

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

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In the interaction between plants and pathogens, carbon (C) resources provide energy and C skeletons to maintain, among many functions, the plant immune system. However, variations in C availability on pathogen associated molecular pattern (PAMP) triggered immunity (PTI) have not been systematically examined. Here, three types of starch mutants with enhanced susceptibility to *Pseudomonas syringae* pv. tomato DC3000 *hrcC* were examined for PTI. In a dark period-dependent manner, the mutants showed compromised induction of a PTI marker, and callose accumulation in response to the bacterial PAMP flagellin, flg22. In combination with weakened PTI responses in wild type by inhibition of the TCA cycle, the experiments determined the necessity of C-derived energy in establishing PTI. Global gene expression analyses identified flg22 responsive genes displaying C supply-dependent patterns. Nutrient recycling-related genes were regulated similarly by C-limitation and flg22, indicating re-arrangements of expression programs to redirect resources that establish or strengthen PTI. Ethylene and NAC transcription factors appear to play roles in these processes. Under C-limitation, PTI appears compromised based on suppression of genes required for continued biosynthetic capacity and defenses through flg22. Our results provide a foundation for the intuitive perception of the interplay between plant nutrition status and pathogen defense.

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 transmitted *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 (cwiInv) 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 (Less et al., 2011).

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Although genetic and physiological evidence is limited in comparison to biochemical and molecular data, several reports support the importance of plant C supply over housekeeping functions in the defense against pathogens. Over-expression of yeast invertase in the apoplast of tobacco reduces plant growth, increases levels of sugars and defense proteins, while enhancing resistance to tobacco mosaic virus (Herbers et al., 1996). Conversely, transgenic suppression of endogenous tobacco cwl_{nv} leads to a delay of immune responses in response to a challenge by the oomycete *P. nicotianae* (Essmann et al., 2008). Furthermore, inhibition of cwl_{nv} with the pseudo-tetrasaccharide acarbose enhances susceptibility of *Arabidopsis* to *Pseudomonas syringae* pv. tomato DC3000 (*Pst*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_{nv} activity or by affecting mitochondrial function (Biemelt and Sonnewald, 2006; Block et al., 2010). Barley alcohol dehydrogenases 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 resistance or susceptibility 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 *starch-free 1*, lacks phosphoglucomutase, a key enzyme in starch biosynthesis accumulating 1-3% of the amount seen in wild type (Caspar et al., 1985). The *starch 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 *starch synthase 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 $\mu\text{mol}^{-1}\text{m}^{-2}\text{s}^{-1}$). Flg22 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* (*Pst*DC3000 *hrcC*) bacterial suspension (OD_{600nm} = 0.0005, 2.5×10^5 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 flg22 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 Quantimix SYBR kit (PhileKorea Technology). UBQ10 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 AtRTPrimer software (<http://atrtprimer.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 flg22, 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 *Pst*DC3000 *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*),

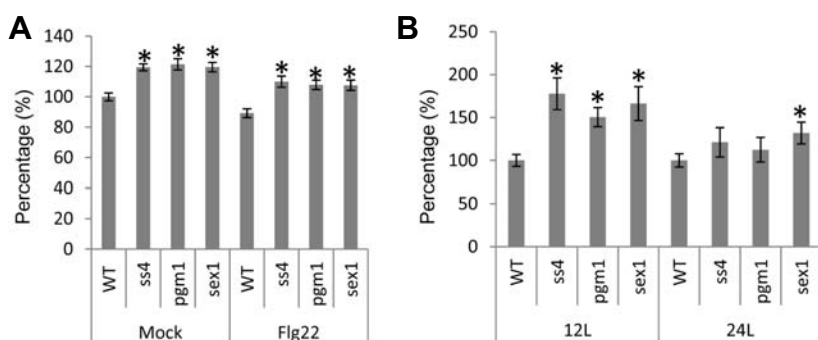


Fig. 1. Susceptibility of starch mutants to *PstDC3000 hrcC*. (A) Plants were grown in 12 L photoperiod for 5 weeks. Leaves were infiltrated with 10 mM $MgCl_2$ without (Mock) or with 1 μM flg22 supplementation (Flg22) and infiltrated with *hrcC* ($OD_{600nm} = 0.0005$) 1 day later. Bacterial growth was measured at 3 dpi. Three independent experiments displayed the same trend. In each experiment, bacterial growth in the mutants was calculated as a percentage of the growth in wild type (WT) (100%). Data from three independent assays were combined (means \pm SE, n =

9). *, Student's *t*-test, $p < 0.05$ vs. wild type (WT). (B) Plants were grown for 5 weeks in 12 L and for 3 weeks in 24 L photoperiod. Leaves were infiltrated directly with *hrcC* and bacterial growth was measured at 3 dpi. In each experiment, bacterial growth in the mutants was calculated as a percentage of the growth in wild type (WT) (100%). Data from two (24 L) or three (12 L) independent assays were combined (means \pm SE, n = 6 to 12). *, Student's *t*-test, $p < 0.05$ vs. wild type (WT).

