Pathogen Associated Molecular Pattern (PAMP)-Triggered Immunity Is Compromised under C-Limited Growth

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INTRODUCTION

Plants have developed various mechanisms to defend themselves against bacterial, fungal, oomycete and viral infections. These defense responses begin with the recognition of the invading pathogen by pattern recognition receptors (PRRs) that connect to pathogen-associated molecular patterns (PAMPs) (Bittel and Robatzek, 2007; Segonzac and Zipfel, 2011). Bacterial flagellin (flg) is the best characterized PAMP and flg22, a peptide corresponding to a strongly conserved stretch of 22 amino acids in the N-terminus of flagellins, has been frequently used as a strong synthetic PAMP inducer (Felix et al., 1999). Different plants recognize different subsets of PAMPs to induce PAMP triggered immunity (PTI). PTI signaling, initiated by formation of PRR-PAMP complexes, is transduced via at least two differential and overlapping signaling cascades mediated by MAP kinases and calcium dependent protein kinases (CDPKs) (Asai et al., 2002; Boudsocq et al., 2010). The resulting changes in the phosphorylation status of downstream regulators and enzymes lead to the production of antimicrobial molecules and signaling molecules, such as reactive oxygen species (ROS), ethylene (ET), jasmonic acid (JA), and salicylic acid (SA). These further amplify the defense responses (Pieterse et al., 2009, Tena et al., 2011).

Triggering defense responses in plants demand significant resources and increased supply of carbon (C)/nitrogen (N) skeletons as well as ATP and NADPH for the synthesis of defense related proteins and metabolites (Berger et al., 2007; Bolton, 2009; Bonfig et al., 2006; Swarbrick et al., 2006). A well-known mechanism to supply sugars for plant defense is an increase of cell wall invertase (cwlInv) activity (Benhamou et al., 1991; Chou et al., 2000; Fotopoulos et al., 2003; Sturm and Chrispeels, 1990). An increase of apoplastic sucrose level as well as stimulation of the oxidative pentose phosphate (OPP) pathway and respiration (that generate NADPH and ATP, respectively) has also been observed in tobacco leaves infected with the oomycete Phytophthora nicotianae (Scharte et al., 2005). Recent meta-analyses based on microarray data clearly show that genes related to the OPP pathway, aerobic respiration [tricarboxylic acid (TCA) cycle and mitochondrial electron transfer], and ATP biosynthesis are generally up-regulated after biotic stress treatments (Lessa et al., 2011).
Types of biotrophic and necrotrophic pathogens. (Chandran et al., 2010). Taken together, the manipulation of plant metabolism at infection sites (Pathuri et al., 2011). Localized plant glycolytic metabolism thereby meeting the energy demands of the pathogen at infection sites (Pathuri et al., 2011). Localized host endo-duplication and enhanced expression of host energy-related genes have been observed in Arabidopsis to Pseudomonas syringae pv. tomato DC3000 (Bonfig et al., 2010).

The mechanisms by which pathogens manipulate host metabolism to capture resources from plants depend on their lifestyle. Some hemibiotrophic pathogens have developed effectors that constrain energy metabolism in plants either by suppressing cwl activity or by affecting mitochondrial function (Biemelt and Sonnewald, 2006; Block et al., 2010). Barley alcohol dehydrogenase 1 and 2 are induced upon infection with the biotrophic fungus Blumeria graminis f.sp. hordei, whereas RNAi-mediated knockdown or inhibition of alcohol dehydrogenase activity increases tolerance to the fungus, suggesting that alcohol dehydrogenase activity supports biotrophy by increasing plant glycolytic metabolism thereby meeting the energy demands of the pathogen at infection sites (Pathuri et al., 2011). Localized host endo-duplication and enhanced expression of host energy-related genes have been observed in Arabidopsis thaliana at the infection sites of the biotrophic fungus Golovinomyces orontii (Chandran et al., 2010). Taken together, the manipulation of plant energy metabolism appears to determine or strongly enhance either susceptibility or resistance to pathogens depending on the types of biotrophic and necrotrophic pathogens.

A large fraction of the carbon fixed by photosynthesis is stored in chloroplasts in the form of starch. Degradation of leaf starch supplies C in a way that stored starch is nearly used up by the end of the night. Consequently, plants defective in either starch biosynthesis and storage or nighttime degradation exhibit growth retardation that is alleviated by growth in constant light (Caspar et al., 1985; Roldan et al., 2007; Yu et al., 2001). To study whether the blocking of energy metabolism could affect PTI responses in Arabidopsis, we selected Arabidopsis mutants with C deficiencies during the dark phase. The pgm1 mutant, also known as stachy tree 1, lacks phosphoglucomutase, a key enzyme in starch biosynthesis accumulating 1-3% of the amount seen in wild type (Caspar et al., 1985). The stachy excess 1-1 (sex1-1) mutant is deficient in α-glucan, water dikinase activity required for starch degradation (Yu et al., 2001). Included is also the stachy syn thase 4-1 (ss4-1) mutant with low amounts of leaf starch accumulation in the light (Roldan et al., 2007). The lower amounts of starch or starch-derived sugars in these mutants, albeit derived from different defects, compared to WT plants generated a C starvation phenotype. These starch mutants were used to elucidate the role of C and energy supply affecting plant defense capacity.

