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by Jana Baldrianová, Martin Černý, Jan Novák, Petr L. Jedelský, Eva Divíšková and Břetislav Brzobohatý

See Journal of Proteomics for the final version: doi:10.1016/j.jprot.2015.02.011

TITLE PAGE

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Running head:

Karrikin-response proteome in *Arabidopsis*

Authors:

Jana Baldrianová[1*], Martin Černý[1*], Jan Novák[1], Petr L. Jedelský[2,†], Eva Divíšková[1] and Břetislav Brzobohatý[1]

(1) Laboratory of Plant Molecular Biology, Institute of Biophysics AS CR, v.v.i. and CEITEC – Central European Institute of Technology, Mendel University in Brno, Zemědělská 1, CZ-613 00 Brno, Czech Republic

(2) Department of Cell Biology, Faculty of Science, Charles University in Prague, Viničná 7, CZ-128 43 Prague, Czech Republic

(†) Present address: Research Department, Faculty of Arts, Charles University in Prague, Náměstí Jana Palacha 2, CZ-116 38 Prague, Czech Republic

*These authors contributed equally to this work.

Jana Baldrianová 51197@mail.muni.cz

Martin Černý martincerny83@gmail.com

Jan Novák novakhonza@atlas.cz

Petr L. Jedelský petr.jedelsky@natur.cuni.cz, petr.jedelsky@seznam.cz

Eva Divíšková diviskova.e@seznam.cz
Břetislav Brzobohatý brzoboha@ibp.cz

Corresponding author:
Name: Břetislav Brzobohatý
Address: Mendel University in Brno, Zemědělská 1, CZ-61300 Brno, Czech Republic
Phone: +420-545133359
Fax: +420-545133295
E-mail: brzoboha@ibp.cz

Number of figures: 6
Number of tables: 6
Supplementary tables: 4
Supplementary figures: 5

ABSTRACT

Karrikins are butenolide plant growth regulators in smoke from burning plant material that have proven ability to promote germination and seedling photomorphogenesis. However, the molecular mechanisms underlying these processes are unclear. Here we provide the first proteome-wide analysis of early responses to karrikin in plants (*Arabidopsis* seedlings).

Image analysis of two-dimensionally separated proteins, Rubisco-depleted proteomes and phosphoproteomes, together with LC-MS profiling, detected >1900 proteins, 113 of which responded to karrikin treatment. All the differentially regulated proteins (except HSP70-3) are novel karrikin-responders, and most are involved in photosynthesis, carbohydrate metabolism, redox homeostasis, transcription control, proteosynthesis, protein transport and processing, or protein degradation. Our data provide functionally complementary information to previous identifications of karrikin-responsive genes and evidence for a novel karrikin signalling pathway originating in chloroplasts. We present an updated model of karrikin signalling that integrates proteomic data and is supported by growth response observations.

Keywords

smoke, proteome, growth regulators, karrikin, butenolide

1. Introduction

Plant growth and development processes are controlled by numerous signalling molecules, both endogenous and exogenous. Some, including auxins, cytokinins, abscisic acid, ethylene and gibberellins, have been known for decades, while others have only been discovered recently and both their functions and signalling mechanisms are poorly understood. The latter include karrikins, butenolides identified in smoke from burning plant material [1, 2]. These heterocyclic molecules, containing a five-membered butenolide ring fused to a six-membered pyran ring, promote germination of fire-following plant species. To date, six compounds with similar action have been identified in smoke, KAR_1 being the most effective [3-5]. Karrikins also induce responses in the model plant *Arabidopsis*, notably stronger promotion of seed germination than gibberelins, 1-aminocyclopropane-1-carboxylic acid and *epi*-brassinolide [6], and seedling photomorphogenesis, including inhibition of hypocotyl elongation and cotyledon expansion [7, 8]. Relatively little is known about karrikin signalling, but it has apparent similarity to signalling mediated by the structurally related plant hormones strigolactones. A proposed model summarized by Waters and co-workers [9] postulates that karrikin binding may induce conformational changes in α/β–fold hydrolase KAI2 and its association with MAX2, an F-box component of E3 ubiquitin-protein ligase, thereby targeting signalling repressor(s) for degradation. SMAX1 and SMAX1-like proteins act downstream of MAX2 and could be the hypothetical repressors. Further, karrikin signalling requires HY5 for full photomorphological responses, induces auxin-response genes and recruits gibberellins by increasing transcription of gibberellin oxidases (GA3ox1, 2). The involvement of proteasome-mediated signalling and similarity to strigolactone signalling indicates that proteome dynamics could mediate at least some karrikin responses. However, no proteome-wide analysis of karrikin signalling has been previously reported. We recently showed that state-of-the-art proteomic analyses can elucidate early links between plant hormone cytokinin signalling and temperature perception [10-12]. We have now applied this approach to identify early karrikin-response proteins and refine the emerging model of karrikin action in *Arabidopsis*.

2. Materials and methods

2.1. Plant material, growth conditions and KAR_1 treatment for proteomic experiments

Unless specified otherwise, seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) were surface-sterilized and sown on Uhelon 120T (Silk & Progress, Brněnec, Czech Republic). Meshes were placed on Murashige and Skoog medium (pH 5.7) solidified with 1% (w/v) agar, stratified at 4 °C for 48 h and cultivated at 21°C/19°C day/night temperatures, with a 16 h photoperiod (90 μmol m^{-2} s^{-1} light intensity) for 7 d in an AR-36L growth chamber (Percival, Perry, IA, USA). For proteome profiling, Uhelon mesh supporting 7-day-old seedlings was transferred onto liquid MS medium supplemented with mock (ddH_2O) or 1 μM KAR_1 (kindly provided by Dr. Martin Vlk, Czech Technical University, Prague, Czech Republic) and incubated for 15 min. Seedlings were then rapidly harvested, dried, flash-frozen and ground in liquid nitrogen. For morphological and chlorophyll fluorescence analysis, a Uhelon mesh supporting 6-day-old seedlings was transferred onto liquid MS medium supplemented with mock (ddH_2O) or 1 μM KAR_1 and cultivation was continued for an additional 24 h under the environmental conditions specified above. Seedlings were divided into two sets for determining chlorophyll fluorescence parameters and morphological variables, root length and cotyledon area (using ImageJ software; http://rsbweb.nih.gov/ij/). The whole experiment was performed in three biological replicates.

2.2. Protein extraction, 2-DE analysis and protein identification

Arabidopsis proteome and sub-proteomes were extracted, prepared and analysed essentially as previously described. Briefly, total protein was extracted by acetone/trichloroacetic acid extraction [10, 13, 14], a Rubisco-depleted proteome was prepared [11, 15], and phosphoroteins were enriched [10]. Portions of each extract, 500 or 150 μg, were loaded onto 18 or 7 cm IPG strips (Bio-Rad, http://www.bio-rad.com/), respectively, isoelectrically focused (linear pH gradient, 4-7), and subsequently resolved by SDS-PAGE. Gels were stained with colloidal Bio-Safe Coomassie G-250 (Bio-Rad), digitally imaged and analysed using Decodon Delta 2D software (http://www.decodon.com). Responses to KAR_1 treatment of proteins corresponding to detected spots were deemed significant if there was an absolute KAR_1/mock spot volume ratio ≥ 1.4, with *t*-test p value<0.05, and similar profiles in two biological replicates of the total protein extract and Rubisco-depleted proteome (three technical replicates per sample), or three biological replicates for phosphoproteome comparison (two technical replicates per sample). Selected protein spots were digested with trypsin. The dried tryptic peptides were each dissolved in 10 µl of 0.1% trifluoroacetic acid and purified using ZipTip C18 tips and eluted directly on sample plate with 10 mg ml^{-1} CHCA in 50% v/v acetonitrile and 0.1% trifluoroacetic acid. Spectra were acquired using 4800 Plus MALDI TOF/TOF analyzer (AB Sciex) equipped with a Nd:YAG laser (355 nm) with firing rate 200 Hz. All spots were measured in MS mode and then up to ten strongest precursors were selected for MS/MS which was performed with 1 kV collision energy and operating pressure of collision cell set to 10^{-6} Torr. MS and MS/MS spectra were searched by local Mascot v. 2.1 (Matrix Science) against TAIR10 database of *Arabidopsis* protein sequences. Database search criteria were as follows: enzyme – trypsin; taxonomy – *Arabidopsis thaliana*; fixed modification – carbamidomethylation; variable modification - methionine oxidation; peptide tolerance - 80 ppm, allowed one missed cleavage; MS/MS tolerance - 0.2 Da.