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 $MgCl_2$ (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 $MgCl_2$ 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 *fls2* 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

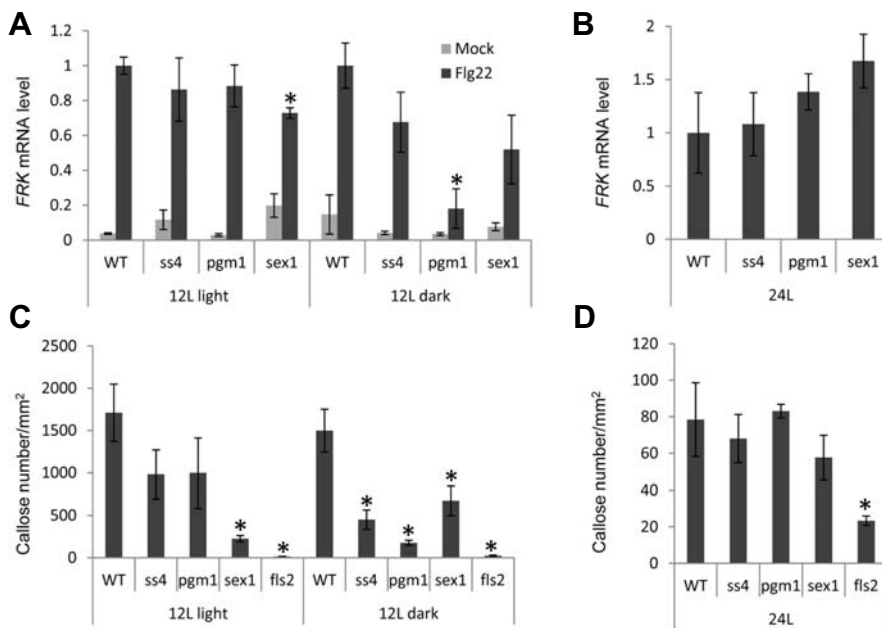


Fig. 2. Comparison of flg22-induced *FRK1* expression and callose deposition in WT and starch mutants. Analyses were performed on five-week-old plants grown under 12 L photoperiod and on three-week-old plants grown under 24 L photoperiod. Mock (10 mM MgCl₂) or flg22 (1 μM in 10 mM MgCl₂) was infiltrated into mature leaves and leaves were harvested 6 h after infiltration for analysis. For 12 L plants, infiltration was performed 2h after light turned on or off. (A, B) qRT-PCR analysis of *FRK1* transcript levels. *FRK1* expression was measured relative to an internal *UBQ10* control levels (means ± SE, n = 3). *, Student's *t*-test, *p* < 0.05 vs. wild type (WT). (C, D) callose analyses in flg22 treated leaves (means ± SE, n = 7-9). *, Student's *t*-test, *p* < 0.05 vs. wild type (WT).

