NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1mM DTT, 1 mM GTP-γS, pH 7.5, for 90 min at room temperature under rotation. Afterwards, buffer 1 was removed and the GTP-γS form of GST–Rab5 was stabilized with buffer (buffer 2) containing 20 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, 1mM DTT, pH 7.5, in the presence of 1 mM GTP-γS for 20 min at room temperature under rotation. Beads were then incubated for 120 min at 4 °C with bovine brain cytosol obtained as follows: 14 bovine brains were homogenized in a blender with buffer 2 and the homogenate was centrifuged at 4,200*g* at 4 °C for 50 min. The resulting postnuclear supernatant was then centrifuged at 100,000*g* at 4 °C for 60 min. The high-speed supernatant was dialyzed against buffer 2 (without nucleotide) before incubation with the affinity column. After incubation with cytosol, beads were washed with ten column volumes of buffer 2 containing 10 μM GTP-γS, ten column volumes of buffer 2 containing 250 mM NaCl final concentration and 10 μM GTP-γS, and one column volume of 20 mM HEPES, 250 mM NaCl, 1 mM DTT, pH 7.5.

Bound proteins were eluted with 1.5 column volumes of a buffer containing 20 mM HEPES, 1.5 M NaCl, 20 mM EDTA, 1mM DTT, 5 mM GDP, pH 7.5, incubated with the beads for 20 min at room temperature under rotation. EDTA was used at this step to remove Mg^{2+} from Rab5 and release the effectors from the column. As EEA1 is a Zn^{2+}-binding protein and EDTA chelates Zn^{2+} (refs 23, 30), our subsequent assays were done in the presence of 1 mM $ZnCl_2$ filtered (0.22 μM) before its use. The GDP form of GST–Rab5 (1 ml) was made as described above for the GTP-γS form of GST–Rab5 with the following changes: all buffers contained GDP instead of GTP-γS, except for the elution buffer which contained GTP-γS (1 mM) instead of GDP. In the case of GST column (100 μl), there was no nucleotide in the buffers.

Fractionation of Rab5 effectors. The eluate (30 ml) from the 20-ml affinity column containing the mixture of Rab5-interacting proteins was first treated twice for 1 h at 4 °C with 1.5 ml glutathione Sepharose beads to remove GST–Rab5 which leaked from the affinity column during the elution step. The sample was then desalted using PD10 columns from Pharmacia in a buffer containing 20 mM HEPES, 150 mM NaCl, 1mM DTT, pH 7.5, and diluted three times with 20 mM HEPES, 1 mM DTT, pH 7.5, resulting in a final volume of 135 ml. The diluted sample was loaded on a 1-ml MQ FPLC column (Pharmacia) and bound proteins were step-eluted with 20 mM HEPES, 1 M NaCl, 1mM DTT, pH 7.5, in a total volume of 1 ml (concentration step). This eluate was fractionated on a 24-ml Superose 6 FPLC gel filtration column (Pharmacia). Fractions of 0.4 ml were collected, aliquoted and frozen at −80 °C.

Endosome docking assay. HeLa cells grown in suspension were harvested, washed with PBS and incubated for 5 min with 20 μg ml^{-1} rhodamine-labelled transferrin at 37 °C for 5 min. Then endosomes were isolated[15] and incubated in a 10 μl reaction for 25 min at 37 °C with reagents mentioned in Fig. 4. At the end of the reaction, samples were put on ice and visualized with a Zeiss Axiophot fluorescence microscope. Images were taken using a Cohu camera. Quantitation of docking was done by taking random pictures, followed by counting of the occupied squares (an indication of organelle area) on a reference grid.

Other preparations. GST–Rab5 and GST proteins were expressed in *Escherichia coli* using the P-GEX vector (Pharmacia). his-Rab GDI[16], his-α-SNAP and his-α-SNAP(L294A)[25] were produced as described. The preparation of recombinant Rabaptin-5/Rabex-5 complex will be described elsewhere (R. Lippe and M. Zerial, manuscript in preparation). Early endosome fusion assay was done as described[16].