**MATERIALS AND METHODS**

**Plant growth conditions and chemicals**

Arabidopsis thaliana lines, Columbia-0, were used. Plants were grown in growth chambers under indicated photoperiods. Illumination was provided by fluorescent light (100-150 μmol m⁻² s⁻¹). Fig22 and monofluoroacetate (MFA) were purchased from Alpha diagnostic International (Cat#FLG22-P-5) and Sigma (31220-1G) respectively.

**Bacterial growth assay**

Three to four leaves of 5-week old Arabidopsis plants were infiltrated with Pseudomonas syringae pv. tomato strain DC3000 hrcC (PstDC3000 hrcC) bacterial suspension (OD₆₀₀ = 0.0005, 2.5 x 10⁵ CFU ml⁻¹ in 10 mM MgCl₂). To minimize leaf position dependent differences in bacterial growth, similar position leaves were selected for infiltration. Leaf discs were ground in 10 mM MgCl₂, diluted serially, plated on King’s medium, and incubated at 28°C for 2-3 days. The number of colonies that formed was counted in each sample. Colony forming units per unit leaf area was calculated.

**RNA extraction and quantitative RT-PCR analysis**

Five-week old Arabidopsis leaves were infiltrated with 1 μM fig22 and collected 6 h after infiltration. Total RNA was isolated using RNeasy Plant Mini kit (Qiagen) and the cDNA was synthesized from 2 μg total RNA using oligo(dt) primer and SuperScript III reverse transcriptase (Invitrogen). qRT-PCR was carried out using CFX96 (Bio-Rad) and Quantimax SYBR kit (PhilieKorea Technology). UBO10 was used as the reference gene for normalization. PCR primer sequences are listed in Supporting Information Table S4. Primers for ACS and NAC genes were designed using AITPrimer software (http://artrprimer.kaist.ac.kr/) (Han and Kim, 2006).

**Callose staining**

Three to four leaves from 3-week old 24 L-grown Arabidopsis plants and 5-week-old 12 L-grown Arabidopsis plants were infiltrated with 1 μM fig22, and collected 6 h after infiltration. Samples were prepared and mounted on glass slides in 50% glycerol. The leaves were viewed under ultraviolet illumination using an Olympus AX-70 fluorescence microscope equipped with DAPI filter. About 8-12 field of views were collected from each leaf and average number of callose deposits per field of view (0.43 mm²) was calculated with QUANTITY ONE software (Bio-Rad).

**Microarray analyses**

Briefly, microarrays were processed by the W. M. Keck Center for Comparative and Functional Genomics in the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Affymetrix GeneChip® 3’ Expression Arabidopsis ATH1 Genome Array was hybridized with antisense RNA prepared from 100 ng of total RNA and scanned with a GeneChip® Scanner model 3000 7G Plus. From the image files, fluorescence intensity (CEL) files were generated and analyzed with Affymetrix Expression Console version 1.1. Raw data was processed with the GCRMA algorithm and clustered using Weighted Gene Co-expression Network Analysis 8.9 (WGCNA). For selected clustered modules, over-representation analyses of Gene Ontology terms and KEGG pathways were performed.

**RESULTS**

Mutants C-limited in starch display enhanced susceptibility to PstDC3000 hrcC

To eliminate interference from ETI responses, we used Pseudomonas syringae pv. tomato DC3000 hrcC (hrcc) that induces PTI but not ETI because of a defect in type III secretion system (Jones and Dangl, 2006; Nicaise et al., 2009). WT Arabidopsis plants and the starch mutants pgm1, sex1-1 (sex1),
and ss4-1 (ss4) were grown under 12 h light/12 h dark cycle (12 L) to impose C starvation on the starch mutants. To test susceptibility to hrcC, plants were infiltrated with 10 mM MgCl₂ (mock) incubated for 1 day and then infiltrated with hrcC. Bacterial growth at 3 days post inoculation (dpi) was greater in all three mutants compared to WT suggesting that general defect in starch metabolism lead to weakened PTI responses (Fig. 1A). When plants were infiltrated with 1 μM flg22 in 10 mM MgCl₂ one day before inoculation with hrcC, bacterial growth in all lines was reduced compared to mock-infiltrated plants. Flg22 is a synthetic PAMP that is often used to induce PTI. Therefore these results indicated that the starch mutants were able to mount PTI like the WT plants. Within the flg22-treated group, bacterial growth was greater in all three mutants compared to WT, similar to the pattern observed within the mock-treated group, confirming that the starch mutants have a weakened PTI response (Fig. 1A, Supplementary Fig. S1). The data suggest that PTI is reduced, but not totally impaired, in the starch mutants.

