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

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**TITLE PAGE**

Title: *Arabidopsis* proteome responses to the smoke-derived growth regulator  
karrikin

**Running head:**

Karrikin-response proteome in *Arabidopsis*

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11 **Number of figures: 6**

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14 **Supplementary figures: 5**

15

1 **ABSTRACT**

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

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

15

16 **Keywords**

17 smoke, proteome, growth regulators, karrikin, butenolide

## 1 **1. Introduction**

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

- 1 signalling and temperature perception [10-12]. We have now applied this approach to identify
- 2 early karrikin-response proteins and refine the emerging model of karrikin action in
- 3 *Arabidopsis*.

## 1 **2. Materials and methods**

### 2 **2.1. Plant material, growth conditions and KAR<sub>1</sub> treatment for proteomic experiments**

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

19

### 20 **2.2. Protein extraction, 2-DE analysis and protein identification**

21 *Arabidopsis* proteome and sub-proteomes were extracted, prepared and analysed essentially  
22 as previously described. Briefly, total protein was extracted by acetone/trichloroacetic acid  
23 extraction [10, 13, 14], a Rubisco-depleted proteome was prepared [11, 15], and  
24 phosphoproteins were enriched [10]. Portions of each extract, 500 or 150 μg, were loaded onto  
25 18 or 7 cm IPG strips (Bio-Rad, <http://www.bio-rad.com/>), respectively, isoelectrically

1 focused (linear pH gradient, 4-7), and subsequently resolved by SDS-PAGE. Gels were  
2 stained with colloidal Bio-Safe Coomassie G-250 (Bio-Rad), digitally imaged and analysed  
3 using Decodon Delta 2D software (<http://www.decodon.com>). Responses to KAR<sub>1</sub> treatment  
4 of proteins corresponding to detected spots were deemed significant if there was an absolute  
5 KAR<sub>1</sub>/mock spot volume ratio  $\geq 1.4$ , with *t*-test *p* value  $< 0.05$ , and similar profiles in two  
6 biological replicates of the total protein extract and Rubisco-depleted proteome (three  
7 technical replicates per sample), or three biological replicates for phosphoproteome  
8 comparison (two technical replicates per sample). Selected protein spots were digested with  
9 trypsin. The dried tryptic peptides were each dissolved in 10  $\mu$ l of 0.1% trifluoroacetic acid  
10 and purified using ZipTip C18 tips and eluted directly on sample plate with 10 mg ml<sup>-1</sup>  
11 CHCA in 50% v/v acetonitrile and 0.1% trifluoroacetic acid. Spectra were acquired using  
12 4800 Plus MALDI TOF/TOF analyzer (AB Sciex) equipped with a Nd:YAG laser (355 nm)  
13 with firing rate 200 Hz. All spots were measured in MS mode and then up to ten strongest  
14 precursors were selected for MS/MS which was performed with 1 kV collision energy and  
15 operating pressure of collision cell set to 10<sup>-6</sup> Torr. MS and MS/MS spectra were searched by  
16 local Mascot v. 2.1 (Matrix Science) against TAIR10 database of *Arabidopsis* protein  
17 sequences. Database search criteria were as follows: enzyme – trypsin; taxonomy –  
18 *Arabidopsis thaliana*; fixed modification – carbamidomethylation; variable modification -  
19 methionine oxidation; peptide tolerance - 80 ppm, allowed one missed cleavage; MS/MS  
20 tolerance - 0.2 Da.

21

### 22 **2.3. LC-MS proteome profiling**

23 Quantitative proteomic analyses were also performed using a gel-free shotgun protocol based  
24 on nano-HPLC and MS/MS. Briefly, two independently grown replicates, each consisting of  
25 approximately 300 *Arabidopsis* seedlings cultivated as described above, were extracted by



1 acetone/TCA and phenol extraction then digested in solution with immobilized trypsin beads  
2 (Promega, <http://www.promega.com/>). The resulting peptides were desalted, dried and  
3 dissolved in 0.5% (v/v) formic acid in 5% (v/v) acetonitrile, then analysed by nanoflow C18  
4 reverse-phase liquid chromatography using a 15 cm Ascentis Express Column (0.1 mm inner  
5 diameter; Sigma-Aldrich) and a Dionex Ultimate 3000 RSLC nano UPLC system (Thermo,  
6 [www.thermoscientific.com](http://www.thermoscientific.com)) directly coupled to a CaptiveSpray nanoESI source (Bruker) and  
7 an UHR maXis impact q-TOF mass spectrometer (Bruker, [www.bruker.com](http://www.bruker.com)). Peptides were  
8 eluted with up to a 180-min, 4% to 40% acetonitrile gradient. Raw files obtained from the MS  
9 analysis were analysed by Profile Analysis 2.1 (Bruker) and MS precursors with significant  
10 differences (absolute ratio  $\geq 1.5$ , with *t*-test *p*-values  $< 0.05$ ) were targeted and identified in  
11 consecutive MS/MS analyses. MS/MS spectra were acquired in an intensity dependent mode  
12 at a rate of 2-20 Hz with a maximum of 20 precursor ions and a MS spectra rate of 2 Hz. Data  
13 from MS/MS data-dependent measurements were processed by DataAnalysis 4.1 (Bruker)  
14 and searched against the TAIR10 *Arabidopsis* database using the Mascot 2.4 (Database search  
15 criteria: trypsin; variable modifications - methionine oxidation, NQ deamidation, ST  
16 phosphorylation; peptide tolerance - 10 ppm; allowed one missed cleavage; MS/MS tolerance  
17 - 0.06 Da) and Bruker's ProteinScape percolator algorithms (target FDR $< 1\%$ ) to identify  
18 source proteins (using high-confidence peptides, *p*  $< 0.05$  with at least one distinct proteotypic  
19 peptide per protein). Quantitative differences were further manually validated by comparing  
20 corresponding peptide ion signal peak areas in Skyline 1.4 (MacCossLab Software;  
21 <https://skyline.gs.washington.edu>).

22

#### 23 **2.4. Proteomic data analysis**

24 Information about functions of identified proteins was collected from available literature and  
25 the UniProt, UniGene (<http://www.ncbi.nlm.nih.gov/unigene>), TAIR ([8](http://www.</a></p></div><div data-bbox=)

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

12

## 1 **3. Results**

### 2 **3.1. Proteomic responses to KAR<sub>1</sub>-treatment**

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

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

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

8 Determination of subcellular locations of the identified differentially regulated  
9 proteins, using the SUBA database (<http://suba.plantenergy.uwa.edu.au/>; [19]), indicated that  
10 62 (50%) and 32 (26%) are chloroplastic and cytosolic, respectively. Corresponding  
11 proportions for all proteins identified by LC-MS were both ca. 30% (Table S3). Thus,  
12 chloroplastic proteins are significantly overrepresented in the karrikin-responsive proteome.

13

### 14 **3.2. Novel links in karrikin responses**

15 Our proteomic analyses detected only one *Arabidopsis* gene identified as being karrikin-  
16 responsive in previous transcriptomic analyses (Heat shock 70kDa protein 3; L23, decreased  
17 after KAR<sub>1</sub>-treatment). This protein is induced during germination [20] and is involved in  
18 protein-protein interactions with several partners, including the deubiquitinating enzyme  
19 AMSH3, which is involved in intracellular trafficking [21], and a ubiquitination target [22].  
20 Further, its close human homolog HSPA8 (>75% identity) participates in transcription control  
21 [23]. The remaining 112 differentially regulated proteins we detected are novel KAR<sub>1</sub>-  
22 responsive factors. However, our protein-protein interaction network and functional  
23 enrichment of metabolic pathways analyses showed that significant proportions of these  
24 proteins have functional matches or complement previously identified karrikin-responsive  
25 genes. These are proteins involved in carbon fixation and photosynthesis, RNA metabolism,

1 lipid metabolism and glutathione metabolism. Categories ‘Citric acid cycle’, ‘Protein  
2 transport’, proteasome mediated processes, and ribosome components were highlighted only  
3 in karrikin-response proteome (Fig. 3). All identified karrikin-responsive proteins were  
4 divided according to their predominant function into the following categories: Photosynthesis  
5 and carbohydrate metabolism; Redox homeostasis; Transcription; Proteosynthesis, protein  
6 transport and processing; and Protein degradation (Tables 1-5, respectively). The remaining  
7 categories (each represented by less than five proteins) are grouped in Table 6.

