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Article Addendum

The common metabolite glycerol-3-phosphate is a novel regulator of plant defense signaling

Srivathsa C. Venugopal, Bidisha Chanda, Lisa Vaillancourt, Aardra Kachroo and Pradeep Kachroo*

Department of Plant Pathology; University of Kentucky; Lexington, KY USA

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Conversion of glycerol to glycerol-3-phosphate (G3P) is one of the highly conserved steps of glycerol metabolism in evolutionary diverse organisms. In plants, G3P is produced either via the glycerol kinase (GK)-mediated phosphorylation of glycerol, or via G3P dehydrogenase (G3Pdh)-mediated reduction of dihydroxyacetone phosphate (DHAP). We have recently shown that G3P levels contribute to basal resistance against the hemibiotrophic pathogen, *Colletotrichum higginsianum*. Since a mutation in the *GLY1*-encoded G3Pdh conferred more susceptibility compared to a mutation in the *GLI1*-encoded GK, we proposed that GLY1 is the major contributor of the total G3P pool that participates in defense against *C. higginsianum*.

Glycerol and its metabolites are involved in a variety of physiopathological processes in both prokaryotes and eukaryotes, most of which appear to be highly conserved,[1] signifying the fundamental importance of these molecules. Glycerol-3-phosphate (G3P), an obligatory component of energy-producing reactions including glycolysis and glycerolipid biosynthesis, participates in the disease-related physiologies of many organisms. In humans, deficiencies in glycerol kinase activity (catalyzing the phosphorylation of glycerol to G3P) result in a variety of metabolic and neurological disorders, while mutations in G3P dehydrogenase (G3Pdh, catalyzing the oxidation of dihydroxyacetone phosphate, DHAP, to G3P) have been linked to sudden infant death syndrome and decreased cardiac Na^{2+} current resulting in ventricular arrhythmias and sudden death.[2,3] Given the fact that glycerol metabolism is conserved between plants and animals, it is conceivable that glycerol and/or G3P might also participate in disease physiology of plants. However, such a role for glycerol and/or G3P remains unexplored.

Previous work from our laboratory and others has shown that *GLY1*-encoded G3Pdh plays an important role in plastidal oleic acid-mediated signaling[4-7] and systemic acquired resistance.[8] This group of enzymes also plays an important role in fungi and it was recently shown that the disruption of a *G3Pdh* gene in *Colletotrichum gloeosporioides* eliminated the ability of the mutant fungus to grow on most carbon sources in vitro, including amino acids and glucose.[9] However, the *G3Pdh* knockout (KO) fungus grew normally in the presence of glycerol. The *G3Pdh KO* fungus also developed normally in its plant host (the round-leaved mallow), prompting the suggestion that glycerol, rather than glucose or sucrose, was the primary transferred source of carbon in planta. This was an unexpected finding, but direct analysis of infected host leaves revealed that their glycerol content did decrease by 40% within 48 hours of infection with *C. gloeosporioides*.[9] Since the hemibiotroph *C. gloeosporioides* appears to be able to utilize glycerol for growth and conidiation in planta, it was possible that glycerol metabolism and associated pathways in the host played an important role in the establishment of infections by *Colletotrichum* fungi. Furthermore, it was possible that the host had evolved to sense these pathogen-mediated changes in glycerol levels and utilize them as signal(s) to initiate defense.

We tested these possibilities by characterizing the role of glycerol metabolism in the Arabidopsis—*C. higginsianum* interaction (Fig. 1). Infection with *C. higginsianum* reduced the glycerol content while concomitantly increasing the G3P content in Arabidopsis plants.[10] Mutations in G3P-synthesizing genes *gly1* (a G3Pdh) and *gli1* (a glycerol kinase),[11] resulted in enhanced susceptibility to *C. higginsianum*. The *gly1* plants were much more susceptible than the *gli1 plants*, suggesting that *GLY1*-encoded G3Pdh played a more important role in basal resistance to *C. higginsianum*. Conversely, the *act1* mutant, which is impaired in the acylation of G3P with oleic acid (18:1) (Fig. 1), was more resistant to the fungus. The phenotypes seen in the infected *gly1* and *act1* plants correlated with pathogen-induced G3P levels; *C. higginsianum* inoculation induced ~2-fold higher accumulation of G3P in the *act1* plants, and ~2-fold lower G3P in the susceptible *gly1* plants, as compared to wild-type plants.[10] To test the hypothesis that G3P synthesized via GLY1 entered the plastidial glycerolipid pathway

