Hypothesis and theory

Retrograde signalling from functionally heterogeneous plastids

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Abstract

Structural and functional components of chloroplast are encoded by genes localized both to nuclear and plastid genomes of plant cell. Development from etioplasts to chloroplasts is triggered by light receptors that activate the expression of photosynthesis-associated nuclear genes (PhaNGs). In addition to photoreceptor-mediated pathways, retrograde signals from the chloroplast to the nucleus activate or repress the expression of nuclear genes involved in acclimatory or stress responses in plant leaves. A plant mesophyll cell contains up to one hundred chloroplasts that function autonomously, raising intriguing questions about homogeneity and coordination of retrograde signals transmitted from chloroplast to nucleus. We have previously demonstrated that the knockout of the chloroplast regulatory protein, CHLOROPLAST NADPH-DEPENDENT THIOREDOXIN REDUCTASE (NTRC) leads to a heterogeneous population of chloroplasts with a range of different functional states. The heterogeneous chloroplast population activates both redox-dependent and undifferentiated plastid-generated retrograde signalling pathways in the mutant leaves. Transcriptome analysis of the *ntrc* knockout lines shows that the induction of a redox signalling pathway depends on light conditions and leads to activation of stress-responsive gene expression. The signals derived from anomalous chloroplasts repress expression of PhaNGs as well as genes associated with light receptor signalling and differentiation of stomata, demonstrating interaction between retrograde pathways and plant development. Furthermore, mutation in a nuclear-encoded chloroplast protein can influence the signalling pathways controlled by light receptors.

Key words: Light signalling, redox signals, nuclear gene expression, stress, differentiation, NTRC

Introduction

Light is the primary environmental factor controlling plant development and acclimation processes, regulating the entire life cycle of plants from seed germination to seed production (Sullivan and Deng, 2003). Light is perceived directly by blue (cryptochromes CRY, phototropins and zeitlupe ZTL) and red light (phytochromes PHY) photoreceptors, which then activate signalling networks to initiate an array of light response processes such as photomorphogenesis, photoperiodic development as well as acclimatory and protective modifications of plants. Light signals are also mediated by chloroplasts to control chloroplast biogenesis and acclimation to changes in light quality, quantity and day length. Transcriptomics studies have demonstrated that between 5 – 25% of Arabidopsis (*Arabidopsis thaliana*) genes are light-regulated, depending on gene content in microarrays and experimental conditions (Jiao et al., 2007; Sharrock, 2008; Li et al., 2012). Recently light receptor-dependent signalling pathways have been suggested to interact with chloroplast retrograde signalling pathways (Ruckle and Larkin, 2009). The mechanisms by which photoreceptor-dependent signals and chloroplast signals interact are not well understood. Here we review recent findings from the study of the light and retrograde signalling pathways and discuss evidence showing interaction of these signalling pathways. We also present a hypothesis proposing that a heterogeneous plastid population leads to formation of distinct retrograde signals from chloroplast to nucleus. The hypothesis is based on our analysis of nuclear gene expression in an Arabidopsis mutant containing both photosynthetically active chloroplasts and non-photosynthetic plastids in a single mesophyll cell.

Light signalling pathways in the control of photosynthetic development of leaf

Light receptors control leaf development in angiosperm species by regulating chloroplast biogenesis. Development of chloroplasts from etioplasts is triggered by light by two primary mechanisms. In the absence of light, nuclear repressor molecules such as CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) and PHYTOCHROME-INTERACTING FACTORS (PIFs) cause degradation of positive light regulators that would activate the expression of light-responsive genes, thereby suppressing light-induced processes and maintaining etiolation-specific processes (see the reviews by Bae and Choi, 2008; Bu et al., 2011; Li et al., 2012). Upon illumination, light-activated phytochromes and cryptochromes move from cytoplasm to the nucleus and drive photomorphogenetic development of seedlings by removing repressors from the nucleus and by enhancing the expression of the positive light regulators like HY 5 (LONG HYPOCOTYL 5), and GOLDEN2-LIKEs (GLKs) proteins (Bae and Choi, 2008; Bu et al., 2011; Waters et al., 2009; Nagy and Schäfer, 2002). The removal of COP1 from the nucleus also stabilizes the positive regulators (Bae and Choi, 2008) which, in turn activate the transcription of genes involved in chloroplast development, cell division and plant growth. Expression of light-induced genes was recently found also to be regulated by epigenetic factors (Li et al., 2012). In angiosperms, chlorophyll is synthesised exclusively in light because the reduction of protochlorophyllide to chlorophyllide is energised by photons absorbed by protochlorophyllide bound to the PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR) enzyme (Reinbothe et al., 1996).

