Microbial Volatile-Induced Accumulation of Exceptionally High Levels of Starch in *Arabidopsis* Leaves Is a Process Involving NTRC and Starch Synthase Classes III and IV

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Submitted 5 May 2011. Accepted 1 June 2011.

Microbial volatiles promote the accumulation of exceptionally high levels of starch in leaves. Time-course analyses of starch accumulation in *Arabidopsis* leaves exposed to fungal volatiles (FV) emitted by *Alternaria alternata* revealed that a microbial volatile-induced starch accumulation process (MIVOISAP) is due to stimulation of starch biosynthesis during illumination. The increase of starch content in illuminated leaves of FV-treated *hy1/cry1*, *hy1/cry2*, and *hy1/cry1/cry2* *Arabidopsis* mutants was many-fold lower than that of wild-type (WT) leaves, indicating that MIVOISAP is subjected to photoreceptor-mediated control. This phenomenon was inhibited by cordycepin and accompanied by drastic changes in the *Arabidopsis* transcriptome. MIVOISAP was also accompanied by enhancement of the total 3-phosphoglycerate/Pi ratio, and a two- to threefold increase of the levels of the reduced form of ADP-glucose pyrophosphorylase. Using different *Arabidopsis* knockout mutants, we investigated the impact in MIVOISAP of downregulation of genes directly or indirectly related to starch metabolism. These analyses revealed that the magnitude of the FV-induced starch accumulation was low in mutants impaired in starch synthase (SS) classes III and IV and plastidial NADP-thioredoxin reductase C (NTRC). Thus, the overall data showed that *Arabidopsis* MIVOISAP involves a photo-controlled, transcriptionally and post-translationally regulated network wherein photoreceptor-, SSIII-, SSIV-, and NTRC-mediated changes in redox status of plastidial enzymes play important roles.

Plants produce starch as the predominant storage carbohydrate to cope with temporary starvation imposed by the environment. In leaves, starch is synthesized in the chloroplast during the day from photoassimilated carbon and degraded throughout the following night to support metabolism. This branched homopolysaccharide is produced by starch synthase (SS) using ADP-

glucose (ADPG) as the sugar donor molecule. Branch points are introduced by branching enzymes, and debranching enzymes are involved in tailoring the branched glucans into a form capable of crystallization. In leaves, starch breakdown is mainly hydrolytic, with maltose and glucose being the major starch-degradation products. As in bacteria, where glycogen acts as a carbon capacitor that regulates the carbon flow through different metabolic pathways in response to physiological needs (Belanger and Hatfull 1999; Guedon et al. 2000; Moreno-Bruna et al. 2001), starch acts as a major integrator of the plant metabolic status, its metabolism involving transcriptional and post-transcriptional regulatory mechanisms (Baena-González et al. 2007; Kleczkowski 1999; Michalska et al. 2009; Sehnke et al. 2001; Smith et al. 2004; Sokolov et al. 1998; Valerio et al. 2010).

Plants perceive biotic stimuli by recognizing a multitude of different signaling compounds originating from the interacting organisms. Some of these substances include carbohydrates, proteins, glycoproteins, peptides, lipids, and sterols that act as elicitors of defense reactions (Hahlbrock et al. 2003). Microbes also synthesize and emit many volatile compounds. Volatile emissions from rhizobacterial isolates of *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937, and *B. cepacia* promote growth in *Arabidopsis* plants by facilitating nutrient uptake, photosynthesis, and defense responses, and by decreasing glucose sensing and abscisic acid levels (Paré et al. 2005; Ryu et al. 2003, 2004; Vespermann et al. 2007; Zhang et al. 2008b, 2009). Recently, Kai and Piechulla (2009) have proposed that CO₂ emitted by these microbes acts as a plant-growth-promoting volatile. Physical contact with microorganisms adjusts plant defense reactions and very often leads to a decrease in photosynthesis and a transition from source to sink status in photosynthetic tissues. In some instances, the sucrolytic enzymes invertase and sucrose synthase (SuSy) are upregulated upon microbial contact (Bonfig et al. 2006; Chou et al. 2000; Fotopoulos et al. 2003; Michel et al. 2006; Scharte et al. 2005), which may serve to reduce sucrose export from the infected leaf to other parts of the plant or to promote cell wall polysaccharide biosynthesis at infection sites (Essmann et al. 2008). Physical contact with the pathogen may also result in downregulation of genes involved in starch metabolism (Cartieaux et al. 2008; Fabro et al. 2008),

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*The e-Xtra logo stands for “electronic extra” and indicates that four supplementary figures and three supplementary tables are published online.

which may facilitate the availability of simple sugars for the pathogen at infection sites.

Most studies on plant-microbe interactions have been carried out under conditions of physical contact between the host plant and the microbe. However, little is known about how microbial volatile emissions may affect plant physiology in the absence of physical contact. Given the lack of knowledge regarding how microbial volatiles may affect reprogramming of cellular metabolism in plants (in particular, primary carbohydrate metabolism), we have recently explored the effect on starch metabolism of volatiles released from different microbial species ranging from gram-negative and gram-positive bacteria to different fungi (Ezquer et al. 2010). We found that all microbial species tested (including plant pathogens and microbes normally not interacting with plants) emitted volatiles that strongly promoted starch accumulation in leaves of both mono- and di-cotyledonous plants when microbes were cultured in minimal media (Ezquer et al. 2010). Analyses of potato leaves exposed for 2 days to fungal volatiles (FV) emitted by the plant pathogen *Alternaria alternata* revealed that this phenomenon, the microbial volatile-induced starch accumulation process (MIVOISAP), was accompanied by dramatic changes in the leaf transcriptome and in activities of starch-related enzymes. To better understand MIVOISAP, in this work, we carried out time-course analyses of starch accumulation in leaves of *Arabidopsis* plants cultured in the presence or in the absence of FV emitted by *A. alternata*. We also analyzed transcriptomic changes in leaves of *Arabidopsis* plants upon FV treatment. In addition, using *Arabidopsis* T-DNA mutants, we investigated the impact in MIVOISAP of downregulation of genes directly involved in starch metabolism and genes whose expression is altered during MIVOISAP. The overall data showed that *Arabidopsis* MIVOISAP involves a photocontrolled, transcriptionally and post-translationally regulated network wherein SS class III- and IV- and NADP-Trx reductase (NTRC)-mediated changes in redox status of plastidial enzymes play important roles. Furthermore, we provide evidence that FV treatment activates plastidial β -amylase-dependent starch degradation during starch overaccumulation.

RESULTS AND DISCUSSION

Microbial volatiles promote transitory starch biosynthesis during illumination.