2.3. LC-MS proteome profiling

Quantitative proteomic analyses were also performed using a gel-free shotgun protocol based on nano-HPLC and MS/MS. Briefly, two independently grown replicates, each consisting of approximately 300 *Arabidopsis* seedlings cultivated as described above, were extracted by acetone/TCA and phenol extraction then digested in solution with immobilized trypsin beads (Promega, http://www.promega.com/). The resulting peptides were desalted, dried and dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, then analysed by nanoflow C18 reverse-phase liquid chromatography using a 15 cm Ascentis Express Column (0.1 mm inner diameter; Sigma-Aldrich) and a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo, www.thermoscientific.com) directly coupled to a CaptiveSpray nanoESI source (Bruker) and an UHR maXis impact q-TOF mass spectrometer (Bruker, www.bruker.com). Peptides were eluted with up to a 180-min, 4% to 40% acetonitrile gradient. Raw files obtained from the MS analysis were analysed by Profile Analysis 2.1 (Bruker) and MS precursors with significant differences (absolute ratio ≥1.5, with *t*-test p-values <0.05) were targeted and identified in consecutive MS/MS analyses. MS/MS spectra were acquired in an intensity dependent mode at a rate of 2-20 Hz with a maximum of 20 precursor ions and a MS spectra rate of 2 Hz. Data from MS/MS data-dependent measurements were processed by DataAnalysis 4.1 (Bruker) and searched against the TAIR10 *Arabidopsis* database using the Mascot 2.4 (Database search criteria: trypsin; variable modifications - methionine oxidation, NQ deamidation, ST phosphorylation; peptide tolerance - 10 ppm; allowed one missed cleavage; MS/MS tolerance - 0.06 Da) and Bruker's ProteinScape percolator algorithms (target FDR<1%) to identify source proteins (using high-confidence peptides, p <0.05 with at least one distinct proteotypic peptide per protein). Quantitative differences were further manually validated by comparing corresponding peptide ion signal peak areas in Skyline 1.4 (MacCossLab Software; https://skyline.gs.washington.edu).

2.4. Proteomic data analysis

Information about functions of identified proteins was collected from available literature and the UniProt, UniGene (http://www.ncbi.nlm.nih.gov/unigene), TAIR (http://www.

Arabidopsis.org), KEGG (http://www.genome.jp/kegg/pathway.html), conserved domains (http://www.ncbi.nlm.nih.gov/Structure/index.shtml), and homology (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases. Protein-protein interactions were evaluated by String 9.1 (http://string-db.org/, [16]). Overrepresentation of Gene Ontology categories was analysed by the BioMaps module of VirtualPlant 1.3 (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/, [17]), using the *Arabidopsis thaliana* TAIR 10 genome, TAIR/TIGR GO biological process assignments, and p-values of over-representation ≤ 0.05. OriginPro 9.0 was then used for PCA analysis. In the presentation of results, numbers following named proteins are spot numbers, while the alphanumerical designations L and P with numbers (e.g. L23 and P58) are codes assigned to differentially expressed proteins detected in the LC-MS and phosphoproteome analyses, respectively.

3. Results

3.1. Proteomic responses to KAR_1-treatment

To obtain insights into early proteomic responses to karrikin, 7-day-old *Arabidopsis* seedlings were treated with 1 μM KAR_1 for 15 minutes. The experimental design is outlined in Figure 1. First, whole proteome changes were monitored by 2-DE. To increase coverage of seedlings' proteomes, we combined standard denaturative acetone/TCA whole protein extraction with native extraction followed by immunodepletion of Rubisco or affinity-based isolation of phosphoproteins. The isolated proteins were subjected to 2-DE (Fig. 2). Image analysis of the resulting proteome maps yielded 114 spots with at least 1.4-fold absolute variation between control and karrikin-treated samples in all biological replicates ($P<0.05$; Fig. 2), which were subjected to protein identification. Altogether, 74 proteins were identified in the 85 spots, including three protein mixtures, by MALDI TOF/TOF MS analysis followed by Mascot database searches against the TAIR 10 database (Additional file 1: Table S1). Of these proteins, 63 and 17 were detected in comparisons of the total protein extract with the Rubisco depleted sub-proteome and phosphoproteome, respectively.

To complement results from the protein-based 2-DE analysis (which provides excellent qualitative data) we applied a more sensitive peptide-based quantitative approach: LC-MS shotgun proteomic analysis of total soluble proteins. In total, we quantified relative peptide abundances in 1890 proteins, detected in at least nine repeated LC-MS experiments, represented by more than 5600 peptides with 3.3 peptides per protein on average and average sequence coverage of 12.3%. Comparisons after Skyline validation of MS spectra and elimination of low-confidence peptides (e.g. peptides susceptible to non-enzymatic modifications [18]) revealed 49 differentially regulated proteins quantified by 109 unique peptides (for details, see Additional files 2, 3).

Combining the 2-DE and LC-MS datasets revealed limited overlaps of the differentially regulated proteins/proteoforms in the sub-proteomes. In total, we detected 113 karrikin-responsive proteins, 15 present in several proteoforms. Only 11 of these differentially regulated proteins were detected in multiple subproteome fractions, most of which probably represent different proteoforms of the same protein (Additional file 4: Table S4). Analysis of the Rubisco-depleted proteome further validated results from total protein extract 2-DE and detected 9 additional karrikin-responsive proteins (Supplementary Fig. S1).

Determination of subcellular locations of the identified differentially regulated proteins, using the SUBA database (http://suba.plantenergy.uwa.edu.au/; [19]), indicated that 62 (50%) and 32 (26%) are chloroplastic and cytosolic, respectively. Corresponding proportions for all proteins identified by LC-MS were both ca. 30% (Table S3). Thus, chloroplastic proteins are significantly overrepresented in the karrikin-responsive proteome.

3.2. Novel links in karrikin responses

Our proteomic analyses detected only one *Arabidopsis* gene identified as being karrikin-responsive in previous transcriptomic analyses (Heat shock 70kDa protein 3; L23, decreased after KAR_1-treatment). This protein is induced during germination [20] and is involved in protein-protein interactions with several partners, including the deubiquitinating enzyme AMSH3, which is involved in intracellular trafficking [21], and a ubiquitination target [22]. Further, its close human homolog HSPA8 (>75% identity) participates in transcription control [23]. The remaining 112 differentially regulated proteins we detected are novel KAR_1-responsive factors. However, our protein-protein interaction network and functional enrichment of metabolic pathways analyses showed that significant proportions of these proteins have functional matches or complement previously identified karrikin-responsive genes. These are proteins involved in carbon fixation and photosynthesis, RNA metabolism, lipid metabolism and glutathione metabolism. Categories ‘Citric acid cycle’, ‘Protein transport’, proteasome mediated processes, and ribosome components were highlighted only in karrikin-response proteome (Fig. 3). All identified karrikin-responsive proteins were divided according to their predominant function into the following categories: Photosynthesis and carbohydrate metabolism; Redox homeostasis; Transcription; Proteosynthesis, protein transport and processing; and Protein degradation (Tables 1-5, respectively). The remaining categories (each represented by less than five proteins) are grouped in Table 6.

3.3. Proteins involved in photosynthesis and carbohydrate metabolism are rapidly regulated in karrikin responses

The most significant overlap between known karrikin-responsive genes and the karrikin-responsive proteins we identified was among those involved in carbohydrate metabolism (7 genes and 17 proteins; Fig. 3). The significantly regulated proteins included 23 enzymes and seven structural components of the photosynthetic apparatus, some present in multiple proteoforms (Table 1). KAR_1-treatment increased levels of enzymes involved in chlorophyll biosynthesis (protoporphyrinogen oxidase 1; porphobilinogen deaminase). However, it appeared to have generally negative effects on photoassimilatory proteins, including: Calvin cycle enzymes (Rubisco - 18|L44|P18|P21|P31; chaperonin 60, required for Rubisco folding – 46; Rubisco activase - P22|P30; glyceraldehyde-3-phosphate dehydrogenase - L6|P25|P26; fructose-bisphosphate aldolase - L13|L34; transketolase – 72; phosphorybulokinase - P20) and enzymes involved in both downstream and upstream metabolic pathways of carbon fixation (carbonic anhydrase) and carbohydrate metabolism (aldolase 1-epimerase, fructokinase, and nucleotide-sugar biosynthesis enzymes). Similarly, it reduced levels of constituents of PSI (light harvesting complex – 36; chlorophyll binding protein – 33; PSI reaction centre - L11|L33), PSII (Oxygen-evolving enhancer proteins – L26|P14|L29) and ATP synthase (L43).

3.4. Proteasome-mediated signalling components in the KAR$_1$ responsive proteome

KAI2, MAX2 and ubiquitin-proteasome signalling is the only reported molecular mechanism of karrikin perception. Thus, proteins involved in ubiquitin-dependent processes in the karrikin-responsive proteome should be particularly interesting. We were not able to detect KAI2 (At4g37470), nor MAX2 on protein level. However, KAR$_1$ treatment increased levels of a proteasome subunit (76) and two deubiquitinating enzymes (9, 82) (Table 2). One, ubiquitin carboxyl-terminal hydrolase 14, is important for development and its mutation results in enlarged endosperm nuclei and defective embryos [24]. The treatment also upregulated the ubiquitin receptor protein RAD23, which reportedly interacts with a proteasome docking subunit and participates in recognition of ubiquitinated substrates [25]. Products of proteasome activity are predominantly peptides up to 12 amino acids long that are subsequently processed by peptidases. Accordingly, we detected significant changes in abundance of two dipeptidases (L38, L49) and one tripeptidase (L30). Our data also indicate more potential links with the ubiquitin-dependent pathway, e.g. the changes in levels of Heat shock 70kDa (ubiquitinated, interacts with deubiquitinating enzyme) described above, but the evidence is less conclusive. An alpha/beta domain-containing hydrolase with an uncertain function and expected mitochondrial location (L31) was also upregulated. However, sequence comparison does not indicate that the hydrolase is a mitochondrial counterpart of KAI2 (identity <12%).