Besides light receptor-driven signalling networks, retrograde signals from chloroplast and mitochondria to the nucleus impact seedling development and plant acclimation to environmental cues (Woodson and Chory, 2008; Pogson et al., 2008; Larkin and Ruckle, 2008; Kleine et al., 2009; Jung and Chory, 2010; Inaba, 2010; Barajas-López et al., 2012,

Leister 2012). Retrograde signals can activate or repress nuclear gene expression, depending on the genes and processes dissected. Several sources of retrograde signals in chloroplast have been identified during last decades, including altered production of tetrapyrrole biosynthesis intermediates, defective expression of plastid genes, production of reactive oxygen species (ROS) in plastids, and the redox state of thylakoid electron transfer components (PET) (Pfannschmidt et al., 1999; Sullivan and Gray 1999; Pursiheimo et al., 2001; Strand et al., 2003; Piippo et al., 2006; Pesaresi et al., 2007; Kim et al., 2008; Muhlenbock et al., 2008; Foyer and Noctor, 2009; Lepistö and Rintamäki, 2012). Redox components at the acceptor side of Photosystem I (PSI) also initiate retrograde signals that modify nuclear gene expression (Pursiheimo et al., 2001;Piippo et al., 2006).

The routes of retrograde signal transmission within the chloroplast, through the cytoplasm and eventually to the nucleus are still fairly unknown, although some components of the signalling pathway have been identified. A genetic screen for potential signalling molecules identified a number of *gun* (*genomes uncoupled*) mutants in which the nuclear gene expression was unresponsive to plastid signals (Mochizuki et al., 2001). This approach identified the *GUN1* gene encoding a chloroplast pentatricopeptide repeat-containing protein (Koussevitzky et al., 2007). GUN1 has been described as a 'switchboard' inside a chloroplast that can receive signals from tetrapyrrole intermediates, chloroplast translation machinery (Koussevitzky et al., 2007; Woodson and Chory, 2008; Cottage et al., 2010) and from the redox state of PET (Inaba, 2010; Sun et al., 2011). Chloroplast proteins EXECUTER 1 and 2 (EX1, EX2) are components of a $^1O_2$ –dependent retrograde signalling route that controls cell death in plants (Wagner et al., 2004; Kim et al., 2008). Recently highly promising candidates mediating the signal from chloroplast to nucleus has been identified. Phosphoadenosine phosphate (PAP) has been suggested to carry information from chloroplast to nucleus (Estavillo et al., 2011). PAP accumulates in chloroplast in response to drought and high light and moves to nucleus, in which it activates the expression of stress-related genes (Estavillo et al., 2011). Sun et al. (2011) also identified a promising candidate for a mediator of retrograde signal from chloroplast envelope to nucleus. The homeodomain transcription factor PTM is attached to the chloroplast envelope. Following a signal from the chloroplast, a peptide is cleaved from the N-terminus of PTM and the peptide translocates to the nucleus where it activates expression of *ABI4*, a nuclear AP2-type transcription factor. ABI4 was previously shown to act downstream of GUN1 in the plastid-derived signalling pathway and to repress the expression of photosynthetic genes by binding to CCAC motif upstream of light-responsive genes (Koussevitzky et al., 2007). Another nuclear transcription factor, GLK2, has been proposed to act downstream from chloroplast retrograde signalling. GLK1 and GLK2 control chloroplast biogenesis and acclimation of a plant to light intensity by preferentially activating the expression of genes in chlorophyll biosynthesis and light-harvesting complexes (Waters et al., 2009). The expression of both *GLK*s genes is regulated by phytochromes (Tepperman et al., 2006), while the expression of GLK2 also responds to plastid-derived signals (Waters et al., 2009).

Acclimation of the photosynthetic structures to light intensity and to the length of diurnal photoperiod

Plants adjust leaf cell morphology and chloroplast ultrastructure according to incident light conditions in order to coordinate absorption of solar energy with the capacity for carbon assimilation. This light acclimation involves adjustments to the photosynthetic apparatus, such as changes in photosystem stoichiometry and the size of light-harvesting antennae, as well as modulation of stromal enzyme activities and antioxidant production (Walters and

Horton, 1995; Vanderauwera et al., 2005; Bartoli et al., 2006; Li et al., 2009). Several reports suggest that the light signal triggering the modification of photosynthetic traits is perceived in chloroplast rather than mediated by cytoplasmic light receptors (Pfannschmidt et al., 1999; Pursiheimo et al., 2001; Piippo et al., 2006; Muhlenbock et al., 2008; Bräutigam et al., 2009; Foyer and Noctor, 2009).