Leaves of plants cultured in a regime of 16 and 8 h of white light and darkness, respectively, accumulated exceptionally

Fig. 1. Time course of starch content in leaves treated of fungal volatiles (FV)-treated and nontreated *Arabidopsis* plants. Diamonds represent the starch content in leaves of FV-treated plants illuminated for 16 h with white light and then subjected to darkness for 56 h. Triangles represent the starch content in leaves of FV-treated plants cultured in darkness. Squares represent the starch content in leaves of FV-nontreated plants illuminated for 16 h. Results are the mean $\pm$ standard error of three independent experiments.

high levels of starch after 2 days of exposure to FV emitted by *A. alternata* (Ezquer et al. 2010). To know whether this phenomenon was the result of enhanced starch biosynthesis during illumination or reduced starch breakdown during the dark, we carried out a time-course analysis of starch content in leaves of FV-treated and nontreated *Arabidopsis* plants cultured in solid Murashige-Skoog (MS)-sucrose for 16 h of illumination under white light. As control, we measured the starch content in FV-treated plants cultured in solid MS sucrose medium under dark conditions. As expected, these analyses revealed that FV-treated plants did not accumulate starch during darkness (Fig. 1). Illuminated, FV-nontreated plants accumulated starch at a rate approximately 18 nmol of glucose transferred to starch per minute $\times$ gram fresh weight (FW) (Fig. 1). Notably, starch overaccumulation in leaves of illuminated plants was rapidly stimulated by FV treatment (Fig. 1). Judicious examination of the process revealed that the rate of starch accumulation during the initial 2 h of FV treatment consisted of glucose transferred to starch at 240 nmol/min $\times$ gram FW. After 2 h of exposure to FV, the rate of starch accumulation increased to glucose transferred to starch at 480 nmol/min $\times$ gram FW, which remained nearly constant during the following 14 h of FV treatment (Fig. 1). Time-course analyses of starch content during darkness in leaves of plants that had previously been cultured for 16 h in the presence of FV under white light revealed that starch content declined for glucose removed from starch at a rate of 450 nmol/min $\times$ gram FW when plants were cultured in darkness in the presence of FV (Fig. 1). After 32 h of darkness in the presence of FV, *Arabidopsis* leaves accumulated very low starch content, which indicates that starch breakdown machinery is active during darkness in FV-treated *Arabidopsis* leaves.

Thus, the overall data showed that *Arabidopsis* MIVOISAP is ascribed to stimulation of starch biosynthesis during illumination.

Arabidopsis MIVOISAP is subjected to photoreceptor-mediated control.

As well as providing the primary source of energy to support photosynthesis, light is one of the most important environmental signals governing many developmental and physiological aspects of plants (Chen et al. 2004). Light acts through photoreceptors to regulate a variety of responses including light-induced reserve starch degradation and carbon partitioning (Appenroth and Gabrys 2003; Keiller and Smith 1989). The blue-light-absorbing phototropins and cryptochromes (CRY), and the red- and far-red-light-absorbing phytochromes (PHY) are the principle families of photoreceptors occurring in higher plants. They direct rapid adaptational changes in gene expression in response to environmental light signals (Monte et al. 2004; Tepperman et al. 2001, 2006), the expression of genes coding for starch enzymes (such as APS1, APL1, SSIV, BAM3, and so on), and nitrogen metabolism (such as NR1) being subjected to PHY-mediated photocontrol (Tepperman et al. 2001). Photoreceptors also direct very rapid cytoplasmic responses (Elzenga et al. 2000; Kinoshita et al. 2001; Rösler et al. 2007; Takagi et al. 2003). Red and far-red light perception by PHY requires the phytochromobilin, which is covalently attached to the PHY apoprotein. In *Arabidopsis*, this chromophore is synthesized by the products of *HY1* and *HY2* genes (Davis et al. 1999; Kohchi et al. 2001). To investigate the possible involvement of light signaling in MIVOISAP, we compared the FV-promoted increase of starch content between the wild type (WT) and *hy2*, *cry2*, *hyl/cry1*, *hyl/cry2*, and *hyl/cry1/cry2*. The starch content increase in *cry2* leaves after 16 h of FV treatment was slightly lower than that of WT leaves (20- to 25-fold more starch in the presence than in the absence

of FV) (Fig. 2A), indicating that, independently, CRY does not exert a major effect on MIVOISAP. In clear contrast, an approximately 15-fold starch content increase occurred in *hy2* leaves, and only a 5- to 10-fold starch content increase occurred in *hyl/cry1*, *hyl/cry2*, and *hyl/cry1/cry2* mutants after 16 h of FV treatment. Thus, the overall data would indicate that MIVOISAP is subjected to the synergic action of both CRY and PHY. We also measured the FV-induced starch content increase in leaves of WT plants cultured under 16 h of continuous red, far-red, and blue light. These analyses revealed that the FV-induced starch content increase in plants cultured under continuous red light was comparable with that of plants cultured in white light (20- to 25-fold more starch in the presence of FV than in the absence of FV), whereas far-red and blue light promoted an approximately 3-fold increase of the normal starch content (Fig. 2B).

Transcriptome profile of *Arabidopsis* leaves during MIVOISAP.

The above results showing that MIVOISAP is subjected to photoreceptor-mediated control would suggest the possibility that MIVOISAP is regulated, at least in part, at the transcriptional level. To test this hypothesis, we carried out time-course analyses of starch content in detached *Arabidopsis* leaves cultured under white light in solid MS medium in the presence and absence of micromolar concentrations of cordycepin. These analyses revealed that this antibiotic exerted a marked inhibitory effect on starch accumulation after 2 to 3 h of FV treatment (Fig. 3), which would indicate that MIVOISAP is regulated, at least partially, at the transcriptional level. We then carried out a high-throughput transcriptome analysis of leaves

from *Arabidopsis* plants cultured in MS medium in the absence or in the presence of FV emitted by *A. alternata* for 16 h using the *Arabidopsis* Gene Expression Microarray 4 × 44K (G2519; Agilent Technologies, Santa Clara, CA, U.S.A.). In all, 1,155 genes were found to be deregulated in the presence of FV (more than 1.6-fold difference relative to control; *P* value < 0.05), 346 of them being annotated as “not function assigned”. Among this population, 418 genes were upregulated and 737 genes were downregulated (Supplementary Table 1 for a selected list of genes). Nearly 15% of the genes whose expression was altered after 16 h of FV treatment are target genes of SnRK1, a central integrator of transcription networks in plant stress and energy signaling (Baena-González and Sheen 2008; Baena-González et al. 2007), which would suggest that some processes occurring during MIVOISAP are regulated by SnRK1. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses of some of the identified genes (Supplementary Fig. 1) validated the results of the array analyses.

