The link between transcript regulation and de novo protein synthesis in the retrograde high light acclimation response of Arabidopsis thaliana

Marie-Luise Oelze†, Meenakumari Muthuramalingam†, Marc Oliver Vogel and Karl-Josef Dietz*

Abstract

Background: Efficient light acclimation of photosynthetic cells is a basic and important property of plants. The process of acclimation depends on transformation of retrograde signals in gene expression, transcript accumulation and de novo protein synthesis. While signalling cues, transcriptomes and some involved players have been characterized, an integrated view is only slowly emerging, and information on the translational level is missing. Transfer of low (8 μmol quanta m⁻² s⁻¹) or normal light (80 μmol quanta m⁻² s⁻¹) acclimated 30 d old Arabidopsis thaliana plants to high light (800 μmol quanta m⁻² s⁻¹) triggers retrograde signals. Using this established approach, we sought to link transcriptome data with de novo synthesized proteins by in vivo labelling with ³⁵S methionine and proteome composition.

Results: De novo synthesized protein and proteome patterns could reliably be matched with newly annotated master gels. Each molecular level could be quantified for a set of 41 proteins. Among the proteins preferentially synthesized in plants transferred to high light were enzymes including carbonic anhydrase, fructose-1,6-bisphosphate aldolase, O-acetyl serine thiol lyase, and chaperones, while low rates upon transfer to high light were measured for e.g. dehydroascorbate reductase, glyceraldehyde-3-phosphate dehydrogenase and CuZn superoxide dismutase, and opposite responses between 10-fold and 100-fold light increment for e.g. glutamine synthetase and phosphoglycerate kinase.

Conclusions: The results prove the hypothesis that transcript abundance is poorly linked to de novo protein synthesis due to profound regulation at the level of translation. This vertical systems biology approach enables to quantitatively and kinetically link the molecular levels for scrutinizing signal processing and response generation.

Background

Fluctuating environmental conditions elicit acclimation responses that occur at different molecular levels and on various time scales. For immediate response to light intensity shifts the acclimation includes rapid posttranslational modifications such as reversible protein phosphorylation for state transition or photochemical quenching, and thiol-disulfide transitions of metabolic enzymes [1]. An intermediate response to alter the proteome is mediated by modification of the transcripts recruited to the ribosomes and allows for fast adjustment of de novo synthesized proteins [2]. Initiated at the same time scale, transcriptional activity is adjusted, but due to the multiple subsequent steps of transcript accumulation, translation and assembly, the response is somewhat delayed compared to the first and second mechanism [3]. Each level of molecular response is subjected to additional regulation such as RNA stability [4] and dynamics and assembly of complexes [5]. Since these reactions occur outside the organelles for most plastidic proteins their initiation and control depends on retrograde signals from the chloroplast. On a longer time scale reorganization of cell structures, epigenetic control of gene activity and changes in morphology realize additional levels of acclimatory modifications.

This work aims for understanding the different levels of molecular acclimation to high light (H-light). Arabidopsis thaliana has been repeatedly used to investigate reactions to H-light. Retrograde signals released within the chloroplast trigger signal transduction pathways that transmit information to the nucleus to modify gene expression

* Correspondence: karl-josef.dietz@uni-bielefeld.de
† Equal contributors
Biochemistry and Physiology of Plants, Faculty of Biology – W5-134, University of Bielefeld, 33501 Bielefeld, Germany
necessary for acclimation. The origin and nature of plastid retrograde signals has been deduced from physiological and genetic experiments. Oxidation of the plastoquinone (PQ) pool activates the expression of chlorophyll-a/b-binding protein genes in the nucleus [6]. Redox changes in the intersystem electron transport chain as experimentally induced by preferential excitation of either photosystem II or photosystem I using light quality variation or by addition of inhibitors affect plastid and nuclear gene expression [7]. The thylakoid-associated protein kinases STN7 and STN8 mediate PQ-dependent regulation in the chloroplast, e.g. photosystem II protein phosphorylation, and in the long term response [8]. Signals originating downstream of photosystem I trigger the acclimation responses in chloroplasts and extrachloroplast compartments, e.g. regulating the expression of nuclear encoded 2-Cys peroxiredoxin [9]. Hormonal signals involved in retrograde signalling include abscisic acid, salicylic acid and 12-oxophytodienoic acid, the precursor of jasmonic acid [10-12]. In some cases signalling components such as transcription factors of the AP2/EREBP family participate in the retrograde signalling response. Few involved signalling elements could already tentatively be aligned. Thus, different operational signals from the chloroplast converge upstream of GUN1 and initiate ABI4-dependent nuclear gene expression [13]. Using genetic approaches, singlet oxygen signalling was associated with FLU and the functional executor isoforms EXE1 and EXE2 [14].

The here employed experimental design uses a differential light acclimation regime of Arabidopsis thaliana grown at low light near the light compensation point (about 8 \( \mu \)mol quanta.m\(^{-2}\).s\(^{-1}\)) and normal growth light (80 \( \mu \)mol quanta.m\(^{-2}\).s\(^{-1}\)) with a subsequent transfer to H-light (800 \( \mu \)mol quanta.m\(^{-2}\).s\(^{-1}\)) as introduced before [11]. In the previous work transcript, protein and metabolite levels, as well as activities of components of the water-water cycle were compared with untreated control plants at 6 h and 24 h after the 10- and 100-fold light shift. In addition the response of marker transcripts described as basic parameters and taken from Oelze et al. [11]: It can be seen that the L-plants from Oelze et al. [11]: It can be seen that the L-plants only had 38% of the fresh weight-related RNA of N-plants, which increased significantly during the H-light treatment, albeit less in the N→H-plants than in the L→H-plants. It should be noted that the photoinhibition was entirely reversible [11].