8

### 9 **3.3. Proteins involved in photosynthesis and carbohydrate metabolism are rapidly** 10 **regulated in karrikin responses**

11 The most significant overlap between known karrikin-responsive genes and the karrikin-  
12 responsive proteins we identified was among those involved in carbohydrate metabolism (7  
13 genes and 17 proteins; Fig. 3). The significantly regulated proteins included 23 enzymes and  
14 seven structural components of the photosynthetic apparatus, some present in multiple  
15 proteoforms (Table 1). KAR<sub>1</sub>-treatment increased levels of enzymes involved in chlorophyll  
16 biosynthesis (protoporphyrinogen oxidase 1; porphobilinogen deaminase). However, it  
17 appeared to have generally negative effects on photoassimilatory proteins, including: Calvin  
18 cycle enzymes (Rubisco - 18|L44|P18|P21|P31; chaperonin 60, required for Rubisco folding –  
19 46; Rubisco activase - P22|P30; glyceraldehyde-3-phosphate dehydrogenase - L6|P25|P26;  
20 fructose-bisphosphate aldolase - L13|L34; transketolase – 72; phosphorybulokinase - P20)  
21 and enzymes involved in both downstream and upstream metabolic pathways of carbon  
22 fixation (carbonic anhydrase) and carbohydrate metabolism (aldolase 1-epimerase,  
23 fructokinase, and nucleotide-sugar biosynthesis enzymes). Similarly, it reduced levels of  
24 constituents of PSI (light harvesting complex – 36; chlorophyll binding protein – 33; PSI

1 reaction centre - L11|L33), PSII (Oxygen-evolving enhancer proteins – L26|P14|L29) and  
2 ATP synthase (L43).

3

#### 4 **3.4. Proteasome-mediated signalling components in the KAR<sub>1</sub> responsive proteome**

5 KAI2, MAX2 and ubiquitin-proteasome signalling is the only reported molecular mechanism  
6 of karrikin perception. Thus, proteins involved in ubiquitin-dependent processes in the  
7 karrikin-responsive proteome should be particularly interesting. We were not able to detect  
8 KAI2 (At4g37470), nor MAX2 on protein level. However, KAR<sub>1</sub> treatment increased levels  
9 of a proteasome subunit (76) and two deubiquitinating enzymes (9, 82) (Table 2). One,  
10 ubiquitin carboxyl-terminal hydrolase 14, is important for development and its mutation  
11 results in enlarged endosperm nuclei and defective embryos [24]. The treatment also  
12 upregulated the ubiquitin receptor protein RAD23, which reportedly interacts with a  
13 proteasome docking subunit and participates in recognition of ubiquitinated substrates [25].  
14 Products of proteasome activity are predominantly peptides up to 12 amino acids long that are  
15 subsequently processed by peptidases. Accordingly, we detected significant changes in  
16 abundance of two dipeptidases (L38, L49) and one tripeptidase (L30). Our data also indicate  
17 more potential links with the ubiquitin-dependent pathway, e.g. the changes in levels of Heat  
18 shock 70kDa (ubiquitinated, interacts with deubiquitinating enzyme) described above, but the  
19 evidence is less conclusive. An alpha/beta domain-containing hydrolase with an uncertain  
20 function and expected mitochondrial location (L31) was also upregulated. However, sequence  
21 comparison does not indicate that the hydrolase is a mitochondrial counterpart of KAI2  
22 (identity <12%).

23

#### 24 **3.5. Transcription regulators involved in KAR<sub>1</sub> responses**

1 Previous transcriptomic analyses have detected five KAR-responsive *Arabidopsis* genes  
2 involved in transcription control, but the proportion of genes covered by the analyses (and  
3 proteins covered by our analyses) is too low to pinpoint any significant over-representation in  
4 this category. However, our supervised analyses detected upregulation of several relevant  
5 proteins (Table 3), including two (DNA topoisomerase-like protein, 48, and Nucleosome  
6 assembly protein, 10) indicating that KAR-treatment induces DNA uncoiling. Another three  
7 proteins are classified as mediators (19, 53) or a probable mediator (L35) of RNA polymerase  
8 II, which directly participates in transcription regulation. Further, all these mediators share  
9 sequence similarity with Heat shock 70kDa protein 3 (> 60% for the first two and >92% for  
10 L35) which is also apparently a transcription mediator (Supplementary Fig. S2). Furthermore,  
11 KAR<sub>1</sub>-treatment affected RNA processing, or at least expression of two RNA helicases — 14  
12 (expected to be involved in nonsense-mediated mRNA decay and ribosome biogenesis) and  
13 56 (pre-mRNA splicing, mRNA export from nucleus) — and ribonucleoprotein At2g37220.  
14 At2g37220 was significantly affected by KAR<sub>1</sub>-treatment in all analysed proteome fractions  
15 (38|L17|P19), contains an RRM domain and plays an expected role in chloroplastic RNA  
16 processing. We also detected posttranslational modification(s) in Cold shock protein 2 (59 -  
17 increased, 77 - decreased), an RNA chaperone that is regulated by cold and developmental  
18 signals [26].

19

### 20 **3.6. The early karrikin-response proteome regulates proteosynthesis**

21 Translation regulates all physiological processes, directly or indirectly. Accordingly, we  
22 detected 28 karrikin-responsive proteins (35% of all differentially expressed proteins)  
23 involved in ribosome assembly (26|L4|L14|L15|L18|L22|L24|L27), translation initiation  
24 (75|L39|P1|P12), aminoacyl t-RNA synthesis (54), elongation (5|13|67|P8|P28), and protein  
25 processing and transport (7|8|12|46|57|L25|L32|L41|L45|L46). Overall, KAR<sub>1</sub>-treatment

1 seemed to inhibit proteosynthesis (at least cytosolic proteosynthesis) and related processes  
2 (Table 4). However, the responses are probably complex. For example, changes in ribosome  
3 composition may have severe effects on plant growth and development [27]. Mutant  
4 phenotypes are known for two KAR-responsive ribosomal proteins identified here: 50S  
5 ribosomal protein L11 (slow growth, pale green; [28]) and 60S ribosomal protein L23  
6 (embryo defective; [29]). Since ribosomal proteins have been implicated in auxin and  
7 cytokinin signalling [11, 30, 31], some KAR-effects are probably results of stimulation or  
8 interference with ribosome-mediated signalling of endogenous hormones.