To determine the biological processes affected by microbial volatiles, an analysis of genes using the MapMan tool (Thimm et al. 2004) was carried out. This study revealed that, consistent with our previous studies using potato leaves (Ezquer et al. 2010), FV promoted changes in the expression of *Arabidopsis* genes involved in multiple processes such as metabolism of carbohydrates, amino acids, and lipids; redox status of the cell; development; cell wall biosynthesis; photosynthesis; secondary metabolism; protein translation and stability; vesicle trafficking; regulation of gene expression; signaling; energy production; plant defense; and stress responses (Fig. 4). No changes were observed in the expression of a number of genes that code for proteins directly or indirectly involved in starch and sucrose metabolism such as plastidial adenylate kinase, plastidial hexokinase, plastidial phosphoglucose isomerase, plastidial phosphoglucomutase, the small and large ADPG pyrophosphorylase (AGP) subunits (APS1 and APL1-4), ADPG pyrophosphatase, sucrose transporters, cytosolic phosphoglucose isomerase, cytosolic phosphoglucomutase, sucrose-phosphate synthase, sucrose-phosphate phosphatase, and invertases. Unlike potato leaves exposed to FV emitted by *A. alternata* (Ezquer et al. 2010), FV treatment did not exert major effects on the expression in leaves of genes coding for Calvin cycle enzymes, plastidial maltose and triose-P/3-phosphoglycerate/Pi translocators, alkaline pyrophosphatase, protease inhibitors, soluble SS, starch branching enzyme, plastidial β -amylases, starch phosphorylases, and plastidial ribosomal proteins.

Fig. 2. *Arabidopsis* is subjected to photoreceptor-mediated control. **A**, Fungal volatile (FV)-induced increase of starch content in leaves of wild-type (WT), *cry2*, *hy2*, *hyl/cry1*, *hyl/cry2*, and *hyl/cry1/cry2* *Arabidopsis* plants cultured for 16 h in solid Murashige-Skoog medium under white light. **B**, FV-induced increase of starch content in leaves of WT plants cultured under continuous white, red, far-red, and blue light. Graphics represent the ratio between starch content in leaves of FV-treated and nontreated plants. Results are the mean $\pm$ standard error of three independent experiments. Starch content in the leaves of FV-nontreated WT plants cultured under white, red, far red, and blue light and of mutants cultured under white light are shown in Supplementary Figure 4.

Fig. 3. Time course of starch content in fungal volatile (FV)-treated detached *Arabidopsis* leaves cultured under white light in solid Murashige-Skoog medium supplemented or not with 30 μ M cordycepin. Diamonds and squares represent the starch content in leaves cultured in the absence or presence, respectively, of cordycepin. Results are the mean $\pm$ standard error of three independent experiments.

3-Phosphoglycerate/Pi ratio and levels of reduced AGP form are enhanced during MIVOISAP.

AGP is involved in the production of ADPG linked to starch biosynthesis. This enzyme is allosterically activated by 3-phosphoglycerate (3PGA) and inhibited by Pi (Kleczkowski 1999). Allosteric modulation of AGP is considered to represent an important determinant of the starch biosynthesis rate in photosynthetic tissues, although several reports failed to show a correlation between stromal 3PGA levels and starch content (Geigenberger et al. 1996; Westrarm et al. 2002). Time-course analyses of FV-treated and nontreated *Arabidopsis* leaves revealed that the total 3PGA/Pi ratio increased after 6 h of FV treatment, which is mainly due to accumulation of 3PGA (Fig. 5). AGP activity is also subjected to redox regulation of the small AGP subunit (APS1) (Hendriks et al. 2003; Kolbe et al. 2005; Michalska et al. 2009). To investigate whether MIVOISAP is accompanied by changes in the redox status of APS1, we carried APS1 immunoblot analyses of proteins from FV-treated and nontreated WT leaves that had previously been extracted and electrophoretically separated under nonreducing

conditions. In these conditions, APS1 is present as a mixture of 50-kDa active (reduced) monomers and 100-kDa inactive (oxidized) dimers formed by intermolecular links involving Cys bridges (Hendriks et al. 2003; Kolbe et al. 2005) (discussed below). Consistent with the findings of Hendriks and associates (2003), these analyses revealed that most of APS1 is largely oxidized (inactive) in FV-nontreated leaves (Fig. 6A). Unlike potato leaves (Ezquer et al. 2010), a two- to threefold increase in the relative content of the monomeric APS1 form was observed when plants were treated with FV, although most of APS1 still remained in the oxidized form (Fig. 6A; Supplementary Fig. 2). Notably, the process of FV-promoted APS1 reduction was rapid (Fig. 6B) and took place in both the presence and absence of light (Fig. 6A).

Western blot analyses of proteins extracted and electrophoretically separated under reducing conditions revealed no apparent difference in APS1 amount between FV-treated and nontreated leaves (Fig. 6C), which is consistent with the transcriptomic analyses showing that MIVOISAP is not accompanied by changes in *APS1* expression.

Fig. 4. Functional categorization of the transcripts differentially expressed in leaves of *Arabidopsis* plants cultured in solid Murashige-Skoog in the presence of fungal volatiles (FV) emitted by *Alternaria alternata*. Transcripts were identified using the *Arabidopsis* Gene Expression Microarray 4 × 44K (G2519; Agilent Technologies). Significantly down- and upregulated transcripts (1.6-fold change) as compared with control were sorted by their putative functional category according to the MapMan software. The number of deregulated genes in each categorical group is presented here. Upregulated genes are in white bars and downregulated genes are in black bars. Genes from the “no hits found” category were not included in the graphic.

NADPH thioredoxin reductase is an important determinant of MIVOISAP.

Regulation of protein function by changes in redox status plays a major role in multiple aspects of plant life such as

Fig. 5. Time course of **A**, 3-phosphoglycerate (3PGA); **B**, Pi; and **C**, 3PGA/Pi ratio in leaves of fungal volatile (FV)-treated (○) and nontreated (●) wild-type *Arabidopsis* plants for 16 h of illumination under white light and 8 h of darkness. Results are the mean ± standard error of three independent experiments.

growth, development, and response to environmental constraints. Illumination results in a very rapid, PHY-mediated activation of proton-pumping plasma membrane ATPases which, in turn, leads to rapid changes of membrane potentials, ion fluxes, and internal cellular redox status (Crane et al. 1991). Illumination also affects the redox status of the photosynthetic electron transport chain in the chloroplast that, in turn, affects the activity of numerous enzymes. Chloroplasts possess two important redox systems that independently regulate plastid metabolism by supplying reducing equivalents to target enzymes. One is based on thioredoxins (Trx) and the other pathway is based on the peculiar NTRC. Whereas the Trx-dependent pathway obtains reducing power from ferre-

Fig. 6. Western blot analysis of ADP-glucose pyrophosphorylase in fungal volatile (FV)-treated and nontreated wild-type leaves whose proteins were extracted and electrophoretically separated under **A** and **B**, nonreducing conditions and **C**, reducing conditions. **A**, Proteins from leaves of illuminated and nonilluminated plants cultured in the presence or absence of FV. Note that, regardless of the presence of light, the relative content of the monomeric (approximately 50 kDa) form increases when plants were exposed to FV. **B**, Proteins from nonilluminated leaves and from leaves illuminated for 30 min, 1 h, and 16 h in the presence or absence of FV. **A** and **C**, Leaves were harvested after 16 h of FV treatment; **A** and **B**, proteins coming from 1 mg of FW were loaded per lane; **C**, 20 µg of protein was loaded per lane.

doxin (Fdx) reduced by the photosynthetic electron chain and mediated by Fdx-dependent Trx reductase (FTR) (Lemaire et al. 2007; Meyer et al. 2008), NTRC uses NADPH as the reducing power source, which can be produced in darkness from

Fig. 7. NADP-thioredoxin reductase C (NTRC) is involved in microbial volatile-induced starch accumulation process (MIVOISAP). Fungal volatile (FV)-induced increase of starch content in leaves of wild-type (WT) and *ntrc* mutants cultured for **A**, 1 and 2 h and **B**, 16 h in solid Murashige-Skoog medium under white light. The graphic represents the ratio between starch content in leaves of FV-treated and nontreated plants. Results are the mean $\pm$ standard error of three independent experiments. **C**, Western blot analysis of ADP-glucose pyrophosphorylase (AGP) in FV-treated and nontreated *ntrc* leaves whose proteins were extracted and electrophoretically separated under nonreducing conditions. Note that, unlike the case of WT leaves, redox status of AGP was not altered upon 16 h of exposure to FV.