In addition to light intensity, the length of the diurnal photoperiod influences on the development of leaf structure and composition of chloroplasts. We have shown that Arabidopsis plants grown under identical light intensities in either short or long photoperiods show both structural and photosynthetic characteristics typical of shade or sun plants, respectively (Lepistö et al., 2009; Lepistö and Rintamäki, 2012). The characteristics modified by the length of the photoperiod include the density of stomata in leaf epidermis, respiration and CO_2 assimilation capacity, the ultrastructure of chloroplast and the chlorophyll a/b ratio in thylakoid membranes (Lepistö et al., 2009; Lepistö and Rintamäki, 2012). Thus the modifications of photosynthetic traits induced by photoperiod length resemble light intensity acclimation strategies. Acclimation of chloroplast ultrastructure to light intensity is largely controlled by chloroplast signals, whereas light receptor signalling associated with the circadian clock regulates the photoperiodic development in plants. The signalling cascade controlling photoperiodic development consists of complex network of multiple, functionally-redundant regulators within a circadian clock (recent reviews see Turck et al., 2008; Harmer, 2009; Imaizumi, 2010; Song et al., 2010). The circadian clock is entrained to a 24-hour cycle by photoperiodic signals transmitted from photoreceptors, and while the light-regulated mechanisms of resetting the clock are still not clear, expression of components of transcriptional feedback loops within the circadian clock is known to be regulated by light (Imaizumi, 2010; Song et al., 2010). Importantly, interaction between the circadian clock and light receptors is complex, since the circadian clock also controls the adaptation of light signalling pathways to the light/dark cycles (Li et al., 2012). Whether signals generated in chloroplasts also regulate the photoperiodic development of photosynthetic structures in leaves, and whether these signalling pathways are independent or interconnected with guiding leaf differentiation under various light regimes, are interesting questions that remain to be answered.

Mutation in chloroplast components as a tool to dissect chloroplast-to-nucleus retrograde signalling

Chloroplast retrograde signalling pathways have largely been investigated by dissecting nuclear gene expression in the *gun* mutants (Mochizuki et al., 2001). In these studies, norflurazon (NF) and lincomycin treatments that induce bleaching of seedlings have been used to generate signals from non-functional plastids (Mochizuki et al., 2001; Strand et al., 2003; Mochizuki et al., 2008; Moulin et al., 2008; Cottage et al., 2010). It is likely, however, that these harsh treatments induce secondary modifications in nuclear gene expression that confound interpretation of the experimental data. On the other hand, mutating chloroplast proteins to impair chloroplast function without inducing plastid bleaching is also an approach to investigate chloroplast retrograde signalling pathways. Some chloroplast mutants exhibiting conditional phenotype that appear only under specific circumstances (Yu et al., 2007; Kim et al., 2008; Sirpiö et al., 2008; Lepistö et al., 2009; Rosso et al., 2009; Tikkanen et al., 2010) can also be used to dissect signalling pathways.

We have employed an Arabidopsis mutant lacking the nuclear-encoded chloroplast regulatory protein, CHLOROPLAST NADPH-DEPENDENT THIOREDOXIN REDUCTASE (NTRC)

to dissect chloroplast retrograde signalling pathway. NTRC is a member of chloroplast thioredoxin family (Serrato et al., 2004). Redox-active cysteines in thioredoxins are used to reduce disulphide bridges in target proteins. NTRC knockout mutants (*ntrc*) have reduced growth and decreased chlorophyll content (Perez-Ruiz et al., 2006; Lepistö et al. 2009) indicating that it is an important component of the chloroplast redox network. NTRC has been shown to regulate the activities of chloroplast proteins involved in ROS scavenging, and in the synthesis of chlorophyll, starch and aromatic amino acids (Perez-Ruiz et al., 2006; Stenbaek et al., 2008; Kirchsteiger et al., 2009; Lepistö et al., 2009; Michalska et al., 2009; Pulido et al. 2010). Intriguingly, *ntrc* mutants possess both normal chloroplasts and irregularly differentiated plastids in a single mesophyll cell (Fig. 1) (Lepistö and Rintamäki, 2012). Some of the chloroplasts in *ntrc* are elongated and possess anomalous terminal appendages (Lepistö, 2011). The mesophyll cells of *ntrc* lines also contain small plastid-like organelles with poorly developed thylakoid membranes (Fig. 1, Lepistö and Rintamäki, 2012), suggesting that NTRC controls early steps of chloroplast differentiation.

The phenotype of the *ntrc* mutant depends on light conditions (Perez-Ruiz et al., 2006; Lepistö et al., 2009), and is most pronounced when plants are grown under short photoperiods (Fig.1), especially under high light. On the other hand, low light and long photoperiods reduce growth defects in *ntrc* lines. In comparison to wild type, 60 and 90 % retardation of the *ntrc* biomass was recorded under long and short photoperiod, respectively (Lepistö et al., 2009). The anomalous *ntrc* chloroplasts were present in seedlings as well as in young developing and mature leaves grown under all light conditions studied (Fig. 1, Lepistö, 2011), suggesting that generally slow growth of *ntrc* plants is primarily due to the defects in chloroplast differentiation in the absence of NTRC. It is likely, however, that the further reduced growth rate under short photoperiods is caused by imbalance in starch metabolism that is more severe in *ntrc* mutants grown under a shorter photoperiod (Lepistö, 2011). Defective starch metabolism (Kirchsteiger et al., 2009; Lepistö, 2011) impaired the utilization of light energy for carbon fixation in *ntrc* lines acclimated to short photoperiod, thereby increasing the reduced state of the components in PET. Accordingly, *ntrc* leaf grown under short photoperiod suffered from chronic photoinhibition of PSII in growth light (Lepistö et al., 2009).