The three starch mutants used in this study exhibit growth retardation that is recovered upon growth under continuous illumination (Caspar et al., 1985; Roldan et al., 2007; Yu et al., 2001). We also observed growth retardation when WT, ss4, pgm1 and sex1 plants were grown under 12 L photoperiod; however, growth under continuous light (24 L) prevented dwarfism (Supplementary Fig. S2). To ascertain whether compromised PTI responses in starch mutants are due to shortage of C supply during the night, we performed bacterial growth assays on plants grown continuously under both 12 L and 24 L. There was no difference in bacterial growth between 12 L-grown and 24 L-grown WT plants. However, bacterial growth was significantly greater in all starch mutants compared to WT grown under 12 L photoperiod. When grown in a 24 L photoperiod a statistically significant increase in bacterial growth was only observed in the sex1 mutant, albeit to a smaller degree compared to 12 L (Fig. 1B). Enhanced susceptibility of pgm1 and ss4 mutants to hrcC in 12L was abolished by growth under continuous light.

**Compromised PTI responses in starch mutants were more significant during the dark portion of a 12 h light/12 h dark diurnal cycle**

Induction of Flg22-induced Receptor-like Kinase 1 (FRK1) transcripts and callose deposition are two well-known PTI responses. To understand the effect of energy deficit on PTI induction, we compared these PTI responses in plants grown under 12 L and 24 L photoperiods. Plants grown in 12 L were sampled separately during the light and dark periods. In 12 L-grown lines, FRK1 expression level was much higher upon flg22 treatment than mock treatment under light and dark, indicating that the starch mutants are able to mount PTI responses. In 12 L plants that received flg22 treatment, a decreasing trend of FRK1 expression was observed in all starch mutants compared to the WT under both light and dark (Fig. 2A). The decrease was more significant during dark than during the light period in plants grown under 12 L and not observed in plants grown under 24 L (Fig. 2B), suggesting that C limitation impedes full induction of FRK1 in response to flg22 treatment during the dark period.

Callose, a cell wall β-1,3-d-glucan polymer, is deposited at infection sites. This is considered to provide a physical barrier against penetration of pathogen (Nishimura et al., 2003). Callose deposits were clearly observed in flg22-infiltrated leaves of 12 L-grown WT plants under both light and dark conditions and also in flg22-infiltrated leaves of 24 L-grown WT plants. As expected, callose deposits were much lower or hardly detectable in flg22-infiltrated leaves of the fs2 mutant under 12 L and 24 L conditions since it lacks FLS2, the pattern recognition receptor for flg22 (Figs. 2C and D). The average number of callose deposits was much lower in flg22-infiltrated leaves of all 12 L-grown starch mutants than in WT under dark and light conditions (Fig. 2C), whereas no significant differences in callose formation distinguished starch mutants and WT when the experiment was performed on 24 L-grown plants (Fig. 2D). Together, the results show that compromised PTI responses in the starch mutants are evident only under growth conditions that require efficient nighttime starch degradation.

**PTI responses are attenuated by inhibiting the TCA cycle**

To clarify the role of energy in PTI, we analyzed bacterial growth and PTI marker gene expression in leaves sprayed with the TCA cycle inhibitor, monofluoroacetate (MFA). WT plants grown under 24 L were used to eliminate the effects of circadian clock and dark-associated starvation. MFA was sprayed on the leaves at time points before and after hrcC infiltration. A nearly 10-fold increase in bacterial growth was observed when leaves were treated with MFA 1 h before hrcC infiltration, and also when MFA treatments were performed 6 h and 12 h after hrcC infiltration (Fig. 3A). No significant increase in bacterial growth was observed when the MFA treatment was performed 24 h
after hrcC infiltration, suggesting that the effect of MFA treatment disappeared after 24 h of hrcC infiltration of the plants. FRK1 induction by flg22 was also blocked in leaves that had been treated with MFA 1 h before flg22 infiltration (Fig. 3B). To confirm a block in energy generation in these MFA-treated leaves, we measured the expression levels of Alternative Oxidase 1a (AOX1a). AOX1a expression is induced when energy generation in mitochondria is blocked (Saisho et al., 1997). AOX1a was not induced by flg22 treatment, but was induced by MFA treatment, indicating blockage of the TCA cycle while the tissues remain functional (Fig. 3C). The results point to the importance of continuous energy supply during early stages of infection as critical for establishing PTI.