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Photosynthetic control of chloroplast gene expression

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Redox chemistry—the transfer of electrons or hydrogen atoms—is central to energy conversion in respiration and photosynthesis. In photosynthesis in chloroplasts, two separate, light-driven reactions, termed photosystem I and photosystem II, are connected in series by a chain of electron carriers[1–3]. The redox state of one connecting electron carrier, plastoquinone, governs the distribution of absorbed light energy between photosystems I and II by controlling the phosphorylation of a mobile, light-harvesting, pigment–protein complex[4,5]. Here we show that the redox state of plastoquinone also controls the rate of transcription of genes encoding reaction-centre apoproteins of photosystem I and photosystem II. As a result of this control, the stoichiometry between the two photosystems changes in a way that counteracts the inefficiency produced when either photosystem limits the rate of the other. In eukaryotes, these reaction-centre proteins are encoded universally within the chloroplast. Photosynthetic control

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of chloroplast gene expression indicates an evolutionary explanation for this rule: the redox signal-transduction pathway can be short, the response rapid, and the control direct.

Photosynthesis and plant growth can be supported by light with a wavelength composition that favours either photosystem II ('light 2') or photosystem I ('light 1')[2,3]. Figure 1 shows the effects of such photosystem-specific light on chloroplast composition and chloroplast gene expression. Figure 1a, b shows the relative transcriptional rates of specific chloroplast genes in 7-day-old mustard plants grown in light 1, in light 2, and after changes between the two regimes. Plants described as 'light 2 → 1' were grown for 5 days under photosystem-II light, before being transferred to photosystem-I light for 2 days; 'light 1' plants were grown under light 1 for 7 days; white-light-grown plants were used as the control; 'light 2' plants were grown under light 2 for 7 days; and 'light 1 → 2' plants were grown for 5 days under photosystem-I light, and then transferred to photosystem-II light for 2 days. Cotyledons of seedlings were collected, and chloroplasts were isolated and used in chloroplast run-on transcription experiments. This method leads to radioactive pulse-labelling of messenger RNA from a gene that was actually being transcribed at the time of chloroplast isolation. The transcriptional rate of the genes for the two major reaction-centre subunits of photosystem I, *psaAB*, shows an incremental increase from left to right (Fig. 1a), as photosystem I changes from being light-saturated to being light-limited. The transcriptional rate for the *psbA* gene, which encodes the D_1 reaction-centre protein of photosystem II, shows broadly the reverse pattern (Fig. 1b): the transcriptional rate of *psbA* is highest when photosystem II is rate-limiting ('light 2 → 1'). The RNA quantities of *psaAB* (Fig. 1c) and, to a lesser extent, *psbA* (Fig. 1d), follow changes in their respective transcriptional rates (Fig. 1a, b).

Figure 1e, f shows that the quantity of functional reaction centres follows the same pattern of response to illumination as the rate of transcription (Fig. 1a, b) and as transcript pool size (Fig. 1c, d). P_{700} is the specialized chlorophyll *a* that is bound to the *psaAB* gene products. It undergoes primary charge separation in photosystem I (ref. 6). P_{700} (Fig. 1e) is least abundant when the light supplied favours its activity (light 2 → 1), and most abundant when it does not (light 1 → 2). Q_A is the species of plastoquinone that is bound to the *psbA* gene product. It acts as a secondary electron acceptor in photosystem II (ref. 7). Q_A (Fig. 1f) is most abundant where illumination favours photosystem I (light 2 → 1), and least abundant where it favours photosystem II (light 2 and light 1 → 2). The chlorophyll *a/b* ratio (Fig. 1g) follows the same pattern as P_{700} and as the rate of transcription of *psaAB*. The ratio of photosystem II to I, measured as the molar ratio of Q_A to P_{700} (Fig. 1h), follows the same pattern as the absolute quantity of Q_A and as the rate of transcription of *psbA*. These results indicate that when either photosystem becomes rate-limiting to photosynthesis, transcription of genes for its reaction-centre proteins is induced. Genes for reaction-centre proteins of the other photosystem, which has surplus photochemical capacity, are simultaneously repressed.