G6P via the oxidative pentose phosphate pathway (OPPP), or obtained in the light from reduced Fdx via Fdx-NADP reductase (Pérez-Ruiz and Cejudo 2009). Trx may regulate starch metabolism through posttranslational redox changes of enzymes involved in starch breakdown such as SEX1 (Mikkelsen et al. 2005) and BAM1 (Sparla et al. 2006; Valerio et al. 2010). NTRC plays important roles in supplying reductant for detoxifying hydrogen peroxide in the dark and in regulating the redox status of AGP (Michalska et al. 2009; Pulido et al. 2010) (discussed above).

One of the most striking alterations in the transcriptome of *Arabidopsis* leaves treated with FV emitted by *A. alternata* involves the activation of plasma-membrane-type proton ATPases, as well as repression of plastidial FTR and various Trx. The former would result in changes of membrane potentials, ion fluxes, and internal cellular redox status. The latter would result in a partial blockage of the main link between light absorbed by chlorophylls and metabolic activity in the plastid (the Fdx/Trx system) which, in turn, would favor the NTRC pathway as an alternate system for transferring reducing equivalents to target enzymes. Whether plastidial Trx and NTRC play a role in *Arabidopsis* MIVOISAP was examined by measuring the starch content in *ntrc* and the plastidial Trx f1, f2, y1, m2, and m3 mutants cultured in the absence or presence of FV for 16 h. These analyses revealed that the FV-induced increase of starch content in the plastidial Trx mutants was comparable with that of WT plants (20- to 25-fold more starch in the presence than in the absence of FV). Notably, FV did not promote starch accumulation in the leaves of *ntrc* mutants during the first 2 h of FV treatment (Fig. 7A), and only an approximately 15-fold increase of starch content occurred in the leaves of *ntrc* mutants after 16 h of FV treatment (Fig. 7B). Thus, the overall data strongly indicated that, i) independently, Trx f1, f2, y1, m2, and m3 do not exert a major control on MIVOISAP; and ii) NTRC is an important determinant of *Arabidopsis* MIVOISAP, especially during the initial 2 h of FV treatment. The fact that FV do not promote starch accumulation in *ntrc* mutants during the initial 2 h of treatment is consistent with the idea that NTRC regulates MIVOISAP at the post-translational level.

Recent studies have shown that NTRC adjusts the redox status of several target proteins involved in different metabolic or signaling pathways (Pulido et al. 2010). Results strongly indicated that FV promote partial AGP reduction (Fig. 6). To investigate whether this phenomenon is mediated by NTRC, AGP Western blot analyses of FV-treated and nontreated *ntrc* mutants were carried out under nonreducing conditions. These analyses revealed that, unlike WT leaves (Fig. 6A), no changes in the redox status of AGP occurred when *ntrc* mutants were exposed to FV (Fig. 7C), the overall data indicating that FV-promoted AGP reduction is exclusively mediated by NTRC.

SS classes III and IV are major determinants of *Arabidopsis* MIVOISAP.

Five distinct classes of SS are known in plants: granule-bound SS (GBSS), which is responsible for the synthesis of amylose; and soluble SS classes I, II, III, and IV (SSI, SSII, SSIII, and SSIV, respectively), which are responsible for the synthesis of amylopectin. Roldán and associates (2007) demonstrated that elimination of SSIV resulted in the accumulation of only one large starch granule in *Arabidopsis* chloroplasts. Furthermore, using different combinations of SS mutations in SSIII and SSIV mutant backgrounds, Szydlowski and associates (2009) have recently shown that autotrophically grown *Arabidopsis* SSIII/SSIV double mutants accumulated null starch levels, whereas SSI/SSII/SSIII triple T-DNA mutants accumulated nearly 20% of the WT starch levels. Their data i)

indicated that both SSIII and SSIV play a key role in starch accumulation, although SSIV is mandatory to render the regular number of starch granule found in WT plants; and ii) suggested that SSIV plays a crucial role in the starch granule initiation process.

Our transcriptome analyses of leaves of FV-treated *Arabidopsis* plants revealed no changes in the expression of *SSI*, *SSII*, *SSIII*, *SSIV*, and *GBSS*, showing that changes in SS-encoding gene expression play a minor role, if any, in *Arabidopsis* MIVOISAP. Whether SS are involved in MIVOISAP was investigated by measuring starch content in leaves of homozygous *GBSS*, *SSI*, *SSII*, *SSIII*, *SSIV*, *SSI/SSIV*, *SSII/SSIV*, *SSIII/SSIV*, *SSI/SSII/SSIII*, and *SSI/SSII/SSIV* T-DNA mutants cultured, for 16 h, in the presence or in the absence of FV emitted by *A. alternata*. These analyses revealed that the FV-induced increase of starch content in leaves of *GBSS*, *SSI*, and *SSII* T-DNA mutants was normal when compared with WT leaves (20- to 25-fold more starch in the presence than in the absence of FV). In clear contrast, the increase of leaf starch content in FV-treated *SSIII*, *SSIV*, *SSI/SSIV*, *SSII/SSIV*, and *SSI/SSII/SSIV* T-DNA mutants was markedly lower than in FV-treated WT leaves (Fig. 8). Furthermore, FV treatment did not promote starch accumulation in *SSIII/SSIV* and *SSI/SSII/SSIII* T-DNA mutants. Thus, the overall data indicated that SSIII and SSIV are important determinants of *Arabidopsis* MIVOISAP. Taking into account that *SSIII* and *SSIV* expression levels do not change upon FV treatment, it is inferred that, similar to AGP (Fig. 6), both enzymes are in excess and largely inactive in FV-nontreated plants. Furthermore, the results indicate that SSIII and SSIV are activated after FV treatment by yet-to-be-identified post-translational mechanisms.

Fungal volatiles promote decay of amino acid biosynthesis in *Arabidopsis* leaves.

Starch and glycogen are the main storage carbohydrates in plants and bacteria, respectively, their metabolism being tightly connected with that of nitrogen (amino acid) by still poorly understood mechanisms. In plants, nitrate represses starch metabolism in leaves (Scheible et al. 1997). In bacteria, a decreased demand in ATP-dependent protein synthesis and breakdown takes place under conditions of limited amino acid provision, with excess ATP and carbon being diverted from protein metabolism toward glycogen biosynthesis (Eydallin et al. 2007; Montero et al. 2009; Wilson et al. 2010).