3.5. Transcription regulators involved in KAR$_1$ responses

Previous transcriptomic analyses have detected five KAR-responsive *Arabidopsis* genes involved in transcription control, but the proportion of genes covered by the analyses (and proteins covered by our analyses) is too low to pinpoint any significant over-representation in this category. However, our supervised analyses detected upregulation of several relevant proteins (Table 3), including two (DNA topoisomerase-like protein, 48, and Nucleosome assembly protein, 10) indicating that KAR-treatment induces DNA uncoiling. Another three proteins are classified as mediators (19, 53) or a probable mediator (L35) of RNA polymerase II, which directly participates in transcription regulation. Further, all these mediators share sequence similarity with Heat shock 70kDa protein 3 (> 60% for the first two and >92% for L35) which is also apparently a transcription mediator (Supplementary Fig. S2). Furthermore, KAR_1-treatment affected RNA processing, or at least expression of two RNA helicases — 14 (expected to be involved in nonsense-mediated mRNA decay and ribosome biogenesis) and 56 (pre-mRNA splicing, mRNA export from nucleus) — and ribonucleoprotein At2g37220. At2g37220 was significantly affected by KAR_1-treatment in all analysed proteome fractions (38|L17|P19), contains an RRM domain and plays an expected role in chloroplastic RNA processing. We also detected posttranslational modification(s) in Cold shock protein 2 (59 - increased, 77 - decreased), an RNA chaperone that is regulated by cold and developmental signals [26].

3.6. The early karrikin-response proteome regulates proteosynthesis

Translation regulates all physiological processes, directly or indirectly. Accordingly, we detected 28 karrikin-responsive proteins (35% of all differentially expressed proteins) involved in ribosome assembly (26|L4|L14|L15|L18|L22|L24|L27), translation initiation (75|L39|P1|P12), aminoacyl t-RNA synthesis (54), elongation (5|13|67|P8|P28), and protein processing and transport (7|8|12|46|57|L25|L32|L41|L45|L46). Overall, KAR_1-treatment seemed to inhibit proteosynthesis (at least cytosolic proteosynthesis) and related processes (Table 4). However, the responses are probably complex. For example, changes in ribosome composition may have severe effects on plant growth and development [27]. Mutant phenotypes are known for two KAR-responsive ribosomal proteins identified here: 50S ribosomal protein L11 (slow growth, pale green; [28]) and 60S ribosomal protein L23 (embryo defective; [29]). Since ribosomal proteins have been implicated in auxin and cytokinin signalling [11, 30, 31], some KAR-effects are probably results of stimulation or interference with ribosome-mediated signalling of endogenous hormones.

3.7. Similarities in response to plant hormones and KAR treatment

Karrikin is not recognized as a plant hormone because it may not be endogenously synthesised, but since it has similar effects to known plant hormones we compared GO-category KAR_1 responses with those elicited by abscisic acid, auxin (IAA), brassinosteroids (BR), cytokinin (CK), ethylene, jasmonic acid (JA), gibberellins (GA) and salicylic acid (SA) (Fig. 4). Our analysis covered 128 karrikin-responsive genes and 113 proteins, and 3453, 1245, 861, 672, 973, 348, 778 and 541 TAIR-annotated genes linked to the respective hormones [32, 33]. Altogether, over 900 GO categories are significantly overrepresented in sets of genes or proteins responding to at least one of the listed substances. The diversity of processes apparently up- or down-regulated by them (number of overrepresented GO categories) decreased in the order JA>SA≥IAA>ethylene>ABA>GA>KAR>CK>BL. The GO category ‘Response to karrikin’ (which does not yet include proteins identified here) is not significantly enriched in sets that reportedly respond to plant hormones, unsurprisingly as no responses to any of the listed phytohormones have been detected for >50% of karrikin-responsive genes. Similarly, only 28 of the proteins identified here reportedly respond to phytohormones: 15, 10, 9, 2 and 1 to ABA, JA, SA, IAA and BR, respectively. Nevertheless, our PCA indicates that the differences between hormonal and KAR_1 responses is not dramatic. The first dimension, covering nearly 67% of total variability, clusters KAR- and BR-responsive proteins in the same group. Unfortunately, we could not include strigolactone in the analysis, as there are too few annotated strigolactone-responsive genes.

3.8. Fluorescence measurements suggest photosynthesis is modulated in karrikin responses

To obtain further insights into karrikin effects on photosynthetic processes we measured chlorophyll fluorescence parameters of *Arabidopsis* seedlings cultivated on medium supplemented with 1 μM KAR_1 for the last 24 h of a 7d cultivation period (Supplementary Fig. S3, S4). Then we measured the seedling's cotyledon surface area, root length and maximum PSII quantum yield in a dark-adapted state (F_v/F_m) to acquire information about photosynthetic processes following light adaptation (Supplementary Fig. S4). Calculated F_v/F_m ratios did not differ between karrikin- and mock-treated seedlings, indicating that properties of PSII and associated processes were not significantly affected. However, as already described, the proteomic analyses indicate that KAR_1 treatment reduced levels of PSI constituents and enzymes involved in carbon photoassimilation. This is likely to repress processes required for efficient light adaptation and hence the quantum efficiency of PSII (ΦPSII). Accordingly, 24 hours after transfer to KAR_1-supplemented medium we detected a slight decrease in this parameter. We also observed an increase in NPQ (non-photochemical quenching), which corresponds to thermal energy dissipation and may correlate with a decrease in energy transfer to PSI. However, the changes in the photosynthetic parameters were not statistically significant at $P<0.05$.

4. Discussion

4.1. Profiling early proteomic responses to karrikin in Arabidopsis

Karrikin responses have been previously examined in bioassays, selected mutants and transcriptomic analyses [3, 6-8, 34-39], but not (to our knowledge) in proteome-wide analyses. However, protein abundance is not dependent only on transcription, and posttranslational modifications can rapidly activate or inactivate enzymes without degradation or *de novo* synthesis (e.g. [40, 41]). Thus, proteome analysis can complement transcriptomic analysis and extend insights into molecular processes. Karrikins were discovered as germination-promoting substances, but their effect is not limited to this developmental stage. They also promote cotyledon expansion and inhibit hypocotyl elongation of *Arabidopsis* seedlings [8], eliciting responses at nanomolar to micromolar concentrations [4], like plant hormones. This prompted us to employ the approach we established for identifying cytokinin-responsive proteins [10, 12], combining affinity depletion of Rubisco and phosphoproteome isolation with 2-DE – MALDI TOF/TOF and LC-MS identification of differentially regulated proteins. Preliminary experiments confirmed that micromolar KAR_1 elicits reported growth responses [8] under experimental conditions similar to those applied in our proteomic experiments, promoting both root and cotyledon growth when applied for the last 24 h of a 7-day cultivation period (Fig. 5).

4.2. Down-regulation prevails in proteomic responses to karrikin

Karrikin treatment reportedly up-regulates transcription of most (>80%) differentially expressed genes [8], but most (69%) early karrikin-response proteins we detected were down-regulated: 97% in the phosphoprotein-enriched fraction and ~62% in sets detected in both the LC-MS and total protein 2-DE (including Rubisco-depleted fraction) analyses. Interestingly, we observed similar proportions in analyses of early cytokinin (~67%) and temperature-shock (~62%) response proteins [10, 12]. We hypothesise that processes that reduce protein/proteoform abundance may be preferentially employed in rapid responses to stimuli because they are faster than protein synthesis.

4.3. Plastids – the site of missing links in karrikin signalling?

Early karrikin-response proteins we detected in seedlings are localized mainly to the chloroplast (50% of the set). The plastidic genome of vascular plants has lost most of its original content and encodes fewer than 100 open reading frames [42, 43]. Thus, plastid development and function are highly dependent on the nucleus and largely under its control. However, plastids also generate signals that modulate nuclear gene expression via several pathways, including redox signalling [42]. Accordingly, we identified 11 differentially expressed proteins involved in redox homeostasis (Table 2). Of these, six have a chloroplast location, including monothiol glutaredoxin-S12 (L12), thioredoxin-like protein CDSP32 (25) and thioredoxin M1 (L1). Thioredoxins (Trx) participate in day/night metabolism switches, including light-dependent regulation of the Calvin–Benson cycle, but they also apparently participate in protein targeting, cell-to-cell trafficking and gene regulation [44] and CK responses [11, 45]. The m-type of Trx is also essential for chloroplast development [46]. Thus, Trx could be the missing link between light signalling and transcription factor HY5.