Two models for retrograde signalling pathways in *ntrc* knockout lines

The *ntrc* lines are valuable in dissecting different aspects of chloroplast-to-nucleus retrograde signalling pathways by; i) showing how heterogeneous population of plastids in a single cell influences the quantity and quality of chloroplast signals and ii) facilitating the study of conditionally induced retrograde signals in chloroplast. Genome-wide transcript profiling of *ntrc* lines revealed two gene expression clusters in mutant plants (Fig. 2, Lepistö et al., 2009). The first cluster contained genes that were repressed in *ntrc* independently of photoperiod length and leaf age, including photosynthetic genes, light signalling genes and the genes regulating the stomatal density in leaf epidermis (Cluster 1 in Fig. 2). The hypocotyl of *ntrc* lines has a weakened response to far-red and low fluence-rate blue light (Lepistö et al., 2009) that is coincident with the repression of the CRY2 gene and a component of the far-red light signalling pathway, respectively (Fig. 2). Furthermore, the *ntrc* lines also have reduced ability to control the stomatal density under light conditions in which the differentiation of epidermal cells to guard cells is reduced in wild type leaves (Lepistö et al., 2009). Accordingly, the expression of the genes encoding the repressors of the development of stomatal guard cells (SDD1 and EPF1) is significantly reduced in *ntrc* lines (Fig. 2). Another sixty genes were also repressed in *ntrc* lines independently of the age or growth light

conditions (Lepistö et al., 2009). Half of these repressed genes encode unknown proteins or proteins with putative domains, while the rest of the repressed genes cannot be categorized to any specific functional groups or linked to visible *ntrc* phenotype.

Because NTRC is a chloroplast-localized protein, the down-regulation of Cluster 1 genes is likely due to a signal from *ntrc* chloroplast to nucleus. These results show that this repressive chloroplast signal not only down-regulates photosynthetic genes, but also controls processes linked to photosynthetic function such as stomatal differentiation. Furthermore, down-regulation of genes responsive to far-red light and low fluence-rate blue light, along with the long hypocotyl phenotype in the mutant, indicate that the chloroplast signal in *ntrc* interacts with signalling pathways controlled by light receptors.

The second cluster contained genes that were conditionally up-regulated in mature leaves of *ntrc* plants (Cluster 2 in Fig. 2), with stronger expression levels coinciding with a stronger *ntrc* mutant phenotype. The cluster 2 includes genes of chlorophyll synthesis that are strongly light-regulated (Matsumoto et al., 2004). In addition, cluster 2 genes encode enzymes in the photorespiration pathway, as well as chloroplast proteases and several heat shock proteins that are involved in stress responses (Fig. 2). Another thirty genes (Lepistö et al. 2009) show expression profile similar to cluster 2 genes in Fig. 2. Interestingly cluster 2 genes were not up-regulated in young *ntrc* seedlings indicating that the regulatory signal generated from the chloroplast may arise from long-term modifications of chloroplast metabolism.

Light conditions have a different effect on the expression of the clusters 1 and 2 genes in *ntrc* lines, suggesting that retrograde signals initiate at different sources. Can these signals be identified and how are they transduced from chloroplasts to the nucleus? Repression of cluster 1 gene expression resembles the expression pattern of genes in treatments abolishing plastid function or plastid gene expression (Sullivan and Gray 1999; Strand et al., 2003; Koussevitzky et al., 2007; Ruckle et al., 2007; Mochizuki et al., 2008). This retrograde signal is therefore likely to be a result of poorly differentiated anomalous chloroplasts in *ntrc* mesophyll cells (Fig. 1). We hypothesize that the poorly differentiated small plastids arise from asymmetric division of a chloroplast in an expanding *ntrc* leaf (Lepistö 2011). The irregular division may result in unequal distribution of resources between daughter plastids that impairs the development of the smaller plastid. Anomalous chloroplasts are present in *ntrc* cotyledons and leaves grown under various light conditions and the abundance even rises in expanded leaves (Table 1). However, cluster 1 genes were equally down-regulated in seedlings and mature leaves of *ntrc*, and their repressed expression was unrelated to the severity of the mutant phenotype, indicating that the regulation of cluster 1 genes does not depend on the abundance of anomalous chloroplasts. This suggests that the regulatory effect is independent of the strength of retrograde signals that are emitted from these plastids. The plastid signal is probably detected by a downstream signalling component inside the chloroplast or in the envelope, which relays the information through the cytoplasm to the nucleus (see the scenario in Figs. 1C and 2 in Leister, 2012), where a nuclear component of the signalling cascade activates expression of the repressor, which in turn controls the expression of target genes (Fig. 3A). The chloroplast retrograde signalling pathway recruiting GUN 1 and/or PTM fulfils the criteria for retrograde signalling pathway repressing the cluster 1 genes in *ntrc* (Fig.3A). Both signalling components have shown to act downstream to chloroplast signal and up-stream to ABI4, a repressor of light-induced genes. The knockout lines of *gun1* and *ptm* under standard growth conditions are indistinguishable from wild type (Mochizuki et al., 2001; Sun et al., 2011). Testing the nuclear gene expression in *ntrc* mutants