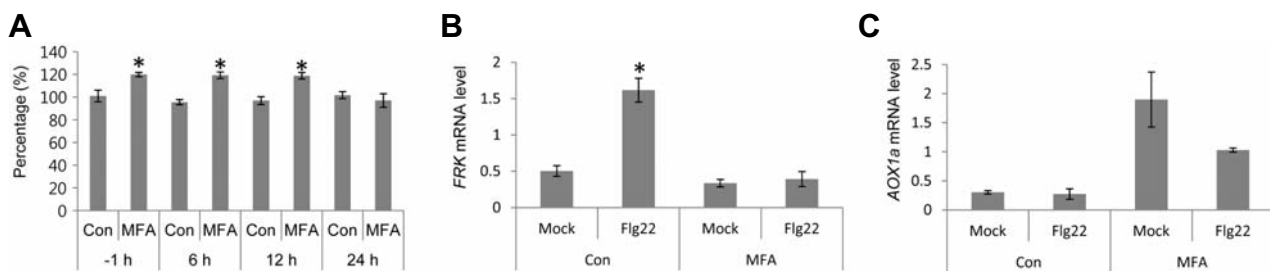


Fig. 3. MFA treatment affects bacterial growth and PTI marker gene expression. Mature leaves of three-week-old plants grown under 24 L were used. (A) 0.01% Tween 20 (Con) or 25 mM MFA in 0.01% Tween 20 (MFA) were sprayed on leaves at indicated times before (-) or after *Pst*DC3000 *hrcC* (OD_{600nm} = 0.0005) infiltration. Bacterial growth was measured at 2 dpi (means ± SE, n = 5). *, Student's *t*-test, *p* < 0.05 vs. control (Con). (B) and (C) qRT-PCR analysis of expression levels of *FRK1* and *AOX1a*, respectively. Expression of these genes was measured relative to an internal *UBQ10* control levels (means ± SE, n = 3). Con or MFA solutions were sprayed on leaves 1 h before Mock (10 mM MgCl₂) or Flg22 (1 μM in 10 mM MgCl₂) infiltration. Leaves were sampled 6 h after infiltration (means ± SE, n = 3). *, Student's *t*-test, *p* < 0.05 vs. control (Con).

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. *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

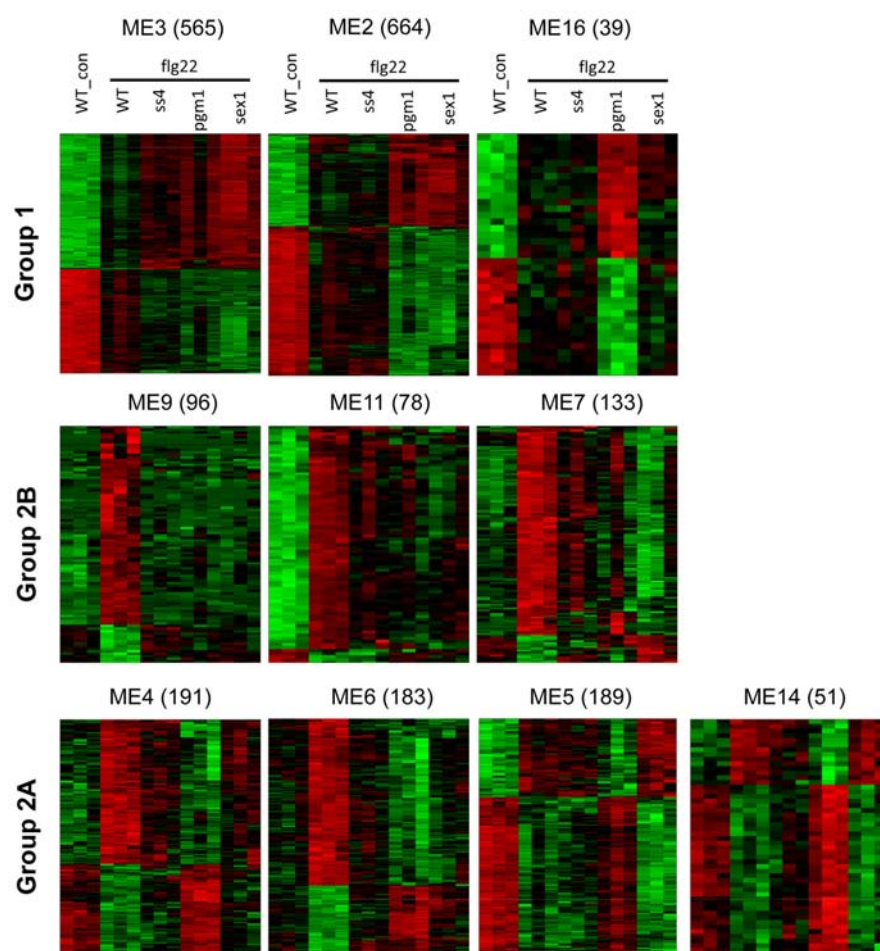


Fig. 4. Identification of modules of co-expressed genes that display carbon supply-dependent differential expression patterns. Module number (ME) and number of genes in the module (in parentheses) are indicated. Each module was divided into up- and down-regulated genes according to the flg22 response in WT. Group 1 modules contain genes whose expression changes in starch mutants_flg vs WT_con are toward the same direction but more dramatic than the expression change in WT_flg vs. WT_con. Group 2 modules contain genes that display less expression change in starch mutants_flg vs. WT_con compared to WT_flg vs. WT_con. Group 2A consists of modules that contain a significant percentage of genes that show similar changes in expression in this *pgm1_flg* vs WT_flg dataset and also in published *pgm1* vs WT datasets. Group2B modules do not contain a significant percentage of genes that show similar changes in expression in this *pgm1_flg* vs WT_flg dataset and published *pgm1* vs WT datasets.