**Microarray analyses to identify C-dependent PTI-related genes that are altered under dark**

Next we performed microarray analyses to identify major molecular players or pathways affected by C deficiency. To minimize side effects due to the growth retardation that occurs in the starch mutants under 12 L growth (Supplementary Fig. S1), all lines were grown under 24 L for 3 weeks and then adjusted to a 12 h light/dark cycle for 3 days (24 L → 12 L). Flg22 was infiltrated into the leaves during the 4th dark period after transfer to 12 L two hours after entering into the dark. Leaves were sampled 6 h after infiltration (means ± SE, n = 3). FRK1 expression levels in response to flg22 treatment were compared in pgm1 and WT grown under 24 L and 24 L → 12 L photoperiods to again confirm that the pgm1 mutant exhibited a compromised PTI response during the dark period in 24 L → 12 L. In 24 L plants, induction of FRK1 expression level by flg22 was the same or slightly higher in the pgm1 mutant compared to WT as observed earlier, indicating a robust and comparable PTI response in both lines (Fig. 2B and Supplementary Fig. S3). In 24 L → 12 L plants, however, induction of FRK1 expression level by flg22 was significantly lower in the pgm1 mutant compared to WT indicating a compromised PTI response (Supplementary Fig. S3). Therefore, samples for microarray analyses were obtained from 24 L → 12 L plants treated in this manner.

We performed global gene expression analyses in triplicate for 5 different samples: WT treated with 10 mM MgCl2...
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(WT_con), WT treated with 1 μM flg22 in 10 mM MgCl₂ (WT_flg), ss4_flg, pgm1_flg, and sex1_flg. Flg22-regulated genes in WT leaves during the dark period were identified by comparison of WT_con with WT_flg. The list of flg22 target genes that showed energy-dependent expression characteristics were identified among these genes by comparison of WT_flg with ss4_flg, pgm1_flg, and sex1_flg. Raw data were preprocessed and filtered using the GCRMA algorithm (Wu and Irizarry, 2005) and 15,846 out of a total of 22,810 probe sets on the array could be used for differential expression analyses (Supplementary Table S1A).

Nearly two-third of known flg22-responsive genes display C supply-dependent alterations in expression
To understand the role of C depletion at the molecular level, we chose 3341 probe sets that showed more than 1.5-fold difference between WT_flg and either ss4_flg, pgm1_flg, or sex1_flg. Flg22-regulated genes in WT leaves during the dark period were identified by comparison of WT_con with WT_flg. The list of flg22 target genes that showed energy-dependent expression characteristics were identified among these genes by comparison of WT_flg with ss4_flg, pgm1_flg, and sex1_flg. Raw data were preprocessed and filtered using the GCRMA algorithm (Wu and Irizarry, 2005) and 15,846 out of a total of 22,810 probe sets on the array could be used for differential expression analyses (Supplementary Table S1A).

We sorted the modules to identify flg22-responsive genes responding to C limitation. Modules ME3, ME9, ME11, and ME4 consisted of genes altered in all flg22-treated starch mutants compared to WT_flg, which would indicate that the change in expression level of these genes is general and sensitive to relatively mild C deficiency. Modules ME2, ME6, and ME7 contained genes significantly altered in pgm1_flg and sex1_flg when compared to those of ss4_flg. Since growth retardation is more severe in pgm1 and sex1 compared to ss4 in 12 L growth conditions, we considered that these genes exhibited some specificity for the starch defect or/and respond only to moderate C limitation (Fig. 4). Modules ME16, ME5, and ME14 consisted of genes altered only in pgm1_flg. These genes appeared to respond only to severe C limitation because pgm1 experiences the most severe C deficiency in the dark period among the three starch mutants. In summary, ten of the eighteen modules analyzed, composed of 2189 probe sets (66% of total 3341), could be sorted according to these criteria indicating that a significant number of flg22-responsive genes responds to C limitation (Fig. 4).

In addition, we further sorted these ten C limitation- and flg22-responsive gene modules (ME2-ME7, ME9, ME11, ME14, ME16) into two groups depending on magnitude and direction of flg22-induced expression changes in the different Arabidopsis lines. Group1 modules (ME3, ME2, and ME16) were found
to contain genes that display expression changes in starch mutants_flg vs WT_con that change in the same direction, but more dramatic than expression changes in WT_flg vs. WT_con (Fig. 4). Thus, Group 1 is likely to contain flg22 responsive genes required to increase the supply of C needed to establish PTI or under C deficiency conditions. Other modules (ME4, ME5, ME6, ME7, ME9, ME11 and ME14) were placed in Group 2 and contained genes that displayed less pronounced changes in expression in starch mutants_flg vs. WT_con compared to WT_flg vs. WT_con. The Group 2 modules could be further classified into two subgroups depending on whether lower expression is general (Group 2A) or preferential forte pgm1 defect (Group 2B).