Leaves of *Arabidopsis* plants exposed to FV accumulated markedly reduced levels of Gly (1.15 ± 0.19 $\mu\text{mol/g}$ FW in the absence of FV versus 0.52 ± 0.08 $\mu\text{mol/g}$ FW in the presence of FV) and Arg (1.35 ± 0.2 $\mu\text{mol/g}$ FW in the absence of FV versus 0.58 ± 0.15 $\mu\text{mol/g}$ FW in the presence of FV), and moderately reduced levels of Ser (2.62 ± 0.23 $\mu\text{mol/g}$ FW in the absence of FV versus 2.13 ± 0.10 $\mu\text{mol/g}$ FW in the presence of FV). MIVOISAP was also accompanied by a marked increase of Pro (2.20 ± 0.27 $\mu\text{mol/g}$ FW in the absence of FV versus 3.63 ± 0.39 $\mu\text{mol/g}$ FW in the presence of FV), an amino acid accumulating in response to environmental stress that plays important roles in stabilizing the redox balance and energy status of the cell (Szabados and Savaure 2009). Nitrate reductase (NR) is a cytosolic enzyme playing a key role in nitrate assimilation and amino acid biosynthesis. It is highly regulated, being inactivated by SnRK1-mediated phosphorylation (Kaiser and Huber 2001; Lillo et al. 2004). Quantitative RT-PCR and array analyses revealed that, similar to potato leaves (Ezquer et al. 2010), *Arabidopsis* MIVOISAP is accompanied by downregulation of *NR1* expression (2.44-fold reduction) which, in turn, was accompanied by a concomitant reduction of NR activity (100.8 ± 8.3 mU/g FW and 59 ± 3.35 mU/g FW in the absence and presence of FV, respectively). These

observations prompted the hypothesis that, similar to bacteria, a decreased demand of ATP and carbon occurring during impairment in internal amino acid provision would result in starch overaccumulation. To investigate this possibility, we measured the starch content in detached *Arabidopsis* leaves cultured in the presence and in the absence of FV emitted by *A. alternata*, and in the presence of different amino acids. Most notably, these analyses revealed that, similar to potato leaves (Ezquer et al. 2010), MIVOISAP was strongly repressed by exogenously added Cys whereas other amino acids such as Ser and Gly did not exert a negative effect on MIVOISAP (Fig. 9). We also measured the starch content in leaves of *NR1::T-DNA* mutants and in leaves of *OAS-A1* and *OAS-B* T-DNA mutants impaired in cytosolic and plastidial Cys biosynthesis, respectively (Bermúdez et al. 2010). These analyses revealed that these mutants accumulated nearly WT starch content in both the presence and absence of FV (Supplementary Figs. 3 and 4). Thus, the overall data would indicate that, whereas MIVOISAP appears to be the consequence, at least in part, of inadequate internal Cys provision, changes in the expression or action of *NR1*, *OAS-A1*, and *OAS-B* do not independently exert a major effect on MIVOISAP.

Trehalose and trehalose-6-phosphate are not involved in *Arabidopsis* MIVOISAP.

Trehalose is a disaccharide that occurs both in a large variety of microorganisms and, in trace amounts, in plants. Its precursor, trehalose-6-P (T6P), is indispensable for the regulation of growth and starch biosynthesis (Kolbe et al. 2005; Lunn et al. 2006). Altering trehalose metabolism results in dramatic pleiotropic effects, such as morphological changes, growth defects, and altered carbon metabolism (Schluepmann et al. 2003). External application of trehalose for 10 days exerts dramatic effects on both growth and starch metabolism in *Arabidopsis* plants, severely curtailing root and shoot growth, and causing starch overaccumulation in cotyledons and leaves (Fritzius et al. 2001; Ramon et al. 2007; Wingler et al. 2000). This phenomenon is accompanied by an increase of *APL3* expression and reduction of the expression levels of genes involved in starch breakdown such as *SEX1* and *BAM3*. Schluepmann and associates (2004) have proposed that T6P rather than trehalose causes the observed effects on carbon metabolism. A positive effect exerted

Fig. 8. Fungal volatile (FV)-induced increase of starch content in leaves of wild-type plants and starch synthase (SS) class *SSIII*, *SSIV*, *SSI/SSIV*, *SSII/SSIV*, *SSIII/SSIV*, *SSI/SSII/SSIV*, and *SSI/SSII/SSIII* T-DNA mutants cultured for 16 h in solid Murashige-Skoog medium under white light. The graphic represents the ratio between starch content in leaves of FV-treated and nontreated plants. Results are the mean $\pm$ standard error of three independent experiments.

by T6P on starch accumulation has been ascribed to its effect on the plastidial Trx-mediated posttranslational redox activation of AGP (Kolbe et al. 2005). We investigated whether changes in trehalose metabolism play a major role in MIVOISAP by measuring starch content in FV-treated and nontreated *Arabidopsis* plants cultured for 16 h under white light in solid MS medium supplemented with either 30 mM trehalose or sucrose. Furthermore, we measured the starch content in FV-treated and nontreated detached *Arabidopsis* leaves cultured directly in contact with solid MS medium supplemented with either 30 mM trehalose or sucrose. These analyses revealed that starch levels in both FV-nontreated detached leaves and leaves of plants cultured for 16 h in MS supplemented with trehalose were comparable with or even lower than those of FV-nontreated detached leaves and leaves of plants cultured in MS sucrose (Fig. 10), indicating that trehalose or T6P metabolism is not involved in MIVOISAP.

Trehalose biosynthesis involves a two-step process catalyzed by T6P synthase (TPS) and T6P phosphatase (TPP). Of the 11 annotated *Arabidopsis* TPS genes, our transcriptomic analyses revealed that *TPS5* expression was upregulated (2.10-fold increase), whereas *TPS8*, *TPS9*, and *TPS11* expression was downregulated (2.01-, 1.63-, and 1.98-fold reduction, respectively) when plants were cultured in the presence of FV emitted by *A. alternata*. We measured the starch content in homozygous *TPS1*, *TPS5*, *TPS9*, and *TPS11* T-DNA mutants cultured in the presence or absence of FV. The FV-induced increase of starch content in these mutants was comparable with that of WT plants (20- to 25-fold increase upon FV treatment). Thus, the overall data would further indicate that changes in trehalose metabolism play a minor role, if any, in MIVOISAP.

Plastidial β -amylase-mediated starch breakdown is activated during MIVOISAP.