Figure 2 shows that the light-induced changes in rate of transcription and quantity of transcript are remarkably fast. For both *psaAB* (Fig. 2a) and *psbA* (Fig. 2b) the change in rate of transcription, whether an increase or decrease, takes place over a timescale of minutes, and is followed by a corresponding change in quantity of

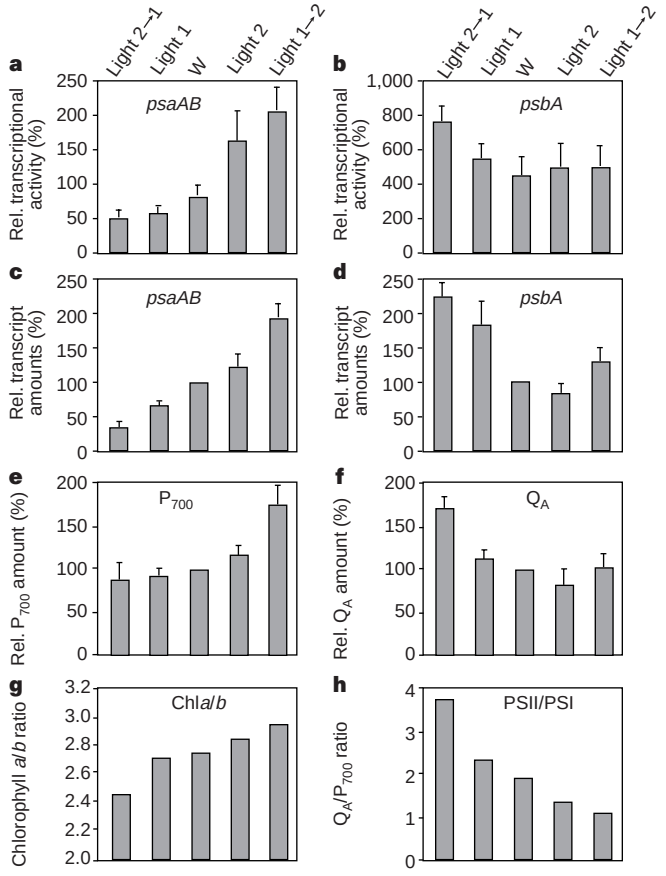

Figure 1 Reaction-centre stoichiometry and transcription. Transcriptional rate (**a**, **b**) and mRNA abundance (**c**, **d**) for the chloroplast reaction-centre genes *psaAB* (photosystem I) and *psbA* (photosystem II), together with quantities of the functional prosthetic groups, P_{700} (**e**) and Q_A (**f**), to which their respective apoprotein gene products bind, and chlorophyll *a/b* (**g**) and photosystem II/I (PS II/PS I) ratios (**h**). Gene expression and functional consequences for photosystem I are arranged in the left-hand column (**a**, **c**, **e**, **g**); those for photosystem II are arranged in the right-hand column (**b**, **d**, **f**, **h**). The PS II/PS I ratios in (**h**) are the ratios of Q_A to P_{700} from (**f**) and (**e**). Values in **a** and **b** are means of between four and five independent experiments. Values in **c**–**f** are means of three independent experiments and are expressed as percentages of the value of the white-light control (W).