in *gun* and *ptm* backgrounds under various light conditions would reveal whether GUN1 and/or PTM mediates a signal generated from an anomalous *ntrc* plastid to nucleus.

We propose that the expression of the cluster 2 genes in *ntrc* is regulated by a different signalling pathway than the one described for cluster 1 genes. The transcript levels of the up-regulated cluster 2 genes in *ntrc* lines were positively correlated with the severity of the mutant phenotype. The short photoperiod that induced the strongest mutant phenotype in *ntrc* also significantly enhanced photoinhibition of PSII in mutant *ntrc* leaves (Lepistö et al., 2009). The short photoperiod also caused a severe imbalance in starch metabolism (Lepistö, 2011) that decreases the utilization of light energy and consequentially increases the redox status of chloroplasts (Lepistö et al., 2009). Thus the signal activating the expression of cluster 2 genes in mature *ntrc* chloroplast may arise from reduced components of the electron transfer chain, likely from the plastoquinone pool or from the acceptor side of PSI (Pfannschmidt et al., 1999; Pursiheimo et al., 2001; Piippo et al., 2006; Pesaresi et al., 2007; Bräutigam et al., 2009; Barajas-López et al., 2012). This redox signal activates expression of genes involved in stress responses, such as heat shock proteins and chloroplast proteases. Photorespiratory genes also respond to this redox signal, likely because photorespiration has been proposed to protect chloroplasts against over-reduction by dissipating excess light energy that cannot be utilized in photosynthetic carbon metabolism (Kozaki and Takeba, 1996). Cluster 2 genes are only slightly up-regulated in *ntrc* plants acclimated to a long photoperiod because of fewer redox signals are produced in the photosynthetically active chloroplasts with less attenuated starch metabolism (Fig. 3B, Lepistö, 2011).

Expression of *HEMA1* and *GUN5*, members of the most important light-regulated gene cluster in tetrapyrrole synthesis (Matsumoto et al., 2004), was also conditionally up-regulated in *ntrc* leaves (Fig. 2). Heme and intermediates of chlorophyll biosynthesis are thought to act as signalling molecules in the chloroplast-derived signalling pathway (Strand et al., 2003; Woodson et al., 2011). In comparison to wild type, *ntrc* lines accumulated higher amount of the chlorophyll biosynthesis intermediate magnesium protoporphyrin IX (Mg-Proto) (Stenbaek et al., 2008). Therefore, tetrapyrrole biosynthesis intermediates may mediate and/or strengthen the redox signal generated by light reactions in *ntrc* lines. Tetrapyrrole intermediates are reported to generate signals repressing PhaNG expression (Woodson and Chory, 2008; Inaba, 2010), but this has been subsequently challenged (Mochizuki et al., 2008; Moulin et al., 2008). On the other hand, Mg-Proto and heme have been shown to stimulate *HSP70* and *HEMA* gene expression in *Chlamydomonas* (Vasileuskaya et al., 2004; von Gromoff et al., 2006 and 2008), which resembles the response observed in *ntrc* leaves. The heme- and Mg-Proto-dependent signalling cascade in *Chlamydomonas* differs significantly from the GUN1-mediated pathway (von Gromoff et al., 2008), suggesting that this signalling route is GUN1-independent, although nuclear factor(s) involved in heme- or Mg-Proto dependent signalling are not known (von Gromoff et al., 2008). With respect to the signal characteristic, conditionally induced retrograde signal in *ntrc* leaves (Fig. 3B) resembles the passive diffusion transport mechanism described by Leister (2012) in Fig. 1C. In this scenario, the chloroplast signal migrates from the chloroplast to the cytoplasm and/or to the nucleus, in which the expression level of cluster 2 genes depends on the concentration of signalling molecule (Fig. 3B). To find components of this signalling pathway, *ntrc* lines can be transformed with a reporter gene fused to the promoter of cluster 2 genes and subsequently mutagenizing these transgenic lines by ethyl methanesulfonate (EMS). Mutants that no longer respond to the conditional chloroplast signal would potentially contain mutations in signalling components of this pathway.