4.4. Posttranslational control in KAR_1 responses

Our 2-DE data provide several indications that targeted proteasome-mediated protein degradation is not the only posttranslational mechanism involved in karrikin signalling. First, differences between theoretical and observed pI/MW values indicate that >50% of significantly regulated proteins detected in our 2-DE analysis are probably posttranslationally modified proteoforms (Additional file 1: Table S1). Identification and characterization of these modifications detected in the total protein extract/Rubisco depleted proteome comparison is beyond the scope of this work. However, our targeted phosphoproteome analysis indicates that changes in phosphorylation status are involved, at least indirectly. We have previously demonstrated that the phosphoprotein enrichment protocol we employed is fairly specific by validation with phospho-specific staining and MS/MS sequencing [10, 12]. Here, we supplemented our previous validation by Western blot analysis (Supplementary Fig. S5). We were able to follow dynamics in phosphoproteome, but the biological relevance of detected phosphorylations mostly remains to be elucidated. For some of them, even the function of a protein itself is not clear. Phosphorylation could be involved in a direct karrikin signalling mechanism, but with the combined sensitivity of 2-DE analysis and MALDI-MS we detected regulatory phosphorylation for only 17 proteins, all of which seem to be influenced indirectly. Literature and a homology search indicate that one detected dephosphorylation (of Rubisco; P18, P21, P31) would be inhibitory [47] while another (of the cytosolic enzyme triosephosphate isomerase; P29) would be activating [48], which correlates with observed regulations in our dataset. Rubisco activase (P22, P30) has multiple phosphorylation sites, some also with a potential on/off function [49]. Elongation factor Tu (P8) is one of four detected phosphoproteins involved in proteosynthesis. Phosphorylation of this chloroplastic protein prevents ternary complex formation [50] and its regulation in response to karrikin likely represents an increased proteosynthesis in chloroplasts. The last phosphorylation which should be discussed is that of chloroplastic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH; P25, P26). Although the prominent role of GAPDH is commonly known to be in carbohydrate metabolism, its homolog also participates in intracellular trafficking. Tisdale (2002) reported that phosphorylation of GAPDH influences microtubule dynamics in the early secretory pathway [51]. In this respect, we can note that chloroplastic proteins like Trigger factor-like protein TIG (L41, protein exporter) or

Chaperone protein ClpC1 (57) indicate that there are changes in chloroplast protein trafficking after karrikin treatment.

4.5. Karrikin signalling – an updated model

Considerable time may be required for manifestation of transcriptional control at the protein level. For example, heat stress induces significant increases in Hsp70 transcript levels, peaking within minutes, but significant changes (gradual rises) in HSP70 protein levels only occur after an hour [52]. Thus, we believe that the protein dynamics revealed by our data cannot correlate with transcript levels and originate in posttranscriptional and/or posttranslational control. This is consistent with recent indications that posttranscriptional, translational, posttranslational and degradation govern protein concentrations at least as strongly as transcription (see e.g. [53]). Proteasome-dependent degradation could explain some observed karrikin responses, and even increases in protein abundance could result from degradation of repressors. However, the sheer number of differentially expressed chloroplastic proteins we detected indicates involvement of an alternative proteasome-independent and chloroplast-based signalling pathway. We did not pinpoint a novel karrikin receptor *per se*, but our data provide evidence for several novel signal transduction pathways. We have already discussed karrikin-responsive proteins involved in ribosome biogenesis (posttranscriptional control) and redox signalling that could participate in retrograde chloroplast-to-nucleus signalling, and the whole concept is illustrated in Fig. 6. Further, we observed a decrease in levels of phospholipase D (42), representing a link to calcium signalling and crosstalk with endogenous hormones like ABA or CK [54, 55].

4.6. Could inhibition of photosynthesis stimulate growth?

Karrikin treatment for 24 h increased root length and cotyledon area in *Arabidopsis* seedlings (Fig. 5), in accordance with reported effects, but seemingly conflicting with indications from our proteome analysis that it reduces photoassimilation. Furthermore, our fluorescence measurements suggest that karrikin reduces photoassimilatory energy transfer and increases thermal energy dissipation (although it does not affect PSII properties), which should reduce photosynthetic efficiency. We hypothesize that for a limited period compensatory mechanisms may be induced by such changes, including cotyledon expansion, which increases plants' photosynthetic area. As karrikin signalling reportedly requires HY5 for full photomorphological responses [8], we examined the abovementioned responses in a *hy5* mutant, which responded largely as wild-type Col-0. Thus, HY5 function is likely not critical for these responses (Supplementary Fig. S3).

5. Conclusions

We have pioneered a proteomic analysis of karrikin responses in the model plant *Arabidopsis thaliana*. Combination of a traditional 2-DE approach with robust LC-MS analysis resulted in identification of numerous novel karrikin-response proteins that provide novel targets for detailed mechanistic studies using, e.g., mutants and transgenic plants. The data obtained fundamentally deepen our understanding of karrikin roles in chloroplast functions. Further, the comparative analysis provided novel indications of a chloroplast-based signalling pathway that operates in parallel to KAI2-mediated karrikin signalling.

Author contributions

JB, MČ, JN, PLJ and ED performed the experiments and analysed the data. BB and MČ conceived the study and wrote the manuscript. All the authors approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgement

We thank Dr. Martin Vlk for supplying KAR_1 and Prof. Johannes van Staden for discussions during preparatory phases of this work, which was supported by grants P305/12/2144 (CSF), TE02000177 (TACR), 1M06030 (MEYS CR), MSM0021620858 (P.L.J.; MEYS CR), and funds from the ERDF for ‘CEITEC–Central European Institute of Technology’ (CZ.1.05/1.1.00/02.0068). Access to the MetaCentrum computing facilities provided under the Projects of Large Infrastructure for Research, Development, and Innovations program (LM2010005), funded by the Ministry of Education, Youth, and Sports of the Czech Republic is highly appreciated. M.Č. was supported by the Operational Program Education for Competitiveness - European Social Fund (Project CZ.1.07/2.3.00/30.0017, for Postdocs in Biological Sciences at MENDELU).

Appendix A. Supplementary data

The data sets supporting the results of this article are included within the article and its additional files.

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Figure Legends

Fig. 1 - Experimental design.

Fig. 2 - Effects of KAR_1 on the proteome and phosphoproteome of *Arabidopsis* seedlings. Average two-dimensional gel electrophoresis maps of total protein extracts and RuBisCO-depleted proteomes (A) and phosphoproteomes (B) of 7-day-old *Arabidopsis* seedlings treated with karrikin/mock buffer for 15 min. Differentially regulated protein spots are indicated. See Tables 1-6 and Additional files for detailed information on the corresponding identified proteins. Proteins were separated in the first and second dimensions by IPG — 7 cm or 18 cm strips, pH 4-7 - followed by 8-20% (7 cm) or 11% (18 cm) SDS–PAGE then visualized by Bio-Safe Coomassie G250 staining. Isoelectric points (pI) and migrating positions of molecular mass (kDa) markers are marked. For details see *Materials and methods*. Decodon Delta 2D was used to compensate for the PAGE background and thus optimize the visibility of all differentially regulated protein spots.

Fig. 3 - Protein–protein interaction network constructed using STRING (http://string-db.org; [16]). Karrikin-responsive genes (TAIR, Nelson and co-workers [8]; blue) and proteins identified here (brown) were analysed for protein-protein interaction networks. String analysis indicates that two sets overlap in several categories (purple). Categories 'Flavonoid biosynthesis' and 'Light induced processes' are extensively regulated according to the transcriptomic data, while significant effects of karrikin on the TCA cycle, protein transport and proteasome-mediated processes are apparent only in our proteomics data.

Fig. 4 - Functional classification of genes and proteins responsive to phytohormones and karrikin. PCA analysis indicates that karrikin has distinct effects, with some similarities to

those of recognised plant hormones (ABA, abscisic acid; BR, brassinosteroids; CK, cytokinin; ethylene; IAA, auxin; JA, jasmonic acid; GA, gibberellins; SA, salicylic acid). Although the overlap between previously found karrikin-responsive genes (K_{TAIR}) and novel karrikin-responsive proteins (K_{Prot}) identified here is minimal (protein L23, Heat shock 70kDa protein 3), the similarity in over-represented GO categories cluster them in the same group. Data for principal component analysis were mined from the TAIR database (9/2014) and large-scale hormonal transcriptomic datasets [32, 33], followed by functional classification using BioMaps [17]. GO categories of interest are marked. For details, see *Materials and Methods*.

Fig. 5 - Effects of prolonged KAR_1 treatment on *Arabidopsis* seedlings.

Selected morphological parameters of 7-day-old Col-0 seedlings exposed for the last 24 h to 1 μM KAR_1. Photographs of representative seedlings for each treatment are shown. Data shown are means of two biological replicates, each with >40 seedlings per replicate, and standard errors (SE; error bars). Asterisks indicate statistically significant (Student's *t*-test $p<0.05$) differences.

Fig. 6 - Integration of karrikin-response proteins into a karrikin signalling model.