*Correspondence to: Pradeep Kachroo; University of Kentucky; 1405 Veterans Drive; 244 Plant Science Building; Lexington, KY 40546 USA; Tel.: 859.257.7445 ext. 80729; Fax: 859.323.1961; Email: pk62@uky.edu

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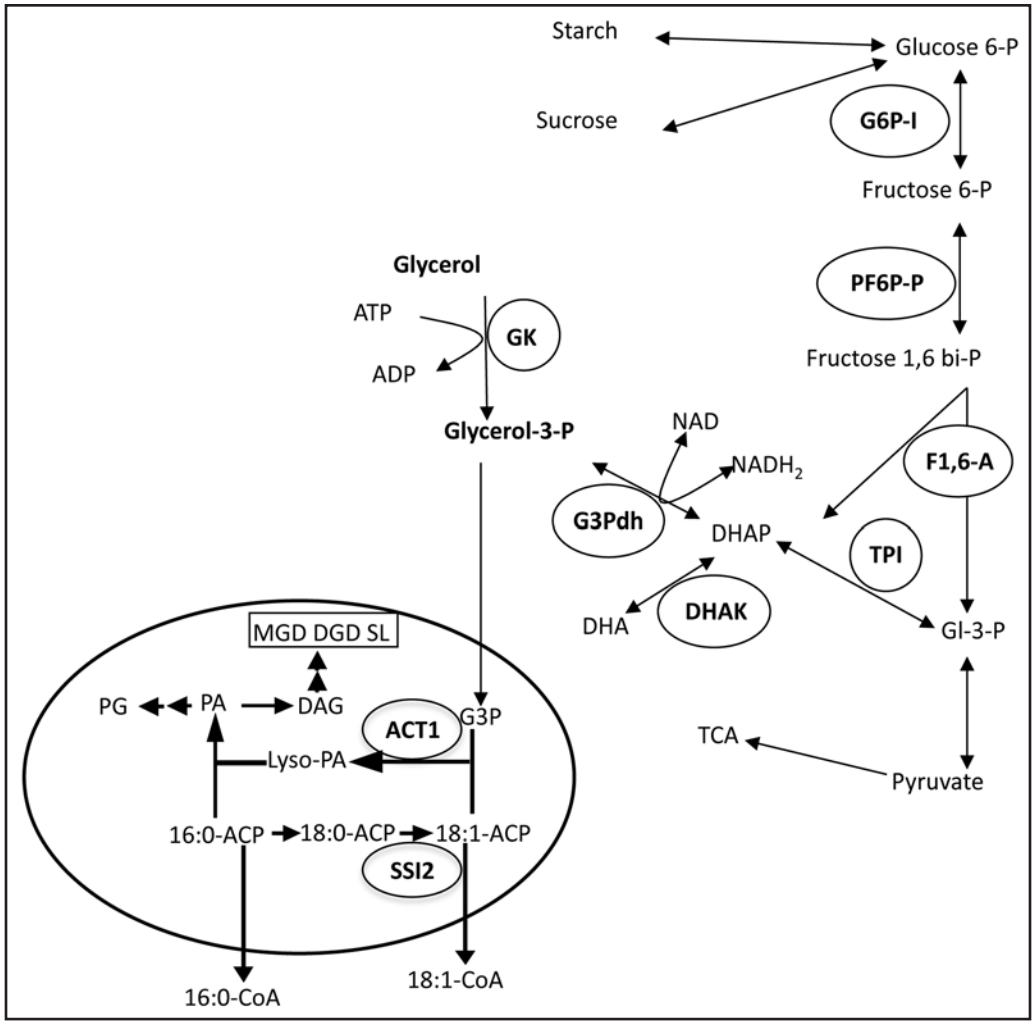