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Table 1. The leaf width, the area of palisade mesophyll cells and the number of chloroplasts in Col-0 and *ntrc* grown under short day (SD) and long day (LD) condition. Data are determined from light microscope cross-sections of leaf (see Lepistö and Rintamäki 2012). The parameters measured for SD plants with different age indicate that in comparison to wild-type the relative proportion of chloroplasts with differentiated thylakoids decreases as the *ntrc* palisade cell and leaf expands. The decrease likely depends on the accumulation of small plastids with poorly developed thylakoids in *ntrc* cells, which are not visible in light microscope cross-sections of leaf. Data are presented as means ± SE of 30 cells in four independent experiments (leaf width, palisade mesophyll cell area and chloroplast number per mesophyll cell).

Growth conditions and age		Leaf number	Leaf width, mm	Palisade mesophyll cell area, μm^2	Chloroplasts per palisade mesophyll cell transection	Chloroplasts per 100 μm^2 of palisade cell area	Chloroplasts per 100 μm^2 of palisade cell area, % of Col-0
SD,	Col-0	1	2.9 ± 0.2	358 ± 12	7.5 ± 0.2	2.09	
10 days	*ntrc*	1	1.7 ± 0.3	199 ± 8	4.4 ± 0.2	2.21	105 %
SD,	Col-0	7	9.4 ± 1.3	1008 ± 40	9.9 ± 0.2	0.98	
4 weeks	*ntrc*	7	4.7 ± 0.3	684 ± 29	4.9 ± 0.2	0.72	73 %
SD,	Col-0	12	11.2 ± 0.9	771 ± 32	9.7 ± 0.3	1.28	
6 weeks	*ntrc*	12	7.2 ± 1.2	857 ± 33	6.8 ± 0.2	0.79	61 %
LD,	Col-0	6	9.4 ± 1.9	1564 ± 60	10.3 ± 0.3	0.66	
3 weeks	*ntrc*	6	7.2 ± 0.6	1788 ± 72	10.1 ± 0.3	0.56	84 %

Figure legends

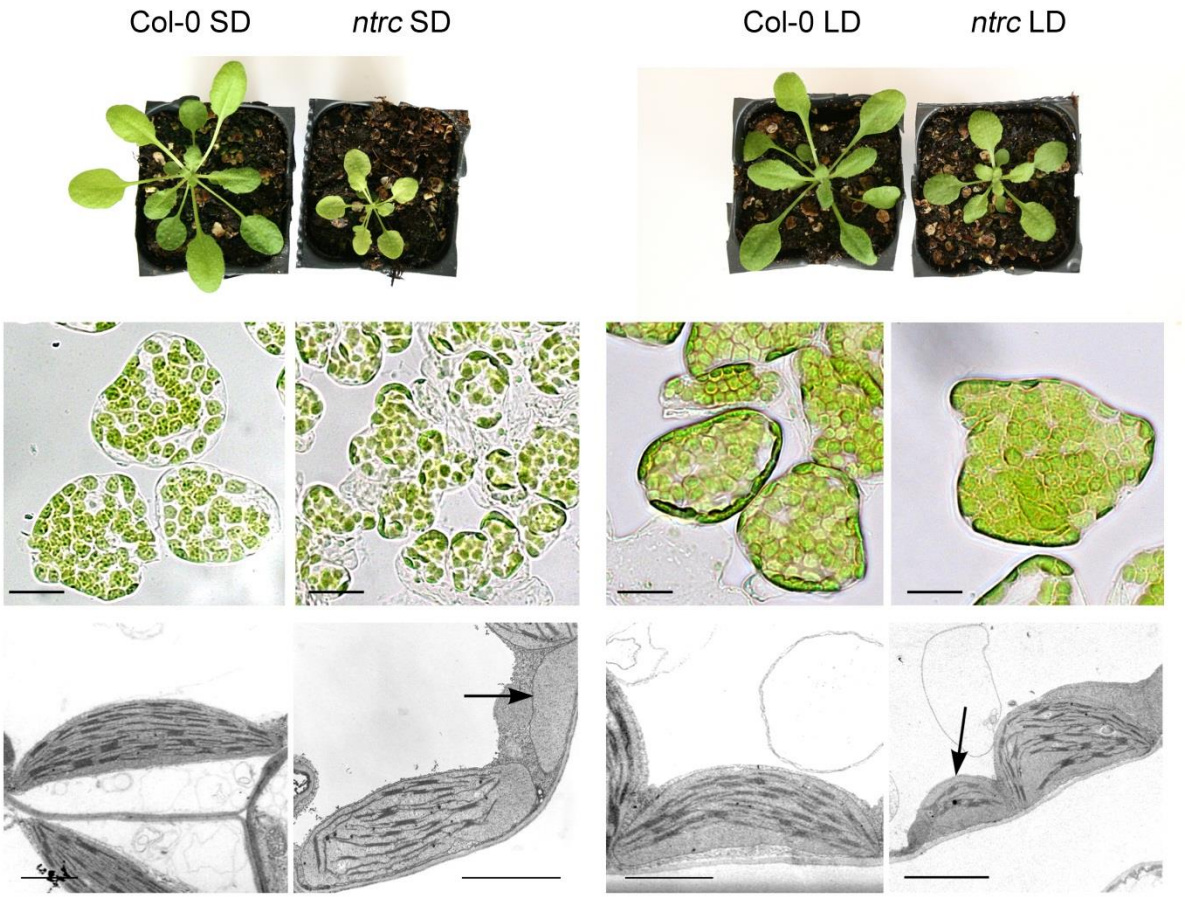
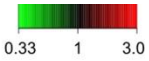