H-light triggers the release of retrograde signals which derive from the chloroplast, modify nuclear gene expression and initiate acclimation responses. L- and N-plants revealed 2,219 transcripts with ≥2-fold difference. The transcriptional regulation following transfer to H-light was almost

### Results

Low (L-) and normal (N-) light-acclimated plants were transferred to the same high (H-) light intensity of 800 \( \mu \)mol quanta.m\(^{-2}\).s\(^{-1}\) which is equivalent to a 100- and 10-fold increase over acclimation light, respectively. The experimental design and the response of the plants have been described in detail by Oelze et al. [11]. Table 1 summarizes four parameters measured as basic parameters and taken from Oelze et al. [11]: It can be seen that the L-plants only had 38% of the fresh weight-related RNA of N-plants, 48% protein and 61% chlorophyll. Protein and RNA tented to increase during the H-treatment, however only in the L→H-light treatment protein content increased significantly. Effective quantum yield of photosynthesis decreased significantly during the H-light treatment, albeit less in the N→H-plants than in the L→H-plants. It should be noted that the photoinhibition was entirely reversible [11].
finished after 6 h, with only 205 transcripts remaining differentially expressed between L→H- and N→H-plants [20]. This experimental system has previously been established in order to follow the acclimation process to H-light in particular with focus on the antioxidant defence system after 6 h of H-light exposure [11] and to address involved signaling pathways in a time-resolved manner [20]. The setup appeared suitable to ask the next question concerning the coupling between transcript regulation and de novo protein synthesis. To this end leaf proteins were extracted from L-, N-, L→H- and N→H-light samples after 6 h of treatment and subjected to 2D gel electrophoresis with silver staining for sensitive visualization of protein pattern (Figure 1). Polypeptides were excised from parallel gels and subjected to mass spectrometric identification (Table 2). Using this information and 2D analysis software a partially annotated master gel was assembled (Figure 2).

In the next step, radioactively labelled 35S-methionine was applied to leaf surfaces of intact plants in the identical experimental setup at 1 pm, i.e. 4 h after the beginning of H-treatment, and the plants were further incubated for two more hours. Thus, harvesting and analysis of de novo synthesized proteins occurred 6 h after transfer to H-light. For analysis protein extracts of labelled leaves equivalent to 10^6 counts per minute were subjected to 2D PAGE and analysed by autoradiography (Figure 3). The four conditions resembled each other in the basic pattern of a large set of proteins, but also revealed significant differences,

Table 1 Basic characterization of plants grown in normal (N) or in low (L) light, or transferred to high (H) light for 6 h (N→H, L→H)

| Parameter       | Treatment | N     | N→H   | L     | L→H   |
|-----------------|-----------|-------|-------|-------|-------|
| Chlorophyll [mg/g fw] |           | 1.22 ± 0.10^a | 1.15 ± 0.10^a | 0.75 ± 0.08^b | 0.80 ± 0.10^b |
| ΦPSII [r.U.]    |           | 0.76 ± 0.01^a | 0.62 ± 0.04^c | 0.72 ± 0.02^b | 0.49 ± 0.05^c |
| Protein [mg/g fw] |           | 10.19 ± 1.01^a | 11.49 ± 0.96^a | 4.94 ± 0.14^b | 5.58 ± 0.33^b |
| RNA [μg/g fw]   |           | 13.92 ± 6.60^a | 17.27 ± 7.91^a | 5.31 ± 3.11^b | 5.89 ± 3.02^b |

Contents of chlorophyll, protein and RNA were determined in leaf samples (n between 3 and 8 independent experiments, m ± SD; different letters mark significance groups according to t-test, p ≤ 0.05). Effective quantum yield of photosystem II as measured by pulse amplitude modulated chlorophyll fluorimetry is shown as m ± SD with n = 30 from 3 independent experiments. Letters mark groups of significant difference according to t-test, with p ≤ 0.01. Data are from [11].
Table 2 Compilation of polypeptides identified both in the silver stained gels and in the autoradiogram