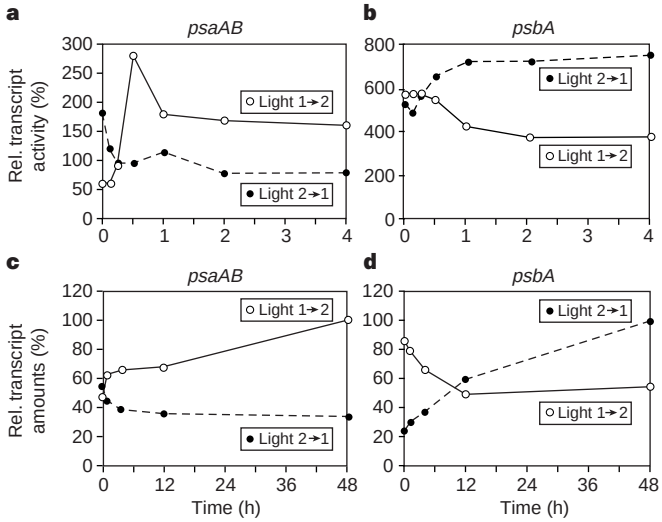

Figure 2 Complementary changes in transcription. Changes in transcriptional rate (**a**, **b**) and mRNA abundance (**c**, **d**) for the chloroplast reaction-centre genes *psaAB* (photosystem I; **a**, **c**) and *psbA* (photosystem II; **b**, **d**), as a function of time after changes in light regime between illumination conditions favouring photosystem I and photosystem II. Each change in illumination took place after 5 days' growth in either light 1 or light 2. Changes in rate of *psaAB* transcription (**a**) are detectable within 15 min, during which time fluorescence-emission changes that report on post-translational events are still taking place (results not shown). Each value is the mean of three independent experiments.

mRNA (Fig. 2c, d). We conclude that changes in the rate of transcription (Fig. 2a, b) arise from light-induced perturbation of the redox state of the plastoquinone pool. The altered rates of transcription result, in turn, in altered mRNA quantities (Fig. 2c, d) and, ultimately, in the altered stoichiometries of the two photosystems of the chloroplast thylakoid membrane (Fig. 1e–h).

The inhibitor 3-(3′,4′-dichlorophenyl)-1,1′-dimethyl urea (DCMU) inhibits electron flow from photosystem II into the plastoquinone pool[8]. A complementary electron-transport inhibitor is 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB), which inhibits oxidation of plastoquinol by photosystem I (ref. 8). Figure 3 shows that the rate of *psaAB* transcription in isolated chloroplasts is decreased in the presence of DCMU or by light 1, and increased in the presence of DBMIB or by light 2. These effects on transcriptionally and photosynthetically active chloroplasts isolated *in vitro* are precisely as predicted if plastoquinone redox control accounts for the responses observed *in vivo* (Figs 1, 2), and demonstrate that the control does not depend upon nuclear or cytosolic components. Taken together with the observation that the *psaAB* transcriptional rate *in vivo* is higher in light 2 than in light 1 (Fig. 1), we conclude that *psaAB* transcription is promoted when plastoquinone is reduced, and retarded when it is oxidized. In contrast, the rate of transcription of *psbA* (Fig. 3) does not respond to light or inhibitors *in vitro* under our experimental conditions, and seems to be a function of plastoquinone redox state only *in vivo* (Figs 1, 2).

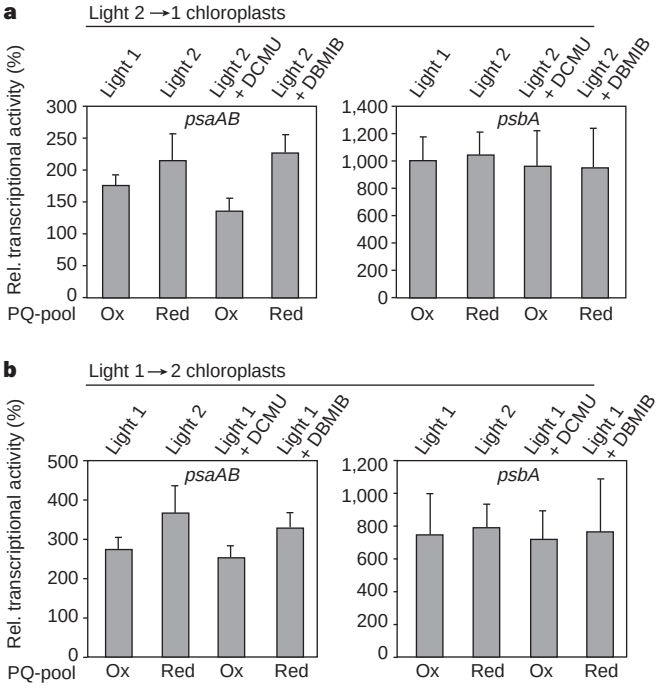

Figure 3 Transcription of reaction-centre genes *psaAB* (photosystem I) and *psbA* (photosystem II) in chloroplasts isolated from 7-day-old light 2 → 1 and light 1 → 2 plants. For *psaAB*, transcription is increased in light 2 relative to that in light 1, consistent with the effects observed *in vivo* (Fig. 2). Light 2 is known to produce a reduced plastoquinone pool. The inhibitor DBMIB also results in net reduction of plastoquinone, by suppressing oxidation of plastoquinol by photosystem I: the effect of DBMIB in increasing transcription of *psaAB* is consistent with redox control at the level of plastoquinone. The inhibitor DCMU causes oxidation of the plastoquinone pool by suppressing its reduction by photosystem II: the effect of DCMU in decreasing *psaAB* transcription supports the conclusion that *psaAB* is transcribed when plastoquinone is reduced, but not when it is oxidized. Redox effects on *psbA* transcription, in contrast, are absent under the conditions of this experiment. Error bars represent variations of four independent experiments.