Proteasome-dependent signalling: Karrikin binding to KAI2 initiates a proteasome-dependent cascade and regulates proteins involved in ubiquitination and a di/tripeptide cleavage (Table 5), accounting for observed reductions in abundance of proteins involved in transcription control (Table 3). Rapid changes in the chloroplastic proteome indicate the presence of an alternative signalling pathway originating in chloroplasts. Karrikin negatively affects proteins involved in photosynthesis and subsequent carbon assimilation (Table 1). Consequently, reductions in ATP fluxes from photosynthesis would likely be compensated (at least temporarily) by increases in pyruvate dehydrogenase activity, TCA cycle fluxes and respiration rates, accompanied by reductions in lipid biosynthesis (Table 6). Alterations in photoassimilation modulate redox homeostasis (Table 2) and through retrograde signalling may influence gene expression. Signalling from both pathways thus converges on proteosynthesis and protein transport (Table 4).

Suppementary data

Table S1 - Differentially regulated proteins detected by 2-DE-based analysis.

Table S2 - Differentially regulated proteins detected by LC-MS-based analysis.

Table S3 - Proteins identified in LC-MS based analysis.

Table S4 - Overlaps of differentially regulated proteins in sets analysed by 2-DE and LC-MS.

Figure S1. Differentially regulated protein spots revealed after Rubisco depletion.

Figure S2. Sequence analysis of HSP70 proteins.

Figure S3. Effects of prolonged KAR_1 treatment on *Arabidopsis* seedlings.

Figure S5. Western blot detection of phosphorylated proteins.

Tables

Table 1 - Proteins involved in photosynthesis and carbohydrate metabolism

Spot/protein no.	AGI	Name	Abundance KAR$_1$:mock
51	AT5G66530	Aldose 1-epimerase family protein	↓
L43	ATCG00120	ATP synthase subunit alpha, chloroplastic	↓↓
22|P5	AT3G01500	Carbonic anhydrase 1	↓|↓↓
33	AT1G29910	Chlorophyll a-b binding protein 2	↓
23	AT5G61410	D-ribulose-5-phosphate-3-epimerase	↑
16	AT1G20020	Ferredoxin-NADP reductase	↑
30|L9	AT1G66430	Fructokinase	↓↓|↓↓
L6	AT1G42970	Glyceraldehyde-3-phosphate dehydrogenase	↓
P25	AT1G42970	Glyceraldehyde-3-phosphate dehydrogenase	↓
P26	AT1G42970	Glyceraldehyde-3-phosphate dehydrogenase	↓
L26	AT5G66570	Oxygen-evolving enhancer protein 1-1	↓
	AT3G50820	Oxygen-evolving enhancer protein 1-2	
P14	AT5G66570	Oxygen-evolving enhancer protein 1-1	↓↓
44	AT1G06680	Oxygen-evolving enhancer protein 2-1	↑↑
L29	AT4G05180	Oxygen-evolving enhancer protein 3-2	↓↓
L7	AT1G56190	Phosphoglycerate kinase 2	↓↓
35	AT2G45790	Phosphomannomutase	↑
P20	AT1G32060	Phosphoribulokinase	↓
36	AT3G61470	Photosystem I light harvesting complex protein	↓
L33	AT4G28750	Photosystem I reaction center subunit IV A	↓↓
L11	AT2G20260	Photosystem I reaction center subunit IV B	↓
L36	AT5G08280	Porphobilinogen deaminase	↑↑
L13	AT2G21330	Probable fructose-bisphosphate aldolase 1	↓↓

28|L34	AT4G38970	Probable fructose-bisphosphate aldolase 2	↑|↓↓
L28	AT4G01690	Protoporphyrinogen oxidase 1	↑
L3	AT1G23740	Quinone oxidoreductase-like protein	↑↑
18|L44	ATCG00490	Ribulose bisphosphate carboxylase large chain	↓|↓
P18	ATCG00490	Ribulose bisphosphate carboxylase large chain	↓
P21	ATCG00490	Ribulose bisphosphate carboxylase large chain	↓↓
P31	ATCG00490	Ribulose bisphosphate carboxylase large chain	↓↓
P22|P30	AT2G39730	Rubisco activase	↓↓|↓↓
72	AT3G60750	Transketolase-1, chloroplastic	↓
L20	AT2G45290	Transketolase-2, chloroplastic	↑↑
P29	AT3G55440	Triosephosphate isomerase, cytosolic	↓
P27	AT3G29360	UDP-glucose 6-dehydrogenase 2	↓

Spot/protein no.	AGI	Name	Abundance KAR_1:mock
21	AT1G07890	L-ascorbate peroxidase	↓↓
64	AT4G08390	L-ascorbate peroxidase	↓
L2	AT1G11840	Lactoylglutathione lyase-like protein	↓↓
L8	AT1G63940	Monodehydroascorbate reductase	↑↑
L12	AT2G20270	Monothiol glutaredoxin-S12	↑
49	AT1G65980	Peroxiredoxin-2B	↑
32	AT1G67280	Probable lactoylglutathione lyase/glyoxalase	↑
24	AT3G10920	Superoxide dismutase [Mn]	↑
25	AT1G76080	Thioredoxin-like protein CDSP32	↑↑
L1	AT1G03680	Thioredoxin M1	↓↓
43	AT2G17420	Thioredoxin reductase 2	↓

Table 2 - Proteins involved in redox homeostasis

Table 3 - Proteins involved in transcription

Spot/protein no.	AGI	Name	Abundance KAR$_1$:mock
59|77	AT4G38680	Cold shock protein 2	↑↑|↓↓
L21	AT3G01540	DEAD-box ATP-dependent RNA helicase 14	↑↑
P9	AT5G11200	DEAD-box ATP-dependent RNA helicase 56	↓↓
48	AT3G15950	DNA topoisomerase-like protein	↑
L23	AT3G09440	Heat shock 70 kDa protein 3	↓
53	AT5G28540	Mediator of RNA polymerase II transcription subunit 37a	↓↓
19	AT5G42020	Mediator of RNA polymerase II transcription subunit 37f	↑↑
P2|P3	AT1G73230	Nascent polypeptide-associated complex NAC	↓↓|↓
10	AT4G26110	Nucleosome assembly protein 1-like 1	↑↑
L35	AT5G02500	Probable mediator of RNA polymerase II transcription subunit 37e	↓↓
38|L17|P19	AT2G37220	Ribonucleoprotein At2g37220	↑|↓|↓

Table 4 - Proteins involved in proteosynthesis, protein transport and processing

Spot/protein no.	AGI	Name	Abundance KAR_1:mock
57	AT5G50920	Chaperone protein ClpC1	↑
46	AT1G55490	Chaperonin 60 subunit beta 1	↓
L27	AT3G63140	Chloroplast stem-loop binding protein of 41 kDa a	↓↓
5	AT5G13650	Elongation factor family protein	↓↓
67	AT4G29060	Elongation factor Ts	↓
13|P8	AT4G20360	Elongation factor Tu	↓|↓↓
P12	AT1G10840	Eukaryotic translation initiation factor 3 subunit H	↓↓
P1	AT1G26630	Eukaryotic translation initiation factor 5A-2	↓
L32	AT4G24280	Heat shock 70 kDa protein 6	↓↓
L45	AT5G49910	Heat shock 70 kDa protein 7	↓↓
L46	AT4G37910	Heat shock 70 kDa protein 9	↓
8	AT1G79920	Heat shock 70 kDa protein 15	↓
L25	AT3G15520	Peptidyl-prolyl cis-trans isomerase CYP37	↓↓
7	AT3G25230	Rotamase FKBP 1	↓↓
P28	AT1G57720	Probable elongation factor 1-gamma 2	↓
54	AT5G52520	prolyl-tRNA synthetase	↓
12	AT2G47470	Protein disulfide-isomerase like 2-1	↓
26	AT5G24490	Putative 30S ribosomal protein	↓
P15|P16	AT1G07140	Ran-binding protein 1 homolog a	↓↓|↓↓
L39	AT5G38640	Translation initiation factor eIF-2B delta subunit-like protein	↑↑
75	AT3G13920	Translational initiation factor 4A-1	↑
L41	AT5G55220	Trigger factor-like protein TIG	↑↑

L18	AT2G37270	40S ribosomal protein S5-1	↓↓
L24	AT3G11510	40S ribosomal protein S14-2	↓↓
L14	AT2G21580	40S ribosomal protein S25-2	↑↑
L4	AT1G32990	50S ribosomal protein L11	↑↑
L15	AT2G27720	60S acidic ribosomal protein P2-1	↓↓
L22	AT3G04400	60S ribosomal protein L23	↓

Table 5 - Proteins involved in protein degradation

Spot/protein no.	AGI	Name	Abundance KAR_1:mock
L49	AT4G30920	Leucyl aminopeptidase 3	↑
11	AT4G38220	Peptidase M20/M25/M40 family protein	↑
39	AT5G38470	Ubiquitin receptor RAD23d	↓↓
L38	AT5G36210	Putative S9 Tyrosyl aminopeptidase	↓↓
76	AT5G35590	Proteasome subunit alpha type-6-A	↑
L30	AT4G20850	Tripeptidyl peptidase 2	↓↓
82	AT1G51710	Ubiquitin carboxyl-terminal hydrolase 6	↑
9	AT3G20630	Ubiquitin carboxyl-terminal hydrolase 14	↓

