An *Arabidopsis* soluble chloroplast proteomic analysis reveals the participation of the Executer pathway in response to increased light conditions

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Abstract

The Executer1 and Executer2 proteins have a fundamental role in the signalling pathway mediated by singlet oxygen in chloroplast; nonetheless, not much is known yet about their specific activity and features. Herein, we have followed a differential-expression proteomics approach to analyse the impact of Executer on the soluble chloroplast protein abundance in *Arabidopsis*. Because singlet oxygen plays a significant role in signalling the oxidative response of plants to light, our analysis also included the soluble chloroplast proteome of plants exposed to a moderate light intensity in the time frame of hours. A number of light- and genotype-responsive proteins were detected, and mass-spectrometry identification showed changes in abundance of several photosynthesis- and carbon metabolism-related proteins as well as proteins involved in plastid mRNA processing. Our results support the participation of the Executer proteins in signalling and control of chloroplast metabolism, and in the regulation of plant response to environmental changes.

Key words: Abiotic stress, acclimation response, chloroplast metabolism, DIGE, light, retrograde signalling, ROS.

Introduction

During evolution, plants have developed an intricate network of signalling pathways to trigger physiological responses as a consequence of diverse environmental stimuli. Recent observations have demonstrated that retrograde communication coordinates the expression of nuclear genes with the metabolic and developmental state of the cell through signals emitted from plastids and mitochondria (Woodson and Chory, 2008; Ng *et al.*, 2014). Particularly, chloroplasts—photosynthetic organelles in plant cells—are the source of specific signalling molecules that relay information to the nucleus (Nott *et al.*, 2006; Pfannschmidt, 2010; Pogson and Albrecht, 2011; Barajas-López *et al.*, 2013; Chi *et al.*, 2013). The plastid signals identified so far can be linked to specific stress conditions, and the best characterized signals are intermediates of the tetrapyrrole biosynthesis pathway, the redox state of the thylakoid membrane, and reactive oxygen species (ROS) (Karpinski *et al.*, 1999; Strand *et al.*, 2003; Gadjev *et al.*, 2006; Koussevitzky *et al.*, 2007; Foyer and Noctor, 2009; Galvez-Valdivieso *et al.*, 2009; Pesaresi *et al.*, 2009; Sun *et al.*, 2011; Woodson *et al.*, 2011; Zhang *et al.*, 2011; Pfalz *et al.*, 2012; Petrillo *et al.*, 2014). Also, the metabolic state of chloroplasts can be sensed by exported metabolites such as carbohydrates,

Abbreviations: CB, Calvin-Benson cycle; CBB, Coomassie Brilliant Blue; DIGE, differential gel electrophoresis; Ex, Executer; HL, high light; NL, normal light; NPO, non-photochemical quenching; PCA, principal component analysis; PFD, photon flux density; ROS, reactive oxygen species; 2D, two-dimensional, WT, wild-type.
reductive power in the form of NADPH, isoprenoid intermediates, xanthophyll derivatives or a phosphonucleotide (Rolland et al., 2006; Pfannschmidt, 2010; Zhang et al., 2010; Estavillo et al., 2011; Ramel et al., 2012; Xiao et al., 2012).

In chloroplasts, ROS are produced as an unavoidable side effect of the photosynthetic light reactions, as a consequence of the spatial and temporal concurrence of electron and energy transfer reactions with photosynthetically generated molecular oxygen (Asada, 2006; Galvez-Valdivieso and Mullineaux, 2010). Whereas electron transfer reactions to molecular oxygen in its ground triplet state lead to the formation of superoxide, hydrogen peroxide and hydroxyl radicals, energy transfer reactions result in the formation of the highly reactive singlet oxygen. ROS generation in chloroplasts is enhanced during environmental stress, and particularly singlet oxygen is the major ROS produced in leaves exposed to high light (Fryer et al., 2002; Krieger-Liszkay et al., 2008). Genetic approaches have shown that the production of singlet oxygen under controlled conditions in the chloroplast plays a significant role in signalling the oxidative response of plants (Apel, 2004). Subsequent work in Chlamydomonas reinhardtii showed that earlier exposure of the cells to low levels of singlet oxygen elicits an acclimation response that protects cells from photooxidative damage (Ledford et al., 2007).