Although various redox effects have been described for translation of chloroplast *psbA*[9], transcription of nuclear genes[10–12] and *de novo* protein synthesis in isolated chloroplasts[13], our results show a new, direct regulatory coupling between the redox state of plastoquinone and transcription of specific chloroplast genes. Figure 4 describes how this coupling supports a novel mechanism for physiological and developmental adjustment of photosystem stoichiometry[14].

There is an additional, evolutionary implication of these results. The ancestor of eukaryotic cells acquired many genes upon its merger[15–17] with the prokaryotic, eubacterial ancestors of chloroplasts[18] and mitochondria[19]. Most of the genes subsequently

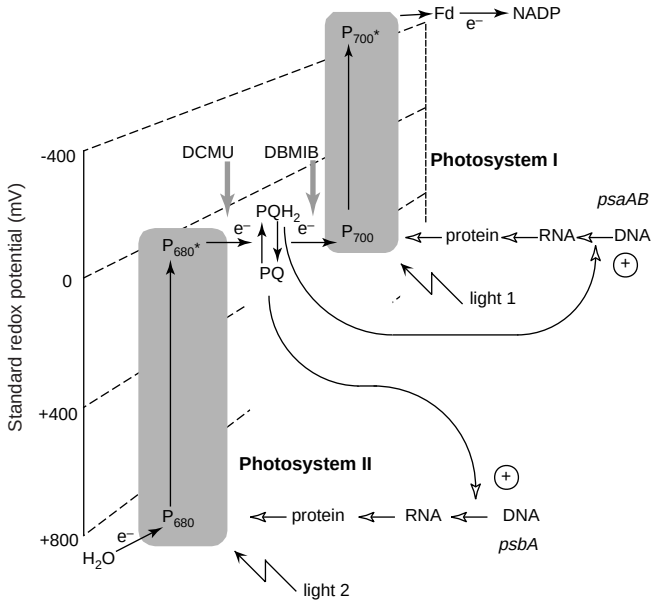

Figure 4 A working hypothesis for photosynthetic control of chloroplast gene expression by redox regulation of transcription. The Hill and Bendall 'Z-scheme' for non-cyclic electron transport in photosynthesis[1] is presented with standard redox potential of electron carriers on the *y*-axis, and with the sequence of electron transfers plotted on a projected *z*-axis, at 30° to the horizontal. The *x*-axis itself schematically represents gene expression. Electron flow from H_2O to $NADP^+$ requires two photosystems. The chemically reduced form of plastoquinone, plastoquinol (PQH_2), is produced by electron flow into the plastoquinone pool from photosystem II, and consumed by outward electron flow to photosystem I. Experimentally, electron flow from photosystem II to plastoquinone can be selected with light 2 and inhibited by addition of DCMU, whereas electron flow from plastoquinol to photosystem I can be selected with light 1 and inhibited by addition of DBMIB. *psaAB*, which encodes reaction-centre apoproteins of photosystem I, is induced when plastoquinone is reduced, and repressed when it is oxidized (PQ). *psbA*, encoding the D_1 reaction-centre protein of photosystem II, is induced when plastoquinone is oxidized and repressed when plastoquinone is reduced. The plastoquinone pool therefore exerts regulatory control over synthesis of the two photosystems in such a way as to balance its own reduction by photosystem II with its own oxidation by photosystem I. This mechanism of redox control determines the stoichiometry of photosystem I to photosystem II (Fig. 1), and adjusts this stoichiometry in response to any change in illumination that favours one or other of the two photosystems. Photosystem stoichiometry adjustment is thus a transcriptional counterpart to the more rapid, post-translational adjustment of the relative light-harvesting function of the two photosystems[4,5], and serves a similar function in maintaining redox homeostasis and photosynthetic efficiency. Although redox signals may also, indirectly, control nuclear gene expression[10–12], location of reaction-centre genes within the chloroplast itself permits transcriptional responses within minutes of perturbation of the redox state of the plastoquinone pool (Fig. 2).