9

### 10 **3.7. Similarities in response to plant hormones and KAR treatment**

11 Karrikin is not recognized as a plant hormone because it may not be endogenously  
12 synthesised, but since it has similar effects to known plant hormones we compared GO-  
13 category KAR<sub>1</sub> responses with those elicited by abscisic acid, auxin (IAA), brassinosteroids  
14 (BR), cytokinin (CK), ethylene, jasmonic acid (JA), gibberellins (GA) and salicylic acid (SA)  
15 (Fig. 4). Our analysis covered 128 karrikin-responsive genes and 113 proteins, and 3453,  
16 1245, 861, 672, 973, 348, 778 and 541 TAIR-annotated genes linked to the respective  
17 hormones [32, 33]. Altogether, over 900 GO categories are significantly overrepresented in  
18 sets of genes or proteins responding to at least one of the listed substances. The diversity of  
19 processes apparently up- or down-regulated by them (number of overrepresented GO  
20 categories) decreased in the order JA>SA≥IAA>ethylene>ABA>GA>KAR>CK>BL. The  
21 GO category ‘Response to karrikin’ (which does not yet include proteins identified here) is  
22 not significantly enriched in sets that reportedly respond to plant hormones, unsurprisingly as  
23 no responses to any of the listed phytohormones have been detected for >50% of karrikin-  
24 responsive genes. Similarly, only 28 of the proteins identified here reportedly respond to  
25 phytohormones: 15, 10, 9, 2 and 1 to ABA, JA, SA, IAA and BR, respectively. Nevertheless,



1 our PCA indicates that the differences between hormonal and KAR<sub>1</sub> responses is not  
2 dramatic. The first dimension, covering nearly 67% of total variability, clusters KAR- and  
3 BR-responsive proteins in the same group. Unfortunately, we could not include strigolactone  
4 in the analysis, as there are too few annotated strigolactone-responsive genes.

5

### 6 **3.8. Fluorescence measurements suggest photosynthesis is modulated in karrikin** 7 **responses**

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

24

## 1 **4. Discussion**

### 2 **4.1. Profiling early proteomic responses to karrikin in Arabidopsis**

3 Karrikin responses have been previously examined in bioassays, selected mutants and  
4 transcriptomic analyses [3, 6-8, 34-39], but not (to our knowledge) in proteome-wide  
5 analyses. However, protein abundance is not dependent only on transcription, and  
6 posttranslational modifications can rapidly activate or inactivate enzymes without degradation  
7 or *de novo* synthesis (e.g. [40, 41]). Thus, proteome analysis can complement transcriptomic  
8 analysis and extend insights into molecular processes. Karrikins were discovered as  
9 germination-promoting substances, but their effect is not limited to this developmental stage.  
10 They also promote cotyledon expansion and inhibit hypocotyl elongation of *Arabidopsis*  
11 seedlings [8], eliciting responses at nanomolar to micromolar concentrations [4], like plant  
12 hormones. This prompted us to employ the approach we established for identifying cytokinin-  
13 responsive proteins [10, 12], combining affinity depletion of Rubisco and phosphoproteome  
14 isolation with 2-DE – MALDI TOF/TOF and LC-MS identification of differentially regulated  
15 proteins. Preliminary experiments confirmed that micromolar KAR<sub>1</sub> elicits reported growth  
16 responses [8] under experimental conditions similar to those applied in our proteomic  
17 experiments, promoting both root and cotyledon growth when applied for the last 24 h of a 7-  
18 day cultivation period (Fig. 5).

19

### 20 **4.2. Down-regulation prevails in proteomic responses to karrikin**

21 Karrikin treatment reportedly up-regulates transcription of most (>80%) differentially  
22 expressed genes [8], but most (69%) early karrikin-response proteins we detected were down-  
23 regulated: 97% in the phosphoprotein-enriched fraction and ~62% in sets detected in both the  
24 LC-MS and total protein 2-DE (including Rubisco-depleted fraction) analyses. Interestingly,  
25 we observed similar proportions in analyses of early cytokinin (~67%) and temperature-shock  
26 (~62%) response proteins [10, 12]. We hypothesise that processes that reduce

1 protein/proteoform abundance may be preferentially employed in rapid responses to stimuli  
2 because they are faster than protein synthesis.

3

#### 4 **4.3. Plastids – the site of missing links in karrikin signalling?**

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

18

#### 19 **4.4. Posttranslational control in KAR<sub>1</sub> responses**

20 Our 2-DE data provide several indications that targeted proteasome-mediated protein  
21 degradation is not the only posttranslational mechanism involved in karrikin signalling. First,  
22 differences between theoretical and observed pI/MW values indicate that >50% of  
23 significantly regulated proteins detected in our 2-DE analysis are probably posttranslationally  
24 modified proteoforms (Additional file 1: Table S1). Identification and characterization of  
25 these modifications detected in the total protein extract/Rubisco depleted proteome

1 comparison is beyond the scope of this work. However, our targeted phosphoproteome  
2 analysis indicates that changes in phosphorylation status are involved, at least indirectly. We  
3 have previously demonstrated that the phosphoprotein enrichment protocol we employed is  
4 fairly specific by validation with phospho-specific staining and MS/MS sequencing [10, 12].  
5 Here, we supplemented our previous validation by Western blot analysis (Supplementary Fig.  
6 S5). We were able to follow dynamics in phosphoproteome, but the biological relevance of  
7 detected phosphorylations mostly remains to be elucidated. For some of them, even the  
8 function of a protein itself is not clear. Phosphorylation could be involved in a direct karrikin  
9 signalling mechanism, but with the combined sensitivity of 2-DE analysis and MALDI-MS  
10 we detected regulatory phosphorylation for only 17 proteins, all of which seem to be  
11 influenced indirectly. Literature and a homology search indicate that one detected  
12 dephosphorylation (of Rubisco; P18, P21, P31) would be inhibitory [47] while another (of the  
13 cytosolic enzyme triosephosphate isomerase; P29) would be activating [48], which correlates  
14 with observed regulations in our dataset. Rubisco activase (P22, P30) has multiple  
15 phosphorylation sites, some also with a potential on/off function [49]. Elongation factor Tu  
16 (P8) is one of four detected phosphoproteins involved in proteosynthesis. Phosphorylation of  
17 this chloroplastic protein prevents ternary complex formation [50] and its regulation in  
18 response to karrikin likely represents an increased proteosynthesis in chloroplasts. The last  
19 phosphorylation which should be discussed is that of chloroplastic protein glyceraldehyde-3-  
20 phosphate dehydrogenase (GAPDH; P25, P26). Although the prominent role of GAPDH is  
21 commonly known to be in carbohydrate metabolism, its homolog also participates in  
22 intracellular trafficking. Tisdale (2002) reported that phosphorylation of GAPDH influences  
23 microtubule dynamics in the early secretory pathway [51]. In this respect, we can note that  
24 chloroplastic proteins like Trigger factor-like protein TIG (L41, protein exporter) or

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

3

#### 4 **4.5. Karrikin signalling – an updated model**

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

23

#### 24 **4.6. Could inhibition of photosynthesis stimulate growth?**

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

12

## 13 **5. Conclusions**

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

21

1 **Author contributions**

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

4

5 **Competing interests**

6 The authors declare that they have no competing interests.

7

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19

20 **Appendix A. Supplementary data**

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

23

1

2 References

3 [1] Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD. A compound from smoke that  
4 promotes seed germination. *Science* 2004;305(5686):977.

5 [2] Van Staden J, Jager A, Light M, Burger B. Isolation of the major germination cue from  
6 plant-derived smoke. *South African J Bot* 2004;70:654-659.

7 [3] Light M, Daws M, Van Staden J. Smoke-derived butenolide: towards understanding its  
8 biological effects. *South African J Bot* 2009;75(1):1-7.

9 [4] Chiwocha SD, Dixon KW, Flematti GR, Ghisalberti EL, Merritt DJ, Nelson DC,  
10 Riseborough JM, Smith SM, Stevens JC. Karrikins: A new family of plant growth regulators  
11 in smoke. *Plant Science* 2009;177(4):252-256.