Table 6 - Proteins involved in diverse cellular processes

Spot/protein no.	AGI	Name	Abundance KAR$_1$:mock
71	AT2G38040	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	↓↓
55	AT5G59880	Actin depolymerizing factor 3	↓
50	AT3G23810	Adenosylhomocysteinase 2	↓
L5	AT1G34430	At1g34430/F7P12_2	↑
L10	AT2G07698	ATP synthase subunit alpha, mitochondrial	↑
2	AT1G48030	Dihydrolipoyl dehydrogenase 1	↓
3	AT3G48990	4-coumarate--CoA ligase-like 10	↓
L47	AT5G14910	Heavy metal transport/detoxification superfamily protein	↓↓
L42	AT5G63890	Histidinol dehydrogenase	↑
15	AT1G53240	Malate dehydrogenase 1, mitochondrial	↑
L40	AT5G41970	Metal-dependent protein hydrolase	↑↑
L37	AT5G26000	Myrosinase 1	↓↓
4	AT5G11670	NADP-dependent malic enzyme 2	↓↓
62	AT1G79690	Nudix hydrolase 3	↓
L16	AT2G28900	Outer envelope pore protein 16-1, chloroplastic	↓↓
42	AT3G15730	Phospholipase D alpha 1	↓↓
56	AT2G35040	Phosphoribosylaminoimidazolecarboxamide formyltransferase	↓
L19	AT2G38410	VHS and GAT domain-containing protein	↑↑
L31	AT4G24160	Hydrolase, alpha/beta fold family protein	↑↑
P24	AT2G38230	Pyridoxal biosynthesis protein PDX1.1	↓↓

65	AT5G09650	Soluble inorganic pyrophosphatase 1	↓
47	AT3G14540	Terpenoid synthase 19	↓
68	AT1G13930	Uncharacterized protein	↑
70	AT2G37660	NAD(P)-binding Rossmann-fold-containing protein	↓
L48	AT3G61260	Remorin-like protein	↑
66	AT1G20260	V-type proton ATPase subunit B3	↓

Figure1

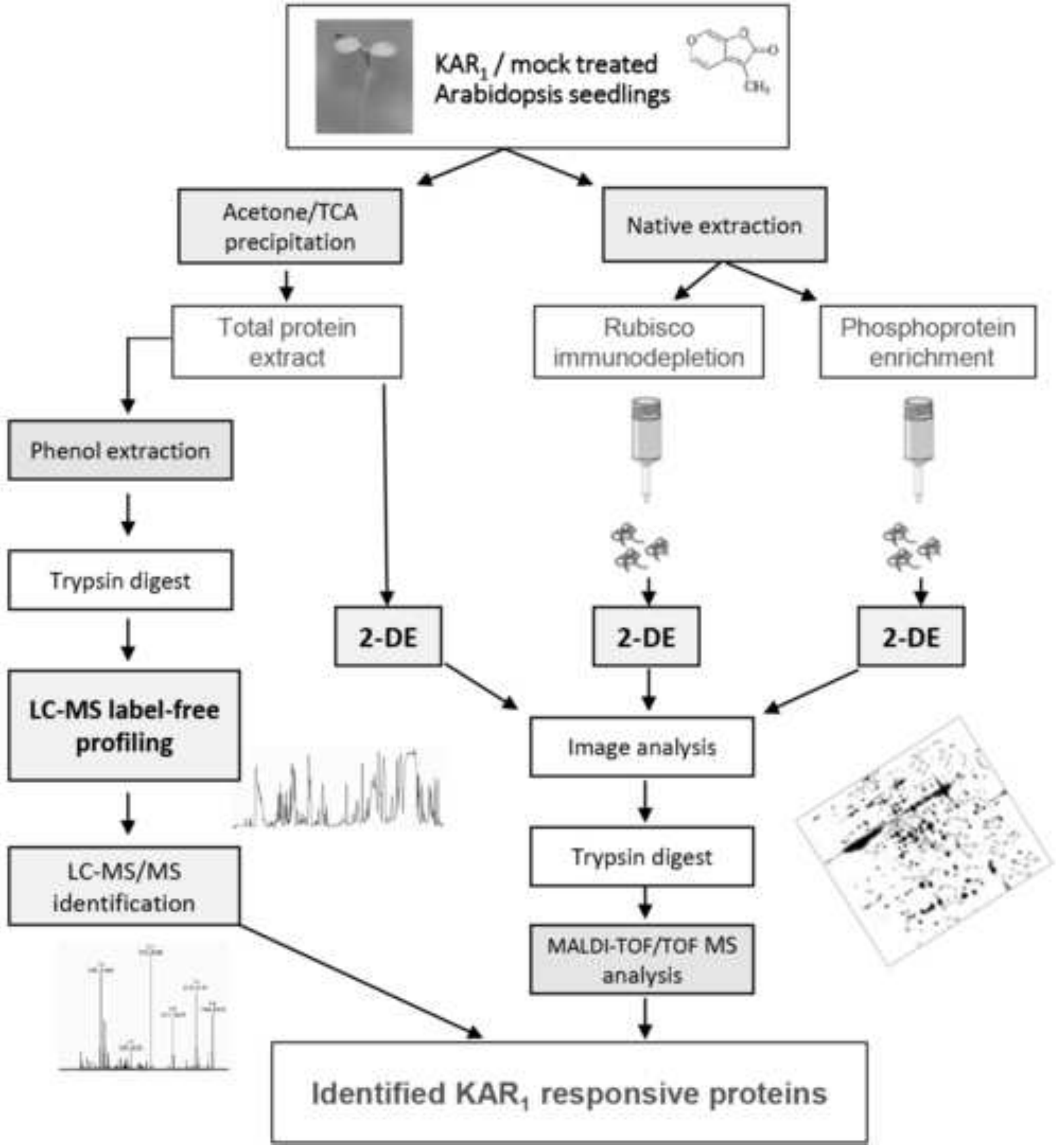
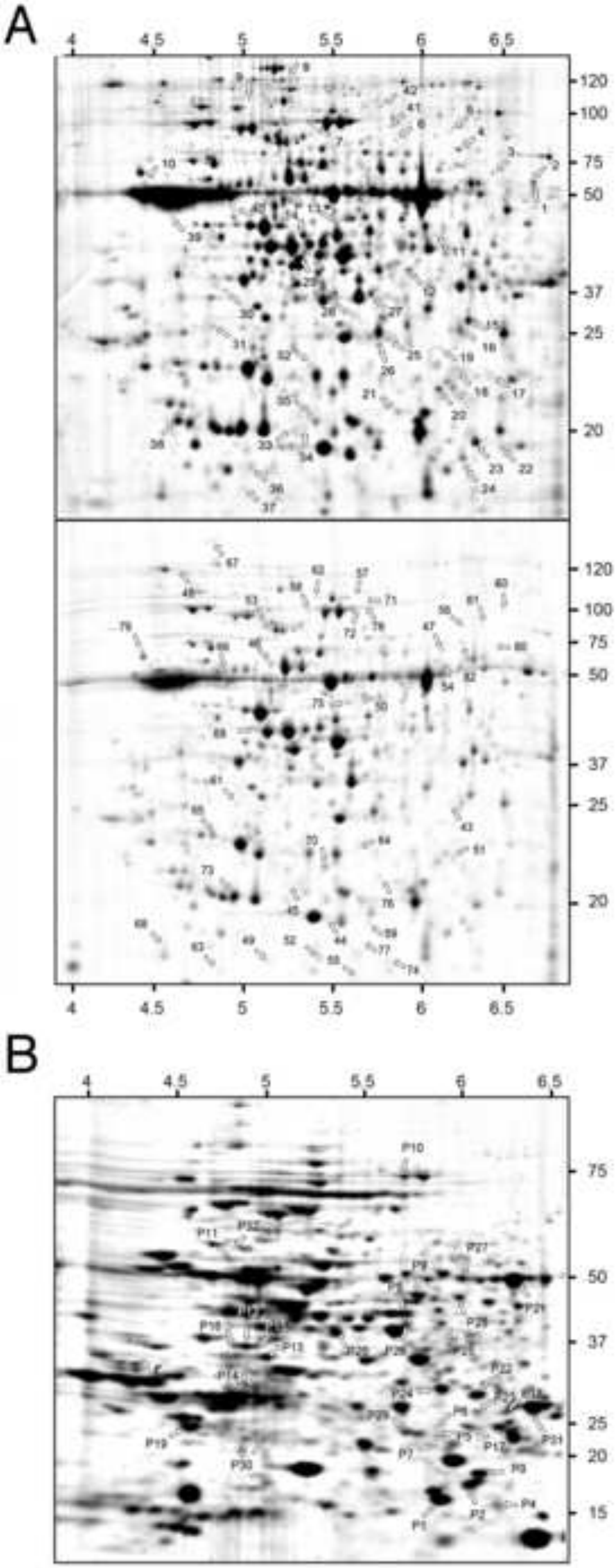