However, the mechanism which transduces the signal from the chloroplast to the nucleus is far from delineated and, in higher plants, appears to undergo significant cross-talk with other signalling pathways controlling such responses as plastid differentiation, plant development and general stress responses (Laloi et al., 2007; Baruah et al., 2009). With the aim of studying the biological activity of singlet oxygen and the mechanisms involved in transduction of redox signalling, the conditional flu mutant of Arabidopsis thaliana was identified (Meskauskienė et al., 2001). The flu mutant accumulates free protochlorophyllide (Pchlide), the immediate precursor of chlorophyllide, in the dark; upon illumination free Pchlide acts as a potent photosensitizer that generates singlet oxygen by transferring light energy to molecular oxygen (Gollnick, 2006). Two stress responses in flu were triggered by the release of singlet oxygen within the plastid compartment during re-illumination of dark-adapted plants: seedling lethality, and cell death and growth inhibition in mature plants (op den Camp et al., 2003). Global gene-expression studies showed that these stress responses were not primarily due to physicochemical damage caused by singlet oxygen during oxidative stress but were attributed to the activation of genetic stress response programs (op den Camp et al., 2003; Gadjev et al., 2006; Laloi et al., 2006; Przybyla et al., 2008). An extensive second-site mutant screen performed in flu identified a group of suppressor mutants named executor (ex). Plants with mutations in Executor1 (Ex1), a plastid protein of unknown function, lost the ability to perceive the presence of singlet oxygen in flu chloroplasts and, consequently, the activation of singlet-oxygen mediated response programs was suppressed (Wagner et al., 2004). Subsequently, a protein homolog to Ex1, named Executor2 (Ex2), was also identified and suggested to be involved in the singlet-oxygen-responsive gene network (Lee et al., 2007).

How the Executor proteins are involved and function in the signalling pathway remains elusive. The loss of function of either of the Executor protein showed no obvious phenotype compared to WT Arabidopsis (Lee et al., 2007; Wagner et al., 2004; Kim and Apel, 2013a). It was found however that exl plants were more resistant than WT to damage upon treatment with low concentrations of 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU) together with high light intensities (Wagner et al., 2004). Also, analysis of the response of WT and exl plants to β-cyclocitrinal (a β-carotene oxidation product) treatments suggested that exl plants were more resistant to photooxidative stress than WT (Ramel et al., 2012). Studies on the hypersensitive response to pathogen infection pointed out that exv2 plants are slightly more resistant to low amounts of pathogens than WT (Mur et al., 2010). Furthermore, a double mutant exl/exv2 was affected in chloroplast development in cotyledons (Kim et al., 2009), and the seedlings of the double mutant exl/exv2 were less susceptible than WT when exposed to a combined low-temperature/high-light treatment (Kim et al., 2012).

The role of Executor proteins in singlet-oxygen mediated signalling is unclear. Because light availability is one of the key factors that modulates acclimation strategies and defence reactions in plants, we aim to analyse how plants adapt to their environment by studying the chloroplast proteome response to a perturbation in light intensity, which promotes ROS production but would not result in oxidative stress or cell death. Here, a differential-expression proteomics approach was used to analyse the impact of light on chloroplast protein abundance in two T-DNA insertional knockout lines (EX1 and EX2). Our study showed changes in abundance of several photosynthesis- and carbon metabolism-related proteins as well as proteins involved in plastid mRNA processing, among others. A good correlation between executor mutants and the changes occurring after exposure of WT plants at a moderate light intensity in the time frame of hours was inferred. It is suggested that Executor proteins participate in signalling in Arabidopsis under growth light conditions, and in the regulation of the response to environmental cues such as light acclimation, likely to avoid the misexpression of defence programs.

Material and methods

Plant materials

For all the experiments, Arabidopsis thaliana plants (WT and mutants) of the ecotype Columbia (Col-0) were used. The SALK_002088C and SALK_021694C lines—harbouring a T-DNA insertion in the EX1 (At4g33630) and EX2 (At1g27510) genes, respectively—were purchased from NASC (Nottingham Arabidopsis Stock Center) (Alonso et al., 2003). Plants were grown on soil or on MS basal salt medium. Genomic DNA was isolated from leaf material using the CTAB extraction protocol adapted from Weigel (2002) and screened for T-DNA insertion by PCR genotyping. The following genomic primers were used: EX1 forward gene specific primer (FP; 5’-CACCCTTCTGAAAAGATC-3’) and EX1 reverse gene specific primer (RP; 5’-TACCCGAATCTTGAATGG-3’) to characterize insertion lines SALK_002088; EX2-FP (5’-CAGGAGCTTGTCATCGGAGG-3’) and EX2-RP (5’-AAATGCTAATGTTG
GCTGGCA-3), to characterize insertion lines SALK_021694. In these experiments, the T-DNA–specific left border (LB) primer LB (5'-ATTITGGCGATTTGGCAAC-3') was also used. To verify PCR products and T-DNA insertion sites, amplified DNA fragments were sequenced.