| Protein name                              | ATG number | MM (kDa) | Localization | Functional role          | Silver | De novo | Mascot score |
|-------------------------------------------|------------|----------|--------------|--------------------------|--------|---------|--------------|
| 1. 2-Cys Peroxiredoxin                    | AT3G11630  | 22.4     | Chloroplast  | Defense response         | +      | +       | 66           |
| 2. 3-Ketoacyl CoA thiolase 3              | AT2G33150  | 48.6     | Peroxisome   | Fatty acid biosynthesis  | +      | +       | 99           |
| 3. Ascorbate peroxidase 1                 | AT1G07890  | 27.6     | Cytosol      | Defense response         | +      | +       | 253          |
| 4. ATP synthase delta-subunit              | AT4G09650  | 22.8     | Chloroplast  | ATP synthesis            | +      | +       | 260          |
| 5. ATP synthase subunit beta               | ATCG00480  | 47.7     | Chloroplast  | ATP synthesis            | +      | +       | 365          |
| 6. Carbonic anhydrase 1                    | AT3G01500  | 25.6     | Chloroplast  | Carbon utilization       | +      | +       | 45           |
| 7. Carbonic anhydrase 2                    | ATSG14740  | 25.1     | Chloroplast  | Carbon utilization       | +      | +       | 72           |
| 8. Chaperonin 60 beta                     | AT1G55490  | 58.1     | Chloroplast  | Protein folding          | +      | +       | 89           |
| 9. Chloroplast HSP 70-1                    | AT4G24280  | 74.6     | Chloroplast  | Stress response          | +      | +       | 528          |
| 10. Chloroplast HSP 70-2                   | ATSG49910  | 74.6     | Chloroplast  | Stress response          | +      | +       | 337          |
| 11. Cu/Zn Superoxide dismutase             | AT2G28190  | 15.7     | Chloroplast  | Defense response         | +      | +       | 81           |
| 12. Cyclophilin Cyp 20-3                  | AT3G62030  | 19.7     | Chloroplast  | Rotamase                 | +      | +       | 207          |
| 13. Dehydroascorbate reductase             | AT1G19570  | 21.7     | Cytosolic    | Defense response         | +      | +       | 93           |
| 14. D-Ribulose-5-P epimerase               | AT5G61410  | 28       | Chloroplast  | Calvin cycle             | +      | ND      | 154          |
| 15. Fructose-bisphosphate aldolase 1       | AT2G21330  | 41.9     | Chloroplast  | Calvin cycle             | +      | +       | 91           |
| 16. Fructose-bisphosphate aldolase 2       | AT4G38970  | 38       | Chloroplast  | Calvin cycle             | +      | +       | 106          |
| 17. GAP C2 subunit                        | AT1G13440  | 36.9     | Cytosol      | Oxidoreductase           | +      | +       | 164          |
| 18. Germin 3 oxalate oxidase               | AT5G20630  | 19.5     | Apoplast     | Defense response         | +      | ND      | 243          |
| 19. Glutamine synthetase 2                | AT5G35630  | 42.5     | Chloro/Mito  | Glutamine biosynthesis   | +      | +       | 141          |
| 20. Glutathione S-transferase F8           | AT2G47730  | 23.9     | Chloroplast  | Stress response          | +      | ND      | 71           |
| 21. Glutathione S-transferase F9           | AT2G30860  | 24.2     | Cytosol      | Stress response          | +      | ND      | 118          |
| 22. Glyceraldehyde-3-P-DH, B subunit       | AT1G42970  | 39.3     | Chloroplast  | Calvin cycle             | +      | +       | 70           |
| 23. HCF 136                               | AT5G23120  | 38.5     | Chloroplast  | Photosynthesis            | +      | +       | 173          |
| 24. Lactate/malate dehydrogenase           | AT1G53240  | 33.2     | Mitochond.   | TCA-cycle                | +      | +       | 65           |
| 25. Malate dehydrogenase                  | AT3G47520  | 34       | Chloroplast  | Redox metabolism         | +      | +       | 107          |
| 26. Manganese SOD                         | AT3G10920  | 22.2     | Mitochond.   | Defence response         | +      | ND      | 78           |
| 27. O-Acetyl serine thiol lyase B          | AT2G43750  | 35.1     | Chloroplast  | Cysteine biosynthesis    | +      | +       | 85           |
| 28. Phosphoglycerate kinase 1              | AT1G79550  | 42.63    | Chloroplast  | Calvin cycle             | +      | +       | 86           |
| 29. Phosphoglycerate mutase                | AT3G08590  | 60.7     | Cytosol      | Glycolysis               | -      | +       | 143          |
| 30. Phosphoribulokinase                    | AT1G32060  | 39.2     | Chloroplast  | Calvin cycle             | +      | +       | 97           |
| 31. Plastid-lipid-associated protein 1     | AT4G04020  | 34.9     | Chloroplast  | Stress response          | +      | +       | 113          |
| 32. Plastocyanin (DRT 112)                 | AT1G20340  | 10.5     | Chloroplast  | Electron transport       | +      | ND      | 169          |
| 33. PSII oxygen evolving complex           | AT5G66570  | 26.5     | Chloroplast  | Photosynthesis            | +      | +       | 114          |
| 34. PSII, subunit PSB-O2                   | AT3G0820   | 35.0     | Chloroplast  | Photosynthesis            | +      | +       | 304          |
| 35. PSII subunit P-1                      | AT1G06680  | 20.2     | Chloroplast  | Photosynthesis            | +      | +       | 183          |
| 36. Ribose 5-phosphate isomerase          | AT3G04790  | 27.1     | Chloroplast  | Calvin cycle             | +      | ND      | 161          |
| 37. Ribosomal protein S1                   | AT5G30510  | 40.5     | Chloroplast  | RNA binding              | +      | +       | 70           |
| 38. RPL12 | ribosomal protein L12-A                 | AT3G27830  | 14        | Chloroplast  | Translation               | +      | +       | 78           |
| 39. Rubisco activase                      | AT2G39730  | 46.2     | Chloroplast  | Calvin cycle             | +      | +       | 462          |
| 40. Rubisco large subunit                  | ATCG00490  | 53       | Chloroplast  | Calvin cycle             | +      | +       | 304          |
| 41. Rubisco small subunit 1A               | AT1G67090  | 14.7     | Chloroplast  | Calvin cycle             | +      | +       | 346          |
| 42. Rubisco small subunit 1B               | AT5G38430  | 14.8     | Chloroplast  | Calvin cycle             | +      | +       | 71           |
| 43. Rubisco small subunit 2B               | AT5G38420  | 14.8     | Chloroplast  | Calvin cycle             | +      | +       | 308          |
| 44. S-Adenosylmethionine synthetase 1      | AT1G02500  | 43.2     | Cytosplasm   | Met adenos.transferase   | +      | ND      | 92           |
particularly between L- and L→H-plants on the one hand and N- and N→H-plants on the other. The most obvious difference was monitored for RubisCO large subunit which was synthesized both in N- and N→H-plants at high rates, but label was almost absent in L-plants and only slightly induced in L-plants upon transfer to H-light. All gels from the three independent experiments were matched to generate a fused master gel image utilizing Delta 2D software (Figure 4) and analyzed for spot response behaviour. In total 129 spots could be identified that revealed differences among the treatments with statistical significance <0.01 (one way ANOVA). The clustered heat map for three experiments with 12 samples and 129 significantly altered spots is depicted in Figure 4B. It shows (a) a consistent regulation for same treatments in the three independent experiments, (b) the contrasting regulatory state of L-plants compared to that of all other treatments, and (c) the efficiency of L→H-plants in adjusting the pattern of de novo synthesized proteins to that of N→H-plants despite the different starting points. Four major cluster types of regulation could be identified: Polypeptides of cluster 1 were synthesized at low de novo rates in N→H- and L→H-plants, polypeptides of cluster 2 were high in N→H- and L→H-plants. Cluster 3 includes polypeptides whose synthesis showed contrasting responses in H-light, i.e. stimulation in N→H and low synthesis in L→H-plants, while cluster 4 showed the opposite. Focusing on proteins being synthesized above ('up-regulated') or below average allowed the generation of a Venn-diagram (Figure 4C), that confirmed the impression from the heat map, namely that the labelling pattern of N-plants was most closely related to the average state with only 26 spots (20%) synthesized above or below average of all treatments, 9 of which were specific to N-plant, 12 overlapped with L-plants and 5 with N→H-plants. Radiolabel of 50% (=64) of the spots in L-plants deviated from average; 47 being specific and only 5 were present in a distinct amount after transfer to H-light. Levels in 22% (28) spots deviated from average in N→H- and L→H-plants.

| Polypeptide Description | Accession Number | Predicted Localization | Functional Role | MASCOT Score |
|-------------------------|------------------|------------------------|-----------------|--------------|
| Sedoheptulose-bisphosphatase | AT3G55800 | Chloroplast | Calvin cycle | + + 229 |
| Stromal APx | AT4G08390 | Chloroplast | Defense response | + + 67 |
| Thioredoxin m1 | AT1G03680 | Chloroplast | Defense response | + + 105 |
| Thioredoxin m2 | AT4G03520 | Chloroplast | Defense response | + + 72 |
| Triose phosphate isomerase | AT2G21170 | Chloroplast | Calvin cycle | + + 133 |

Table 2 Compilation of polypeptides identified both in the silver stained gels and in the autoradiogram (Continued)

Shown are the specific details about size, predicted localization, the functional role of the proteins and the MASCOT score. MG #: number in annotated master gel; #: unequivocally identified by mass spectrometry with at least two peptides; +-: tentatively identified by one peptide; ND: not detected. Polypeptides #5 and 40 are plastome-encoded.