KAR1 / mock treated
Arabidopsis seedlings
CH3
O
Acetone/TCA precipitation
Native extraction
Total protein extract
Rubisco immunodepletion
Phosphoprotein enrichment
Phenol extraction
Trypsin digest
2-DE
2-DE
2-DE
LC-MS label-free profiling
Image analysis
Trypsin digest
LC-MS/MS identification
MALDI-TOF/TOF MS analysis
Identified KAR1 responsive proteins

Figure2


A
4
4.5
5
5.5
6
6.5
120
100
75
50
37
25
20
1
2
3
4
5
6
7
8
9
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11
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64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
B
4
4.5
5
5.5
6
6.5
75
50
37
25
20
15
P1
P2
P3
P4
P6
P7
P8
P9
P10
P11
P12
P13
P14
P15
P16
P17
P19
P20
P21
P22
P23
P24
P26
P27
P28
P29
P30
P31
P32
P33

Figure3

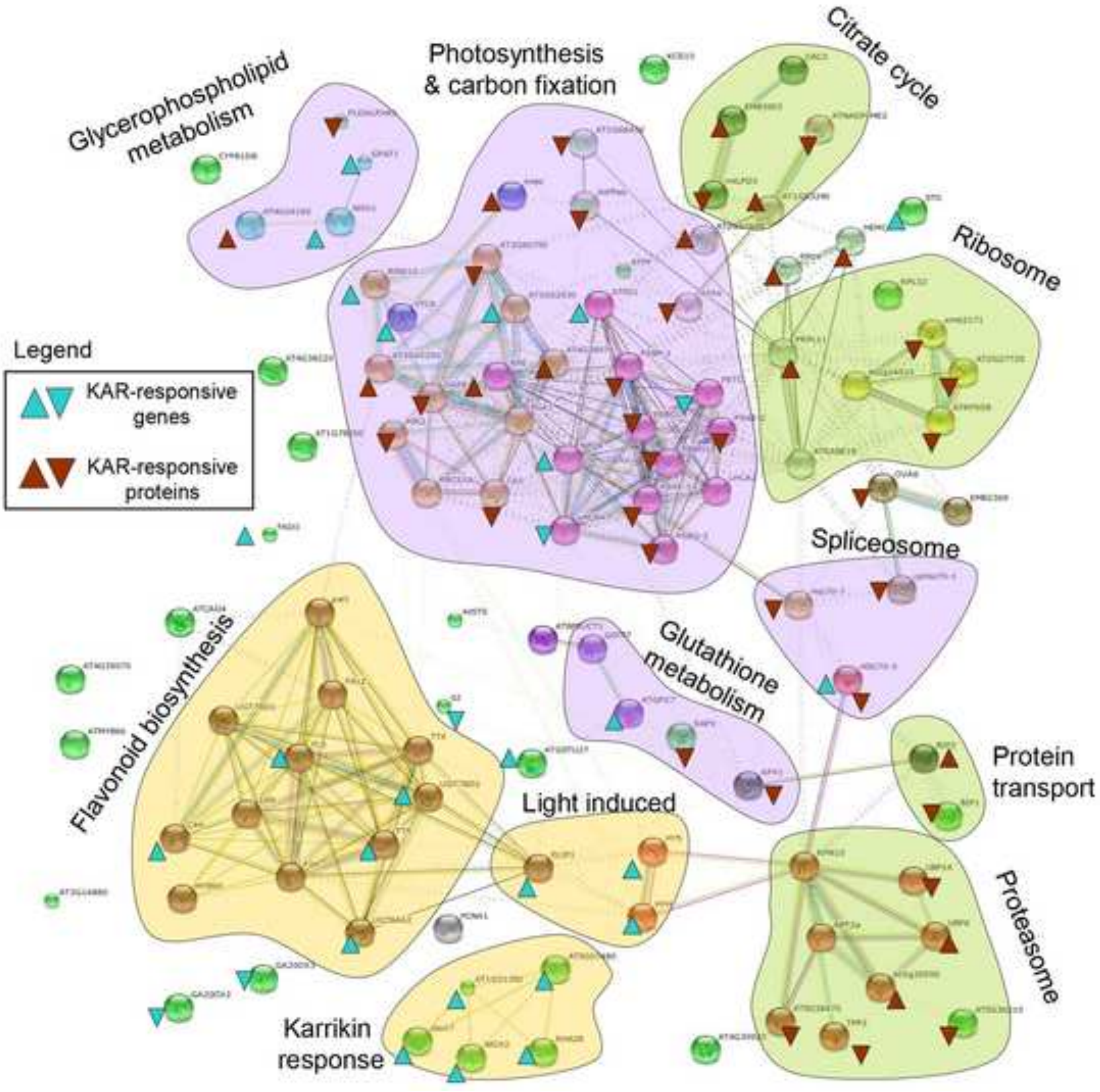
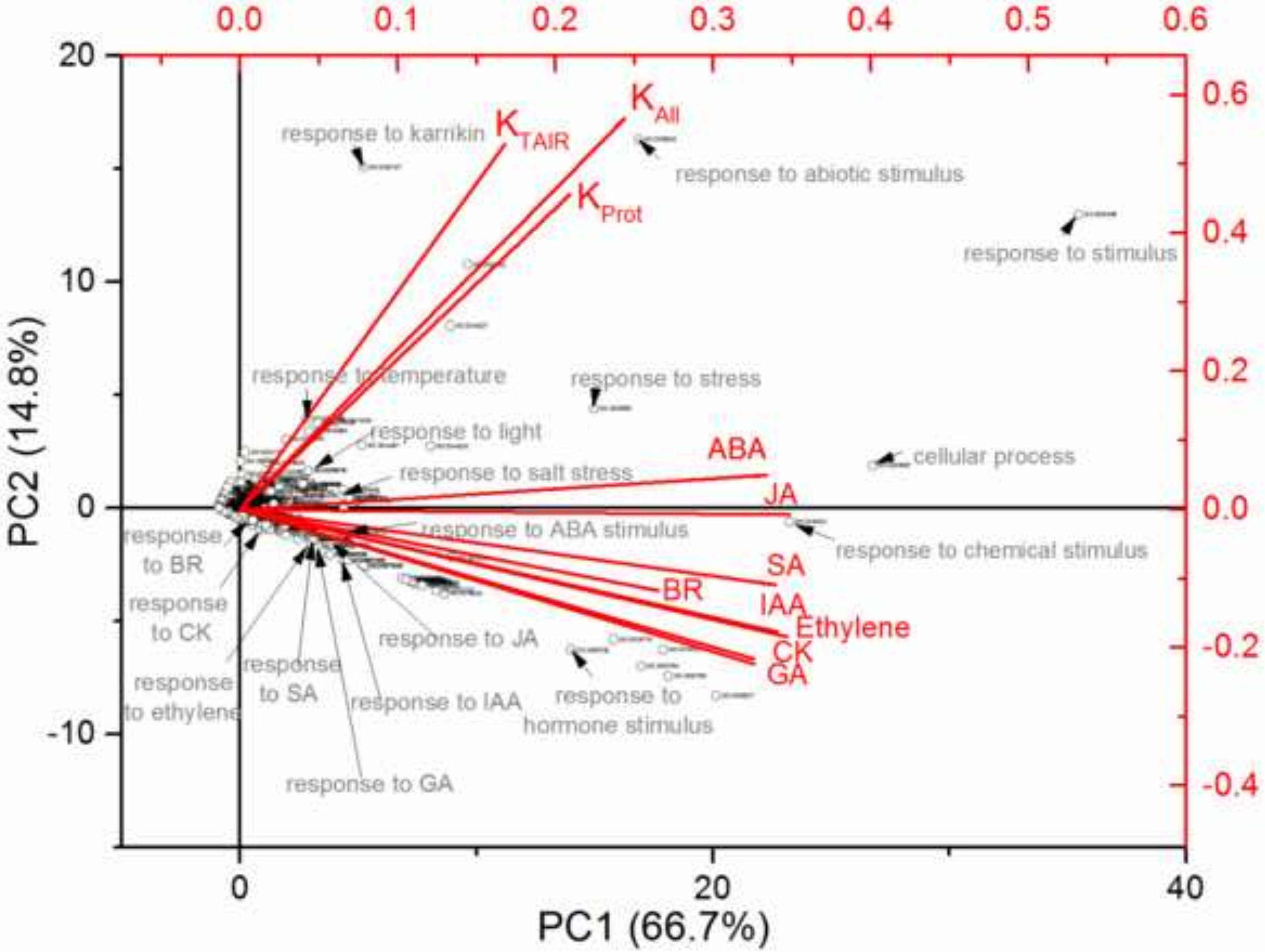

Glycerophospholipid metabolism
Photosynthesis & carbon fixation
Citrate cycle
Ribosome
Legend
▲▼ KAR-responsive genes
▲▼ KAR-responsive proteins
Spliceosome
Glutathione metabolism
Flavonoid biosynthesis
Light induced
Protein transport
Proteasome
Karrikin response