(WT_con), WT treated with 1 μ M 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*, or *pgm1_flg*, or *sex1_flg* among the 5252 probe sets in Group 0 that constitute flg22-induced genes (WT_con vs. WT_flg). Clustering analyses using the WGCNA algorithm were employed to generate modules based on expression differences between the five samples (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). The 3341 genes were clustered into a total of 19 modules and module numbers (ME) were assigned in descending order according to the number of probe sets in each module. Thus, ME1 contained the largest number of probes (711) and ME18 contained

the smallest (27) number (Supplementary Table S1B).

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 for *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; interchromatin 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 biosynthesis) 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 (*GLI1/NHO1*, *FRK1*, *RPM1/RPS3*, *CERK1/LYSM*, *RIN4*, 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*. *HELIPR4*, 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* included 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 *PED1*) were induced by *flg22* in WT while some genes (*AOC4*, *AMI1*, and *JR2*) 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*, *AMI1*, and *PED1*) 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

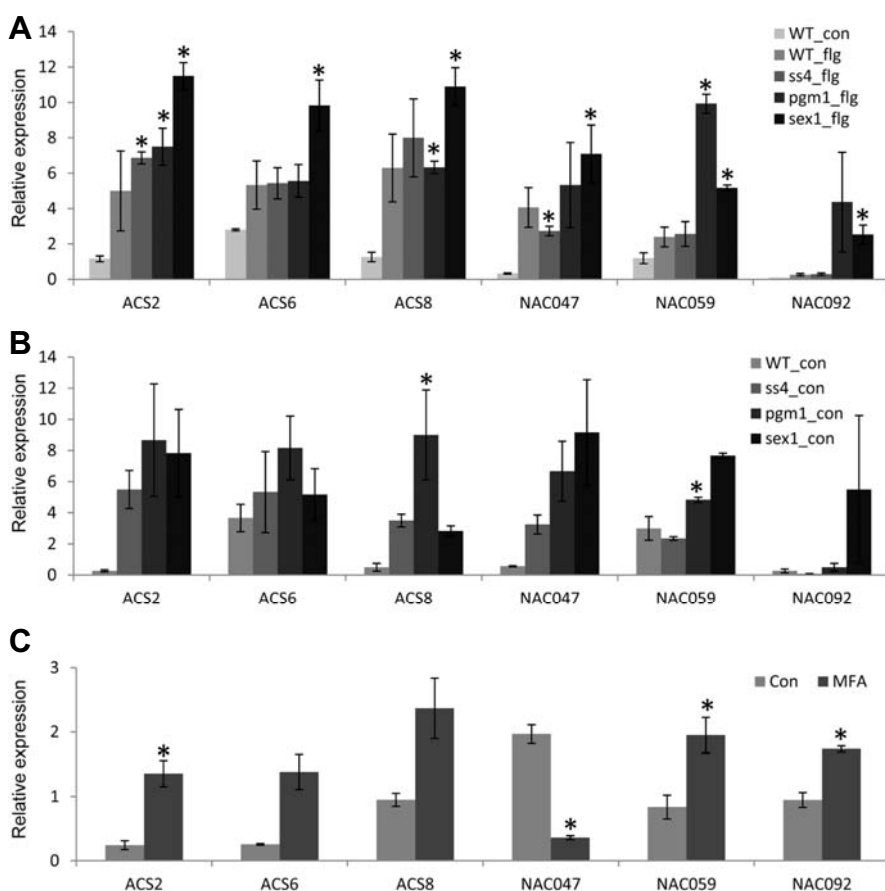


Fig. 5. Comparison of the expression patterns of ACS and NAC genes in WT and starch mutants. (A) Effect of flg22 on target gene expression. Samples used for microarray analyses in Fig. 5 were analyzed by qRT-PCR to verify the reliability of microarray results. *, Student's *t*-test, $p < 0.05$ vs. WT_con. (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 \pm SE, $n = 3$). *, Student's *t*-test, $p < 0.05$ vs. Con.

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 indicating that C depletion was the major cause of weakened PTI responses rather than defective starch metabolism (Figs. 1 and 2). However, an additional factor may be involved in the case of *ss4* because this mutant displayed similar level of PTI defect even though *ss4* experiences minor C starvation effect due to slow starch degradation during the night compared to *pgm1* and *sex1*. A recent report showed photooxidative damage to occur in the photosynthetic apparatus of *ss4*, which will then reduce C supply in the light (Ragel et al., 2013). The increase in susceptibility to *hrcC* and reduced induction of the PTI marker, *FRK1* associated with inhibition of the TCA cycle in WT plants also indicates the importance of energy supply for PTI (Fig. 3). Recently, Engelsdorf et al. (2013) showed that the susceptibility to *Colletotrichum higginsianum* was enhanced in *Arabidopsis* in prolonged dark conditions and in a starch mutant background.