retained have now been removed to the cell nucleus, but a small and relatively constant sub-set of genes has remained *in situ*, within the organelle. So why do chloroplasts and mitochondria retain any genes at all? One proposal is that chloroplast and mitochondrial genetic systems are required to permit direct redox regulation of gene expression[20,21]. Our results are consistent with this hypothesis, and identify plastoquinone or a near neighbour as the site of a rapid and direct redox control of chloroplast transcription. The transcriptional responses shown here are specific and are many times more rapid (Fig. 2a) than comparable effects on nuclear genes[10–12]. These kinetics are reminiscent of transcriptional control in prokaryotic systems. Such rapid and direct regulatory coupling may depend upon the genes concerned being present in the same intracellular compartment as the electron-transport chain that regulates their expression, and upon the persistence there of prokaryotically derived, redox signal-transduction pathways to provide the means of control. □

Methods

Plant material and illumination conditions. Mustard seedlings (*Sinapis alba* L.) were grown on vermiculite at 22 °C under light 1, light 2, white light (7 days) or light 1/light 2, light2/light 1 (5/2 days) in a 16:8 h light–dark cycle at 30–35 $\mu E\,m^{-2}\,s^{-1}$. Light 1 was provided by 40-W incandescent light bulbs and filtered through a red filter giving 50% transmittance at 650 nm (Lee Filters, 027 Medium Red); light 2 was provided by 30-W cool-white fluorescent strip lamps and filtered through an orange-yellow filter giving 50% transmittance at 560 nm (Strand Lightning Filters, 405 Orange). For white light, a combination of incandescent bulbs and cool-white fluorescent strips was used without additional spectral filtering. Measurements of the modulated chlorophyll-fluorescence emission confirmed the selective effects of light 1 and light 2 on photosystems I and II, respectively[22]: light 1 oxidized plastoquinol and induced the transition to light-state 1, whereas light 2 reduced plastoquinone and induced the transition to light-state 2 (refs 3–5).

Chloroplast isolation and spectroscopy. Chloroplasts and thylakoid membranes were prepared by a method based on ref. 23. Concentrations of Q_A and P_{700} were determined spectroscopically according to ref. 24 using the extinction coefficients of 11 $mM^{-1}\,cm^{-1}$ for Q_A (ref. 7) and 64 $mM^{-1}\,cm^{-1}$ for P_{700} (ref. 6). Chlorophyll concentrations of plant cell subfractions and of total plant material were determined spectroscopically after extraction in 80% (*v/v*) buffered acetone using the extinction coefficients of ref. 25.

Chloroplast run-on transcription. Run-on assays were performed essentially as described[26]. Between 2.5×10^7 and 3×10^7 plastids were used in the standard assay. DNA probes representing mustard chloroplast genes for detection of labelled transcripts in run-on transcription experiments were *psaAB* (pSA224-EBH1.9)[27], *psbA* (pSA452a)[28] and *rrn16* (pBSH895)[29]. Gene-specific expression rates were quantified in phosphorimaging analyses. Values were normalized to the value for *rrn16*, which was found not to be influenced by the light sources, and are means from between four and five independent experiments. In inhibitor and light-effect experiments, plastids were pre-incubated with 0.5 μM DCMU or 0.2 μM DBMIB in the presence of 20 mM $Na_4P_2O_7$ and 10 mM $NaHCO_3$ and exposed for 30 min to light 1 or light 2. They were then subjected to the standard procedure for run-on transcriptional assay, as described above.

Northern analysis. Isolation of total RNA and northern analyses were done using standard protocols[30]. Hybridization probes for *psaAB* and *psbA* gene transcripts were generated by *in vitro* transcription with T3 and T7 RNA polymerases[30] of plasmids pSA224-EBH1.9 (ref. 27) and pSA452a (ref. 28).

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