Of the nine β -amylase-like proteins encoded in the *Arabidopsis* genome (BAM1 to BAM9), only BAM1 to BAM4 are plastidial and, thus, have direct access to starch (Fulton et al. 2008; Lao et al. 1999; Sparla et al. 2006). BAM1 degrades starch during the day in both mesophyll and in guard cells subjected to heat shock and osmotic stress (Kaplan and Guy 2004; Valerio et al. 2010). BAM3 is a major determinant of leaf starch degradation during the night (Fulton et al. 2008), also

playing an important role in starch degradation during the day upon cold shock (Kaplan and Guy 2005). BAM4 is a noncatalytic protein required for starch breakdown, acting upstream of BAM1 to BAM3 (Fulton et al. 2008). Unlike plastidial β -amylases, the precise roles of extraplastidial β -amylases and the plastidial α -amylase AMY3 in starch metabolism are still unknown. Although Laby and associates (2001) showed that mutants impaired in BAM5 accumulate normal starch levels, more recent studies have shown that *bam5* mutants can display a starch-excess phenotype (Kaplan and Guy 2005). Furthermore, although Yu and associates (2005) provided evidence that AMY3 is not required for transitory starch breakdown, Kaplan and Guy (2005) showed that *amy3* mutants accumulate more starch than WT leaves.

Isoamylase 3 (ISA3) is a debranching enzyme that participates in β -amylolytic starch degradation by releasing maltodextrins into the stroma (Wattebled et al. 2005). Our transcriptome analyses revealed that FV promote the expression of *ISA3* (1.72-fold increase). These analyses also revealed a marked increase of *BAM5* expression upon FV treatment (4.81-fold increase), which was accompanied by a 3.1-fold increase of the total β -amylase activity (11.8 ± 2.1 and 37.0 ± 3.2 U/g FW in the absence and presence, respectively, of FV for 16 h). Therefore, FV-induced enhancement of total β -amylase activity can be ascribed to upregulation of *BAM5*. Whether plastidial amylases or BAM5 are major determinants of MIVOISAP was investigated by measuring the starch content in leaves of FV-treated and nontreated homozygous *BAM4*, *BAM5*, and *AMY3* T-DNA mutants cultured for 16 h under white light. We also measured the starch content in *sex1* mutants lacking a plastidial α -glucan, water dikinase that catalyzes the phosphorylation of starch necessary for normal β -amylase-dependent starch breakdown (Edner et al. 2007). FV-nontreated *bam5* and *amy3* leaves accumulated WT starch content, whereas FV-nontreated *bam4* and *sex1* leaves accumulated approximately fivefold more starch than WT leaves (Fig. 11A). Unlike *amy3* and *bam5* leaves accumulating WT starch levels upon 16 h of FV treatment (20- to 25-fold more starch in the presence than in the absence of FV), FV-treated *sex1* and *bam4* leaves accumulated approximately 30-fold more starch than WT leaves (Fig. 11A), suggesting the possible occurrence of plastidial β -amylase-mediated starch breakdown during MIVOISAP. To in-

Fig. 9. Microbial volatile-induced starch accumulation process is strongly repressed by exogenously added Cys. Fungal volatile (FV)-treated and nontreated detached *Arabidopsis* leaves were cultured in solid Murashige-Skoog supplemented with the indicated concentrations of amino acids. After 16 h of culture, leaves were harvested and starch content was measured. Results are the mean $\pm$ standard error of three independent experiments.

investigate this hypothesis, we measured the content of maltose (the product of the β -amylase reaction) in WT and *BAM4* T-DNA mutants cultured in the presence and absence of FV. Notably, these analyses revealed that maltose rapidly accumulated after 2 h of FV treatment in WT leaves and, to a much lesser extent, in *bam4* leaves (Fig. 11B). Thus, the overall data strongly indicated that i) plastidial β -amylase-dependent starch degradation is rapidly activated upon FV treatment and ii) starch biosynthesis and breakdown simultaneously occur during MIVOISAP. Because expression levels of plastidial β -amylases do not change upon FV treatment, it is inferred that plastidial β -amylase is in excess and largely inactive in FV-nontreated plants, and activated after FV treatment by as-yet-to-be-identified post-translational mechanisms.

Additional remarks and proposal of an integrative model of starch metabolism during MIVOISAP.

Plants have evolved multiple defense mechanisms against microbial pathogens. Contact with microorganisms or elicitors very often leads to very rapid activation of ion fluxes, phos-

phorylation or dephosphorylation of proteins, and production of signaling molecules that lead to the regulation of gene expression and the induction of defense responses, including production and accumulation of secondary metabolites and transition from source to sink status in photosynthetic tissues (Berger et al. 2007). Indications of the induction to a sink status in infected leaves are the upregulation of SuSy (Eckey et al. 2004; Michel et al. 2006), which may serve to allocate sucrose into callose deposition and to promote cell wall polysaccharide biosynthesis at infection sites (Essmann et al. 2008), although a recent report has provided evidence that SuSy is not required for cell wall biosynthesis in *Arabidopsis* (Barratt et al. 2009). Furthermore, contact with microorganisms or elicitors may also result in downregulation of primary N-assimilation genes such as *NR* (Pageau et al. 2006) and in the activation of starch breakdown enzymes that facilitate the availability of simple sugars for the microbes at infection sites. Consistently, in this work we have observed that microbial volatile-induced accumulation of exceptionally high levels of starch is accompanied by activation of starch breakdown pathways (Fig. 11) and downregulation of *NR* in *Arabidopsis*. Microarray analyses also revealed that FV treatment resulted in enhanced *SUS1* expression (1.69-fold increase), which was accompanied by a concomitant increase of SuSy activity (182.5 ± 18.3 and 280 ± 24 mU/g FW in the absence and presence of FV, respectively).

Fig. 10. Trehalose is not a major determinant of the microbial volatile-induced starch accumulation process. **A**, Starch content in leaves of fungal volatile (FV)-treated and nontreated wild-type *Arabidopsis* plants cultured for 16 h in solid Murashige-Skoog (MS) supplemented with 30 mM sucrose or 30 mM trehalose. **B**, Starch content in FV-treated and nontreated detached *Arabidopsis* leaves cultured for 16 h directly in contact with solid MS supplemented with 30 mM sucrose or 30 mM trehalose. Results are the mean $\pm$ standard error of three independent experiments.

Fig. 11. Plastidial β -amylase-mediated starch breakdown is activated during the microbial volatile-induced starch accumulation process. **A**, Starch content in leaves of fungal volatile (FV)-treated and nontreated wild-type (WT), *SEX1*, *BAM4*, *BAM5*, and *AMY3* T-DNA *Arabidopsis* mutants after 16 h of illumination under white light. **B**, Time course of maltose content in FV-treated and nontreated WT plants ($\circ$ and $\bullet$, respectively) and *BAM4* ($\square$ and $\blacksquare$, respectively) T-DNA mutants. Results are the mean $\pm$ standard error of three independent experiments.