12 [5] Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD. Identification of alkyl substituted  
13 2 H-furo [2, 3-c] pyran-2-ones as germination stimulants present in smoke. *J Agric Food*  
14 *Chem* 2009;57(20):9475-9480.

15 [6] Nelson DC, Riseborough J, Flematti GR, Stevens J, Ghisalberti EL, Dixon KW, Smith  
16 SM. Karrikins discovered in smoke trigger Arabidopsis seed germination by a mechanism  
17 requiring gibberellic acid synthesis and light. *Plant Physiol* 2009;149(2):863-873.

18 [7] Waters MT, Smith SM. KAI2- and MAX2-mediated responses to karrikins and  
19 strigolactones are largely independent of HY5 in Arabidopsis seedlings. *Mol Plant*  
20 2013;6(1):63-75.

21 [8] Nelson DC, Flematti GR, Riseborough J, Ghisalberti EL, Dixon KW, Smith SM.  
22 Karrikins enhance light responses during germination and seedling development in  
23 Arabidopsis thaliana. *Proc Natl Acad Sci U S A* 2010;107(15):7095-7100.

24 [9] Waters MT, Scaffidi A, Sun YK, Flematti GR, Smith SM. The karrikin response system of  
25 Arabidopsis. *Plant J* 2014;79(4):623-631.



- 1 [10] Černý M, Dyčka F, Bobál'ová J, Brzobohatý B. Early cytokinin response proteins and  
2 phosphoproteins of *Arabidopsis thaliana* identified by proteome and phosphoproteome  
3 profiling. *J Exp Bot* 2011;62(3):921-937.
- 4 [11] Černý M, Kuklová A, Hoehenwarter W, Fragner L, Novák O, Rotková G, Jedelský PL,  
5 Žáková K, Šmehilová M, Strnad M, Weckwerth W, Brzobohatý B. Proteome and metabolome  
6 profiling of cytokinin action in *Arabidopsis* identifying both distinct and similar responses to  
7 cytokinin down- and up-regulation. *J Exp Bot* 2013;64(14):4193-4206.
- 8 [12] Černý M, Jedelský PL, Novák J, Schlosser A, Brzobohatý B. Cytokinin modulates  
9 proteomic, transcriptomic and growth responses to temperature shocks in *Arabidopsis*. *Plant*  
10 *Cell Environ* 2014;37(7):1641-1655.
- 11 [13] Hradilová J, Rehulka P, Rehulková H, Vrbová M, Griga M, Brzobohatý B. Comparative  
12 analysis of proteomic changes in contrasting flax cultivars upon cadmium exposure.  
13 *Electrophoresis* 2010;31(2):421-431.
- 14 [14] Lochmanová G, Zdráhal Z, Konečná H, Koukalová S, Malbeck J, Soucek P, Válková M,  
15 Kiran NS, Brzobohatý B. Cytokinin-induced photomorphogenesis in dark-grown *Arabidopsis*:  
16 a proteomic analysis. *J Exp Bot* 2008;59(13):3705-3719.
- 17 [15] Černý M, Skalák J, Kurková B, Babuliaková E, Brzobohatý B. Using a commercial  
18 method for Rubisco immunodepletion in analysis of plant proteome. *Chemické listy*  
19 2011;105:640-642.
- 20 [16] Franceschini A, Szklarczyk D, Frankild S, Kuhn M, Simonovic M, Roth A, Lin J,  
21 Minguéz P, Bork P, von Mering C, Jensen LJ. STRING v9.1: protein-protein interaction  
22 networks, with increased coverage and integration. *Nucleic Acids Res* 2013;41(Database  
23 issue):D808-15.

- 1 [17] Katari MS, Nowicki SD, Aceituno FF, Nero D, Kelfer J, Thompson LP, Cabello JM,  
2 Davidson RS, Goldberg AP, Shasha DE, Coruzzi GM, Gutiérrez RA. VirtualPlant: a software  
3 platform to support systems biology research. *Plant Physiol* 2010;152(2):500-515.
- 4 [18] Černý M, Skalák J, Cerna H, Brzobohatý B. Advances in purification and separation of  
5 posttranslationally modified proteins. *J Proteomics* 2013;92:2-27.
- 6 [19] Heazlewood JL, Verboom RE, Tonti-Filippini J, Small I, Millar AH. SUBA: the  
7 Arabidopsis Subcellular Database. *Nucleic Acids Res* 2007;35(Database issue):D213-8.
- 8 [20] Sung DY, Vierling E, Guy CL. Comprehensive expression profile analysis of the  
9 Arabidopsis Hsp70 gene family. *Plant Physiol* 2001;126(2):789-800.
- 10 [21] Isono E, Katsiarimpa A, Müller IK, Anzenberger F, Stierhof Y, Geldner N, Chory J,  
11 Schwechheimer C. The deubiquitinating enzyme AMSH3 is required for intracellular  
12 trafficking and vacuole biogenesis in *Arabidopsis thaliana*. *Plant Cell* 2010;22(6):1826-1837.
- 13 [22] Saracco SA, Hansson M, Scalf M, Walker JM, Smith LM, Vierstra RD. Tandem affinity  
14 purification and mass spectrometric analysis of ubiquitylated proteins in Arabidopsis. *Plant J*  
15 2009;59(2):344-358.
- 16 [23] Yahata T, de Caestecker MP, Lechleider RJ, Andriole S, Roberts AB, Isselbacher KJ,  
17 Shioda T. The MSG1 non-DNA-binding transactivator binds to the p300/CBP coactivators,  
18 enhancing their functional link to the Smad transcription factors. *J Biol Chem*  
19 2000;275(12):8825-8834.
- 20 [24] Doelling JH, Yan N, Kurepa J, Walker J, Vierstra RD. The ubiquitin-specific protease  
21 UBP14 is essential for early embryo development in *Arabidopsis thaliana*. *Plant J*  
22 2001;27(5):393-405.
- 23 [25] Lin Y, Sung S, Tsai H, Yu T, Radjacommare R, Usharani R, Fatimababy AS, Lin H,  
24 Wang Y, Fu H. The defective proteasome but not substrate recognition function is responsible

1 for the null phenotypes of the Arabidopsis proteasome subunit RPN10. *Plant Cell*  
2 2011;23(7):2754-2773.

3 [26] Sasaki K, Kim M, Imai R. Arabidopsis COLD SHOCK DOMAIN PROTEIN2 is a RNA  
4 chaperone that is regulated by cold and developmental signals. *Biochem Biophys Res*  
5 *Commun* 2007;364(3):633-638.

6 [27] Rosado A, Sohn EJ, Drakakaki G, Pan S, Swidergal A, Xiong Y, Kang B, Bressan RA,  
7 Raikhel NV. Auxin-mediated ribosomal biogenesis regulates vacuolar trafficking in  
8 Arabidopsis. *Plant Cell* 2010;22(1):143-158.

9 [28] Pesaresi P, Varotto C, Meurer J, Jahns P, Salamini F, Leister D. Knock-out of the plastid  
10 ribosomal protein L11 in Arabidopsis: effects on mRNA translation and photosynthesis. *Plant*  
11 *J* 2001;27(3):179-189.

12 [29] Meinke DW, Meinke LK, Showalter TC, Schissel AM, Mueller LA, Tzafrir I. A  
13 sequence-based map of Arabidopsis genes with mutant phenotypes. *Plant Physiol*  
14 2003;131(2):409-418.

15 [30] Brenner WG, Schmülling T. Transcript profiling of cytokinin action in Arabidopsis roots  
16 and shoots discovers largely similar but also organ-specific responses. *BMC Plant Biol*  
17 2012;12:112.