Figure4



0.0
0.1
0.2
0.3
0.4
0.5
0.6
20
10
0
-10
0.6
0.4
0.2
0.0
-0.2
-0.4
PC2 (14.8%)
0
20
40
PC1 (66.7%)
response to karrikin
K_{TAIR}
K_{All}
K_{Prot}
response to abiotic stimulus
response to stimulus
response to temperature
response to stress
response to light
ABA
cellular process
response to salt stress
JA
response to ABA stimulus
response to chemical stimulus
response to BR
SA
BR
IAA
Ethylene
response to CK
CK
GA
response to JA
response to ethylene
response to SA
response to IAA
response to hormone stimulus
response to GA

Figure5

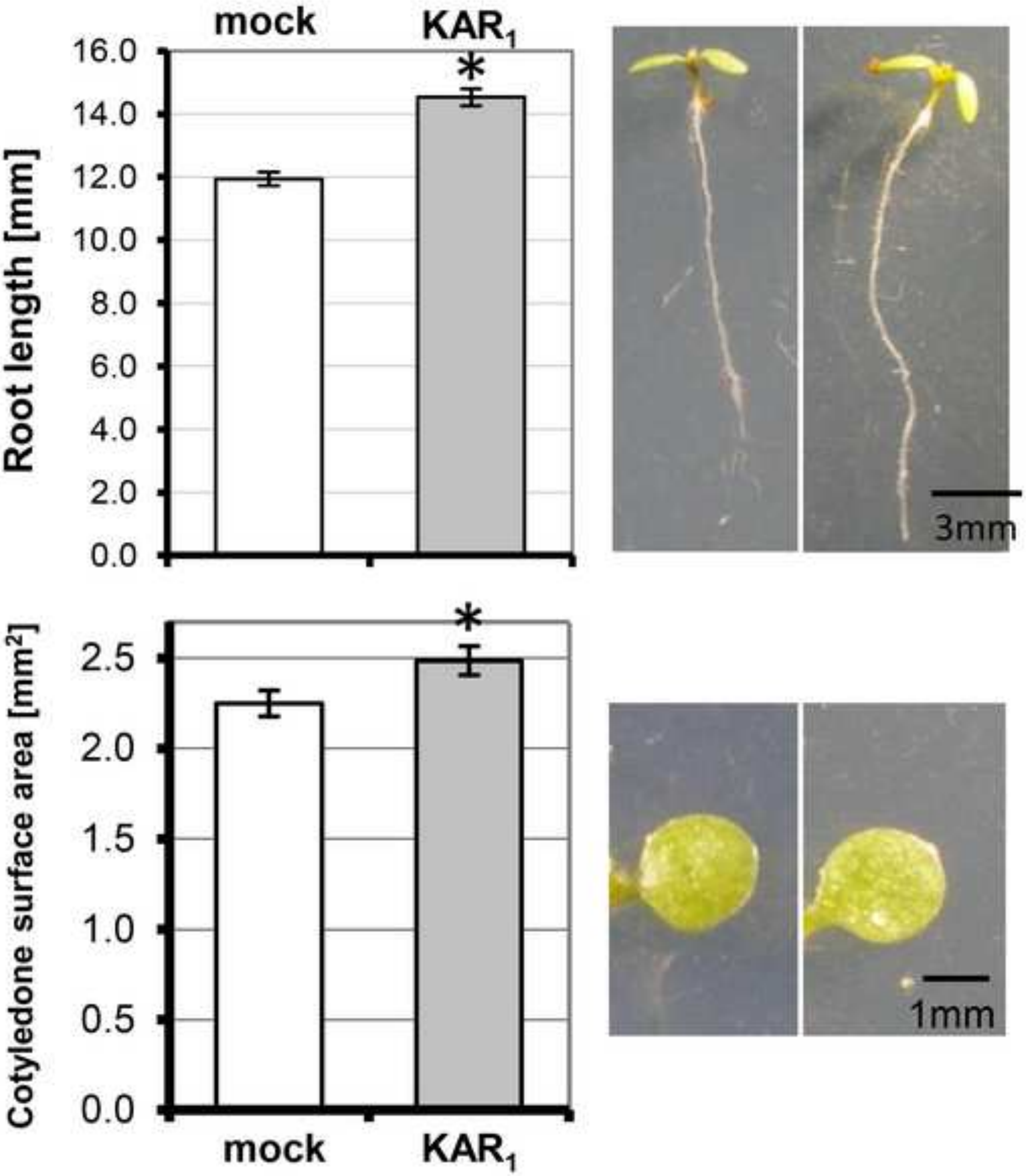
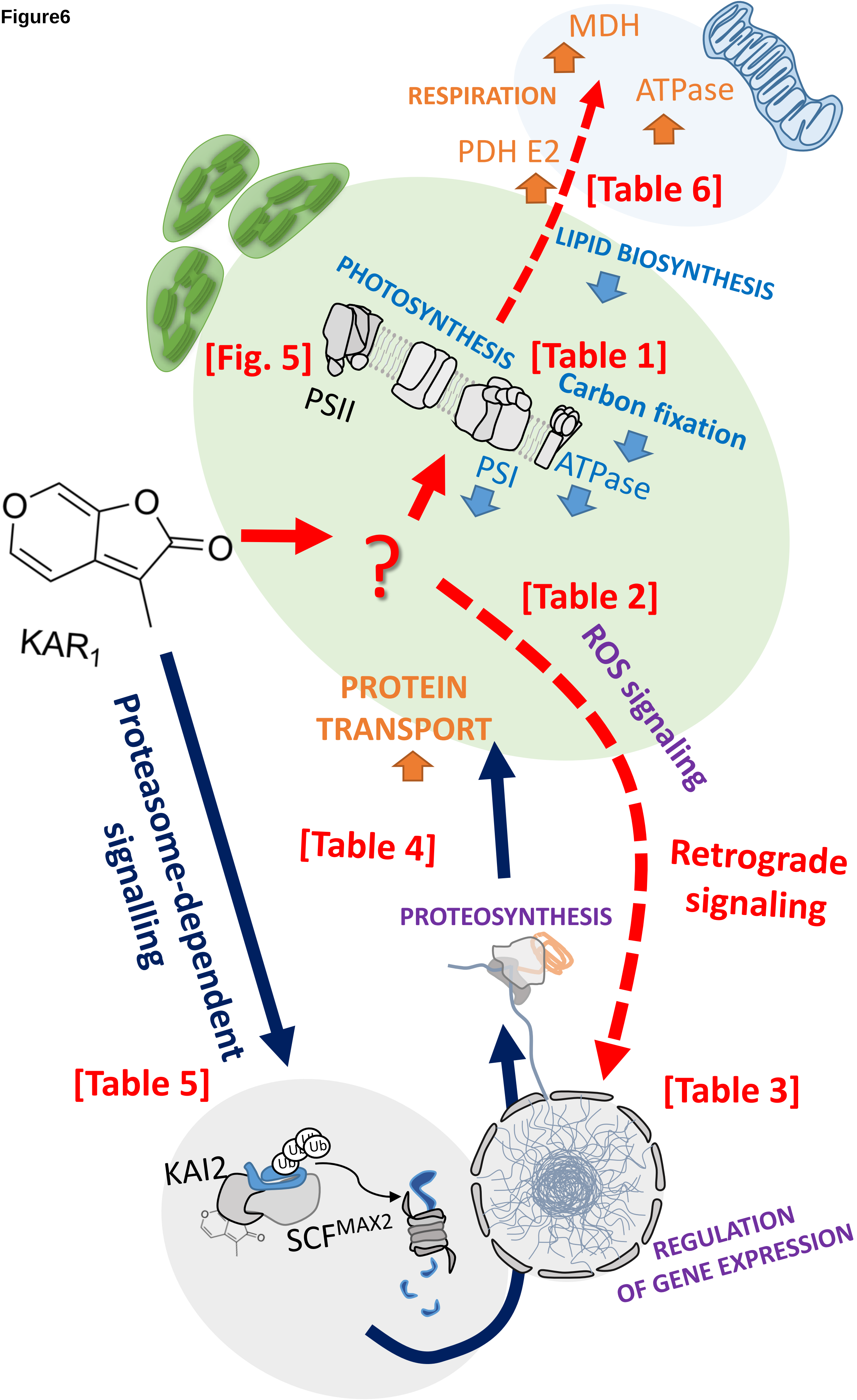

mock
KAR$_1$
Root length [mm]
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
3mm
Cotyledone surface area [mm^2]
2.5
2.0
1.5
1.0
0.5
0.0
*
1mm
mock
KAR$_1$

Figure6


MDH
RESPIRATION
ATPase
PDH E2
[Table 6]
LIPID BIOSYNTHESIS
PHOTOSYNTHESIS
[Fig. 5]
[Table 1]
PSII
Carbon fixation
PSI
ATPase
KAR$_1$
?
[Table 2]
ROS signaling
PROTEIN
TRANSPORT
Proteasome-dependent
signalling
[Table 4]
Retrograde
signaling
PROTEOSYNTHESIS
[Table 5]
[Table 3]
KAI2
Ub
Ub
Ub
Ub
SCF^{MAX2}
REGULATION
OF GENE EXPRESSION