**Group 1 modules are enriched in nutrient recycling-related genes and group 2B modules in defense-related genes**

In addition, we identified over-represented pathways (PATHs) and cellular components (CCs) (p-value < 0.01). Analyses of up-regulated genes showed that three PATHs (arginine and proline metabolism; cysteine and methionine metabolism; fatty acid metabolism) and two CCs (peroxisome; endosome granule) were over-represented in Group 1 modules (Supplementary Tables S2A and S3A). CCs related with peroxisome and endosome (glyoxysomal membrane; endosome membrane; endosomal part; early endosome) were further identified (p-value < 0.05). These results suggest that energy recycling mechanisms are activated in Group 1 because two of the over-represented PATHs (arginine and proline metabolism; fatty acid metabolism) and CCs (peroxisome and endosome) are intimately involved in nutrient recycling. In Group 2A, three over-represented PATHs (protein processing in endoplasmic reticulum; ribosome biogenesis in eukaryotes; phenylpropanoid bio-synthesis) and 17 over-represented CCs characteristic of different types of lumen were identified (Supplementary Tables S2B and S3B). In Group 2B modules, plant-pathogen interaction, phenylpropanoid biosynthesis, and arginine and proline metabolism were identified as over-represented PATHs (Supplementary Table S2C). Well-known defense genes (GLU1/NOHI, FRK1, RPM1/RPS3, CERK1/LY5M, RN4, and WRKY22) and phenylpropanoid genes (CAD-C, REF1, and UGT72E1) were included here. Over-represented CCs in Group 2B included endomembrane system, plasma membrane, cell periphery, integral to membrane, and exocyst (Supplementary Table S3C). Considered together with earlier analyses of our microarray data, which showed that Group 2B modules contain genes regulated by flg22 in an energy dependent manner, this result allows for the conclusion that defense-related genes that are regulated by flg22 to establish PTI in an energy dependent manner are over-represented in Group 2B modules.

Next, we examined Group 2B genes and identified kinases, defense-related, and transporter genes as three major classes that are observed among 305 up-regulated genes (Supplementary Tables S1C, S1D, and S1E). A total of 43 protein kinase genes were identified of which about half (21 genes) encode leucine-rich repeat (LRR) kinases and 8 genes encode cysteine-rich receptor-like kinases (CRK). Other classes of putative defense related genes encoded LRR receptors (8 genes), WRKY transcription factors (WRKY7, WRKY11, WRKY17, WRKY22, and WRKY47), MLO proteins (MLO2, MLO3, and MLO12), exocyst subunits (EXO7H1, EXO7H2, and EXO7E1), and chitinases (AT1G02360, AT3G54420, and AT4G01700). Twenty one genes encoding different types of transporters (sugar, nitrogen, iron, peptide, phospholipid, phosphate, calcium, cadmium, Lys/His, molybdate) were identified. Among these genes those encoding sugar and nitrogen transporters (6 and 4 genes, respectively) were particularly over-represented. It is worth noting that three genes that encode transporters of nitrate, urea, and ammonium, the major N sources, are highly energy dependent.

**C limitation negatively regulates flg22-induced SA related genes but positively regulates flg22-induced ET related genes**

SA, JA, and ET are phytohormones that regulate overlapping and distinct defense responses for different types of pathogen attack. Their roles in PTI have been characterized recently (Tsuda et al., 2009). Therefore, we analyzed the effect of starch metabolism mutations on the expression of genes involved in the biosynthesis of and signaling by these phytohormones in the plant response to flg22.

Genes required for biosynthesis of and signaling by SA and ET were significantly induced by flg22 treatment in WT plants (FRD p-value < 0.05). The induced genes encoded proteins involved in SA biosynthesis and its regulation (SID2, EDS5, EDS1 and SAG101), SA signaling (NPR3, NPR4, WRKY70, TGA1, TGA3, and TGA5), ET biosynthesis (ACS2, ACS6, ACS7, ACS8, and ACO4), ET signaling (CTR1) and ET receptors (ERS1 and EIN4) (Supplementary Table S1F). Only ACS10 and EIL1 were down-regulated by flg22 in WT plants. Except TGA1 which was overexpressed in flg22-treated starch mutants, all of the SA-related genes induced by flg22 in WT were less induced in starch mutants. ET related genes such as ACS2, ACS6, and ACS8 were overexpressed in starch mutants_flg compared to WT_flg. *HELIPE4*, a defense gene regulated by the ET/JA pathway, was induced by flg22 and displayed higher expression levels in starch mutants. A group of ET related genes that were not induced significantly by flg22 in WT (≤ 1.5-fold) were expressed highly in starch mutants_flg including receptors (ETR2 and ERS2), transcription factors (ERF1, ERF2, and ERF4), and ET/JA targets (PDF1.2 and β-CHI). Thus ET signaling is induced under C limitation suggesting that ET may mediate biological responses to C starvation. No clear pattern was evident among JA-related genes. Some genes (OPCL1, ACX1, and PEDI) were induced by flg22 in WT while some genes (AC04, AM11, and IR2) were reduced. Expression levels of some JA biosynthesis genes (LOX2, LOX6, AOS, AOC4, and OPCL1) and two genes encoding JA pathway targets (JR1 and JR2) were lower in starch mutant_flg vs WT_flg than in WT_flg vs WT_con, while the levels of other JA biosynthesis genes (LOX1, ACX5, AM11, and PEDI) were higher.

