shown.

Table 1. Shoot biomass of mock- and virus-inoculated *Arabidopsis* plants

Wild-type plants were grown in a diurnal light (WT-d) or continuous (WT-c) light regime. Mutant *pgm1* plants were also grown under continuous illumination. Plants were mock-inoculated or inoculated with virus (CMV, TVCV or CaMV) at 17 days post-seeding (WT-d) or at 12 days post-seeding (WT-c and *pgm1*). After a further 17 days (WT-d) or 12 days (WT-c and *pgm1*), the aerial parts of individual plants were harvested and weighed (shoot biomass). Each value is the mean biomass ± SEM of 25–60 plants and the probability (*P*; Student's *t*-test) of mock- and virus-inoculated plants being from the same population is shown in parentheses.

Line	Shoot biomass (mg)			
	Mock	TVCV	CMV	CaMV
WT-d	290 ± 23.3	215 ± 18.1 (0.015)	192 ± 18.5 (0.002)	203 ± 12.0 (0.006)
WT-c	123 ± 17.2	157 ± 22.6 (0.219)	119 ± 15.1 (0.886)	174 ± 21.2 (0.060)
pgm	140 ± 19.2	143 ± 12.9 (0.901)	159 ± 14.8 (0.433)	225 ± 15.2 (0.001)

et al., 1997). Under continuous light, TVCV infection induced distinctly different symptoms in wild-type compared with *pgm1* mutant plants: no chlorosis was observed in the mutant plants and leaf shape was rounded, rather than elongated, compared with the mock-inoculated plants (Fig. 2a). In wild-type *A. thaliana*, the Fny strain of CMV induced stunting, leaf distortion, chlorosis and, in some plants, symptoms included premature inflorescence production (bolting) (Fig. 2a; Mayers *et al.*, 2005). The impact of CMV on leaf appearance was more pronounced in wild-type plants grown in continuous light (Fig. 2a). By contrast, in *pgm1* mutant plants chlorosis was less apparent and, as with the plants infected with TVCV, CMV-infected mutant plants produced rounded rather than elongated leaves (Fig. 2a). Consistent with previous reports (Cecchini *et al.*, 1998; Love *et al.*, 2005), we observed that infection of wild-type plants with the Cabb-B JI strain of CaMV caused distorted leaf shape and chlorosis in older leaves and vein-clearing and stunting of newly emerged leaves (Fig. 2a). Chlorosis, vein-clearing and distortion were more apparent on CaMV-infected wild-type plants grown under continuous light, but less severe on *pgm1* mutant plants, which also appeared less stunted (Fig. 2a).

The wild-type, virus-infected plants grown under continuous light did not show a significant degree of growth inhibition compared with mock-inoculated plants, which was in contrast to the inhibition of growth seen in plants infected and grown under diurnal conditions (Table 1). Most notably, whereas CaMV infection of wild-type plants grown in a day/night regime decreased shoot biomass significantly, CaMV-inoculated wild-type and *pgm1* plants under continuous light grew well. This was mirrored by increases of ~60 and ~40 % in shoot biomass for CaMV-inoculated *pgm1* and wild-type plants, respectively, compared with mock-inoculated controls grown under continuous illumination. These differences were statistically significant ($P=0.001$, *pgm1*) or approaching statistical significance ($P=0.060$, wild-type). Consistent with recent findings by Dodd *et al.* (2005), it appears that in this case, the disruption of the circadian system of the plant combined with infection by CaMV significantly perturbs the accumulation of shoot biomass.

To determine if growth under continuous light or inhibition of starch accumulation affected the yield of virus, total soluble protein was extracted from virus- or mock-inoculated plants at 12 or 17 days p.i., for plants grown under continuous or diurnal light regimes, respectively. Protein was extracted, quantified and subjected to Western blotting analysis, as described previously (Chivasa *et al.*, 1997). Rabbit polyclonal antisera against the coat proteins (CP) of TVCV (using anti-TMV CP serum; Wong *et al.*, 2002), CMV or CaMV were used as appropriate and antibody binding was detected using an anti-rabbit IgG horseradish peroxidase conjugate and an enhanced luminol-based chemiluminescence reagent mix (Perkin-Elmer). We found that accumulation of TVCV, CMV (Fig. 2b) and CaMV (data not shown) was similar in wild-type and *pgm1* plants and was not affected by the light regime. These results demonstrate that for all three viruses the effects of infection on symptoms or growth shown in Fig. 2(a) or Table 1 cannot be attributed to differences in viral load.

It is apparent from the data that a combination of environmental and metabolic factors is differentially influencing the susceptibility of *A. thaliana* to viral attack. In plants grown under a normal day/night cycle, such as the virus-infected wild-type *A. thaliana* plants in this study (Fig. 1), CMV-infected marrow cotyledons (Técsi *et al.*, 1994, 1996) or CaMV-infected turnip and *A. thaliana* (Love *et al.*, 2005), changes in starch deposition may reflect perturbations in photosynthetic, biosynthetic and respiratory pathways. Conceivably, this might affect the supply of fixed carbon for synthesis of virus-specific gene products within the virus-infected cells and the import of fixed carbon into virus-infected tissues from uninfected areas of the plant. In this study, a starch-depleted mutant line of plants cannot exhibit such changes, yet the accumulation of three different viruses was unaffected. This demonstrates unequivocally that catabolism of starch is not required to meet the demand for carbon for biosynthesis of virus-specific products. In CMV-infected marrow cells, starch accumulation required direct illumination of those cells, and not the import of photosynthates from neighbouring uninfected cells, yet virus accumulation was unaffected in the dark (Técsi *et al.*, 1994). The susceptibility to several viruses of

pgm1 supports these findings, in that it is not the build-up of starch per se which is a prerequisite for a successful infection, nor is it increased availability of free sugars in tissues where starch synthesis is not possible (as in *pgm1*; this study) or starch is broken down (as in dark-grown plants; Técsi *et al.*, 1994).

Free sugars can be potent signals in plant metabolism, gene expression and development (Rolland *et al.*, 2006), and in *pgm1* mutant plants the conversion of these potential signals to starch is inhibited. Recent work by Love *et al.* (2005), using *A. thaliana* sugar response mutants, indicated that sugar signalling does not play a simple role in the modulation of symptoms in response to CaMV, at least in plants grown under diurnal (16 h day/8 h night) conditions. Nevertheless, we have shown that an environmental influence on symptom severity, in this case continuous light, is being modulated, directly or indirectly, by blocking the incorporation of sugars into starch and that this occurred in plants infected by three diverse viruses. The findings raise the possibility that cross-talk between proposed disease-signalling pathways (Cecchini *et al.*, 2002) and sugar signalling occurs, but only in plants grown under conditions of stress, in which additional stress-related signalling pathways are operating.

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