**Light treatment**

Plants were grown on soil in a growth chamber at 8 h-light/16 h-darkness (20°C) for 7–8 weeks under a photon flux density (PFD) of 120 μmol m⁻² s⁻¹ and relative humidity of 70%. For the high light (HL) experiments, plants were transferred 1 h after the onset of the light period to a growth chamber under PFD of 600–700 μmol m⁻² s⁻¹. As control material one set of plants was maintained at 120 μmol quanta m⁻² s⁻¹, representing normal light (NL) conditions.

**Protein extraction**

After 6 h light treatment (HL or NL), plant leaves were harvested for chloroplast isolation and purification according to Hall et al. (2011). Briefly, 20 g of plant material was homogenized using a blender in ice-cold extraction buffer (20 mM Tricine-NaOH pH 8.4, 300 mM sorbitol, 10 mM KCl, 10 mM Na-EDTA, 0.25% BSA, 4.5 mM sodium ascorbate and 5 mM L-cysteine). Cell debris was removed by a nylon mesh (22 μm), and chloroplasts were pelleted by centrifugation for 2 min at 1000 x g. Chloroplasts were washed and ruptured by osmotic shock in 10 mM Na-pyrophosphate-NaOH pH 7.8 buffer. Following centrifugation at 100 000 x g for 1 h at 4°C, the supernatant containing the soluble stromal proteins was concentrated using an Amicon Ultra-15 10 K ultrafiltration device. Protein concentration was determined using the Bradford assay (Bradford, 1976) and bovine serum albumin as reference. Seppro® Rubisco Spin Columns (Sigma) were used to reduce Rubisco abundance in the stroma samples.

**Two-dimensional gel electrophoresis (2D-DIGE)**

Chloroplast stroma samples were precipitated with ice cold acetone. Protein pellets were solubilized in DIGE labelling buffer (10 mM Tris-HCl pH 8.5, 2 M thiourea, 7 M urea, 2% (w/v) CHAPS). Remaining insoluble material was removed by centrifugation for 10 min at 21 000 x g. The final protein concentration for G-Dye labelling was 5 μg/μl. Solubilized protein samples were separately labelled with G-Dye100, G-Dye200 and G-Dye300 dyes (DyeAGNOSTICS) at a ratio of 400 pmol dye/50 μg protein extract for 30 min in darkness on ice. Labelling was quenched by addition of lysine. In general, samples were labelled using G-Dye200 and G-Dye300 dyes while an internal standard (consisting of a pooled sample comprising an equal amount of all samples in the experiment) was labelled with G-Dye100. Details of labelling and the subsequent combination of differentially labelled samples used are shown in the experimental design presented in Table 1. For each immobilized pH gradient (IPG) strip, equal amounts of G-Dye100, G-Dye200 and G-Dye300 labelled samples were combined, typically 50 μg protein per sample. Prior to iso-electric focusing the mixed samples were diluted with rehydration solution containing 2 M thiourea, 7 M urea, 2% (w/v) CHAPS, 20 mM DTT, 0.002% (w/v) bromophenol blue and 0.5% (v/v) IPG buffer pH 3–11 NL (GE Healthcare, Uppsala, Sweden). Samples were thereafter applied to 24cm Immobiline Dry Strips pH 3–11 NL (GE Healthcare, Uppsala, Sweden) before being separated by 2D-gel electrophoresis as described for DIGE gels above. Preparative gels were fixed in 30% ethanol, 10% acetic acid and were stained by Coomasie Brilliant Blue (CBB).