Figure 2 Annotated reference gel ('master gel') for the light shift experiment. 100 μg of total protein was separated by 2D gel electrophoresis. Spots were excised and 90 polypeptides were identified by mass spectrometric analysis.
Figure 3 Two-dimensional autoradiograms of de novo-synthesized proteins in leaves from L-, L→H-, N- and N→H-light treated plants. 

$^{35}$S-methionine was applied to the leaf surface at $t = 4$ h after transfer to H-light and the controls. Leaves were harvested at $t = 6$ h. Samples equivalent to $10^6$ counts per minute were loaded on each gel. The gels were prepared for autoradiography and x-ray films exposed for 48 h at -80°C. The experiments were conducted three times and representative autoradiograms are shown.

Figure 4 Analysis of the autoradiograms for changes in reliably detected de novo synthesized proteins.

Three autoradiograms for each condition from independent experiments were analysed with the Delta 2D software. (A) The results from three gels were fused and spots color-coded: N = blue, N→H = orange, L = green, L→H = red. (B) A heat map was automatically constructed as described above based on the set of 129 reliably detected changes that were classified as significant with one way ANOVA ($p \leq 0.01$). The lanes of the three identical conditions were placed next to each other. The four clusters were categorized according to the automatically generated cluster tree depicted on the left hand side. (C) Venn diagram of the significantly up-regulated spot intensities representing the overlaps among treatments.
Autoradiographs were digitalized and the spot landscape warped to the master gel image. The protein pattern of de novo synthesized and, thus, radiolabelled polypeptides differed considerably from silver- or Coomassie-stained 2D patterns (Figures 1, 2 and 3). Despite these differences, both patterns could reliably be matched since many spots served as unambiguous landmarks. In the next step all 12 gels from four conditions with three experiments were matched, the spot volume as well the greyness quantified and the annotated polypeptides were confirmed manually. The results of these 49 polypeptides are given in Table 2 which lists the AT' number, molecular mass, localization, detection in the silver stained gels or autoradiograms and the MASCOT score. Eight polypeptides detected in the annotated gels were not found in the autoradiograms. The vast majority of polypeptides, namely 80% showed a proven or predicted chloroplast localisation. De novo protein synthesis of these proteins was investigated for its response to the treatment and assigned to the four major response clusters (Table 3). In cluster 1 “change in de novo protein synthesis down in both H-treatments” appeared polypeptides with function in photosynthetic electron transport and antioxidant defence. Chaperones and proteins of redox homeostasis were found in cluster 2 “up in both H-treatment”. Metabolic enzymes predominated cluster 3 “up in N→H and down in L→H-plants”, and cluster 4 “N and N→H low” with ascorbate peroxidases and redox regulatory elements such as cyclophilin Cyp20-3 and malate dehydrogenase.

As reported before, RNA was isolated from leaves treated as above (L-, N-, L→H, N→H) at t = 6 h. ATH1 whole genome arrays were hybridized from three experiments [20]. Raw data were processed with ROBIN (MPI Golm, Germany) and normalized on total intensity of all spots (RMA normalisation [21]). Means and corrected standard error (p < 0.005) were calculated [22,23]. Transcripts identified in the autoradiograms and silver stained gels were selected from the list of transcripts and ratios of change were calculated. Figure 5 summarizes the results for the protein, de novo-synthesized and transcript level by heat map representation. Total protein was unrelated to transcript levels and de novo protein synthesis rates. However, also changes in transcript levels were unrelated to de novo synthesis for most genes. The changes upon the 10- and 100-fold light shift in transcript amounts were related to the changes in de novo protein synthesis and plotted in a diagram (Figure 6).

Discussion
Reorganization of the leaf proteome in light acclimation
Sun and shade acclimation depends on structural and functional reorganization of photosynthetic organs [24,25]. Total leaf protein amount related to fresh weight differed between plants grown under L- or N-light conditions more than twofold. Two possible reasons might exist, namely either a similar protein complement at lower level or a profound qualitative difference that explains the lower level. Since plasmatic compartments such as cytosol, matrix and stroma contain about 25% (w/v) protein, e.g. 10 mg protein/40 µl chloroplast volume [26,27], a twofold difference clearly indicates that the volumes of plasmatic compartments is strongly decreased after the 10 d L-light acclimation [11]. But in addition to a general decrease in volume, polypeptide composition also changes qualitatively. The best established example of light acclimation-dependent differences in protein composition concerns the increase in D1 protein and the decrease in light harvesting complex proteins (LHClI) with increasing growth light [28]. Changes in the photosynthetic apparatus are instrumental to adjust energy conversion and growth and are also important for optimized resource allocation, e.g. in dependence on light and nitrogen availability [29]. Protein patterns of silver-stained electropherograms differed between L- and N-acclimated plants. Many polypeptides appeared to be less abundant in N-light plants than in L-plants. This may be explained by normalization of each spot on total intensities in the gels. Due to the high RubisCO amount in extracts from N-plants, the intensities of most other bands will appear to be lower. But considering the low fresh weight-related protein contents of L-plants it becomes clear that the polypeptide abundance in silver gels would need some correction if polypeptide abundance should be related to fresh weight. Abundance of only few proteins changed during the 6 h period of H-light treatment. RubisCO was among the significantly accumulating proteins in the L→H-plants. It should be noted that the combined evaluation of both light shift treatments appeared justified despite in some cases different starting points due to the mostly similar response of protein abundance (82% similar response) and transcript regulation (100% similar response). This regulation leads to a highly similar transcriptome state after 6 h H-light [20].