Fig. 1. Rosette phenotypes, bright field images and electron micrographs of the mesophyll cells in wild type and *ntrc* line. The plants were grown under short (SD) and long (LD) photoperiod of 8 hours and 16 hours light, respectively. Plastid like organelles with poorly developed thylakoid membranes are indicated by arrows. Scale bars: 20 μm in light microscope images and 2 μm in electron micrographs, respectively.



		GO cellular component	AGI code
CLUSTER 1 GENES			
PhaNGs	FZL; Thylakoid membrane organization-like	chloroplast	AT1G03160
	LHCB1.4	chloroplast	AT2G34430
	LHCB1.5	chloroplast	AT2G34420
	LHCB2.2	chloroplast	AT2G05070
	LHCB3	chloroplast	AT5G54270
	RuBisCO small subunit 1A	chloroplast	AT1G67090
Light signalling-related genes	CRY2; Cryptochrome 2		AT1G04400
	FRS3; Far1-related sequence 3		AT2G27110
Stomatal development	SDD1; Stomatal density and distribution	secretion	AT1G04110
	EPF1; Epidermal patterning factor 1	secretion	AT2G20875
CLUSTER 2 GENES			
Tetrapyrrole biosynthesis	HEMA1; glutamyl-tRNA reductase	chloroplast	AT1G58290
	GUN5; Genomes uncoupled 5	chloroplast	AT5G13630
Photorespiration	CAT2; Catalase 2	peroxisome	AT4G35090
	GGT1; Alanine-2-oxoglutarate aminotransferase1	peroxisome	AT1G23310
	HPR; Hydroxypyruvate reductase	peroxisome	AT1G68010
	SHM1; Serine hydroxymethyltransferase 1	mitochondrion	AT4G37930
	CDCP1; glycine decarboxylase P-protein 1	mitochondrion	AT4G33010
	FdGOGAT; Fd-dependent glutamate synthase 1	chloroplast	AT5G04140
Proteases	ATPREP1/ATZNMP; Zinc metalloprotease pitrilysin subfamily A	chloroplast / mitochondria	AT3G19170
	CLPC1; Clp protease, Heat shock protein 93-V	chloroplast	AT5G50920
	TPP2; Tripeptidyl peptidase II	chloroplast	AT4G20850
	SPP; Stromal processing peptidase	chloroplast	AT5G42390
Heat shock proteins + stress responses	HSP70; Heat shock protein 70		AT3G12580
	HSP81-3; Heat shock protein 81-3	secretion	AT5G56010
	HSP70-3, Heat shock cognate 70 kDa protein 3		AT3G09440
	HSP81-1; Heat shock protein 81-1		AT5G52640
	HSP17.6-CII; 17.6 kDa class II heat shock protein		AT5G12020
	AT-HSP17.6A; Heat shock protein 17.6A		AT5G12030
	BIP; Luminal binding protein	secretion	AT5G42020
	GPX7; Glutathione peroxidase	chloroplast	AT4G31870
	RCD1; Radical-induced cell death 1		AT1G32230

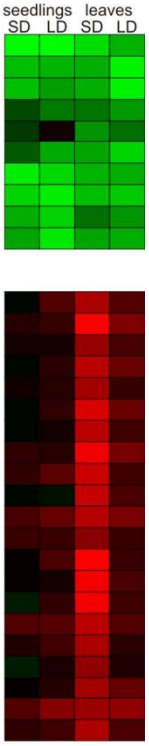


Fig. 2. Differentially expressed cluster 1 and cluster 2 genes in *ntrc* relative to wild type Arabidopsis in 10-d-old seedlings and rosette leaves. The plants were grown under short (SD) and long (LD) photoperiod of 8 hours and 16 hours light, respectively. Values are the means ± SE of three independent biological replicates. For standard errors, p-values and for a complete list of differentially expressed genes, see Lepistö et al., 2009 (Supplemental Table S2 online; www.plantphysiol.org).