Figure 1. A condensed scheme of glycerol metabolism in plants. Glycerol is phosphorylated to glycerol-3-phosphate (G3P) by glycerol kinase (GK; GLI1). G3P can also be generated by G3P dehydrogenase (G3Pdh) via the reduction of dihydroxyacetone phosphate (DHAP) in both the cytosol and the plastids (represented by the oval). G3P generated by this reaction can be transported between the cytosol and plastid stroma. In the plastids G3P is acylated with oleic acid (18:1) by the *ACT1*-encoded G3P acyltransferase. This ACT1-utilized 18:1 is derived from the stearoyl-acyl carrier protein (ACP)-desaturase (SSI2)-catalyzed desaturation of stearic acid (18:0). The 18:1-ACP generated by SSI2 either enters the prokaryotic lipid biosynthetic pathway through acylation of G3P, or is exported out of the plastids as a coenzyme A (CoA)-thioester to enter the eukaryotic lipid biosynthetic pathway. Other abbreviations used are: PA, phosphatidic acid; Lyso-PA, acyl-G3P; PG, phosphatidylglycerol; MGD, monogalactosyldiacylglycerol; DGD, digalactosyl-diacylglycerol; SL, sulfolipid; DAG, diacylglycerol; DHA, dihydroxyacetone; Gl-3-P, glyceraldehyde-3-phosphate; TCA, tricarboxylic acid cycle. Enzymes as abbreviated as: ACT1, G3P acyltransferase; SSI2, stearoyl acyl carrier protein desaturase; GK, glycerol kinase; G3Pdh, G3P dehydrogenase; TPI, triose phosphate isomerase; DHAK, dihydroxyacetone kinase; F1,6-A, fructose 1,6-biphosphate aldolase; PF6P-P, pyrophosphate fructose-6-phosphate phosphotransferase; G6P-I, glucose-6-phosphate isomerase.

via the ACT1 catalyzed reaction, we generated *act1 gly1* plants. The results supported the hypothesis, as *act1 gly1* plants were as susceptible to *C. higginsianum* as *gly1* plants.

More supporting evidence for the role of G3P in defense against *C. higginsianum* was obtained by overexpressing *GLY1* in wild-type plants (Fig. 2A). Similar to *act1*, overexpression of *GLY1* led to a ~2-fold increase in G3P levels after pathogen inoculation, and these plants were also more resistant to *C. higginsianum* (Fig. 2B–D). Furthermore, plants overexpressing *GLY1* or carrying a mutation in *ACT1* exhibited enhanced resistance to *C. higginsianum* in the *pad3* mutant background (Fig. 3).[10] The *pad3* plants are compromised in camalexin synthesis, and are hypersusceptible to necrotrophic pathogens.

Exogenous glycerol application increased endogenous G3P and significantly enhanced the ability of the host to resist *C. higginsianum*.[10] Glycerol-triggered synthesis of G3P also caused a decrease in 18:1 levels, which is known to induce defense signaling, resulting in enhanced basal resistance.[4-7] However, the glycerol-triggered increase in G3P precedes the reduction in 18:1 levels and confers resistance even at time points when low 18:1-mediated signaling is not induced, suggesting that the enhanced resistance after glycerol treatment was due to elevated G3P levels and not to the reduction in 18:1.