It has been previously shown that, despite marked changes in their transcript levels, the amount of starch enzymes in *Arabidopsis* leaves does not change appreciably through the diurnal cycle, which can be ascribed to the occurrence of active protein turnover (Lu et al. 2005; Smith et al. 2004). This showed that day-length and circadian control of starch biosynthesis is exerted primarily through modulation of enzyme activity, and indicated the importance of post-transcriptional control in regulating starch metabolism. In this work, we have provided evidence that, as schematically illustrated in Figure 12, FV-induced accumulation of exceptionally high levels of starch would involve both transcriptional and post-translational regulation of enzymes directly or indirectly related to starch metabolism. Unlike potato leaves, where FV alter the expression of a number of starch-

related genes (Ezquer et al. 2010), FV treatment affected the expression of few genes directly involved in starch metabolism in *Arabidopsis*, indicating that transcriptional control of MIVOISAP would be indirect. Thus, downregulation of *NR1* expression occurring upon FV treatment would result in a decreased demand of ATP and carbon for amino acid biosynthesis, with excess ATP and carbon being diverted toward starch biosynthesis. Also, upregulation of SuSy may partially contribute to increase the production of ADPG linked to starch biosynthesis (Bahaji et al. 2011; Muñoz et al. 2005).

Results of Figure 1 showing sudden starch accumulation upon FV treatment strongly indicated the occurrence of rapid signaling that, as judged from the results shown in Figures 2, 6, and 7, would involve photoreceptor- and plastidial Fdx/Trx/

Fig. 12. Suggested model for microbial volatile-induced starch accumulation process (MIVOISAP) in *Arabidopsis* leaves involving a photocontrolled, transcriptionally and post-translationally regulated network wherein photoreceptors, starch synthase (SS) class SSIII and SSIV, and NADP-thioredoxin reductase C (NTRC)-mediated changes in redox status of plastidial enzymes play important roles in the conversion of CO_2 (both atmospheric and produced by the microorganism) into starch. Light activates photoreceptors, which interact with as-yet-to-be-identified plasma membrane fungal volatile (FV) receptors to produce signaling molecules that rapidly promote i) ferredoxin (Fdx)-, thioredoxin (Trx)-, and NTRC-mediated reduction of plastidial enzymes involved in starch metabolism; ii) proton ATPase-mediated changes in the plasma membrane potential; and iii) activation of SnRK1 (a central integrator of transcription networks in plant stress and energy signaling), thus altering the expression of genes involved in plant defense and carbon metabolism. Reduction of nitrate reductase (NR) activity as a consequence of SnRK1-mediated inhibition of NR and downregulation of NR expression promote decay of plastidial amino acid biosynthesis, the resulting excess ATP and carbon being channeled towards starch biosynthesis. Upregulation of proton-ATPase-encoding genes further contributes to maintain changes in plasma membrane redox status that, in turn, promote Fdx-, Trx-, and NTRC-mediated changes in the redox status of plastidial enzymes. Enhancement of sucrose synthase (SuSy) activity would promote the production of ADP-glucose (ADPG) and UDPglucose linked to the synthesis of starch and cell wall components, respectively. FV treatment promotes isoamylase 3 (*ISA3*) expression which, together with plastidial β -amylase, catalyzes starch breakdown. Starch breakdown products (maltose and G6P) are then transported to the cytosol by means of MEX1 and GPT2 (maltose and G6P/Pi transporters, respectively). Red and blue arrows indicate up- and downregulated functions, respectively, during MIVOISAP.

NTRC-mediated changes in the redox status of enzymes directly or indirectly involved in starch metabolism such as AGP (Fig. 12). Photoreceptors direct rapid activation of plasma membrane proton ATPases in response to light which, in turn, leads to rapid changes of membrane potentials, ion fluxes, redox status, and cytosolic metabolism that would likely favor accumulation of exceptionally high starch levels by as-yet-to-be-identified signals. Notably, FV treatment enhances the expression of various plasma membrane proton ATPases, which would guarantee long-term membrane potentials favoring starch overaccumulation. We must emphasize that previous studies have provided evidence that cytosolic sugars such as trehalose or T6P can act as signaling molecules that affect starch metabolism through regulation of AGP redox status (Geigenberger et al. 2005; Lunn et al. 2006). However, the fact that i) trehalose treatment for 16 h does not promote starch overaccumulation (Fig. 10), and ii) various *tps* mutants display normal MIVOISAP tend to indicate that i) trehalose or T6P are not signaling molecules involved in MIVOISAP and ii) FV-promoted changes in AGP redox status shown in Figure 6 are not ascribed to changes in trehalose metabolism. Further studies will be necessary to identify the signals connecting photoreceptor-mediated FV sensing with Fdx/NTRC-mediated reduction and activation of starch enzymes.

Stimulation of β -amylase-mediated starch degradation in leaves under stress is necessary to both directly support respiration and protect cell structures by raising the levels of maltose as an osmoprotectant (Kaplan and Guy 2004; Valerio et al. 2010). Consistently, results presented in Figure 11 showing FV-promoted maltose accumulation in illuminated WT leaves clearly indicated that plastidial β -amylase-dependent starch degradation is activated during MIVOISAP. Therefore, MIVOISAP involves simultaneous synthesis and breakdown of starch that may entail advantages such as rapid metabolic channeling toward various pathways in response to physiological and biochemical needs. Because expression levels of plastidial β -amylases do not change upon FV treatment, it is inferred that these enzymes are in excess and largely inactive in FV-nontreated plants, and activated after FV treatment by as-yet-to-be-identified post-translational mechanisms. A striking alteration in the transcriptome of FV-treated *Arabidopsis* leaves involves the upregulation of a gene, *GPT2*, encoding a plastidial G6P/Pi translocator (Kammerer et al. 1998; Kang and Rawsthorne 1996) (8.76-fold increase). Similar enhancement of *GPT2* expression was observed in leaves of FV-treated potato plants (Ezquer et al. 2010) and in leaves of *Arabidopsis* plants exposed to *B. subtilis* GB03 volatiles (Zhang et al. 2007). This is surprising because, although *GPT2* has been shown to be essential for photosynthetic acclimation (Athanasios et al. 2010), G6P/Pi transport is mainly restricted to heterotrophic tissues (Kammerer et al. 1998), where the imported G6P can be used for the synthesis of starch and fatty acids (Kang and Rawsthorne 1996; Zhang et al. 2008a) or to drive the OPPP (Bowsher et al. 2007). Thus, it is tempting to speculate that, as schematically illustrated in Figure 12, *GPT2* would play a role in exporting G6P molecules derived from the starch breakdown when leaves are subjected to microbial volatile emissions.