18 [31] Rosado A, Li R, van de Ven W, Hsu E, Raikhel NV. Arabidopsis ribosomal proteins  
19 control developmental programs through translational regulation of auxin response factors.  
20 *Proc Natl Acad Sci U S A* 2012;109(48):19537-19544.

21 [32] Nemhauser JL, Hong F, Chory J. Different plant hormones regulate similar processes  
22 through largely nonoverlapping transcriptional responses. *Cell* 2006;126(3):467-475.

23 [33] Goda H, Sasaki E, Akiyama K, Maruyama-Nakashita A, Nakabayashi K, Li W, Ogawa  
24 M, Yamauchi Y, Preston J, Aoki K, Kiba T, Takatsuto S, Fujioka S, Asami T, Nakano T,  
25 Kato H, Mizuno T, Sakakibara H, Yamaguchi S, Nambara E, Kamiya Y, Takahashi H, Hirai

1 MY, Sakurai T, Shinozaki K, Saito K, Yoshida S, Shimada Y. The AtGenExpress hormone  
2 and chemical treatment data set: experimental design, data evaluation, model data analysis  
3 and data access. *Plant J* 2008;55(3):526-542.

4 [34] Waters MT, Nelson DC, Scaffidi A, Flematti GR, Sun YK, Dixon KW, Smith SM.  
5 Specialisation within the DWARF14 protein family confers distinct responses to karrikins and  
6 strigolactones in *Arabidopsis*. *Development* 2012;139(7):1285-1295.

7 [35] Soós V, Sebestyén E, Juhász A, Light ME, Kohout L, Szalai G, Tandori J, Van Staden J,  
8 Balázs E. Transcriptome analysis of germinating maize kernels exposed to smoke-water and  
9 the active compound KAR1. *BMC Plant Biol* 2010;10:236.

10 [36] Soós V, Sebestyén E, Juhász A, Pintér J, Light ME, Van Staden J, Balázs E. Stress-  
11 related genes define essential steps in the response of maize seedlings to smoke-water. *Funct*  
12 *Integr Genomics* 2009;9(2):231-242.

13 [37] Nelson DC, Flematti GR, Ghisalberti EL, Dixon KW, Smith SM. Regulation of seed  
14 germination and seedling growth by chemical signals from burning vegetation. *Annu Rev*  
15 *Plant Biol* 2012;63:107-130.

16 [38] Nelson DC, Scaffidi A, Dun EA, Waters MT, Flematti GR, Dixon KW, Beveridge CA,  
17 Ghisalberti EL, Smith SM. F-box protein MAX2 has dual roles in karrikin and strigolactone  
18 signaling in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*  
19 2011;108(21):8897-8902.

20 [39] Light ME, Burger BV, Staerk D, Kohout L, Van Staden J. Butenolides from plant-  
21 derived smoke: natural plant-growth regulators with antagonistic actions on seed germination.  
22 *J Nat Prod* 2010;73(2):267-269.

23 [40] Nelson CJ, Li L, Jacoby RP, Millar AH. Degradation rate of mitochondrial proteins in  
24 *Arabidopsis thaliana* cells. *J Proteome Res* 2013;12(7):3449-3459.

- 1 [41] Černý M, Doubnerová V, Müller K, Ryšlavá H. Characterization of  
2 phosphoenolpyruvate carboxylase from mature maize seeds: properties of phosphorylated and  
3 dephosphorylated forms. *Biochimie* 2010;92(10):1362-1370.
- 4 [42] Chi W, Sun X, Zhang L. Intracellular signaling from plastid to nucleus. *Annual review of*  
5 *plant biology* 2013;64:559-582.
- 6 [43] Heldt HW, Piechulla B. *Plant Biochemistry*, 4th Edition. London: Academic Press; 2010.
- 7 [44] Meyer Y, Belin C, Delorme-Hinoux V, Reichheld J, Riondet C. Thioredoxin and  
8 glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional  
9 significance. *Antioxid Redox Signal* 2012;17(8):1124-1160.
- 10 [45] Bhargava A, Clabaugh I, To JP, Maxwell BB, Chiang Y, Schaller GE, Loraine A, Kieber  
11 JJ. Identification of cytokinin-responsive genes using microarray meta-analysis and RNA-Seq  
12 in *Arabidopsis*. *Plant Physiol* 2013;162(1):272-294.
- 13 [46] Wang P, Liu J, Liu B, Feng D, Da Q, Wang P, Shu S, Su J, Zhang Y, Wang J, Wang H.  
14 Evidence for a role of chloroplastic m-type thioredoxins in the biogenesis of photosystem II in  
15 *Arabidopsis*. *Plant Physiol* 2013;163(4):1710-1728.
- 16 [47] Kaul R, Saluja D, Sachar R. Phosphorylation of small subunit plays a crucial role in the  
17 regulation of RuBPCase in moss and spinach. *FEBS letters* 1986;209(1):63-70.
- 18 [48] Lee D, Pan Y, Kanner S, Sung P, Borowiec JA, Chowdhury D. A PP4 phosphatase  
19 complex dephosphorylates RPA2 to facilitate DNA repair via homologous recombination. *Nat*  
20 *Struct Mol Biol* 2010;17(3):365-372.
- 21 [49] Boex-Fontvieille E, Davenport M, Jossier M, Hodges M, Zivy M, Tcherkez G.  
22 Phosphorylation pattern of Rubisco activase in *Arabidopsis* leaves. *Plant Biol (Stuttg)*  
23 2014;16(3):550-557.

1 [50] Alexander C, Bilgin N, Lindschau C, Mesters JR, Kraal B, Hilgenfeld R, Erdmann VA,  
2 Lippmann C. Phosphorylation of elongation factor Tu prevents ternary complex formation. J  
3 Biol Chem 1995;270(24):14541-14547.

4 [51] Tisdale EJ. Glyceraldehyde-3-phosphate dehydrogenase is phosphorylated by protein  
5 kinase C $\alpha$  and plays a role in microtubule dynamics in the early secretory pathway. Journal  
6 of Biological Chemistry 2002;277(5):3334-3341.

7 [52] Palmblad M, Mills DJ, Bindschedler LV. Heat-shock response in *Arabidopsis thaliana*  
8 explored by multiplexed quantitative proteomics using differential metabolic labeling. J  
9 Proteome Res 2008;7(2):780-785.

10 [53] Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic  
11 and transcriptomic analyses. Nature Reviews Genetics 2012;13: 227-232.

12 [54] Romanov GA, Kieber JJ, Schmölling T. A rapid cytokinin response assay in *Arabidopsis*  
13 indicates a role for phospholipase D in cytokinin signalling. FEBS Lett 2002;515(1-3):39-43.

14 [55] Guo L, Mishra G, Markham JE, Li M, Tawfall A, Welti R, Wang X. Connections  
15 between sphingosine kinase and phospholipase D in the abscisic acid signaling pathway in  
16 *Arabidopsis*. J Biol Chem 2012;287(11):8286-8296.

17  
18  
19

1 **Figure Legends**

2 **Fig. 1** - Experimental design.

3

4 **Fig. 2** - Effects of KAR<sub>1</sub> on the proteome and phosphoproteome of *Arabidopsis* seedlings.

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

15

16 **Fig. 3** - Protein-protein interaction network constructed using STRING (<http://string-db.org>;  
17 [16]). Karrikin-responsive genes (TAIR, Nelson and co-workers [8]; blue) and proteins  
18 identified here (brown) were analysed for protein-protein interaction networks. String analysis  
19 indicates that two sets overlap in several categories (purple). Categories 'Flavonoid  
20 biosynthesis' and 'Light induced processes' are extensively regulated according to the  
21 transcriptomic data, while significant effects of karrikin on the TCA cycle, protein transport  
22 and proteasome-mediated processes are apparent only in our proteomics data.