Understanding the precise roles of G3P will require in-depth analysis of real-time alterations in its levels on a cellular level during pathogenesis. This is complicated by the presence of

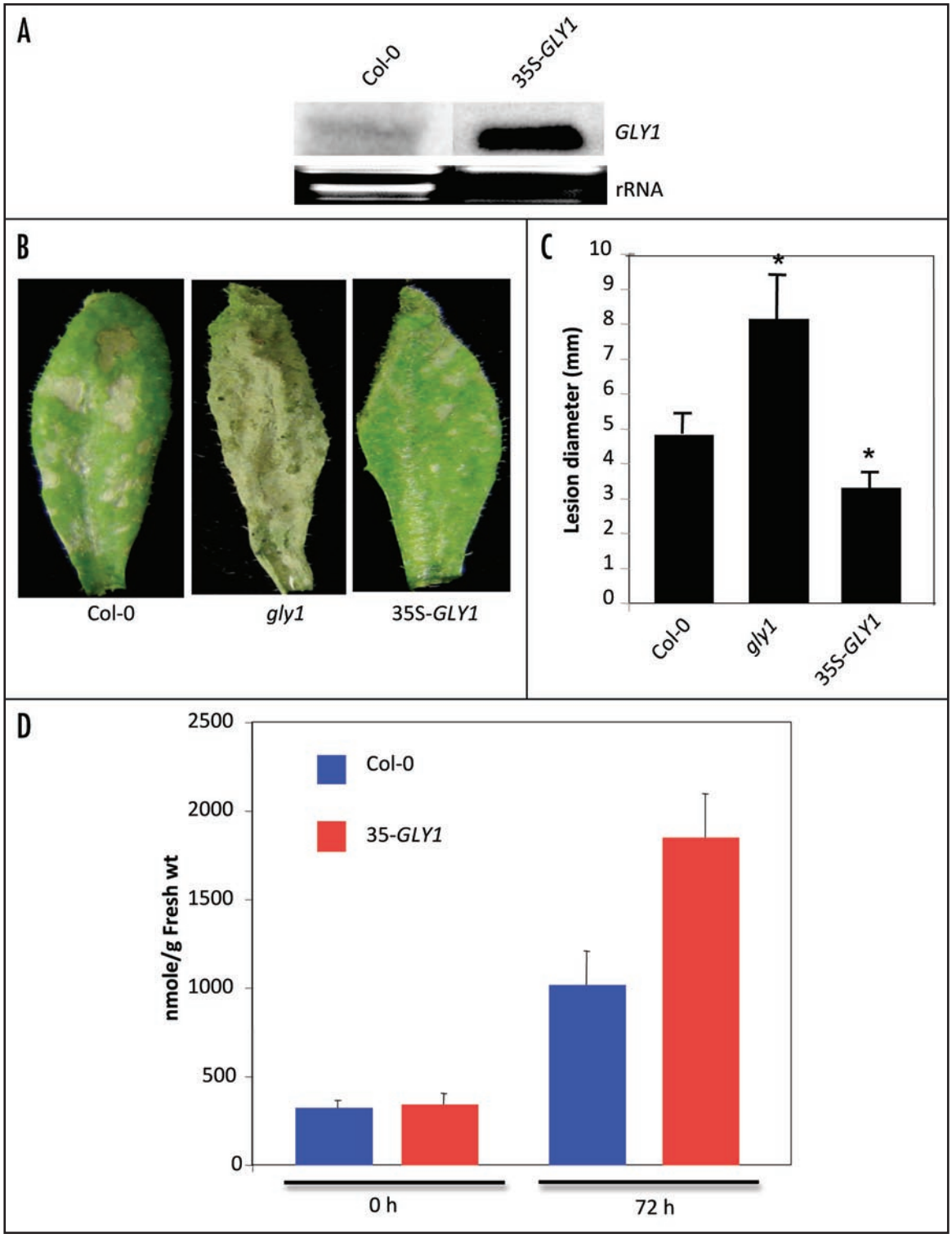

Figure 2. Pathogen response and G3P levels in transgenic lines overexpressing *GLY1*. (A) Expression of the *GLY1* gene in wild-type or 35S-*GLY1* transgenic plant. RNA gel blot analysis was performed on ~7 μg of total RNA. Ethidium bromide staining of rRNA was used as a loading control. (B) Disease symptoms in *C. higginsianum*-inoculated Col-0, *gly1* or 35S-*GLY1* plants at 5 dpi. The plants were spray-inoculated with 10^6 spores/ml of *C. higginsianum*. (C) Lesion size in spot-inoculated genotypes. The plants were spot-inoculated with water or 10^6 spores/ml and the lesion size was measured from 20–30 independent leaves at 6 dpi. Statistical significance was determined using Students t-test. Asterisks indicate data that is statistically significant from that of control (Col-0) ($p < 0.05$). Error bars indicate SD. (D) G3P levels in Col-0 and 35S-*GLY1* plants at 0 and 72 h post-inoculation.