**Gel image analysis**

G-Dye labelled samples were visualized using a Typhoon™ 9400 Variable Mode Imager (GE Healthcare, Uppsala, Sweden). All gel images were scanned at 100 μm resolution using a photomultiplier tube (PMT) voltage optimal for maximal pixel intensity without spot saturation. Prior to image analysis the gel images were cropped using ImageQuant v.5.2 (GE Healthcare, Uppsala, Sweden) in order to remove extraneous areas. DIGE analysis was performed using Redfin 3 software (Ludesi) as was matching of preparative CBB stained gels to DIGE gels. Images of CBB-stained gels were acquired using an image scanner and the Labscan software (GE Healthcare, Uppsala, Sweden). Spot detection, matching and statistical analysis was performed using the Redfin 3 program (www.ludesi.com). A principal component analysis (PCA) was performed to separate the gel samples according to their expression variation. One-way analysis of variance (ANOVA; p<0.001) and Mann-Whitney (p<0.05) tests were conducted to assess differential expression of protein abundance between the different groups. Minimum protein volume was set at 200 and differentially expressed proteins with a change in average spot volume of at least 2.0-fold were selected.

**In-gel digestion and protein identification**

Spots of interest were excised from preparative gels using an Etan Spotpicker™ spot picking station fitted with a 1.4 mm picker head. Gel plugs were dehydrated and destained by incubation with a solution containing 20 mM ammonium hydrogen carbonate in 35% acetonitrile. The solution was removed and gel pieces were dried by addition and removal of neat acetonitrile twice. Dried gel plugs were rehydrated on ice with 20 mM ammonium hydrogen carbonate and 10% acetonitrile containing 2ng/μl trypsin (Promega).
In-gel digestion was performed overnight at 37°C. Mass spectrometry analysis of in-gel digests was performed on a MALDI-TOF Voyager-DE™ STR Bio Spectrometry Workstation (Applied Biosystems). Database searches were performed on a local Mascot server licensed to Umeå University by Matrixscience (www.matrixscience.com), using Arabidopsis TAIR9 and Swiss-Prot databases. For searches a peptide mass error tolerance of 50 ppm was accepted and carbamidomethylation of cysteine and oxidation of methionine were specified as variable modifications.

Chlorophyll fluorescence measurements

In vivo chlorophyll a fluorescence was measured using a Dual-PAM-100 chlorophyll fluorescence photosynthesis analyser (Heinz Walz) on attached rosette leaves. After dark acclimation of the plants (15 min), the measuring light (9 µmol photons m⁻² s⁻¹) was turned on, and minimal fluorescence (F₀) was determined. Leaves were exposed to a pulse of saturating light (3000 µmol photons m⁻² s⁻¹) to determine the maximum fluorescence in the dark-adapted state (Fₘ). Subsequently, leaves were illuminated with actinic red light at 660 µmol of photons m⁻² s⁻¹ for 20 min to measure the maximum fluorescence in the light-adapted state (Fₘ'). The maximum PSII efficiency was expressed as Fₘ/Fₚ = (Fₘ' - F₀)/Fₘ', and the PSII operating efficiency as Φₚₛₛᵢ = (Fₘ' - F₀)/Fₘ' (Genty et al., 1989). The NPQ coefficient was calculated using the Stern–Volmer equation, NPQ = (Fₘ - F₀)/Fₘ', 1-qP was calculated as (Fₘ' - Fₚₚₚ)/(Fₘ' - F₀) (Bilger and Bjorkman, 1990).

Results

The aim of the present study was to examine the collective response of the soluble chloroplast proteome of 8-week-old Arabidopsis plants following a transition from normal light (NL) to moderate high light (HL, 5-fold increase in PFD) in the time scale of hours. Furthermore, using Arabidopsis mutant plants, the link between the Executor pathway and the acclimation response upon exposure of plants to high light was investigated.

Photosynthetic performance in ex1 and ex2 single mutants is comparable to WT

The role of Executor in chloroplast light response was analysed using two independent T-DNA insertion lines inactivating EXECUTER1 (ex1) and EXECUTER2 (ex2), respectively. Absence of Executor transcripts was confirmed in the T-DNA insertion lines indicating them to represent true knockout lines (Supplementary Fig. S1). As previously reported (Wagner et al., 2004; Lee et al., 2007), ex1 and ex2 plants showed no obvious alterations of growth and development. Because Executor heterozygote plants have been associated with PSII (Kim and Apel, 2013b), PSII performance was assessed by measurement of chlorophyll-a fluorescence at room temperature in intact leaves from WT and executor mutant plants treated at the two different PFD (NL and HL) for 6h. No differences could be detected between WT and mutant plants, as derived from the ratio of variable to maximum fluorescence (Fᵥ/Fₚₚₚ), the quantum yield of PSII (Φₚₛₛᵢ), the degree of non-chemical quenching (NPQ) and the excitation pressure of PSII (1-qP) (Table 2). HL treatment resulted in a 5% decline of Fᵥ/Fₚₚₚ for both WT and mutant plants.