23

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

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

9

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

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

16

17

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

19 Proteasome-dependent signalling: Karrikin binding to KAI2 initiates a proteasome-dependent  
20 cascade and regulates proteins involved in ubiquitination and a di/tripeptide cleavage (Table  
21 5), accounting for observed reductions in abundance of proteins involved in transcription  
22 control (Table 3). Rapid changes in the chloroplastic proteome indicate the presence of an  
23 alternative signalling pathway originating in chloroplasts. Karrikin negatively affects proteins  
24 involved in photosynthesis and subsequent carbon assimilation (Table 1). Consequently,  
25 reductions in ATP fluxes from photosynthesis would likely be compensated (at least



1 temporarily) by increases in pyruvate dehydrogenase activity, TCA cycle fluxes and  
2 respiration rates, accompanied by reductions in lipid biosynthesis (Table 6). Alterations in  
3 photoassimilation modulate redox homeostasis (Table 2) and through retrograde signalling  
4 may influence gene expression. Signalling from both pathways thus converges on  
5 proteosynthesis and protein transport (Table 4).

6

7

8

9

1

2 **Supplementary data**

3

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

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

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

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

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

9 Figure S2. Sequence analysis of HSP70 proteins.

10 Figure S3. Effects of prolonged KAR<sub>1</sub> treatment on *Arabidopsis* seedlings.

11 Figure S5. Western blot detection of phosphorylated proteins.

12

## Tables

**Table 1** - Proteins involved in photosynthesis and carbohydrate metabolism

Spot/protein no.	AGI	Name	Abundance KAR <sub>1</sub> :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	↓

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Spot/protein no.	AGI	Name	Abundance KAR <sub>1</sub> :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 <sub>1</sub> :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 <sub>1</sub> :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	↓

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**Table 5 - Proteins involved in protein degradation**

Spot/protein no.	AGI	Name	Abundance KAR <sub>1</sub> :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 <sub>1</sub> :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	↓