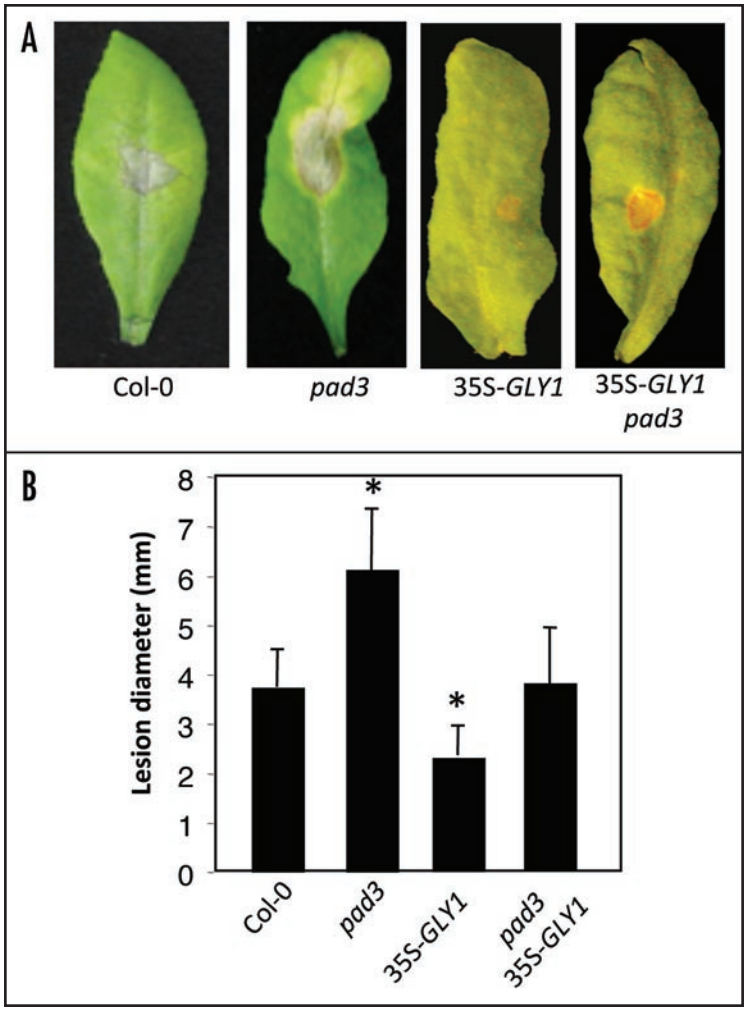

Figure 3. Pathogen response in *C. higginsianum*-inoculated 35S-*GLY1* plants in *pad3* background. (A) Disease symptoms on Col-0, *pad3* or 35S-*GLY1* or 35S-*GLY1 pad3* plants spot-inoculated with 10^6 spores/ml of *C. higginsianum*. The leaves were photographed at 7 dpi. (B) Lesion size in spot-inoculated Col-0, *pad3* or 35S-*GLY1* or 35S-*GLY1 pad3* plants. The lesion size was measured from 20–30 independent leaves at 7 dpi. Asterisks indicate data that is statistically significant from that of control (Col-0) ($p < 0.05$). Error bars indicate SD.

multiple isoforms of *G3Pdh* that contribute to the total G3P pool, and by the lack of appropriate tools for monitoring precise changes in intracellular G3P. Systematic analysis of various *G3Pdh* mutants, in combination with each other and with *gli1*, should yield novel insights into pathway(s) and steps regulating levels of G3P in the cell.

Acknowledgements

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