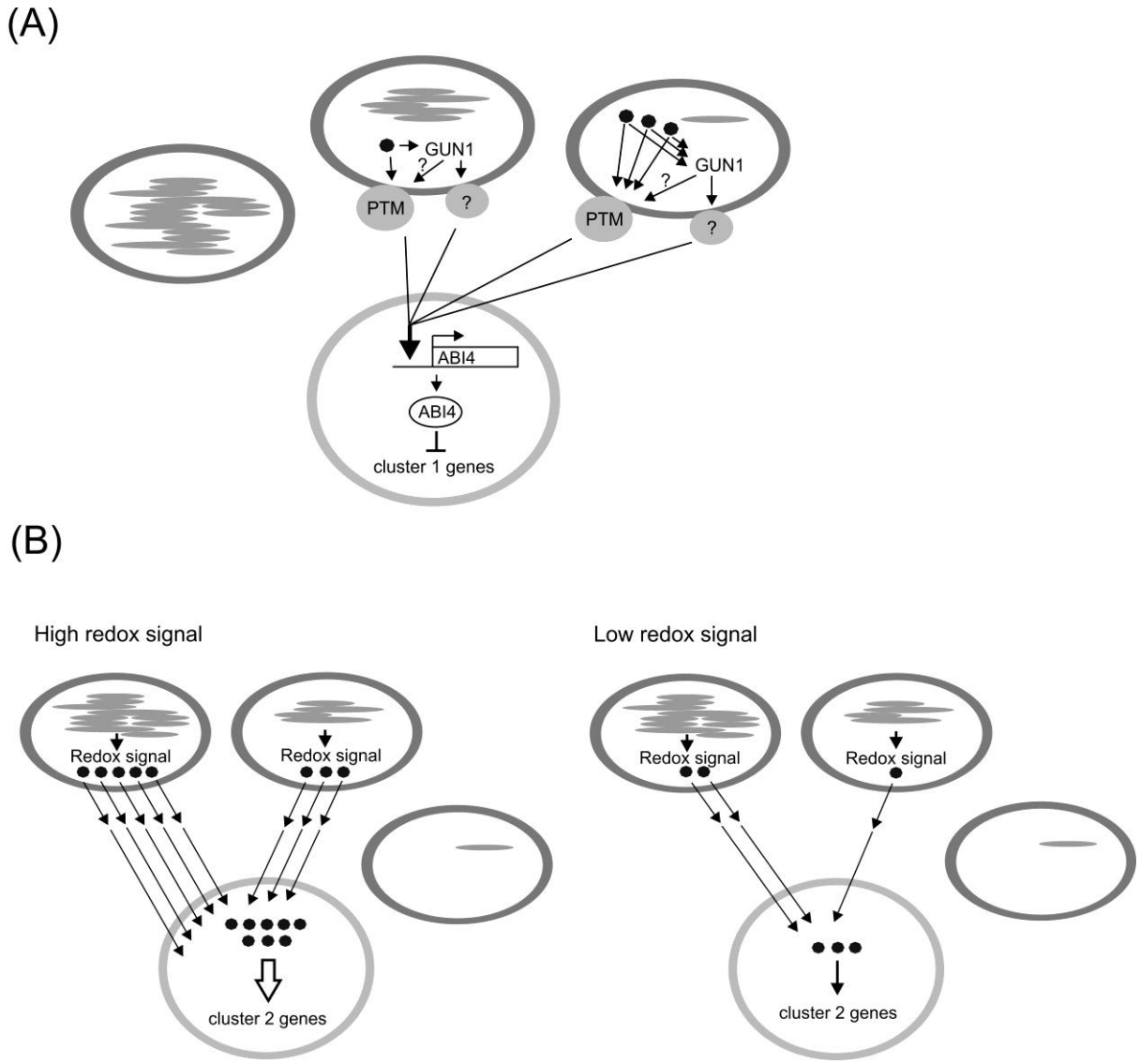


Fig. 3. Models for the plastid-to-nucleus retrograde signalling pathway initiated from plastids in *ntrc* mesophyll cell. **(A)** Signal (•) derived from anomalous plastids in *ntrc* leaves. This signal is mediated by GUN1 and/or PTM to nucleus, where the N-terminal fragment of PTM induces the *ABI4* expression. ABI4, in turn, represses the expression of cluster 1 genes (Fig. 2). The expression level of cluster 1 genes does not correlate with the abundance of the signal originally generated in the plastids. **(B)** Redox-dependent retrograde signalling pathway in *ntrc* mesophyll cell. Redox signal (•) is conditionally generated in *ntrc* chloroplast containing functional thylakoids. The abundance of the signal is high in chloroplasts with low capacity to utilize absorbed light energy in carbon fixation. The signal exits from chloroplast and interacts with the downstream component(s) in cytosol or in nucleus, where the expression of cluster 2 genes is activated. For details, see the text.