Previous studies have shown that continuous exposure to high CO_2 causes increase of starch content in leaves (Larios et al. 2001; Nafziger and Koller 1976). Because *A. alternata* produces CO_2 , it is likely that part of the starch accumulating in *Arabidopsis* leaves during FV exposure is ascribed to short-term exposure to elevated CO_2 . Results presented in Figures 5 to 7 showing FV-promoted enhancement of 3PGA/Pi ratio and rapid NTRC-dependent reduction of AGP would indicate that post-translational modulation of AGP is partially involved in MIVOISAP. However, enhancement of total 3PGA/Pi ratio

only occurs after approximately 6 h of FV treatment (Fig. 5), which does not explain the rapid accumulation of starch occurring upon FV exposure (Fig. 1). Furthermore, recent studies carried out in our lab have shown that previously termed “starch-less” or “zero-starch” *adgl-1* and *aps1* AGP mutants are capable of accumulating almost WT starch levels and high levels of ADPG when exposed to FV (Bahaji et al. 2011), which would indicate that i) FV promote the expression of sources, other than AGP, of ADPG, and ii) AGP is not the main or sole target regulatory enzymatic step during MIVOISAP, although it is an essential enzyme for normal starch biosynthesis. Based on the results of Figure 8 showing that FV promote only a 10-fold increase of starch content in *SSIII* and *SSIV* mutants, it is inferred that *SSIII* and *SSIV* are important targets of signaling cascades occurring upon FV sensing in the illuminated leaf. In this respect, it is important to note that *SSIII* contains a consensus motif for phosphoserine- or threonine-binding 14-3-3 proteins participating in environmentally responsive phosphorylation-regulated regulatory functions in plants (Sehnke et al. 2001). Furthermore, SS activity depends on redox status (Wieweg and de Fekete 1976). We are currently investigating the possible mechanisms regulating SS activity during MIVOISAP.

MATERIALS AND METHODS

Plants, growth conditions, and sampling.

The work was carried out using plants of *Arabidopsis thaliana* (‘Columbia’) and potato (*Solanum tuberosum* L. ‘Desirée’) cultured in solid MS medium which, unless otherwise indicated, was supplemented with 90 mM sucrose. *Arabidopsis* mutants used in this work and their sources are listed in Supplementary Table 2. Plants were grown in growth chambers with a photoperiod of 16 h of white light (photons at $90 \mu\text{mol s}^{-1} \text{m}^{-2}$) and 8 h of darkness at a constant temperature of 22°C . Plants examined for their response to continuous monochromatic red (600 to 700 nm), far-red (680 to 740 nm), and blue (400 to 520 nm) light were grown for 16 h in a growth chamber equipped with a Son-T Agro 400-W halogen lamp (Royal Philips Electronics, Amsterdam) filtered through one layer of either one of the following 0.3-mm polycarbonate filters: Supergel 19 for red light, Supergel 27 for far-red light, and Supergel 69 for blue light (Rosco Ibérica SA, Madrid). The halogen lamp provided a photosynthetically active radiation of $560 \mu\text{E m}^{-2} \text{s}^{-1}$. The photosynthetically active radiation under the different monochromatic lights was 283, 80, and $76 \mu\text{E m}^{-2} \text{s}^{-1}$ for red, far red, and blue filters, respectively. *Alternaria alternata* was cultured in petri dishes containing solid MS medium supplemented with 90 mM sucrose. Synchronized microbial cultures without lids and petri dishes containing fully developed plants were placed into sterile plastic boxes and sealed as indicated by Ezquer and associates (2010). At the indicated incubation periods, leaves were harvested for biochemical and transcriptome analyses. As negative control, petri dishes containing plants were cultured in sealed plastic boxes together with petri dishes containing sterile microbial culture media. Harvested leaves were immediately freeze clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar.

Western blot analyses.

For immunoblot analyses of AGP, protein samples were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose filters, and immunodecorated by using antisera raised against maize AGP as primary antibody and a goat anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis) as secondary antibody. Samples were extracted and

separated on SDS-PAGE under both reducing and nonreducing conditions essentially as described by Hendriks and associates (2003) and Kolbe and associates (2005).

Enzyme assays.

All enzymatic reactions were carried out at 37°C. Frozen powder (1 g) (discussed above) was resuspended at 4°C in 5 ml of 100 mM HEPES (pH 7.5), 2 mM EDTA, and 5 mM dithiothreitol. The suspension was desalted and assayed for enzymatic activities. SuSy was assayed as described by Baroja-Fernández and associates (2009). β -Amylase was assayed as described by Liu and associates (2005). NR was assayed following the method of González and associates (2001). One unit (U) is defined as the amount of enzyme that catalyzes the production of 1 μ mol of product per minute.

Analytical procedures.

Starch was measured by using an amyloglucosydase-based test kit (Boehringer Mannheim, Mannheim, Germany). 3PGA and Pi were determined as described by Lytovchenko and associates (2002). Maltose was measured as described by Ezquer and associates (2010). Determination of amino acid (Ser, Gly, Pro, and Arg) content was performed as described by Loiret and associates (2009). The results shown in the text were the mean $\pm$ standard error of three independent experiments.

Microarrays.

Total RNA was extracted from frozen *Arabidopsis* leaves using the Trizol method according to the manufacturer's procedure (Invitrogen), following purification with RNeasy kit (Qiagen, Hilden, Germany). RNA amplification, labeling, and statistical data analysis were performed basically as described by Adie and associates (2007). *Arabidopsis* Gene Expression Microarray 4 $\times$ 44K (G2519; Agilent Technologies) were used for hybridization. Labeling and hybridization conditions were those described in the manual two-color microarray-based gene expression analysis of Agilent Technologies. Three independent biological replicates were hybridized for leaves from microbe-treated plants and from controls. Images from Cy3 and Hyper5 channels were equilibrated for intensity differences and captured with a GenePix 4000B scanner (Axon, Union City, CA, U.S.A.). Spots were quantified using GenePix software (Axon) and normalized using the Lowess method. The mean of the three replicate log-ratio intensities and their standard deviations were calculated and the expression data was statistically analyzed using the LIMMA Package (Smyth and Speed 2003). Functional characterization of the differentially expressed genes was done using the MapMan tool.

Real-time quantitative PCR.

Total RNA was extracted from *Arabidopsis* leaves as for microarray experiments. RNA was treated with RNase-free DNase (Takara, Shiga, Japan). RNA (1.5 μ g) was reverse transcribed using polyT primers and the Expand reverse transcriptase kit (Roche, Branchburg, NJ, U.S.A.) according to the manufacturer's instructions. RT-PCR reaction was performed using a 7900HT sequence detector system (Applied Biosystems, Foster City, CA, U.S.A.) with the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each reaction was performed in triplicate with 0.4 μ l of the first-strand cDNA in a total volume of 20 μ l. The specificity of the PCR amplification was checked with a heat dissociation curve (from 60 to 95°C). Comparative threshold values were normalized to 18S RNA internal control and compared to obtain relative expression levels. The specificity of the obtained RT-PCR products was controlled on 1.8% agarose gels. Primers used for RT-PCR are listed in Supplementary Table 3.

ACKNOWLEDGMENTS

J. Li and I. Ezquer contributed equally to this work. This research was partially supported by the grants BIO2007-63915 and BIO2010-18239 from the Comisión Interministerial de Ciencia y Tecnología and Fondo Europeo de Desarrollo Regional (Spain), and by Iden Biotechnology S.L. I. Ezquer acknowledges a pre-doctoral fellowship from the Spanish Ministry of Education and Science. M. Ovecka was partly supported by grant number 2/0200/10 from the Grant Academy VEGA. We thank C. Hannah (University of Florida), who kindly provided us the antibody raised against maize AGP; and F. J. Cejudo and C. Gotor (IBVF, Sevilla), who kindly provided seed of *Arabidopsis* mutants used in this study.

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