2D-DIGE analysis of the soluble chloroplast proteome highlights molecular differences between executor mutants and WT plants

To examine whether chloroplasts are affected by the loss of Executor, the effect of light was investigated on the soluble chloroplast proteome of Arabidopsis. First, plants were grown at 120 µmol m⁻² s⁻¹. After 8 weeks, plants were exposed to high light intensity (700 µmol m⁻² s⁻¹) during 6h and chloroplasts were isolated from rosette leaves. The soluble protein extract from chloroplasts was analysed by 2D-DIGE, following a strategy as depicted in Table 1. The DIGE analysis revealed significant differences between WT and mutant plants, both under normal light (WTNL, ex1NL and ex2NL, respectively) and in response to high light (WTHL, ex1HL and ex2HL, respectively). The differences in the stroma proteome between WT, ex1 and ex2 are supported by PCA (Fig. 1). Based on the first principal component (PC1)—that represents the direction of highest variability after gel data dimensionality reduction—there is a large variation between WT in normal conditions versus high light treatment. Differences between executor mutants and WT under normal conditions are also evident (Fig. 1).

Proteome remodelling as a consequence of executor loss of function resembles light treatment in WT

Comparative analysis of WT and ex1 and ex2 Arabidopsis plants in the two light conditions (NL and HL) showed a number of soluble chloroplast proteins that significantly altered their expression level (Supplementary Table S1; fold-change >2, p<0.001). The majority of the observed changes in the proteome were upregulations, irrespectively of genotype or treatments, with the largest changes detected in ex2 plants.

In NL conditions, changes in 54 and 94 protein spots were detected in the ex1NL versus WTNL and ex2NL versus WT NL comparative groups, respectively (Supplementary Table S1). When the responsive spots of the two mutant plants in NL versus WTNL were compared, 41 spots (37+4) were exclusively detected in ex2NL plants, as shown in a Venn diagram analysis in Fig. 2A. Upon exposure of WT Arabidopsis plants to high light (WTHL), 28 protein spots (5+1+18+4) displayed significant expression changes in response to the treatment (Supplementary Table S1). Actually, 23 light-responsive spots, which represent more than 82%, were detected in the ex1NL vs. WTNL and/or ex2NL vs. WTNL groups (1+18+4 in Fig. 2A). When ex1 plants were exposed to high light for 6h (ex1HL), 27 protein spots (23+4 in Fig. 2B) were found to be differentially expressed relative to ex1NL. Interestingly, the behaviour of 23 spots (not present in ex1NL) was shared between ex1HL and ex2NL groups (Fig. 2B). Few differences were detected between ex2NL versus WTNL and ex2HL versus WTNL groups, with more than 91% common protein spots (Fig. 2C). In general, it was observed that a higher number of unique spots were significantly altered in ex2 plants compared to WT, and the changes were already significant in normal light conditions (37 and 18 spots in Figs 2A and 2B, respectively).
Chloroplast protein synthesis and homeostasis

Alpha and beta subunits of Cpn60—a molecular chaperone that participates in protein folding in chloroplasts—increased in executor mutants plants and, to a lesser extent, in WTHL. Two peripheral ATP synthase subunits (alpha and beta) were significantly affected in executor mutants: an increase abundance of the alpha subunit (ATP-A) in the stromal compartment was detected, but a multi-expression pattern (spots 44, 62 and 69) was observed for the beta subunit (ATP-B) and therefore it could not be classified simply into the up- or down-regulated group. On the other hand, the stromal ATP synthase delta subunit (ATP-C) accumulated in a light-dependent manner.