**ET biosynthesis and NAC genes are induced under C or energy limited growth**

Our analyses suggested that genes in the Group 1 modules were required to cope with C deficit, but are not involved in flg22-induced PTI (Fig. 4). Some ET biosynthesis and signaling genes (Supplementary Table S1F), and eleven NAC transcription factor genes were exclusively identified in Group 1, but not in Group 2 modules. ET- and NAC-mediated signaling pathways regulate senescence (Balazadeh et al., 2008). Because cellular processes induced during senescence and starvation are similar, we considered the induction of these ET- and NAC-mediated signaling pathways to supply the C/energy needed to establish PTI. To test this hypothesis and validate our microarray results, we performed qRT-PCR analyses of three ACC synthase genes (ACS2, ACS6, and ACS8) and three NAC genes (NAC047, NAC059/ORS1, and NAC092/ORE1) on the
same sample set used for microarray analyses (Fig. 5). ACS6 was computationally excluded from the target gene list for clustering analyses and does not belong to any group. However, it shows typical expression pattern of Group 1 genes (Supplementary Table S1F) and was included in the qRT-PCR analysis.

All target genes were induced by flg22 treatment in the WT and expression levels in starch mutants_flg were higher or similar compared to those in WT_flg in accordance with microarray results (Fig. 5A). Next we checked expression levels of these genes in mock-treated plants to eliminate flg22 effects. All ACS and NAC genes were expressed at higher levels in the starch mutants compared to WT (Fig. 5B), supporting our inference that most Group 1 genes would be induced under C deficit even in the absence of flg22. Lastly, treatment with the TCA cycle inhibitor, MFA, induced expression of all three ACS genes and two NAC genes (NAC059 and NAC092) whereas NAC047 was repressed (Fig. 5C). Our results suggest that these ET- and NAC-mediated signaling pathways may have a role in response to general C or energy deficiency.

**DISCUSSION**

*Timely supply of energy resources is essential to establish healthy PTI*

Plants store photosynthetic assimilates as leaf starch during the day and obtain the C supply to sustain metabolism and growth during night by degrading this starch. Thus nighttime starch degradation is a mechanism for avoiding C starvation under dark conditions. Because even weak starvation causes growth retardation (Smith and Stitt, 2007; Stitt et al., 2007), mutants defective in starch biosynthesis or degradation display dwarfism that can be alleviated by growth under continuous light (Caspar et al., 1985; 1991; Roldan et al., 2007). That is, the growth defect of starch mutants is not because of the alteration of starch metabolism per se, but because of the lack of C supply at night. To test our hypothesis that lack of C supply or C starvation would negatively impact PTI, we compared the PTI responses of starch mutants with those of WT plants under different growth conditions. We observed a dark-period dependent increase in susceptibility to *hrcC*, compromised FRK1 expression, and reduced callose deposition in three different types of starch mutants compared to WT (Fig. 5B), supporting our inference that most Group 1 genes would be induced under C deficit even in the absence of flg22. Lastly, treatment with the TCA cycle inhibitor, MFA, induced expression of all three ACS genes and two NAC genes (NAC059 and NAC092) whereas NAC047 was repressed (Fig. 5C). Our results suggest that these ET- and NAC-mediated signaling pathways may have a role in response to general C or energy deficiency.

Fig. 5. Comparison of the expression patterns of ACS and NAC genes in WT and starch mutants. (A) Effect of flg22 on target gene expression. Samples used for microarray analyses in Fig. 5 were analyzed by qRT-PCR to verify the reliability of microarray results. *, Student’s t-test, p < 0.05 vs. WT_con. (B) Flg22-independent effects on target gene expression. Mock treated ‘12 L Dark’ samples are the same as those used for the qRT-PCR analyses shown in Fig. 2A. *, Student’s t-test, p < 0.05 vs. WT_con. (C) Effect of MFA on target gene expression in WT. Mock treated samples are the same as those used for the qRT-PCR analyses depicted in Fig. 3B. The expression of these genes was measured relative to an internal UBQ10 control levels (means ± SE, n = 3). *, Student’s t-test, p < 0.05 vs. Con.
FRK1 expression and reduced deposition of callose are also observed in the starch mutants during the light period in 12 L growth (Fig. 2). However, the defects in PTI responses are stronger during the dark period than during the light period in 12 L and are absent in 24 L growth. This suggests that the starch mutants do not have innate defects in their photosynthetic apparatus or immune systems. Our microarray data suggest that photosynthesis may be affected in the starch mutants under 12 L. Data-mining analyses of down-regulated genes in Group 1 modules, which contains genes that display greater repression in starch mutants_flg compared to WT_flg, indicated overrepresentation of chloroplast-related CCs. This agrees with published microarray analyses showing that all genes encoding the small subunit of Rubisco and many genes encoding chlorophyll binding proteins decrease at the end of the night in the pgm1 mutant (Gibon et al., 2004). During the light period, pgm1 and ss4 display reduced photosynthesis (Gibon et al., 2004; Ragel et al., 2013). The changes in photosynthesis may produce lower amounts of carbon assimilates and energy during the day. A more rapid increase of soluble sugars during the early part of the light period in pgm1 and sex1 compared to WT is followed by stabilization or decrease towards the end of the daily light period in pgm1 and sex1 compared to a steady increase in WT (Caspar et al., 1991; Gibon et al., 2004). The phenomenon results from inhibition of C utilization during the early part of the daily light cycle and its recovery towards the end of the light period. Although a time course experiment has not been reported, sugar levels are higher in ss4 compared to WT at midday and this has been attributed to defective starch synthesis in ss4 (Roldan et al., 2007). These observations suggest that weakened PTI responses during the day as it is observed in the starch mutants may be caused by reduced photosynthetic activity or inhibition of C utilization as a result of nighttime C starvation.