Strengths and drawbacks of in vivo labelling of de novo synthesized proteins
Acclimation responses to environmental conditions are most frequently analysed at the level of specific transcripts or of genome-wide transcriptomes [30]. The matching of annotated silver-stained or Coomassie-stained 2D gels with autoradiograms was expected to allow for protein assignments of de novo synthesized polypeptides. But the labelling method needs some discussion. Labelling of intact plant tissue with 35S-methionine requires time for uptake and incorporation, and in some studies it was achieved by wounding [31], in others by feeding via the transpiration stream [18] or by application to tissue surfaces. We chose the application to the cuticular surface of the
Table 3 Clustering of *de novo* synthesized proteins with identified functional assignment

| Cluster | Response pattern | Transcripts/genes | Functional role |
|---------|------------------|-------------------|----------------|
| 1       | L→H & N→H low   | DHAR              | Antioxidant defence |
|         |                  | GAPDH B subunit   | Photosynthesis |
|         |                  | PSI1 subunit O-2  | Photosynthesis |
|         |                  | PSI1 subunit P-1  | Photosynthesis |
|         |                  | Ribose 5-P isomerase A | Photosynthesis |
|         |                  | RPL12, ribosomal protein | Protein synthesis |
|         |                  | SOD, Cu/Zn       | Antioxidant defence |
|         |                  | Thioredoxin m2    | Redox regulation |
| 2       | L→H & N→H high  | Carbonic anhydrase 2 | Photosynthesis |
|         |                  | Chaperonin 60 beta | Protein folding |
|         |                  | FBP aldolase1     | Photosynthesis |
|         |                  | FBP aldolase 2    | Photosynthesis |
|         |                  | HCF 136           | Photosynthesis |
|         |                  | HSP 70-1, cp      | Stress response |
|         |                  | Lactate/malate DH | Respiration |
|         |                  | O-Acetyl serine thiol lyase B | Sulfur metabolism |
| 3       | N→H high, L→H low | 3-Ketoacyl CoA thiolase 3 | Fatty acid metabolism |
|         |                  | GAP C2 subunit    | Photosynthesis |
|         |                  | Glutamine synthetase 2 | Nitrogen metabolism |
|         |                  | Phosphoglycerate kinase 1 | Photosynthesis |
|         |                  | Phosphoribulo kinase | Photosynthesis |
|         |                  | Rubisco activase  | Photosynthesis |
|         |                  | Plastid-lipid-associated protein 1 | Stress response |
|         |                  | Rubisco SU 1A     | Photosynthesis |
|         |                  | SBPase            | Photosynthesis |
|         |                  | SAM synthetase 1  | Sulfur metabolism |
| 4       | N, N→H-high; L, L→H-low | ATP synthase beta | Photosynthesis |
|         |                  | ATP synthase delta | Photosynthesis |
|         |                  | APX 1             | Antioxidant defence |
|         |                  | APx, stromal, cp  | Antioxidant defence |
|         |                  | Carbonic anhydrase 1 | Photosynthesis |
|         |                  | Cyclophilin Cyp 20-3 | Redox regulation |
|         |                  | Malate DH cyt     | Redox regulation |
|         |                  | PSI1 OEC          | Photosynthesis |
|         |                  | 2-Cys Peroxiredoxin | Antioxidant defence |
|         |                  | Germin 3 oxalate oxidase | Stress defence |
|         |                  | GST F8            | Stress defence |
|         |                  | GST F9            | Stress defence |
|         |                  | HSP 70-2, cp      | Stress defence |
|         |                  | Malate DH, cp     | Redox regulation |
|         |                  | Mn SOD            | Antioxidant defence |
|         |                  | Phosphoglycerate mutase | Glycolysis |
|         |                  | Plastocyanin (DRT 112) | Photosynthesis |
|         |                  | Ribosomal protein S1 | Protein synthesis |

No peculiar group pattern
youngest fully expanded leaves because neither application to the transpiration stream e.g. by injection or wounding, appeared suitable for our purpose of undisturbed but sensitive labelling of newly synthesized proteins. Labelling de novo synthesized leaf proteins by feeding the labelled amino acid to roots unlikely would allow for sufficiently strong incorporation within 2 h, but this could be compared in the future. The experimental design required incubation time for sufficient incorporation. Nevertheless, radiolabelling still is the only method at hand that allows for rapid, sensitive and reliable labelling of the de novo synthesized protein. It may be expected that with further advancement of mass

The proteins were clustered using the Delta 2D-software package and assigned to four major types of regulation (Clusters 1 to 4) as outlined.

Table 3 Clustering of de novo synthesized proteins with identified functional assignment (Continued)

| Cluster | Silver stained gels | de novo protein synthesis | Transcript changes |
|---------|---------------------|--------------------------|-------------------|
| 1       | Rubisco large subunit | Photosynthesis | ND |
| 2       | Rubisco small subunit | Photosynthesis | ND |
| 3       | Thioredoxin m1 | Redox regulation | ND |
| 4       | Triosephosphate isomerase | Photosynthesis | ND |

Figure 5 Comparison of light-dependent changes in spot intensity in silver stained gels, autoradiograms and in transcript levels.

Changes in spot intensities of silver gels and autoradiograms were taken from the three independent experiments similar to Figures 1, 2 and 3. Transcript data were extracted from three independent sets of array hybridization. Changes calculated as value at higher light intensity divided by intensity at lower light intensity were colour-coded as indicated in the colour bar at the bottom (asterisks indicate significant difference of changes, t-test (p < 0.1 for de novo synthesis, p < 0.05 for transcript).
spectrometric analysis, stable isotopes will offer alternative methods to study protein turnover also for eukaryotic multicellular organisms similar to unicellular organisms. The employed method should be added to the portfolio of potential options that can be employed. Starting 4 h after transfer to H-light appeared suitable because many transcriptional changes had been shown to reach a new steady state at this time, e.g. sAPX [11] or monodehydroascorbate reductase, ABA-dependent cold regulated 47 (COR47), pyruvate kinase related protein (PKRP) [20]. Thus, the labeling that starts after translocation of 35S-methionine through the cuticle to the mesophyll reflects a transcriptional state similar to 6 h after transfer to H-light for which the transcript analysis has been performed.