Primary and secondary metabolism

Six Calvin-Benson (CB) enzymes (transketolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglyceraldehyde kinase, phosphoribulokinase, rubisco activase and fructose-bisphosphate aldolase) accumulated in mutant plants compared with WTNL. However, ribulose-5-phosphate 3-epimerase, ribose 5-phosphate isomerase and fructose-bisphosphate aldolase 1 were down-regulated. For these enzymes, except glyceraldehyde-3-phosphate dehydrogenase B, a similar direction of response was detected in WTHL. The stromal enzymes glutamine synthetase 2 (involved in nitrogen metabolism) and glutamate-1-semialdehyde 2,1-aminomutase (tetrapyrrole synthesis) were upregulated in a genotype- and treatment-manner. Carbonic anhydrase 1 showed increased relative abundance in mutant plants, and also a slight increase upon light treatment. Glycerate 3-kinase, a participant of the photorespiratory cycle, was particularly affected in WTHL. Two peripheral ATP synthase subunits (alpha and beta) were significantly affected in executor mutants: an increase abundance of the alpha subunit (ATP-A) in the stromal compartment was detected, but a multi-expression pattern (spots 44, 62 and 69) was observed for the beta subunit (ATP-B) and therefore it could not be classified simply into the up- or down-regulated group. On the other hand, the stromal ATP synthase delta subunit (ATP-C) accumulated in a light-dependent manner.

Chloroplast protein synthesis and homeostasis

Alpha and beta subunits of Cpn60—a molecular chaperone that participates in protein folding in chloroplasts—increased in executor mutants plants and, to a lesser extent, in WTHL as compared with WTNL. Interestingly, a group of proteins

### Table 2. PSII performance of intact leaves from NL- or HL-treated WT and executor mutant plants was determined by chlorophyll-a fluorescence measurements using a PAM-fluorometer. The maximum quantum yield of PS II (Fv/F0), the effective quantum yield of PSII (ΦPSII), the degree of non-photochemical quenching (NPQ) and the excitation pressure of PSII (1-qP) was deduced. Data represents mean±standard error. No significant differences were found between plants in the same light condition (ANOVA).

| Parameter | WTNL | WTHL | ex1NL | ex1HL | ex2NL | ex2HL |
|-----------|------|------|-------|-------|-------|-------|
| Fv/Fm     | 0.82±0.01 | 0.78±0.02 | 0.83±0.01 | 0.78±0.01 | 0.83±0.01 | 0.75±0.04 |
| NPQ       | 1.79±0.10 | 1.53±0.25 | 1.53±0.13 | 1.22±0.18 | 1.63±0.16 | 1.24±0.03 |
| ΦPSII     | 0.18±0.02 | 0.16±0.02 | 0.15±0.03 | 0.19±0.02 | 0.18±0.02 | 0.16±0.04 |
| 1-qP      | 0.72±0.04 | 0.73±0.04 | 0.76±0.05 | 0.68±0.02 | 0.72±0.02 | 0.72±0.05 |
related to mRNA metabolism—the chloroplast stem-loop binding proteins of 41 kDa (CSP41A, CSP41B), and plastid-specific ribosomal protein 2 (PSRP-2)—were significantly upregulated in mutant plants. The accumulation of two chloroplast ribonucleoproteins, namely CP29B and CP31A, was affected both by genotype and light treatment.

Other
The abundance of two chloroplast proteins of unknown function (Table 3) varied in a genotype-dependent manner. Spots 122 and 183 corresponded to a protein containing a tetratricopeptide repeat (TPR) region based on protein sequence analysis (Ishikawa et al., 2005). Spot 200 was identified as a thylakoid lumen protein of 19 kDa (TL19) (Schubert et al., 2002).

Discussion
Plants are exposed to a variety of environmental changes, such as light availability, that can compromise their metabolism, growth and development. Therefore, plants have developed a variety of mechanisms for sensing environmental fluctuations and, accordingly, adjust their developmental programs, metabolic processes and defence reactions. Upon changing light conditions, plants exhibit adaptation and acclimation strategies in order to optimize their photosynthetic performance and to avoid imbalance between energy absorption and utilization, which could promote ROS production in the chloroplasts (Apel, 2004; Kangasjärvi et al., 2009; Karpinski et al., 2013). The comparison of a five-fold increase in PFD over growth intensity, not enough for...
light-saturated photosynthesis, was chosen to challenge the acclimation response of WT and executer mutant Arabidopsis plants to irradiance. Chlorophyll fluorescence measurements performed under different conditions showed that the maximum quantum efficiency of PSII photochemistry remained unchanged between WT and mutants, with a moderate decrease in plants under moderate light intensity. Similar effects were detected in the light-dependent thermal dissipation component of NPQ and photochemical efficiency. It was concluded that the photochemical activity of executer plants was not affected relative to WT plants. Moreover, there was no significant loss of PSII efficiency during the chosen exposure time to increased light. Therefore, our experimental conditions would not promote photo-oxidative stress, but likely rapid adjustments in photosynthesis and chloroplast composition for acclimation to a change in the light environment. A double purpose is envisaged, that is, oxidative stress avoidance and molecular adjustments that would facilitate plants utilizing the additional light to improve their photosynthetic performance.