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Figure 1

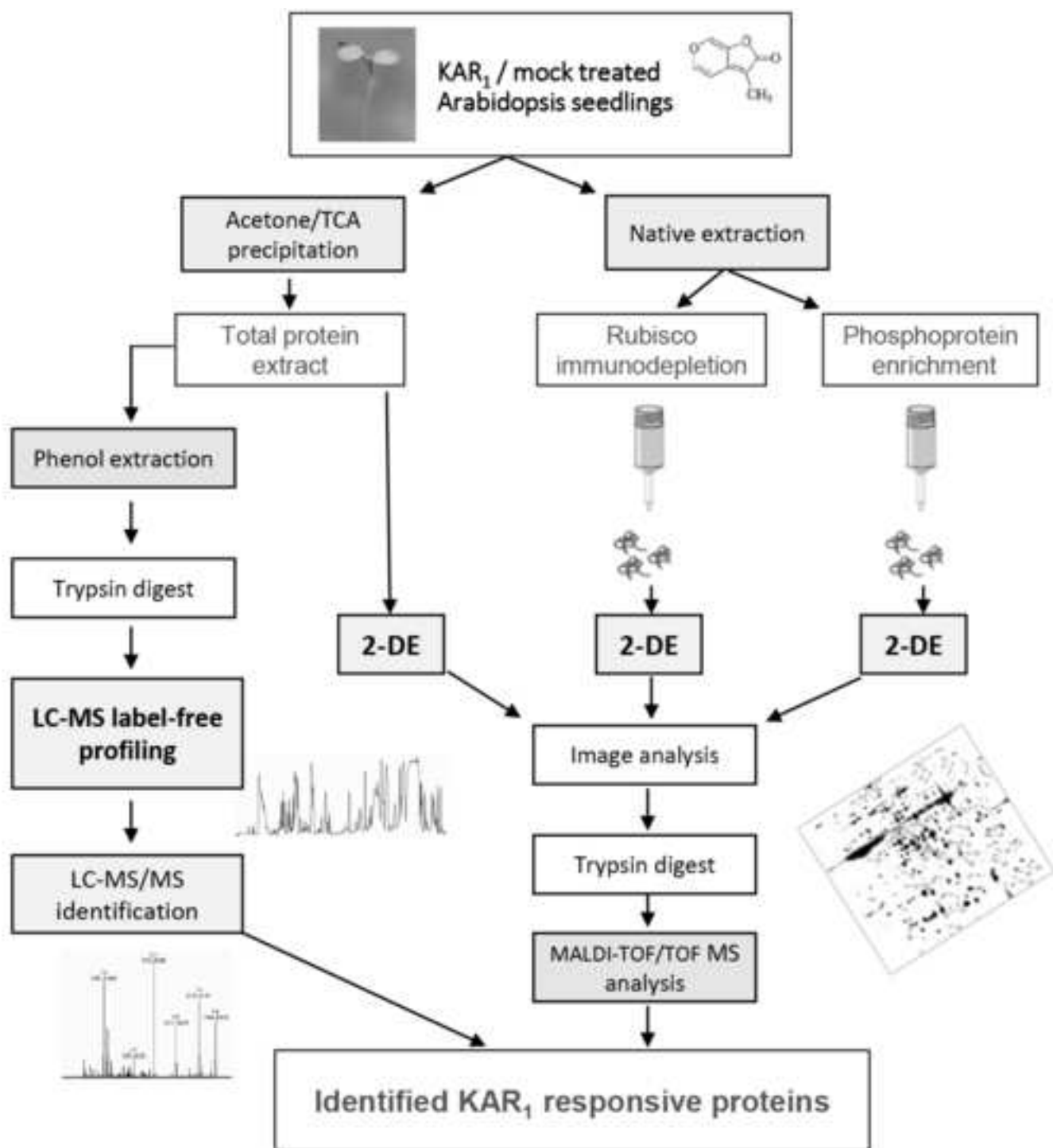


Figure2

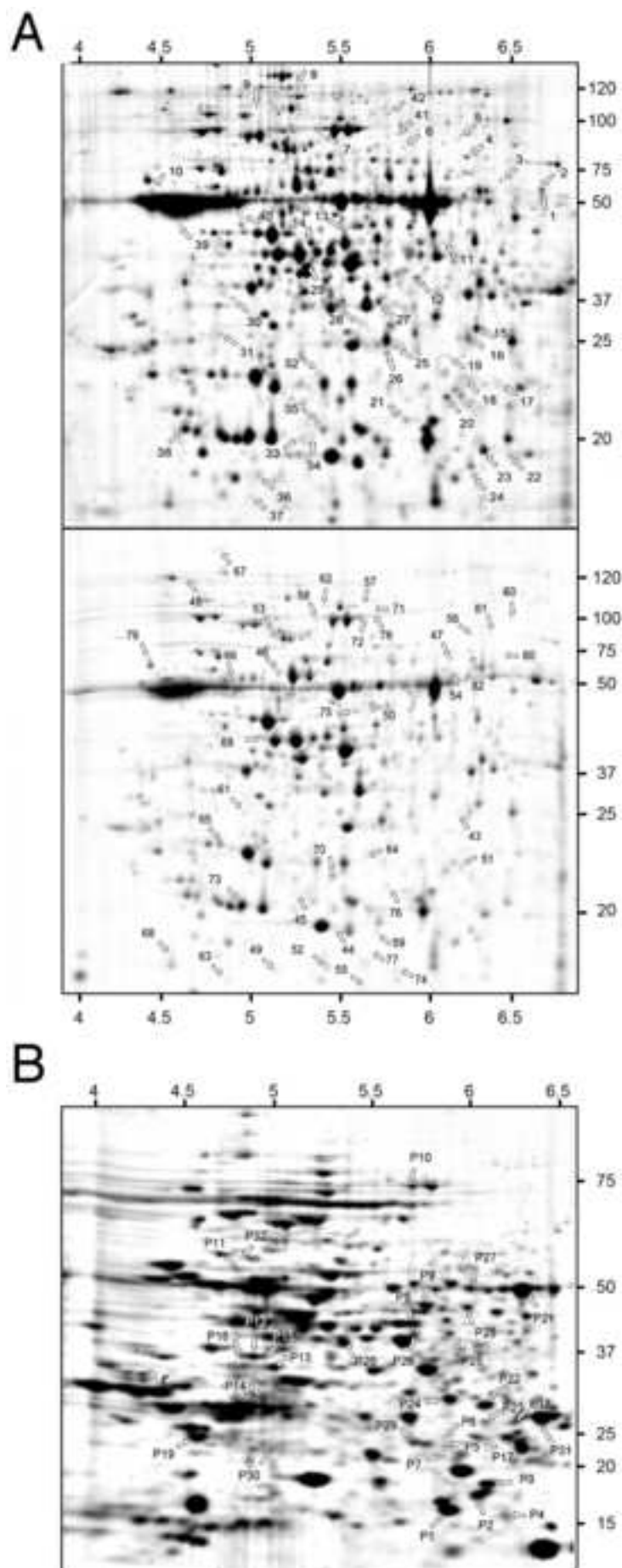


Figure3

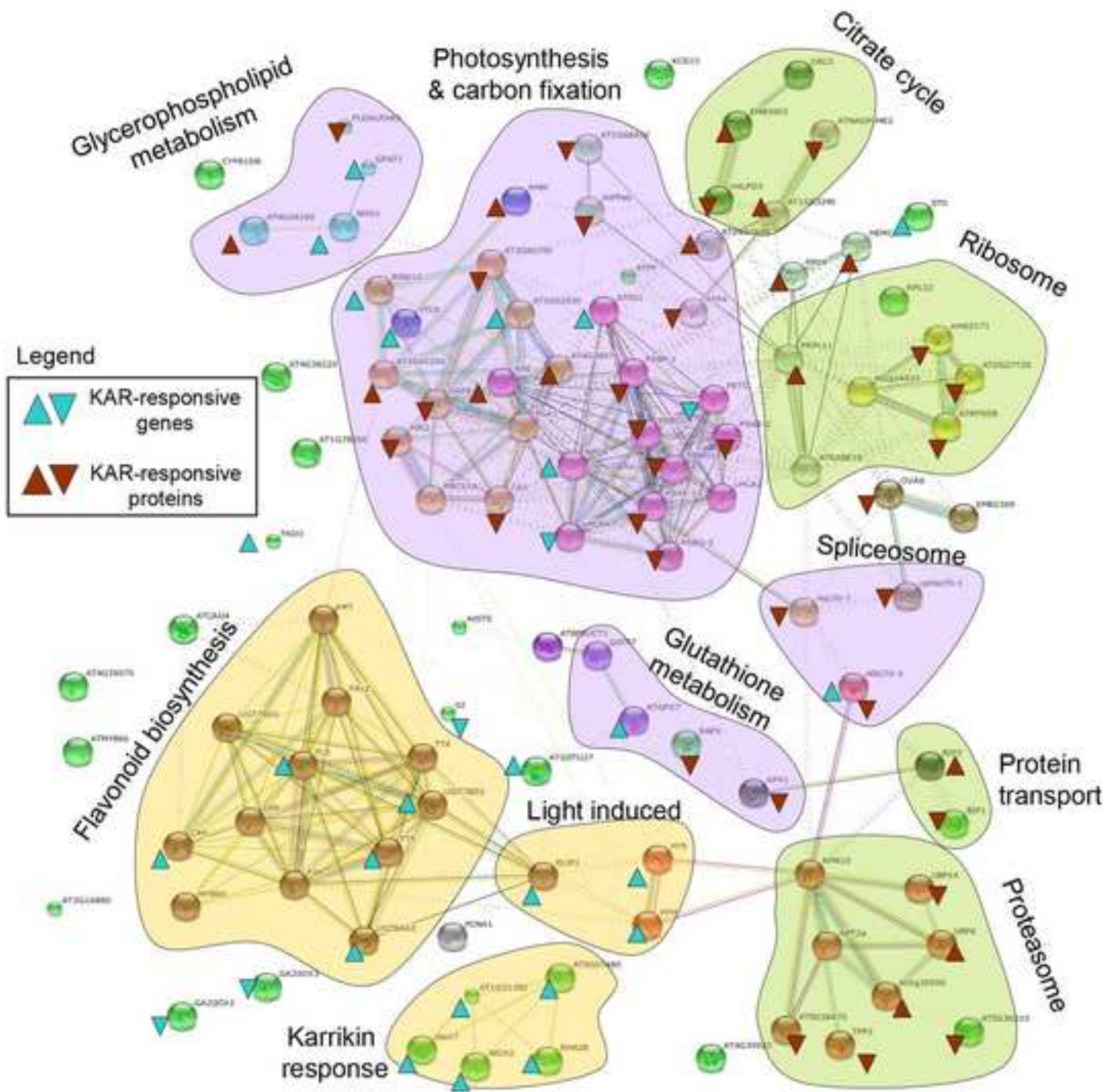


Figure4