The difference between compatible and incompatible plant-pathogen interactions is quantitative rather than qualitative, suggesting that an immediately available supply of energy resources is important to establish immune responses (Bonfig et al., 2006; Tao et al., 2003). We observed that flg22 pretreatment reduces the extent of hrcC growth in starch mutants as in WT, though the starch mutants still display higher susceptibility to hrcC compared to WT (Fig. 1A). This response contrasts with the complete blockage of the PTI response observed in Arabidopsis mutants that lack PAMP receptors or PTI signaling pathway components (Boutrot et al., 2010; Laluk et al., 2011; Lu et al., 2010; Zipfel et al., 2004). Analyses of FRK1 expression and callose deposition show that significant amounts of PTI responses are observed in starch mutants (Fig. 2). The partial inhibition of hrcC growth in starch mutants can therefore be explained as follows. Delayed or reduced supply of C and weakened PTI responses in the starch mutants during the dark period of the daily light/dark cycle will enhance bacterial growth in the starch mutants compared to WT. However a certain level of PTI will be established in starch mutants grown in a 24 h cycle, with or without flg22 pretreatment. The resulting PTI will inhibit bacterial growth partially.

**Coordinated rearrangement of gene expression occurs to provide resources to cope with starvation and to establish PTI**

Activation of the plant immune system is a costly process that utilizes energy and other resources of the host plant to produce and transport defense proteins and metabolites or to induce structural changes. Our physiological and molecular analyses showed that delayed or reduced supply of C compromises PTI responses. Microarray analyses further clarified the effects of C deficiency at the molecular level. Comparative clustering analyses between genes expressed in WT_flg and three different types of starch mutants_flg allowed classification of flg22-regulated genes into three subgroups: Group 1, Group 2A, and Group 2B.

Group 1 genes were induced by flg22 treatment in WT during night and their expression levels were higher in starch mutants_flg compared to WT_flg. Since the starch mutants experience C starvation during the night compared to WT, group 1 genes are likely induced by C depletion. Because our microarray analyses did not include mock-treated starch mutants, we tested this possibility by analyzing publicly available pgm1 microarray data (Thimm et al., 2004). A total of 3065 probe sets were found in common between these two microarray datasets. Of these, 1731 probe sets displayed more than 1.5-fold change in pgm1 vs. WT and the distribution of 1731 sets among the 18 modules was analyzed. The results show that the three Group 1 modules ME16, ME2, and ME3 contained the largest percentage of genes (97%, 73% and 54%, respectively) that exhibited similar changes in expression in the pgm1 mutant vs. WT_flg and pgm1 vs. WT datasets. This result shows that expression levels of most Group 1 genes are altered before flg22 treatment and this trend is maintained after flg22 treatment.

Rapid consumption of C to set up flg22-induced PTI or ‘hijacking’ of C by the pathogen may generate starvation status in the host plant and induce Group 1 genes involved in increasing C supply and/or reducing C usage. PATHs such as amino acid metabolism, valine/leucine/isoleucine degradation, plant hormone signal transduction, and endocytosis (p < 0.05, Supplementary Table S2A) were enriched in Group 1. Altered amino acid metabolism leads to mobilization of both C and N resources and recycling of cell wall components by endocytosis increases available C resources. Induction of hormone biosynthesis and signal transduction is commonly observed under different kinds of biotic and abiotic stress conditions (Less et al., 2011). Specifically our targeted analyses showed overall up-regulation of ET biosynthesis and signaling by flg22 treatment, which was manifested more strongly in starch mutants_flg (Supplementary Table S1F). Enhancement of ET signaling is observed under prolonged night periods and also under stress conditions (Less et al., 2011; Thimm et al., 2004). Accordingly, an increase of ACS gene expression was observed in starch mutants_flg grown in the dark and also when the TCA cycle was inhibited in WT (Figs. 5B and 5B). ET signaling may play important role in coping with various kinds of starvation conditions. Therefore, compromised PTI responses that have been reported in EIN2 mutants (Boutrot et al., 2010) may be attributed in part to the energy-supplying role of ET.