Apparent absence of coupling between transcript regulation and de novo protein synthesis

The comparison of transcript regulation with differences in de novo synthesized protein demonstrates the flexible coupling between transcript regulation and translation (Figure 6). Piques et al. [34] compared transcript levels, ribosome occupancy, enzyme protein amount and activity at different times of day. Their scatter analysis revealed a poor dependency of ribosome loading on total amount of investigated transcripts. The Pearson’s correlation coefficient was 0.065 in the dark period and 0.102 in the light period [34]. Here, transcript analysis revealed efficient regulation following transfer to H-light. In sum 27 out of 42 transcripts of identified proteins, i.e. 64%, had log2-fold differences ≥0.5 between N- and L-light grown plants prior to H-light treatment. The size of this group of differentially regulated transcripts decreased to only 2 genes after 6 h of H-light. Thus, transcriptional regulation in response to H-light was almost completed after 6 h H-light.

In most cases regulation of transcript amounts was more pronounced than regulation of de novo protein synthesis. Regulation of 6 proteins occurred much stronger at the level of de novo protein synthesis. Several translation factors have been identified as target of posttranslational regulation including thiol-disulfide transitions [35], glutathionylation [36], phosphorylation [37] and S-nitrosylation [38]. Among the targets researchers identified several ribosomal proteins (RPL S1, S6, L13, L30), elongation factors (EF-Tu, EF-G, EF-2, EF-1α) and enzymes such as nucleoside diphosphate kinase III and tRNA synthetases which all are involved in translation. Redox changes, ROS production and activation of phosphorylation cascades have been implicated in retrograde signalling. The protein kinases STN7 and STN8 mediate light-dependent reorganization of the photosynthetic apparatus [39]. ROS waves adjust nuclear gene expression in excess light acclimation [40]. ROS and redox feed into the mitogen activated protein kinase pathway [41]. Translational activity is strongly altered by ROS in yeast [42]. Thus, translation in plants is a prime but hitherto not sufficiently explored target of retrograde signalling as underlined by the data presented in this paper. The reader is also referred to the metaanalysis by Schwarzländer et al. [43] who observed that transcripts encoding for proteins involved in protein synthesis are significantly affected by retrograde signals released from the mitochondrion.

Functional implications of translational control of identified targets

Control of posttranscriptional processes accelerates the speed and versatility of stress acclimation. The high
significance of specific transcript recruitment to ribosomes in plants has best been demonstrated for acclimation to hypoxia [44]. The authors showed hypoxia-specific changes of transcriptome and translatome at the global, organ- and cell-specific level. Preferential ribosome association was observed for sucrose transporters, heat shock factors and transcription factors [45]. Here, expression of six genes was more strongly regulated at the level of protein synthesis than of transcript accumulation. It may be assumed that the gene product functions are needed after transfer to H-light. Despite down-regulation at the transcript level, 35S-methionine incorporation into HSP70-1 still occurred at high rates. In a converse manner, HSP70-2 was synthesized at similar rates despite a large increase in transcript amount. Chloroplast HSP70s facilitate protein import into the chloroplasts, a function which is of eminent importance during environmental transition such as exposure to excess excitation energy [45]. High chlorophyll fluorescence HCF136 was identified in a screen for genes with function in assembly of functional photosystem II [46]. FBP aldolase as part of the Calvin cycle, O-acetyl serine thiol lyase with its function in cyanide synthesis, carbamic anhydrase which facilitates equilibration between carbonate and CO2 as substrate of the Calvin cycle and 3-ketoacyl CoA thiolase 3 involved in fatty acid synthesis showed stimulated de novo synthesis. This type of regulation may easily be reconciled with their metabolic functions which are important for H-light acclimation. Arguments appear less straightforward when it comes to explain the low level of de novo protein synthesis observed for 16 genes. They mostly function in metabolism such as sedoheptulose-1,7-bisphosphatase which is suggested to limit Calvin cycle activity [47], large and small subunits of RubisCO, RubisCO activase, phosphoglycerate mutase, phosphoglycerate kinase and ribose-5-phosphate isomerase. Others are involved in redox homeostasis and antioxidant defence (malate dehydrogenase, dehydroascorbate reductase, superoxide dismutase, stromal ascorbate peroxidase and the regulator of chloroplast cysteine synthase complex cyclophilin Cyp20-3 [12]. It may be hypothesized that these proteins are present at sufficient amounts prior to H-light treatment and that the low ratio of de novo synthesis-to-transcript amount merely reflects such mechanisms of yet un-understood feedback control. It should be noted that photoreceptor-dependent signaling might contribute to the transcriptional and translational responses described in this paper, albeit previous work largely excluded a major role of photoreceptors in this particular experimental setup [7,11].

Conclusions

Translational control is still poorly investigated particularly in plants: Initiation, elongation and pausing contribute to transcript selection and efficiency of translation. De novo labelling as used here determines the outcome of all these processes and, therefore is a better readout of protein synthesis than ribosome loading eventually combined with ribosome footprinting [48]. The latter technique allows for profiling of RNA sequences by deep sequencing that are protected from degradation by associated ribosomes. Our study adds a novel method to the portfolio available to investigate posttranscriptional regulation. The results show that H-light acclimation involves translational control as decisive part of retrograde signalling and concerns a large fraction, namely almost 2/3 in the set of identified proteins. Furthermore the rate of de novo protein synthesis cannot directly be predicted from transcript levels.

Methods

Plant growth and treatment

Arabidopsis thaliana was grown in a growth chamber in a mix of 50% soil, 25% Perlite and 25% Vermiculite, supplemented with one dose of Lizetan (Bayer, Germany). Following seed stratification for 2 d at 4°C, plants were grown for 30 d in 80 μmol quanta-1 m-2 (N-light) with a 14 h light and 10 h dark phase. Subsequently, plants were transferred to 8 μmol s-1 m-2 (L-light) for 10 d prior to the experiment with transfer to 800 μmol s-1 m-2 (H-light; 100-fold light increase). The L-plants have been shown to be entirely shade acclimated [11]. Another set of plants was grown in N-light for the whole period of 40 d and also transferred to 800 μmol s-1 m-2 (10-fold light increase). Control plants were kept in L- and N-light, respectively, and harvested in parallel to the H-light rosettes. Harvest time was always at 3 pm. Chlorophyll, protein and RNA contents and effective quantum yield of photosystem II by pulse amplitude modulation (PAM) were determined as described in Oelze et al. [11].