Compromised PTI responses such as reduced induction of

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 publically 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 exhibit similar changes in expression in the *pgm1*_flg 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 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 (*NAC083/VNI2*, *NAC092/ORE1*, *NAC059/ORS1*, *NAC053/NTL4*) encode functionally characterized senescence regulators (Balazadeh et al., 2011;

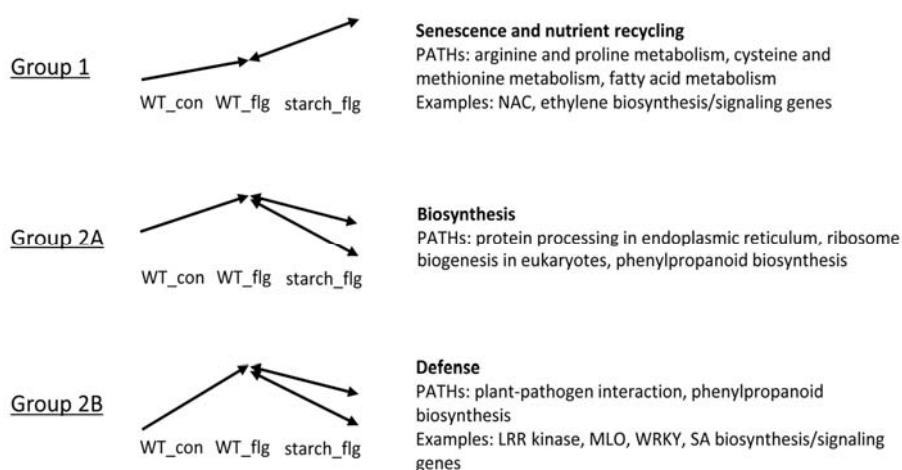


Fig. 6. Summary of microarray results explaining the effects of C deficiency on defense responses. Gene expression patterns and major over-represented pathways in each group are indicated. Genes related to senescence and nutrient recycling in Group 1 may provide resources to establish PTI; however, continual 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.

Lee et al., 2012; Oh et al., 1997; Yang et al., 2011). *NAC092/ORE1* is regulated by miR164 via ET signaling (Kim et al., 2009), suggesting that ET signaling may activate starvation responses by regulating NAC transcription factors. Six *WRKY* genes were identified in Group 1 as well. Of these, *WRKY26*, *WRKY25* and *WRKY33* have been shown to protect plants against heat stress (Li et al., 2011) and plants overexpressing these genes exhibit enhanced susceptibility to a bacterial pathogen (Zheng et al., 2005). Abiotic stress conditions that lead to global down-regulation of photosynthetic activity lead to starvation conditions in the plant (Less et al., 2011). Therefore our results suggest that the activation of nutrient recycling or senescence by increased expression of these *WRKY* proteins may enable plants to sustain their survival under heat stress but enhance sensitivity to pathogen because reduced biosynthetic capacity and increased catabolism might compromise PTI based on our results, discussed below. From this, it follows that temporal regulation of flg22-induced energy supplying pathways will be important for mounting an effective PTI. Activation of energy generating pathways at the initial stages of infection may be helpful to establish PTI; however, prolonged induction is likely to compromise PTI and promote disease (Fig. 6).

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_flg (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 *sex1* 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_flg 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).

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 metabolites such as SA. Treatment of *Arabidopsis* with a specific PAL inhibitor reduces plant SA levels and enhances susceptibility to *Peronospora parasitica* (Mauch-Mani and Slusarenko, 1996). Accordingly, our results showing three *PAL* genes (*PAL1*, *PAL2*, and *PAL4*) induced by flg22 treatment above the level seen in WT in the starch mutants (Supporting Information Table S1A) suggest that compromised induction of these genes will contribute to the reduction of SA biosynthesis and signaling in starch mutants (Supplementary Table S1F).

Many defense related proteins (Receptor-like proteins with/without LRR or with/without kinase domain, MLO proteins, WRKY transcription factors, and exocyst subunits) in Group 2B are predicted to play roles in establishing the immune system at the level of the plasma membrane or in endomembranes. FRK1 and RLK (AT2G37710) are receptor-like kinases induced by PAMPs and SA, respectively (Asai et al., 2002; Blanco et al., 2005) while CERK1/LYSM recognizes chitin oligosaccharide elicitor to induce PTI (Miya et al., 2007). *RPM1* encodes a NB-LRR protein and is known to mediate resistance to phytopathogenic *P. syringae* strains (Kiedrowski et al., 1992). The role

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 by 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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