The acclimation response would involve changes in the relative abundance of a number of proteins (Kosová et al., 2011). Therefore, a subcellular fractionation approach in combination with 2D-DIGE was followed to analyse the dynamics of the soluble chloroplast proteome of Arabidopsis to changing light. It has been shown that Executer proteins are necessary to transmit the signal produced by singlet oxygen from the plastid to the nucleus. Yet little is known about the role that Executers play under normal circumstances. In order to investigate the putative contribution of the Executer pathway to the plant response, a comparative proteome analysis of soluble chloroplast extracts of the two executer mutants was carried out. Our results showed very similar molecular phenotypes between ex1 and ex2 under growth light conditions, though the change was more significant in the latter. Interestingly, a clear separation among the WT and executer mutant groups was observed.

Table 3. Summary of differentially expressed proteins (light treatment and/or genotype effect versus WT-NL) identified by MS. The functional classification (GROUP) and gene accession number (GENE ID) are shown. The fold change in protein abundance is indicated in colour code; values >1 or <1 indicate an increase or decrease in protein abundance, respectively. A colour version of this figure is available at JXB online.
when plants were challenged with increased light. As expected, light treatment significantly affected the soluble chloroplast proteome in WT, but the effect was less pronounced in mutant plants, that otherwise show more similarities to WTHL. The consistent phenotype observed in our proteomic experiments for the two *executor* mutant plants indicates that Ex1 and Ex2 might participate in the same signalling pathway. Moreover, as previously proposed from genetic studies, their functions might not be redundant as the presence of one protein cannot compensate the absence of the other.