In vivo labelling of de novo synthesized proteins

L-[^35]S-methionine (NEG009T, Perkin Elmer, MA, USA) was supplemented with 0.1% (v/v) Triton X-100 and applied to leaf surfaces with a radioactivity of 20 μCi per leaf. For each treatment 20 μCi were administered to fully expanded leaves from three different rosettes 4 h after transfer to H-light. After 6 h, the leaves were excised from the rosettes, washed first with 0.1% (v/v) Triton X-100 and then with 0.5 mol/L Tris-Cl, pH 6.8.

2D-gel electrophoresis

Leaves were ground with a pestle in 1 mL acetone/trichloroacetic acid/β-mercaptopoethanol (89.93:100:0.07% v/v) according to Méchin et al. [49]. Following precipitation at -20°C for at least 1 h and subsequent centrifugation, the pellet was washed and sedimented thrice with ice-cold acetone/β-mercaptopoethanol, dried and resuspended in lysis buffer [50]. For radioactive samples, incorporated 35S
was quantified by precipitating aliquots on Whatman filter followed by scintillation counting. For silver-stained gels, protein amounts were quantified at 595 nm with the BioRad protein assay. Separation in the first dimension was achieved with Immobiline™ DryStrips (pH range 3-10 NL, 18 cm, GE Healthcare, Uppsala, Sweden). Sample equivalent to 100 μg protein or 10⁶ cpm was dissolved in 340 μL complete rehydration buffer (8 mol/L urea, 2% (w/v) CHAPS, 0.002 bromophenolblue, 0.3% amphotelyte, 1.4% (w/v) dithiothreitol) and applied to the Immobiline strips. The rehydration and isoelectric focusing protocol consisted of the steps as follows: 1 h 0 V, 12 h 30 V, 2 h 60 V, 1 h 500 V, 1 h 1000 V, 1000-8000 V for variable time to reach 42000 Vh. Separation in the second dimension was performed on a 12% (w/v) SDS-PAGE of 18 cm length at 40 mA. Silver staining was performed according to Blum et al. [51] and autoradiography as described in Dietz and Bogorad [52].

**Analysis of 2D-gels and heat map construction**

Delta 2D software (Decodon, Greifswald, Germany) with its SmartVectors Technology was used to align the gel images to each other to allow for efficient and reliable spot matching. A fusion image was generated containing all spot positions. Each gel was matched with this master gel. Spot boundary detection, pixel intensity quantification and statistical analysis (one way ANOVA) were performed with the built in TIGR MeV tool. Before constructing the heat map, the data set was standardized to zero mean and unit variance. Clustering was achieved using the euclidean distance and complete linkage- default settings of the delta 2D software (DECODON, Greifswald, Germany).

**ATH1-genome array hybridisation and analysis**

Isolated total RNA was sent to KFB-company (Competence Centre for Fluorescence Bioanalytics, Regensburg, Germany), processed, and derived fluorescent probes hybridized against the 25mer oligonucleotide ATH1-genome array (Affimetrix, Santa Clara, USA). Glyceraldehyde-3-phosphate dehydrogenase, actin and ubiquitin were used as reference transcripts. The raw data were fed into ROBIN (MPI Golm, Germany). Statistical evaluation of the data was based on the corrected p-value [22,23].

**Protein identification by mass spectrometry**

Corresponding areas of interest were excised from the 2D gels and washed with (a) two times a solution containing trifluoroacetic acid (0.1% w/v) and acetonitrile (60% v/v), (b) acetonitrile (50%), (c) acetonitrile (50%)/50 mM NH₄ HCO₃ for 0.5 h, and (d) acetonitrile (50%)/10 mM NH₄ HCO₃ at 21°C for 0.5 h each. Dried gel slices were resuspended in trypsin solution (0.013 mg sequencing quality trypsin (Promega, Mannheim, Germany) in 10 mM NH₄ HCO₃ pH 8.0) at 4°C for 0.5 h and afterwards at 37°C for about 15 h. Digestion solutions were supplemented with cyan-4-hydroxy-cinnamic acid at a 60:40% ratio. Mass spectra were determined using a Biflex III matrix-assisted laser desorption/ionisation-time of flight mass spectrometer (MALDI-TOF)-MS (Bruker, Bremen, Germany) (previously described [53]). The peptide mass fingerprints (PMF) obtained by tryptic digested proteins were analyzed by MALDI-TOF-MS and proteins were identified by Mascot (Multiple-Access Space-Time Coding Testbed) software and the National Center for Biotechnology Information (NCBI) protein database. The program compares the peptide masses obtained from experimental digestion to the predicted peptide masses from the theoretical digestion of proteins.

**Correlation of de novo protein synthesis and transcript regulation during H-light treatment**

The obtained values of the spot intensities for the autoradiograms by Delta 2D were used to calculate the ratios between the different treatments (N/L, N→H, L→H, L→H/N→H). The ratios were recalculated as log₂-fold change values, to be easily comparable to the obtained log₂-fold change values of the microarray experiments by ROBIN.

For the comparison of de novo protein synthesis and transcriptional regulation the maximum reactions (up or down regulation) for both H-light treatments (L→H, N→H) were used as reference. Each value (FPOI)o ft h e reaction (FPOI) of the different targets was divided by the appropriate maximum reaction (FExt; up-regulation was divided by maximum positive reaction while down regulated targets were divided by the maximal negative reaction) for each treatment (N→H or L→H) and for both methods (de novo protein synthesis or transcript regulation). Afterwards the calculated values for both de novo protein synthesis reactions (N→H or L→H light shift) or for both transcriptional regulations were summed up to give the response factor R.

\[
R = \frac{F_{L→H\text{(POI)}}}{F_{L→H\text{(Ext)}}} + \frac{F_{N→H\text{(POI)}}}{F_{N→H\text{(Ext)}}}
\]

Therefore, the maxima of regulation would fit in the range between -2 and 2. To evaluate the relationship between de novo synthesis and transcriptional regulation, the calculated values were plotted in a diagram where deviation from the diagonal ≤0.5 was set as a cutoff (gray shaded area) and only larger deviations (outside this area) were accepted to indicate distinct regulation between transcript and de novo protein synthesis.