Reprogramming of starvation-induced gene expression appears to compromise PTI

Cellular processes induced during starvation and senescence are very similar. NAC and WRKY are major transcription factor families that regulate senescence (Balazadeh et al., 2008). Therefore, NAC and WRKY family transcription factors are likely to be induced by starvation as well as by PAMPs to cope with a shortage and heightened demand for C resources, respectively. Eleven NAC genes were identified in Group 1 modules, while no NAC genes were identified in the other groups. Four of the eleven NAC genes (NAC035/VNI2, NAC032/ORE1, NAC039/ORS1, NAC033/NTL4) encode functionally characterized senescence regulators (Balazadeh et al., 2011;
Changes in expression in the dark were observed in Group 1 genes while weak or no differences were observed in Group 1 modules in the percentage of genes down-regulated in the dark in starch mutants_flg relative to WT_flg, agree with previous reports which show that anabolism is repressed and catabolism is activated under various types of direct starvation conditions (Baena-Gonzalez et al., 2007; Contento et al., 2004; Thimm et al., 2004; Wang et al., 2007). In summary, we infer that induction of PATHs and CCs related to protein synthesis, protein processing, protein export, and phenylpropanoid biosynthesis by flg22 produce proteins and metabolites required for PTI. In contrast, reduced expression of these PATHs and CCs under starvation, as in starch mutants grown under 12 L photoperiod, will lead to compromised PTI responses (Fig. 6).

\section*{Reduced biosynthetic capacity contributes to compromised PTI responses in starch mutants}

Group 2A genes were induced in WT_flg vs. WT_con and expression levels were generally lower in starch mutants_flg vs WT_flag (Fig. 6). The difference was greatest in pgm1 that faces the most severe C depletion in the dark while weak or no difference was observed in ss4 and ssx1 mutants that are under weaker starvation conditions. In analyzing pgm1 microarray data, Group 2A modules (ME14, ME6, ME4 and ME5) ranked just below the Group 1 modules in the percentage of genes (53%, 44%, 35% and 29%, respectively) that showed similar changes in expression in the pgm1_flg vs. WT_flag and pgm1 vs. WT datasets. This result suggests that Group 2A modules contain a significant number of genes that also responded directly to C deficiency and that the response of these genes to C limitation is opposite to their response to flg22 treatment. PATHs and CCs related to protein synthesis, protein processing, protein export, and phenylpropanoid biosynthesis were over-represented in this group (p < 0.05, Supplementary Table S2B). The genes involved in protein and phenylpropanoid biosynthesis are over-represented Group 2A, that also contains genes down-regulated in the dark in starch mutants_flg relative to WT_flg, agree with previous reports which show that anabolism is repressed and catabolism is activated under various types of direct starvation conditions (Baena-Gonzalez et al., 2007; Contento et al., 2004; Thimm et al., 2004; Wang et al., 2007). In summary, we infer that induction of PATHs and CCs related to protein synthesis, protein processing, protein export, and phenylpropanoid biosynthesis by flg22 produce proteins and metabolites required for PTI. In contrast, reduced expression of these PATHs and CCs under starvation, as in starch mutants grown under 12 L photoperiod, will lead to compromised PTI responses (Fig. 6).

\section*{The induction of defense related genes necessary for establishing PTI requires C supply}

Group 2B contains genes that are induced by flg22 in a C-dependent manner (Fig. 6). Well-known defense genes and phenylpropanoid genes, protein kinases and transporter genes were contained in this group (Supplementary Tables S1C-S1E). Induction of sugar, nitrate, urea, ammonium and peptide transporter genes shows that C and N transporting mechanisms are activated by flg22 in order to meet increased demands by the plant. Induction of phenylpropanoid genes aids in reinforcing the cell wall and in the synthesis of defense-related molecules such as SA. Treatment of Arabidopsis with a specific PAL inhibitor reduces plant SA levels and enhances susceptibility to 

\textbf{Fig. 6.} Summary of microarray results explaining the effects of C deficiency on defense responses. Gene expression patterns and major over-represented pathways in each group are indicated. Genes related to senescence and nutrient recycling in Group 1 may provide resources to establish PTI; however, continual over-induction of these genes in starch mutants may compromise PTI responses by degrading defense related molecules. Group 2A and 2B contain genes related to biosynthesis and defense, respectively. Group 2 genes were weakly expressed in starch_flg compared to WT_flg, which contribute to weakened PTI responses in starch mutants.
of MLO2 and MLO12 in defense against powdery mildew has been characterized (Consonni et al., 2006; 2010). WRKY transcription factors play diverse roles in establishing immune system responses by inducing or repressing downstream genes (Eulgem and Somssich, 2007). EXO70B2 and EXO70H1 encode two exocyst subunits that are induced by elongation factor 18 (elf18), another PAMP, and play positive roles in defending against P. syringae pv. maculicola (Pecenkova et al., 2011). The induction of all Group 2B defense related genes discussed above for flg22 was compromised in starch mutants, which accounts for the weakened PTI responses of the starch mutants (Fig. 6).

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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