Examination of differentially accumulated proteins revealed that the proteins that underwent differential expression upon light treatment in WT *Arabidopsis* were related to metabolic pathways (mainly carbon metabolism), protein synthesis and energy production (Fig. 4). We detected an increase in abundance of FNR-2, which has a critical role in the redistribution of photosynthetically derived electrons to various reducing pathways, such as carbon fixation, nitrogen metabolism and chlorophyll biosynthesis (Lintala *et al.*, 2009). Also, the abundance of three peripheral thylakoid ATP synthase subunits was responsive to light; it has been shown that ATP synthase in chloroplasts is regulated by light and metabolite factors, and particularly ROS showed a direct influence in its activity (Buchert and Forreiter, 2010; Buchert *et al.*, 2012; Kohzuma *et al.*, 2013). Our results showed the alteration of a number of CB enzymes that catalyse readily reversible reactions and are not susceptible to ‘fine’ regulation, such as aldolase, transketolase, epimerase and isomerase (Raines, 2003; Michelet *et al.*, 2013). In our experiments, a different expression pattern for two aldolase isoforms (FBA1 and FBA2) was detected, which could indicate some functional specialization. The importance of aldolase and transketolase activities in photosynthetic carbon flux control and for the acclimation of photosynthesis to changing environmental conditions has been reported (Haake *et al.*, 1998, 1999; Henkes *et al.*, 2001; Raines, 2003; Uematsu *et al.*, 2009).
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2012). Furthermore, their substrates and products act as precursors of associated metabolic processes, such as amino acid and fatty acid synthesis, and therefore variations in the abundance would affect other chloroplast pathways (Henkes et al., 2001; Tewlow et al., 2005). On the other hand, epimerase and isomerase decreased significantly in response to light. Our study showed that phosphoribulokinase (PRK), which plays an important role in regulating the flow of sugar through the Calvin cycle, was upregulated by light. PRK can become limiting when plants grown under low irradiance are exposed to high light (Paul et al., 2000). Two enzymes with a defined role in carbon fixation modulation, rubisco activase (Portis, 2003) and beta-carbonic anhydrase 1 (CA1) (Fett and Coleman, 1994), also increased upon light treatment. Other metabolic proteins such as glutamine synthetase-2—a central enzyme in nitrogen metabolism with a role in maintaining the balance of carbon and nitrogen (Miflin and Habash, 2002)—and a GSA nitrogen metabolism with a role in maintaining the balance of proteins such as glutamine synthetase-2—a central enzyme in carbon fixation modulation, rubisco activase (Portis, 2003) and beta-carbonic anhydrase 1 (CA1) (Fett and Coleman, 1994), also increased upon light treatment. Other metabolic proteins such as glutamine synthetase-2—a central enzyme in nitrogen metabolism with a role in maintaining the balance of carbon and nitrogen (Miflin and Habash, 2002)—and a GSA nitrogen metabolism with a role in maintaining the balance of proteins such as glutamine synthetase-2—a central enzyme in carbon fixation modulation, rubisco activase (Portis, 2003) and beta-carbonic anhydrase 1 (CA1) (Fett and Coleman, 1994), also increased upon light treatment. Two CB enzymes (GapB and phosphoglycerate kinase) significantly increased in the mutant plants, particularly in ex2 plants, in parallel to the alterations of CB enzymes detected upon light treatment. A subset of proteins of OEC accumulated in the soluble fraction of executor chloroplasts compared to WT (Ettinger and Theg, 1991; Bricker et al., 2012). Two ribonucleases from the CSP41 family (Qi et al., 2012) and a plastid-specific ribosomal protein (PSRP-2) (Yamaguchi and Subramanian, 2003) were more abundant in executor plants, which might indicate a modified transcriptional and/or translational activity compared to WT under normal growth light conditions. Two chloroplast proteins of unknown function—a protein with a predicted TPR motif and a thylakoid lumen protein that belongs to PsbP-superfamily—showed higher expression levels in mutant plants. Our results revealed significant differences between WT and mutant plants, but an interesting overlap was found between WTHL and executor plants. Although Executor proteins are dispensable for normal growth, our work has detected a molecular perturbation at the basal level in executor mutant plants. It seems that the loss of function of Executor results in a reorientation in chloroplast central metabolism that resembles the activation response of moderate light acclimation. A plausible interpretation of these data is that the Executor proteins set the light intensity threshold that triggers the high light response. Therefore, in the executor mutants, this threshold is lower than in wild type plants. It is thus proposed that Executor form part of a regulatory network for the coordination between environmental stimuli and metabolic adaptation and determine the acclimation response in chloroplasts, although the exact role of the Executor proteins is yet to be defined. Interestingly, our results showed that the absence of Ex2 has a stronger impact in chloroplasts than Ex1. Our findings are consistent with other studies that observed an Executor-dependent stress acclimation in green leaves of mature plants, such as suppression of cell death in ex1 plants treated with DCMU (Wagner et al., 2004), the resistance to photodoxidative stress of ex1 plants (Ramel et al., 2012), or the slight increase to pathogen resistance in ex2 plants (Mur et al., 2010).

In conclusion, despite the demonstration that Executor proteins are necessary to transmit the signal produced by singlet oxygen from the plastid to the nucleus, little is known about the role that Executors play under normal circumstances. In an effort to elucidate the biological activity of Executor proteins as putative mediators of the singlet oxygen response in chloroplasts, we used proteomics to analyse the role of Executor in chloroplasts of Arabidopsis exposed to different light regimes. The analysis of the soluble chloroplast protein profile in WT has provided a deeper insight into changes associated with the acclimation response to light. Six hours of high light exposure triggered responses in chloroplast of WT Arabidopsis; not surprisingly, many proteins involved in photosynthesis and carbon metabolism were affected, which reflects an increase in the efficiency of photosynthesis. Under normal light growth conditions, our experiments detected significant changes in the soluble chloroplast proteome as a consequence of the loss of function of Executors that, interestingly, resembled the acclimation response of the plant to increased light. Our results suggest that Executor proteins form part of the signalling network for the perception of environmental perturbation in plants, and might participate in the basal repression of defence responses in chloroplasts under normal irradiance.

Supplementary material

Supplementary material is available at JXB online.

Supplementary Fig. S1. Verification of two A. thaliana T-DNA lines homozygous for an insertion within EX1 and EX2, respectively.

Supplementary Table S1. Number of spots differentially expressed (up or down-regulated) in response to genotype (WT, ex1 and ex2 plants) and light treatment (NL and HL).

Supplementary Table S2. List of differentially expressed proteins identified by mass spectrometry in the SwissProt/TAIR9 databases.

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