**Abbreviations**

2D: Two dimensional; ABA: Abscisic acid; ABI: ABA insensitive; GUN: Genome uncoupled; H: High light; L: Low light; MS: Mass spectrometry; N: Normal light; PAGE: Polyacrylamide gel electrophoresis; PQ: Plastoquinone;
ROS: Reactive oxygen species; Rubisco: Ribulose-1,5-bisphosphate carboxylase oxygenase.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MLO, MM, MOV: Experimental acquisition, analysis and interpretation of data, KD: Design and discussion of study and writing paper. All authors read and approved the final manuscript.

Acknowledgements
This work was conducted within FOR 804, SPP1710 and project DI 346. MLO acknowledges support by the NRW International Graduate School on Bioinformatics and Genome Research. Authors also thank Dr. Peter Klein for his discussion in computational work.

Received: 24 January 2014 Accepted: 24 April 2014
Published: 30 April 2014

References
1. Dietz KJ, Pfannschmidt T: Novel regulators in photosynthetic redox control of plant metabolism and gene expression. Plant Physiol 2011, 155:1477–1485.
2. Wobbe L, Billemeze O, Schwarz C, Mussgnug JH, Nickelsen J, Kruse O: Cysteine modification of a specific repressor protein controls the transcriptional status of nucleus-encoded LHClI mRNAs in Chlamydomonas. Proc Natl Acad Sci U S A 2009, 106:13290–13295.
3. Baena-Gonzalez E: Energy signaling in the regulation of gene expression during stress. Mol Plant 2010, 3:300–313.
4. Narsai R, Howell KA, Millar AH, O’Toole N, Small I, Whelan J: Novel regulators in photosynthetic redox control nuclear gene expression. Proc Natl Acad Sci U S A 2010, 107:13228–13233.
5. Oelze M, Vogel MO, Oelze ML, Moore M, Stingl N, König K, Friedman H, Mueller MJ, Dietz KJ: Kinetics of retrograde signalling initiation in the high light response of Arabidopsis thaliana. Philos Trans R Soc Lond B Biol Sci, in press.
6. Escoubas JM, Lomas M, LaRoche J, Falkowski PG: Megadalton complexes in the plastoquinone pool. Proc Natl Acad Sci U S A 1996, 93:1179–1182.
7. Leister D: Photosystem II core phosphorylation and photosynthetic plastoquinone pool. J Biol Chem 2007, 282:5318–5328.
8. Benjamini Y, Hochberg Y: Controlling the false discovery rate. A practical and powerful approach to multiple testing. J R Stat Soc B 1995, 57:289–300.
9. Hornstein Y, Anderson JM: Adaptation of the thylakoid membranes of pea chloroplasts to light intensities. II. Regulation of electron transport capacities, electron carriers, coupling factor (CF1) activity and rates of photosynthesis. Photosynth Res 1984, 5:117–138.
10. Hinkson IV, Elias JE: The dynamic state of protein turnover: it EXECUTER1- and EXECUTER2-dependent contributions.
11. Rouhier N, Villarejo A, Srivastava M, Gelhaye E, Keech O, Droux M, Ecker J, Hasegawa K: Novel regulators in photosynthetic ribulose-1,5-bisphosphate carboxylase oxygenase.
12. Bartnik RA, Bolstad BM, Collin F, Cope LM, Hobbins B, Speed TP: Summary of Affymetrix Gene Chip probe level data. Nucleic Acids Res 2003, 31:e15.
13. Hinkson IV, Elias JE: The dynamic state of protein turnover: it’s about time. Trends Cell Biol 2011, 21:293–303.
14. Hinkson IV, Elias JE: The dynamic state of protein turnover: it’s about time. Trends Cell Biol 2011, 21:293–303.
39. Herbstová M, Tietz S, Kinzel C, Turkina MV, Kirchhoff H: Architectural switch in plant photosynthetic membranes induced by light stress. Proc Natl Acad Sci U S A 2012, 109:20130–20135.
40. Galvez-Valdivieso G, Mullineaux PM: The role of reactive oxygen species in signalling from chloroplasts to the nucleus. Physiol Plant 2010, 138:430–439.
41. Pitschke A, Hirt H: Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signaling. Plant Physiol 2009, 149:606–615.
42. Grant CM: Regulation of translation by hydrogen peroxide. Antioxid Redox Signal 2011, 15:191–203.
43. Schwarzländer M, König AC, Sweetlove LJ, Finkemeier I: The impact of impaired mitochondrial function on retrograde signalling: a meta-analysis of transcriptomic responses. J Exp Bot 2012, 63:1735–1750.
44. Mustroph A, Zanetti ME, Jang CJ, Holtan HE, Repetti PP, Galbraith DW, Girke T, Bailey-Serres J: Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in Arabidopsis. Proc Natl Acad Sci USA 2009, 106:18843–18848.
45. Latijnhouwers M, Xu XM, Møller SG: Arabidopsis stromal 70-kDa heat shock proteins are essential for chloroplast development. Planta 2010, 232:567–578.
46. Meurer J, Plücken H, Kowallik KV, Westhoff P: A nuclear-encoded protein of prokaryotic origin is essential for the stability of photosystem II in Arabidopsis thaliana. EMBO J 1998, 17:5286–5297.
47. Lefebvre S, Lawson T, Zakhleniuk OV, Lloyd JC, Raines CA, Fryer M: Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. Plant Physiol 2005, 138:451–460.
48. Ingolia NT, Ghaemmaghami S, Newman JR, Weissman JS: Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science 2009, 324:218–223.
49. Méchin V, Consoli L, Le Guilloux M, Damerval C: An efficient solubilization buffer for plant proteins focused in immobilized pH gradients. Proteomics 2003, 3:1299–1302.
50. Blum H, Beier H, Gross HJ: Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 1987, 8:93–99.
51. Dietz KJ, Bogorad L: Plastid development in Pisum sativum leaves during greening: I. A Comparison of plastid polypeptide composition and in organello translation characteristics. Plant Physiol 1987, 85:808–815.
52. Ströher E, Dietz KJ: The dynamic thiol-disulphide redox proteome of the Arabidopsis thaliana chloroplast as revealed by differential electrophoretic mobility. Physiol Plant 2008, 133:566–583.

doi:10.1186/1471-2164-15-320
Cite this article as: Oelze et al.: The link between transcript regulation and de novo protein synthesis in the retrograde high light acclimation response of Arabidopsis thaliana. BMC Genomics 2014 15:320.

Submit your next manuscript to BioMed Central and take full advantage of:

• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit