Electron transport pathways in isolated chromoplasts from *Narcissus pseudonarcissus* L.

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Significance statement

Chromorespiration takes place via two pathways, one depends on FNR, ferredoxin, the cytochrome b_6f complex, the other one depends on the NDH complex and is ferredoxin independent. We propose an electron transport via the cytochrome b_6f complex that does neither involve a Q-cycle nor a high potential electron transport chain.

Summary

During daffodil flower development, chloroplasts differentiate into photosynthetically inactive chromoplasts having lost functional photosynthetic reaction centers. Chromoplasts exhibit a respiratory activity reducing oxygen to water and generating ATP. Immunoblots revealed the presence of the plastid terminal oxidase (PTOX), the NAD(P)H dehydrogenase (NDH) complex, the cytochrome b_6f complex, ATP synthase and several isoforms of ferredoxin-$NADP^+$ oxidoreductase (FNR) and of ferredoxin (Fd). Fluorescence spectroscopy allowed the detection of chlorophyll *a* in the cytochrome b_6f complex. Here we characterize the electron transport pathway of chromorespiration by using specific inhibitors for the NDH complex, the cytochrome b_6f complex, FNR and redox-inactive Fd in which the iron was replaced by gallium. Our data suggest an electron flow via two separate pathways, both reducing plastoquinone and using PTOX as oxidase. The first oxidizes NADPH via FNR, Fd, and cytochrome b_h of the cytochrome b_6f complex and does not result in the pumping of protons across the membrane. In the second, electron transport takes place via the NDH complex using both, NADH and NADPH, as electron donor. FNR and Fd are not involved in this pathway. The NDH-complex is responsible for the generation of the proton gradient. We propose a model for chromorespiration which may also be relevant for the understanding of chlororespiration and for the characterization of the electron input from Fd to the cytochrome b_6f complex during cyclic electron transport in chloroplasts.

Introduction

Chromoplasts, the plastids of fruits and flowers, are essentially chlorophyll-free and characterized by the accumulation of carotenoids. In most cases, chromoplasts differentiate from chloroplasts or chloroplast-like precursors. The morphological changes taking place during the chloroplast-chromoplast transition have been investigated in detail in case of the daffodil flower (*Narcissus pseudonarcissus*) (Liedvogel et al., 1976). The dismantling of the thylakoid membranes and the *de-novo* formation of a complex concentrically stacked membrane system represent highly energy demanding processes involving several anabolic pathways like massive carotenoid and lipid synthesis (Kleinig and Liedvogel, 1980; Angaman et al., 2012). In the absence of photosynthesis, the required energy is thought to be imported from the cytoplasm or mitochondria into the stroma in the form of ATP and NADPH by the phosphate and malate/dicarboxylate translocators (Renné et al., 2003; Flügge et al., 2011; Bailleul et al., 2015). However, contributing to the energy balance of the system, chromoplasts exhibit a respiratory activity associated with their membrane system. The chromoplast respiratory chain uses NADH or NADPH as electron donor, involves plastoquinone and generates a proton motive force which is used to synthesize ATP (Mayer et al., 1990; Nievelstein et al., 1995; Morstadt et al., 2002; Pateraki et al., 2013). Chromoplasts from different species use either NADPH or NADH as the preferred electron donor. Addition of NADH resulted in higher respiratory activity in tomato chromoplasts than NADPH (Renato et al., 2014), while the opposite was found with daffodil chromoplasts (Nievelstein et al., 1995). Chromorespiration may be related to a similar process in chloroplasts, chlororespiration, first been described by Bennoun (1982) as a light-independent electron transport pathway from NAD(P)H to O_2

The tomato chromoplast proteome revealed the presence of NDH complex subunits and of the cytochrome b_6f complex (Barsan et al., 2012; Wang et al., 2013) rendering these two likely components of the respiratory electron transport chain. Furthermore, it has been suggested that a type II NDH contributes to the reduction of the PQ pool (Renato et al., 2014). The participation of the cyt b_6f complex in chromorespiration is corroborated by the partial sensitivity of ATP synthesis to DBMIB, an inhibitor of the cyt b_6f complex (Renato et al., 2014). In contrary to chloroplast electron transport reactions, cytochrome c_6 instead of plastocyanin was proposed to act as electron acceptor of the high potential chain of the cyt b_6f complex to donate electrons to a putative cyt c_6 oxidase which would act as terminal oxidase (Renato et al., 2014; 2015). However in plastids, there is no evidence for the presence of a

classical mitochondrial-type cyt *c* oxidase and evidence for a high potential electron transport chain is lacking. In tomato chromoplasts, the respiratory activity depends to a large extent on the plastid terminal oxidase PTOX (Renato et al., 2014). PTOX is not only involved in the respiratory activity of these organelles but is also required for carotenoid biosynthesis in photosynthetically inactive chromoplasts. It regenerates the cofactor of the phytoene and ζ-carotene desaturases, plastoquinone, which is reduced upon carotene desaturation (Carol et al., 1999; Gemmecker et al., 2015; Brausemann et al., 2017).

Open questions pertain to the composition of the chromorespiratory electron transport chain and the pathway of electron flux. The implication of the cyt b_6f complex remains obscure in the absence of evidence for a high potential electron transport chain involving the Rieske protein. In addition, the question arises on the pigment bound to the cyt b_6f complex. Chromoplasts seem to be completely devoid of chlorophyll (Liedvogel et al., 1976), while chlorophyll *a* is a constituent of the chloroplast b_6f complex in chloroplasts that is replaced by protochlorophyll in etioplasts (Reisinger al., 2008). This indicates that either chlorophyll itself or a chlorophyll derivative is required to attain structural integrity and functionality of the cyt b_6f complex. Clarification is also needed on the nature of the NAD(P)H electron acceptor. In chloroplasts, electrons enter the chlororespiratory chain through reduced ferredoxin (Fd_{red}) to subsequently be delivered to cyt b_6f and the NDH complex (Yamamoto et al., 2011; Peltier et al., 2016).

We used daffodil chromoplasts to further characterize the chromorespiratory pathway and to investigate the roles of the cyt b_6f and NDH complexes in establishing the proton motive force. We also scrutinized the role of Fd in chromorespiration by replacing it with a redox-inactive version in which Fe of the FeS cluster is replaced by Ga (Mutoh et al., 2015). Based on the differential effects of a variety of inhibitors we propose a model in which two separate electron transport pathways coexist. Of these, one is non-proton-pumping. It involves NADPH and ferredoxin-$NADP^+$ oxidoreductase (FNR)/ferredoxin (Fd) and the cyt b_6f complex while the other represents an NAD(P)H-dependent proton-pumping pathway that employs the NDH complex, but is independent of FNR/Fd.

Results

Two distinct redox pathways involved in chromorespiration?

'Grade A' chromoplasts isolated from the inner coronae of fully opened daffodil flowers show an NADPH-dependent respiratory activity that is linked to an alkalization of the medium (Figure 1). Addition of nigericin, which exchanges H^+ for K^+ across the membrane, reversed the alkalization of the medium, as expected, and increased the respiratory activity. To demonstrate the generation of a proton gradient by the respiratory activity, 9-aminoacridine fluorescence was measured. In thylakoids, 9-aminoacridine fluorescence is quenched upon the generation of a proton gradient because the protonated form of the fluorophore is sequestered into the lumen (Schuldiner et al., 1972). As shown in Fig. 2 (top left trace), addition of chromoplasts to the NADPH containing buffer led to a fluorescence quenching that was reversed by adding nigericin. This methods shows qualitatively the formation of a proton gradient but cannot be used for quantitative analyses since the size and the kinetics of the fluorescence quenching observed upon the addition of the chromoplasts varied between different preparations. In the presence of ocylgallate (OG), a known PTOX inhibitor (Josse et al., 2003), no fluorescence quenching and no O_2-consumption was observed (Fig. 2, Table 1). These data show that a proton gradient is built up in chromoplasts and that it depends on PTOX activity. 9-aminoacridine fluorescence quenching was observed in the presence of DNP-INT, an inhibitor of the cyt b_6f complex (Trebst et al., 1979), and was inhibited by rotenone, an inhibitor of the mitochondrial complex I and of the cyanobacterial NDH complex (Hu et al., 2013). Oxygen consumption, however, was strongly inhibited by DNP-INT, while rotenone had only a minor effect. When NADH was used as a substrate instead of NADPH, rotenone completely abolished oxygen consumption (Table 1). These observations led us to suspect the presence of two distinct electron transport pathways involved in the overall respiratory activity, one leading to the formation of a transmembrane proton gradient required for ATP synthesis, while the other representing a futile pathway using reduction equivalents to generate heat.

Possible redox-active constituents of chromorespiration

Immunoblots were carried out to identify proteins that may be involved in chromorespiration. As shown in Fig. 3, the NDH complex subunit H (NDH-H), PTOX, the ATP-synthase β-subunit, the cyt b_6f complex subunits cyt *f*, cyt b_6 and the Rieske protein,

ferredoxin (Fd), the ferredoxin-NADP$^+$ oxidoreductase (FNR) and the protein gradient regulator-like protein PGRL1 (Hertle et al., 2013) were all well detected in mature chromoplasts. Additional immunoblots served for differentiating between FNR and Fd isoforms (Fig. 4). Arabidopsis contains two functionally redundant leaf-type FNR isoforms (L-FNR1 and L-FNR2; Lintala et al., 2007; 2009) and two distinct leaf-type Fd isoforms (AtFd1 and AtFd2) (Green et al., 1991; Hanke et al., 2004, 2005; Hanke and Mulo, 2013). In addition, two root-type FNRs (R-FNR1 and R-FNR2) and a root-type Fd (AtFd3) are enriched in root plastids (Morigasaki et al., 1993; Hanke et al., 2004). R-FNRs accept electrons from NADPH to reduce root-type Fd, the latter providing reducing power to a number of activities, such as fatty acid biosynthesis and redox regulation (Hanke and Mulo, 2013). Intriguingly, the leaf- and root-type specific FNR and Fd antibodies used recognized several protein bands (Fig. 4) indicating that chromoplasts contain at least three distinct FNR and two to three Fd isoforms, most probably representing leaf- and root-type isoforms. The two L-FNR isoforms were distributed between the soluble (S) and membrane (M) chromoplast subfractions (Fig. 4a; see Supplementary material Fig. S1 for details on the detection of L-FNR and R-FNR) similar to the situation found in chloroplasts (Hanke et al., 2005; Lintala et al., 2007). In contrast, R-FNR appeared as soluble protein only (Fig. 4a). Moreover, Blue Native gel electrophoresis followed by immunoblotting revealed that FNR existed in several large protein complexes in chromoplasts, resembling the pattern reported for thylakoid membranes (Fig. 4b; Benz et al., 2009). Chromoplast membranes also contained the TROL protein, which is known in chloroplasts to exist in a thylakoid protein complex together with L-FNR (Fig. 4c; Juric et al., 2009).

The FNR-, Fd- and cyt *b*$_6$*f*-dependent branch of chromorespiration

To show the participation of Fd in the chromorespiratory pathway, a redox-inactive Fd from *Thermosynechoccus elongatus* in which the FeS cluster iron is replaced by gallium (Fd-Ga; Mutoh et al., 2015) was added to the chromoplasts. Redox-inactive Fd has recently been used to study Fd binding to photosystem I blocking Fd reduction (Mignée et al., 2017). It can be used as a general tool for the study of Fd-dependent electron transport reactions. As shown in Fig. 5, the addition of Fd-Ga inhibited O_2 consumption up to 80% with a Ki ≈100 nM. Addition of an excess of *T. elongatus* Fd-Fe to the Fd-Ga-inhibited samples restored the original activity. When DBMIB, an inhibitor of the cyt *b*$_6$*f* complex (see below), was added in addition to Fd-Ga, no further inhibition was observed. These data show that the main

respiratory pathway in chromoplasts involves Fd as the electron donor. Diphenylene iodonium (DPI) inhibited the diaphorase activity of FNR, isolated from *Synechocystis* PCC6803 (Supplementary material Fig. S2), as expected for an inhibitor of flavoproteins (O'Donnell et al., 1993). Its inhibitory effect on the O_2-consumption by chromoplasts (Table 1b) demonstrates that FNR acts as a Fd-reducing partner in the Fd-dependent chromorespiratory pathway. However, when NADPH was replaced by NADH, DPI had almost no inhibitory effect in chromoplasts (Table 1b), in accordance with the high specificity of FNR for NADPH (Morigasaki et al., 1990; Medina et al., 2001).

To assess whether the cyt b_6f complex is involved in the ferredoxin dependent electron pathway, the effects of its specific inhibitors DBMIB and DNP-INT and the effects of the ferredoxin-quinone reductase (FQR)-inhibitor antimycin A were studied. As shown in Fig. 6, the main respiratory pathway takes in fact this route since all inhibitors tested inhibit O_2 consumption to 70-80 %. DBMIB is the most effective, inhibiting up to 80 % of the O_2 consumption with Ki ≈ 2 µM. DNP-INT inhibited about 70 % with a Ki ≈ 25 µM. DNP-INT did not inhibit the formation of a proton gradient measured by 9-aminoacridine (Fig. 2, bottom left trace). DBMIB could not be used in this assay, since it led to high turbidity of the samples. Addition of Fd-Ga did not result in further inhibition of the O_2 consumption (Fig. 6a, cross symbol). The same effect was found when respiration was inhibited with Fd-Ga followed by the addition of DBMIB (Fig. 5, cross symbol). Antimycin A, which inhibits cyclic electron flow in chloroplasts depending on the FQR (Bendal and Manasse, 1995; Munekage et al., 2002; Hertle et al., 2013), inhibits chromorespiration to about 60 % with a Ki ≈ 25 µM, indicating that PGRL1/PGR5 may be involved in the electron transport reaction although we could only detect PGRL1 in the immunoblots (Fig. 3). The formation of a proton gradient was observed in the presence of antimycin A (Fig. 2, bottom right trace). All inhibitors tested have in common that none is capable in completely abolishing the respiratory activity. This indicates that the remaining activity is neither Fd-dependent nor does it involve the cyt b_6f complex.

The NDH complex-dependent branch of chromorespiration

To further distinguish between the two electron transport pathways, NADH was used as electron donor, in addition to NADPH (Table 1b). The respiratory activity was 25% lower when 200 µM NADH instead of 200 µM NADPH was used. The NADH-dependent pathway was not sensitive to DBMIB, only marginally sensitive to DPI but it was strongly inhibited by

Cibacron Blue 3G-A and completely blocked by rotenone (Table 1b). Cibacron Blue 3G-A mimics adenine nucleotides like NADH and binds to the nucleotide-binding sites of several enzymes (Prestera et al., 1992). Rotenone is a well-known inhibitor of the complex I of mitochondria, and it inhibits NADPH-oxidation by the cyanobacterial NDH complex competitively (Hu et al., 2013). The inhibitory effect of rotenone and Cibacron Blue 3G-A on the NADH-dependent pathway (Table 1b, Fig. 2) shows that the electron transport proceeds via the NDH complex. Most importantly, rotenone inhibition was also accompanied by the complete absence of medium alkalization i.e. proton gradient formation (Table 1a; Fig. 2, middle trace right) suggesting that this branch is responsible for proton translocation. This pathway utilizes the NDH complex but does not employ neither the cyt b_6f complex nor Fd. Addition of FdGa did not inhibit quenching of 9-aminoacridine and therefore did not interfere with proton gradient formation (Fig. 2). Using amino-NADH instead of NADH allows distinguishing between type I and type II NADPH dehydrogenases (Matsushita et al., 1987). Since the chromorespiratory activity was the same with both substrates (Supplementary Material, Table S1), a participation of type II NDH in chromorespiratory electron transport can be excluded. Neither the proton-pumping nor the non-proton-pumping pathway was affected by KCN (Table 1b) or by myxothiazol (Supplementary Material, Table S1) documenting the absence of mitochondrial contaminations in the chromoplast preparations used.

Presence of chlorophyll *a* in chromoplasts

Functional cyt b_6f complex requires the presence of chlorophyll *a* or protochlorophyll (see Introduction). The absorption spectra of chromoplast membranes were dominated by carotenoid absorption and showed no absorbance characteristic for chlorophyll, even at very high sample concentrations. However, when fluorescence was measured, an emission was observed with a maximum at 684 nm (Fig. 7), identical to the one observed with the isolated cyt b_6f complex from the green alga *B. cortulans* (Zuo et al., 2006). Upon acetone extraction, the fluorescence spectrum shifted by 16 nm toward shorter wavelength with a maximum of 668 nm that is characteristic for chlorophyll a (Fig. 7, black line). According to these data, chromoplast membranes contain chlorophyll *a* with similar characteristics as in the cyt b_6f complex.

Discussion

The chromorespiratory pathway as a whole involves the plastid terminal oxidase PTOX, as witnessed by the pronounced inhibitory effect of OG on both oxygen consumption and alkalization (Table 1). However, apart from this common constituent, we provide evidence for the presence of two co-existing sub-branches with only one presumably capable of pumping protons. In daffodil chromoplasts, NADPH-dependent O_2-consumption proceeds via both branches while NADH serves as substrate for only one. The selectivity for the reduction equivalents is one clear-cut distinction that can be made, and it is reflected in the different molecular constituents involved in each pathway.

A pathway that involves the cyt b_6f complex

FNR, Fd, cyt b_6f and finally quinones and PTOX are central to the pathway that shows high selectivity for NADPH (Table 1). The involvement of these complexes is inferred by the strong effect of the FNR inhibitor DPI, Fd-Ga and cyt b_6f inhibitors (Table 1, Figs. 5, 6). Constituents of this pathway were also well detectable by immunoblots in these fully developed chromoplasts (Figs 3, 4) and by the presence of chlorophyll *a* (Fig. 7).

In daffodil chromoplasts, several FNR and Fd isoforms were detected (Fig. 4), raising questions on their specificity to donate electrons from NADPH to the non-proton pumping branch of the electron transport chain. From a thermodynamic and structural perspective (Shinohara et al., 2017), the root FNR:Fd complex favors the electron transfer from NADPH to Fd while the leaf FNR:Fd complex facilitates the reverse. It is conceivable that the two detected FNR types (Fig. 4a) may both function in NADPH oxidation, but there is currently no knowledge on specific interactions with distinct ferredoxin isoforms, on the roles of membrane-bound and soluble FNRs and on the composition of the FNR-containing protein complexes that may exhibit modulated specificity or activity.

By activity, this branch represents the dominating one when 200 µM NAD(P)H is used as substrate. In the presence of inhibitors of the cyt b_6f complex, respiration was affected by 70-80% (Fig. 6, Table 1). However, the alkalization of the medium and the proton gradient remained almost unaffected (Table 1, Fig. 2). This non-proton pumping part of the chromorespiratory pathway via the cyt b_6f complex may generate the heat needed to dissipate volatile terpenoids synthesized by flower chromoplasts (Mettal et al., 1988; Dobson et al.,

1997) serving as pollinator attractants. A similar function has been attributed to the electron transport in mitochondria involving the alternative oxidase AOX for example in *Arum maculatum* (Wagner et al., 2008).

A pathway that involves the NDH complex

We assign the remaining 20-30% respiration found in the presence of cyt b_6f complex inhibitors to a second branch that depends on the NDH complex. We show that this remaining activity can use NADH as an electron donor. The NADH-dependent activity is sensitive to Cibacron Blue 3G-A and rotenone. Rotenone, a well-known inhibitor of the mitochondrial complex I, has been shown to also inhibit isolated NDH complex from *Thermosynechococcus elongatus* (Hu et al., 2013). This proton-pumping branch is affected by neither the FNR-inhibitor DPI, Fd-Ga, nor DBMIB and DNP-INT implying that this pathway is independent of FNR/Fd/cyt b_6f.

This observation contradicts at first glance the data reported on NDH-dependent electron transport in thylakoid membranes where Fd has been identified as electron donor to the NDH complex (Yamamoto et al., 2011). In this study, NDH activity was measured using chlorophyll fluorescence by following the post illumination fluorescence rise in freshly ruptured chloroplasts in the presence of externally added Fd. Fd-Ga should be able to compete with Fd-Fe in chromoplasts if the observed respiration was dependent on electron donation from Fd_{red} to the NDH complex, comparable to the situation in chloroplasts. This was clearly not the case. The protein composition of the chromoplast NDH complex may differ from that of the chloroplast and an NAD(P)H-oxidizing module different from FNR:Fd seems to be present in the daffodil chromoplasts, a module which would be absent in the chloroplast enzyme. This is in line with data reporting differences in the protein composition and enzymatic activity between NDH complexes from etioplasts and chloroplasts with a comparatively higher NADH dehydrogenase activity found with the former (Guéra et al., 2000).

According to our data, we propose that the NDH complex-dependent pathway pumps protons protons yielding the proton gradient to drive ATP synthesis via the H^+-ATP synthase complex, as previously demonstrated with daffodil chromoplasts (Morstadt et al., 2002).

This suggestion is in line with findings on the highly efficient proton pumping activity of the NDH complex in chloroplasts (Strand et al., 2017). Our suggestion is mainly based on the observation that rotenone, inhibiting specifically this branch (while leaving the other one unaffected) concomitantly inhibits the alkalization of the medium and the formation of a proton gradient. In an analogy to the mitochondrial complex I, this branch would be expected to pump 4 protons per NADH oxidized.

In etioplasts, Fd_{red} has been suggested as substrate for the NDH complex (Kambakam et al., 2016), an electron transport pathway which can be ruled out for daffodil chromoplasts since Fd-Ga did not inhibit the NDH-dependent pathway. However, we cannot exclude that etiorespiration as well as chlororespiration might in fact use Fd_{red} as electron donor for the NDH complex. The effect of Fd-Ga on these pathways has not yet been investigated.

The model and remaining questions

We propose that chromorespiration utilizes two different pathways. According to our model, the proton-pumping branch consists of the NDH complex transferring electrons from NAD(P)H to quinone and implies the efficient proton-pumping activity of this complex (Strand et al., 2017). The non-proton pumping branch relies on electron transfer from NADPH to Fd catalyzed by FNR (Fig. 8) and further electron transfer from Fd_{red} via the cyt b_6f complex to plastoquinone. Both pathways are interlinked at the stage of plastoquinol oxidation by PTOX as evidenced by their sensitivity to OG. In the experimental conditions used here, the activity of the non-proton pumping pathway was more important than that of the proton-pumping one when substrate concentrations of 200 µM NADPH were used. It should be noted that in vivo the relative importance of the two pathways may be different, depending on the available NADH and NADPH concentrations. Indeed, Renato and coworkers (2014; 2015) have also suggested a branched electron transport in tomato chromoplasts. They proposed one branch accepting electrons from NADH through NDH and the other branch accepting electrons from NADPH through a type II NADPH dehydrogenase delivering electrons to cyt b_6f. The latter pathway is contrast to our data showing that NADPH is mainly oxidized by FNR/Fd/cyt b_6f. Furthermore, chromoplasts from *N. pseudonarcissus* do not contain a functional type II dehydrogenase since replacing NADH by amino-NADH did not alter the activity of chromorespiration (Figure 2 and Supplementary Material Table S1).

The main question related to the model presented here pertains to the function of the cyt b_6f complex in the non-proton pumping branch. Cytochrome b_6f complexes are known to participate in establishing the proton motive force upon the traveling of electrons through the complex according to the Q-cycle model. We propose that only the stroma-facing side of the cyt b_6f complex including the Qi site, cyt b_h and potentially cyt c_i and/or PGR5/PGRL1 is operating (Fig. 9). Electrons from NADPH would reach the Qi-site quinone via heme c_i and b_h and after two turnovers a quinol could leave the site to become oxidized by PTOX. In chromoplasts, an active high potential chain via cyt *f* and plastocyanin does not exist since there is no photosystem I (Suppl. data, Fig. S3). KCN, a potent inhibitor of heme-copper oxidases did not inhibit the electron transport in daffodil chromoplasts (Table 1), and cytochrome c oxidase is not detectable in immunoblots (Suppl. data, Fig. S4). Therefore, a participation of the mitochondrial cytochrome *c* oxidase as a putative partner of a high potential chain can be excluded. According to our model (Fig. 9), PQ at the Qo site and Cyt b_l do not participate in the chromorespiratory electron transport, no Q-cycle takes place and, consequently, no protons are translocated. In chromoplasts, significantly higher concentrations of DBMIB and DNP-INT than in chloroplasts are necessary to inhibit the electron transport activity. Here we determined a K_I of 2 μM for DBMIB, which is significantly higher than the values reported for Qo site inhibition in chloroplasts (K_I=10-20 nM, depending on the chlorophyll concentration; Graan and Ort, 1986). Binding of the herbicides to the Qo site may affect the redox properties of the Qi site and inhibit thereby quinone reduction in this site. Alternatively these quinone-analogs may enter the Qi site at higher concentrations and directly interfere with quinone reduction. We imagine electron flow from NADPH to occur via FNR, Fd and heme c_i towards heme b_h and finally to the quinone bound in the Qi –site, similar to the model suggested by (Yamashita et al., 2007; Iwai et al., 2010) for cyclic electron flow in chloroplasts. The current view of the function of the cyt b_6f complex is still incomplete since pathways involving primarily the Qi site and heme c_i (such as cyclic electron transport) are yet not understood. The suggested pathway could be tested in future experiments using an vitro approach with isolated cyt b_6f complex, pre-reduced Fd, PQ and following changes in the reduction state of the cytochromes by absorption difference spectra and by EPR spectroscopy.

Alternatively, other types of electron acceptors might exist like for example some types of cytochrome c and alternative oxidase that replace in chromoplasts the high potential chain of chloroplasts. At the present state of knowledge on the protein composition of *N.*

pseudonarcissus chromoplasts we have no indication for the presence of such a pathway but cannot entirely discard this possibility. However, since the alkalization of the medium was not or only slightly affected by DCMU and DNP-INT but was fully inhibited by OG, we favor the pathway suggested in Fig. 9.

The second question concerns the implication of PGR5/PGRL1 in chromorespiration. Antimycin A, which was reported to inhibit the FQR-dependent cyclic electron flow in chloroplasts, inhibits chromorespiration, indicating possible involvement of PGRL1/PGR5 in addition to the cyt b_6f complex. It has been proposed by Hertle and coworkers (Hertle et al., 2013) that PGRL1 can reduce PQ without involvement of the cyt b_6f complex. Since the chromorespiratory activity was inhibited by several known cyt b_6f inhibitors, we discard the option that PGRL1 reduces PQ directly although these inhibitors could also block the quinone binding site of PGRL1. However, we do not exclude PGRL1 participation, potentially forming together with PGR5 the electron input module of cyt b_6f, allowing electron transfer from Fd_{red} to the quinone pool.

Concluding remarks

In daffodil chromoplasts, according to our model, chromorespiration takes place via a proton-pumping pathway employing the NDH complex using NAD(P)H as substrate and a non-proton-pumping pathway depending on NADPH/FNR/Fd/Cyt b_6f complex. These two electron input modules may function in a similar way in chloroplasts in the dark (chlororespiration). Chromoplasts are well suited to study these types of respiratory pathways since their membranes lack functional photosystems, thus removing a substantial portion of the electron transfer complexity present in chloroplasts. This experimental system may serve to answer the question of the importance of the contribution of cyclic electron flow via the cyt b_6f complex in chloroplasts to the proton motif force. Furthermore, Fd-Ga may serve as a unique tool to study the role of Fd in cyclic electron flow via both, the NDH complex and the cyt b_6f complex, in chloroplasts.

Experimental Procedures

Chromoplast preparation

Chromoplasts of the inner coronae of fully opened flowers of daffodil (*Narcissus pseudonarcissus* L. cv Dutch Master), developmental stage IV (Al-Babili et al. 1996), were isolated according to the method of Liedvogel et al. (1976) using a step gradient in which chromoplasts accumulate at the 15%/30% ('grade A') and 30%/40% ('grade B') sucrose interphases. The isolated chromoplasts were suspended in 100 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 1 mM 1,4-dithioerythritol. The preparations were stored at -80°C. Freezing of the chromoplasts damaged only little the membrane integrity of the concentrically arranged membrane layers. O_2-consumption was stimulated by about 10% when frozen samples were compared with fresh samples, showing that a small amount of the membranes became uncoupled upon freezing and thawing. Since the chromoplast preparation was time-consuming, measurements were performed with frozen material despite this small loss in coupling.

The protein content of the chromoplast preparation was determined using amido black. For all experiments only 'grade A' chromoplast preparations were used. To show that the preparation did not contain mitochondria as impurity, an immunoblot was performed using antibodies directed against the cytochrome c oxidase (Supplementary material, Fig. S4). For subfractionation, chromoplasts were lysed with shock buffer (10 mM HEPES-KOH, pH 7.6, 5 mM Sucrose, 5 mM $MgCl_2$), and soluble and membrane fractions were separated by centrifugation, as described by Grabsztunowicz et al. (2015).

Protein extraction

Total proteins of leaf and root material were extracted from hydroponically grown Narcissus plants as described by Lehtimäki et al. (2014) and Raorane et al. (2016), respectively.

Chromorespiration

The respiratory activity of chromoplasts was measured with a Liquid-Phase Oxygen Electrode Chamber (Hansatech Instruments, Norfolk, UK) at 20°C. For simultaneous measurements of O_2-consumption and pH, a pH-electrode was inserted on the top of the O_2-electrode chamber. Chromoplasts were resuspended in a low strength buffer (0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$) to allow measuring alkalization of the medium.

Determination of the formation of a proton gradient by 9-aminoacridine fluorescence

The fluorescence at 420-550 nm was recorded using a DUAL-PAM-100 fluorimeter (Walz, Effeltrich, Germany) at room temperature using a stirred 3 ml quartz cuvette. First the fluorescence of the buffer (0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$) containing 15 µM NADPH was recorded. Both, 9-aminoacridine and NADPH fluorescence are detected simultaneously. Therefore, the reaction was started not by adding the substrate but by adding 8 µg ml^{-1} chromoplasts to the assay solution. After a stable fluorescence level was reached, 100 nM nigericin was added to dissipate the proton gradient.

SDS-PAGE, Blue Native-PAGE and Western Blotting

Chromoplast proteins were separated using SDS–PAGE (8% or 12% acrylamide, as indicated) and the gels were blotted onto a nitrocellulose (Anti-PTOX; Anti-ATPase; anti-FNR; anti-NDH-H; anti-Fd; anti-PGRL1 and anti-PGR5) or a PVDF membrane (anti-TROL; anti AtFd1; anti AtFd3; anti-L-FNR; anti-R-FNR), blocked with 5% milk, and proteins immunodetected using the ECL system (GE Healthcare). For separation of protein complexes, BN–PAGE was performed as given in Sirpiö et al. (2007). BN gels were electro-blotted onto the PVDF membrane and used for immunodetection. The following polyclonal antibodies were used: Anti-PTOX (provided by M. Kuntz, CEA Grenoble), anti-ATPase (β-subunit of ATP synthase; Agrisera, Vännäs, Sweden), anti-FNR (ferredoxin–NADP reductase, provided by W. Oettmeier, Ruhr Universität Bochum, Germany), leaf-type and root-type FNR antibodies (provided by P.E. Jensen, University of Copenhagen, Denmark and T. Hase, Osaka University, Japan, respectively), anti-NDH-H (provided by D. Rumeau, CEA Cadarache, France), anti-Fd (provided by B. Lagoutte, CEA Saclay, France; anti-AtFd3 provided by G. Hanke, Queen Mary University of London, UK; AtFd1 Agrisera, Vännas, Sweden), anti Rieske protein (Rokka et al. 2000), anti-PGRL1 and anti-PGR5 (provided by T. Shikanai, University of Kyoto, Japan) and anti-TROL (provided by G. Hanke, Queen Mary University of London, UK). For distinguishing between leaf-type and root-type FNR, the same blot was incubated first with the specific leaf-type antibody and afterwards a second time with the root-type antibody which detects all FNR isoforms. This way, it is possible to superimpose the two developed films. The three FNR isoforms run close to each other on the gel and it is not possible to identify them with certainity by comparing two membranes from different runs.

Fluorescence spectra

Fluorescence was monitored with a CARY Eclipse spectrophotometer (Varian, Agilent, Santa Clara, CA, USA). Samples were excited at 435 nm, and the emission was recorded from 650 to 800 nm. Chromoplasts (40 µg ml^{-1} protein) were suspended in either 100 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$ or in 100% acetone. The acetone suspension was vortexed for pigment extraction, centrifuged at 12000 x g for 3 min and the supernatant was collected. The chl *a* content was determined with the aid of a calibration curve made with chl *a* solutions in acetone (ε=78.78x10^3 l $mol^{-1}cm^{-1}$ in 100% acetone). Isolated Chl *a* was provided by H. Paulsen, University of Mainz, Germany.

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Table 1

O_2-consumption and alkalization of the medium by chromoplasts. (a) NADPH-dependent respiratory activity in chromoplasts was measured simultaneously with a pH and an oxygen electrode. The assay contained chromoplasts (300 µg protein ml^{-1}), 200 µM NADPH, 0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$. O_2-consumption in the absence of inhibitors was $8.1 \times 10^{-8} \pm 1.7 \times 10^{-8}$ mol O_2 min^{-1} mg $protein^{-1}$) and the pH increased by $6.5 \times 10^{-8} \pm 1.8 \times 10^{-8}$ mol H^+ min^{-1} mg $protein^{-1}$). (b) NADPH- and NADH-dependent respiratory activity in chromoplasts. When 200 µM NADH instead of 200 µM NADPH is used, the respiratory activity is $6.1 \times 10^{-8} \pm 1.1 \times 10^{-8}$ mol O_2 min^{-1} mg $protein^{-1}$. Tables show mean values ±SD, n=3.

(a)

Sample	O_2-Consumption (%)	Alkalization
No addition	100	100
20 µM Ocylgallate	0	0
0.5 µM Nigericin	155 ±35	0
20 µM DBMIB	23 ± 5	84 ± 5
80 µM DNP-INT	25 ± 6	93 ± 8
0.5 µM Rotenone	75 ± 10	0

(b)

Sample	NADPH	NADH
No addition	100	100
20 µM Octylgallate	0	0
20 µM DBMIB	23 ± 5	100
0.5 µM Rotenone	80 ± 10	0
50 µM Cibacron	90 ± 4	32 ± 7
1 mM KCN	100	100
50 µM DPI	10 ± 7	92 ± 5

Figure Legends

Fig. 1

NADPH-dependent respiratory activity in chromoplasts.

Changes in pH in the assay medium and oxygen consumption were measured simultaneously using a pH-electrode inserted into the chamber of a Clark-type O_2-electrode. The assay contained chromoplasts (300 µg protein ml^{-1}), 0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$. The low buffer strength was required to be able to detect pH changes. Addition of NADPH (200 µM) and nigericin (100 nM) is indicated by arrows. HCl was added for calibration. Representative traces are shown.

Fig. 2

Proton gradient formation in chromoplasts using 9-aminoacridine fluorescence. Quenching of 9-aminoacridine fluorescence as an indicator of a ΔpH formed upon the addition of chromoplasts to a NADPH-containing buffer. The first arrow indicates the addition of the chromoplasts (8 µg protein ml^{-1}), the second arrow the addition of nigericin (100 nM). The assay contained 100 µM NADPH, 5 µM 9-aminoacridine, 0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$. When indicated, 20 µM OG, 5 µM FdGa, 20 µM DNP-INT, 5 µM rotenone or 100 µM antimycin A were added to the assay before starting the measurement.

Fig. 3

Proteins of the respiratory chain of the chromoplast. Western blots were conducted with antibodies directed against the subunit H of the NDH complex (NDH-H), the plastid terminal oxidase (PTOX), the β subunit of the ATP-synthase (ATPb), cytochrome *f* (Cyt *f*), cytochrome b_6, Rieske protein, ferredoxin (Fd), ferredoxin-NADP oxidoreductase (FNR) and against PGRL1. Total chromoplast protein was loaded as indicated.

Fig. 4

Analysis of FNR and Fd isoforms in chromoplasts. A. Total (T), soluble (S) and membrane bound (M) chromoplast proteins were separated by SDS-PAGE (12%) and immunodetected

first using a leaf-type FNR antibody followed by immunodetection using the root-type (arrowhead) FNR antibody. B. Chromoplast protein complexes were separated by BN gel electrophoresis, electroblotted and subjected to immunoblotting using leaf-type FNR antibody. C. Total proteins from leaf (L), root (R) and chromoplasts (C) of *Narcissus* plants were separated by SDS-PAGE (12%) and immunodetected with ferredoxin 3 (AtFd3, root-type), ferredoxin 1 (AtFd1, leaf-type) and TROL antibody. MW indicates the nearest molecular weight marker band.

Fig. 5

Inhibition of NADPH-dependent respiratory activity in chromoplasts by Ferredoxin-Ga (Fd-Ga). Black squares: increasing amounts of Fd-Ga inhibit chromorespiration; open squares: Fd-Fe (3.75 µM) restored the respiratory activity when added to samples inhibited with 0.5, 0.7 and 1 µM Fd-Ga. The cross denotes the addition of 10 µM DBMIB that did not result in further inhibition. The assay contained chromoplasts (300 µg protein ml^{-1}), 200 µM NADPH, 0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$.

Fig. 6

Inhibition of NADPH-dependent respiratory activity in chromoplasts by inhibitors of the cytochrome b_6f complex and by Antimycin A. A, Inhibition by DBMIB; B, by DNP-INT; C, by Antimycin A. The cross in A denotes the addition of 0.8 µM Fd-Ga that did not result in further inhibition. The assay contained chromoplasts (300 µg protein ml^{-1}), 200 µM NADPH, 0.1 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$.

Fig. 7

Chlorophyll fluorescence spectra. Gray line, fluorescence of native chromoplasts (40 µg protein ml^{-1}) in 100 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$; black line, acetone extract of the same chromoplast concentration. The wavelengths of the fluorescence maxima are indicated. The fluorescence intensity obtained from native chromoplasts was multiplied by four.

Fig. 8

Putative model of the respiratory electron transport in chromoplasts. Two independent pathways reduce the plastoquinone pool which is oxidized by PTOX (yellow). In the first pathway electrons are donated from NADPH via FNR, Fd to the cytochrome b_6f-dependent pathway which reduces PQ and does not lead to the formation of a proton gradient. In the second pathway (blue) NAD(P)H donates electrons to the NDH complex which reduces PQ and pumps protons fueling the ATP-Synthase. Specific inhibitors of the different proteins/protein complexes are given in red.

Fig. 9

Putative model of electron transport via the cytochrome b_6f complex. PQ binds to the Q_i-site close to cyt b_h and cyt c_i. Fd_{red} donates an electron to the b_6f complex and reduces heme c_i or cyt b_h. In a second step the next Fd_{red} delivers a second electron to the complex and the two electrons reduce PQ to PQH_2 which leaves the binding site. Both, the high potential chain and the Q-cycle are proposed to be nonfunctional in the chromorespiratory pathway. The cofactors not participating in the electron transport are shown in gray. PGR5/PGRL1 as a putative electron input module is omitted in this figure.