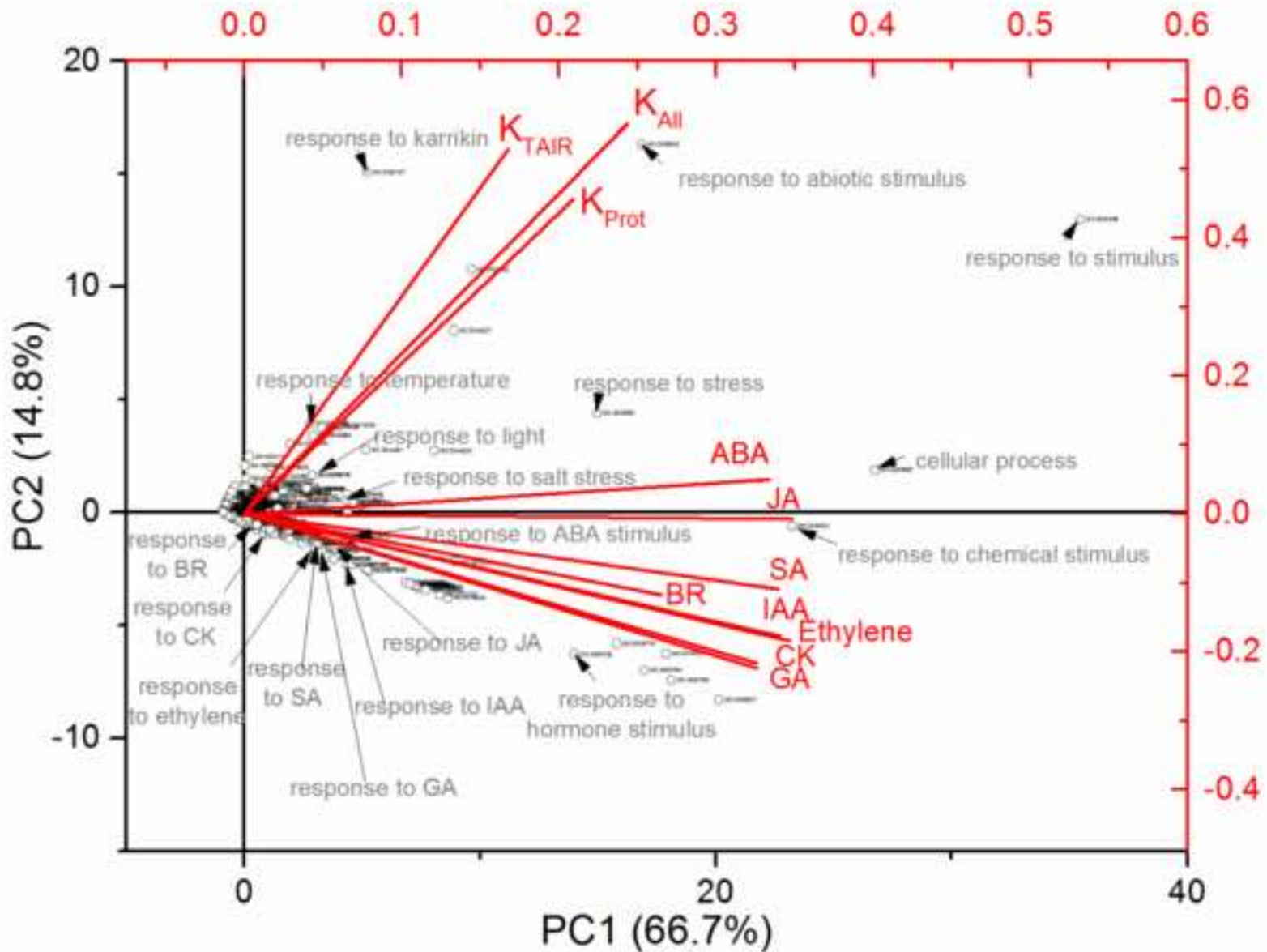




Figure5

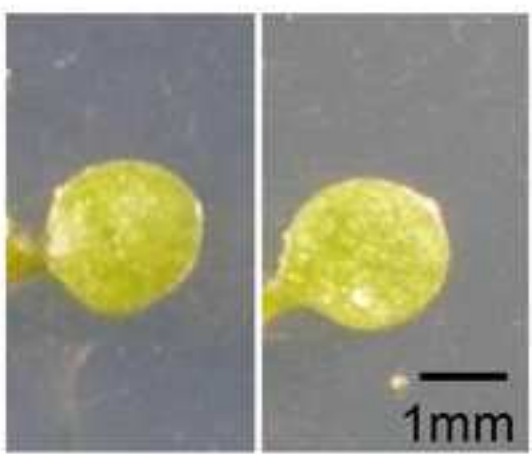
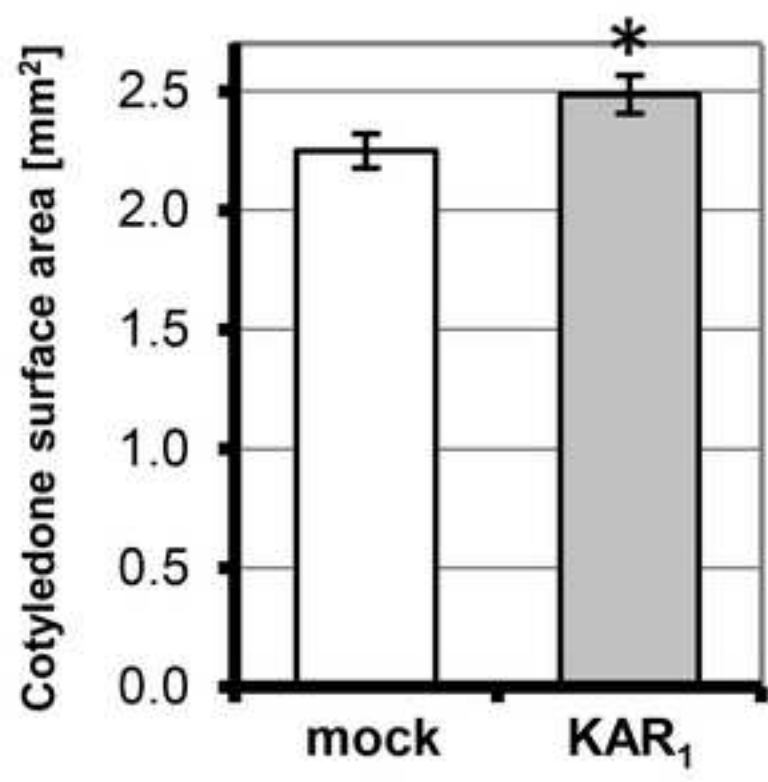
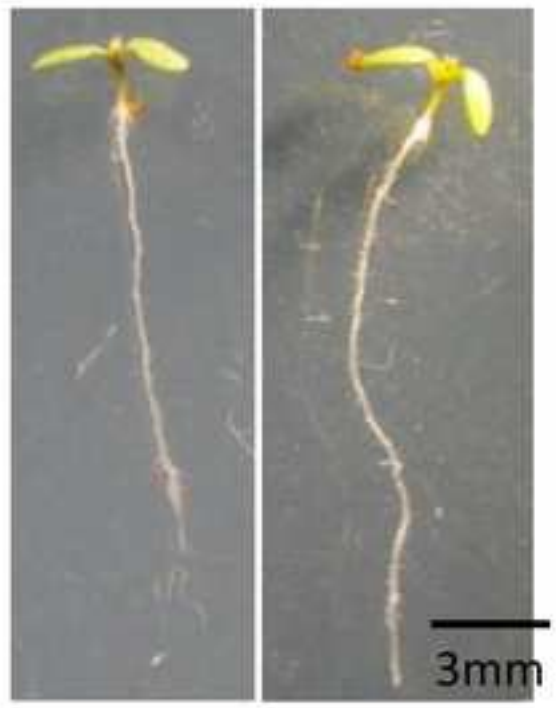
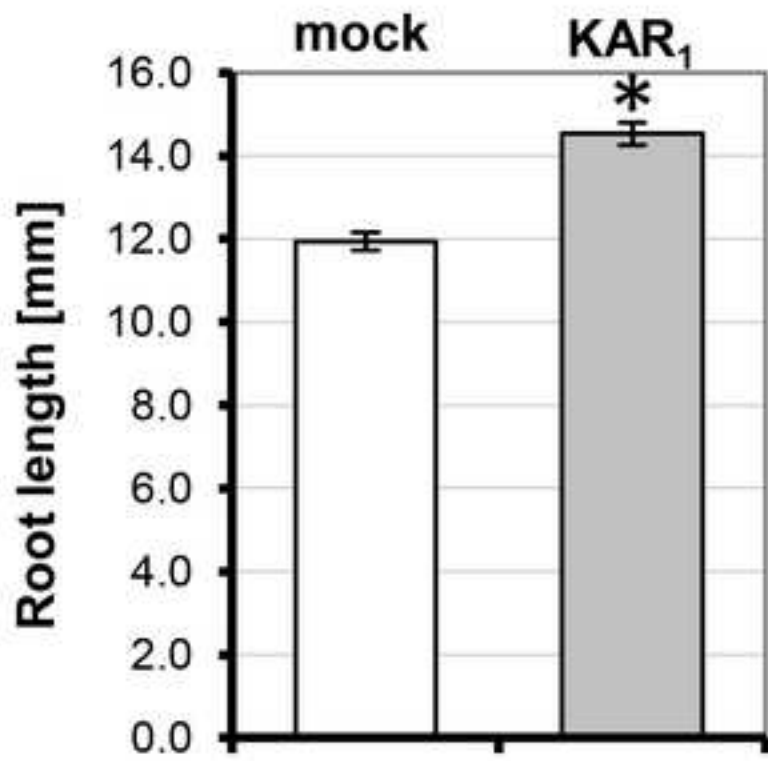


Figure 6

