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REVIEW

Chloroplast avoidance movement: a novel paradigm of ROS signalling

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Abstract

The damaging effects of supra-optimal irradiance on plants, often turning to be lethal, may be circumvented by chloroplast avoidance movement which realigns chloroplasts to the anticlinal surfaces of cells (parallel to the incident light), essentially minimizing photon absorption. In angiosperms and many other groups of plants, chloroplast avoidance movement has been identified to be a strong blue light (BL)-dependent process being mediated by actin filaments wherein phototropins are identified as the photoreceptor involved. Studies through the last few decades have identified key molecular mechanisms involving Chloroplast Unusual Positioning 1 (CHUP1) protein and specific chloroplast-actin (cp-actin) filaments. However, the signal transduction pathway from strong BL absorption down to directional re-localization of chloroplasts by actin filaments is complex and ambiguous. Being the immediate cellular products of high irradiance absorption and having properties of remodelling actin as well as phototropin, reactive oxygen species (ROS) deemed to be more able and prompt than any other signalling agent in mediating chloroplast avoidance movement. Although ROS are presently being identified as fundamental component for regulating different plant processes ranging from growth, development and immunity, its role in avoidance movement have hardly been explored in depth. However, few recent reports have demonstrated the direct stimulatory involvement of ROS, especially H_2O_2, in chloroplast avoidance movement with Ca^{2+} playing a pivotal role. With this perspective, the present review discusses the mechanisms of ROS-mediated chloroplast avoidance movement involving ROS-Ca^{2+}-actin communication system and NADPH oxidase (NOX)—plasma membrane (PM) H^+-ATPase positive feed-forward loop. A possible working model is proposed.

Keywords Actin filaments · Blue light · Calcium (Ca^{2+}) · Chloroplast avoidance movement · Reactive oxygen species (ROS)

Abbreviations

Asc	Ascorbate
DAB	3,3′-Diaminobenzidine
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DPI	Diphenyleneiodonium chloride
EGTA	Ethylene glycol bis (2-aminoethyl) N, N, N′, N′-tetra acetic acid
ETC	Electron transport chain
GSH	Glutathione reduced
MV	Methyl viologen
NBT	Nitro blue tetrazolium chloride
ROS	Reactive oxygen species
TIBA	2,3,5-Triiodobenzoic acid
TMB	3,3′,5,5′-Tetramethylbenzidine

Introduction

Sensible management and utilization of energy obtained from the incident light is fundamental for plant growth and development. While sub-optimal light leads to alteration in photosynthesis and photomorphogenesis, absorption of excess energy beyond light saturation point of photosynthesis would cause photoinhibition. Functioning both as efficient photo-absorptive as well as photo-protective mechanism (through accumulation and avoidance movements, respectively), directional chloroplast photorelocation

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has been evolved as an ideal model of plant-environment (irradiance) interactions. The phenomenon of chloroplast movements ensures maximum photosynthesis and minimum photo-damage at any given incident light intensity (fluence) (Kong and Wada 2014). In the context of present global climate change, chloroplast avoidance movement helps in overcoming one major abiotic stress encountered by plants viz. high incident light (Kasahara et al. 2002) and also serves as sensitive indicator of relative water content (RWC) thereby effectively showing the plants' response towards drought (Nauš et al. 2016). By realigning chloroplasts to anticlinal side of cell or profile position parallel to the incident light, avoidance movement minimizes high irradiance absorption and evades the harmful consequences of such event, like production of excess reactive oxygen species (ROS) leading to damage of D1 protein of PSII (Derks et al. 2015; Kale et al. 2017). On the contrary to chloroplast accumulation, avoidance movement is fast enough to help in quick vacating of most of the cell surface so that absorption of excess light (by chloroplasts) comes to a minimum.

Rigorous research on chloroplast avoidance movements, carried out for last few decades, has identified some key components/properties of the phenomenon including the importance of shape of palisade cells and size of chloroplasts for their locomotion (Dutta et al. 2017; Gotoh et al. 2018). Among the three phases of the process, mechanisms of light perception and ultimate movement of the organelles are well reported. Being a strong BL-induced process, chloroplast avoidance involves phototropins as the photoreceptor (Sztatelman et al. 2016) and actin cytoskeleton is found to be the motor-machinery mediating chloroplast streaming (Samardakiewicz et al. 2015; Wada and Kong 2018, 2019). Chloroplast outer envelope located *Ch*loroplast *U*nusual *P*ositioning 1 (CHUP1) protein is reported to anchor chloroplasts to the plasma membrane and also interact with or help in polymerization of actin filaments thereby allowing directional chloroplast movements (Oikawa et al. 2008). Interestingly, *chup1* mutants have been shown to lack exclusive chloroplast-actin (cp-actin) filaments which mediate chloroplast locomotion by polymerization/depolymerisation at different sides of chloroplasts depending on light intensity (Kadota et al. 2009). Proteins involved in the regulation of cp-actin dynamics are also identified e.g. KAC, PMI1, PMIR1, PMIR2, THRUMIN1, JAC1 and WEB1-PMI2 (Kodama et al. 2010; Whippo et al. 2011; Suetsugu et al. 2015; Suetsugu and Wada 2016; Wada and Kong 2018). However, the signal transduction mechanism linking high light absorption and chloroplast locomotion (avoidance movement) through modulation of actin organization is complex and not unambiguous. Several models have been proposed in this regard involving different signalling components having enhancing/decreasing effects e.g. sugar (Banaś and Gabryś 2007), hormone (ABA) (Eckstein et al. 2016). Since ROS are the immediate products of high irradiance and have actin remodelling capability (Szymańska et al. 2017; Li et al. 2017), they are deemed to be more fit (also prompt) than any other signalling agent to trigger chloroplast avoidance movement for evading high fluence-induced damage. Surprisingly, despite being the most likely agent for signalling/initiating avoidance movement, roles of ROS have not been properly explored or recognized and reports available in this regard are scanty. Although Wen et al. (2008) and Majumdar and Kar (2016) have demonstrated the positive regulation of chloroplast avoidance movement by ROS [specially H_2O_2 (hydrogen peroxide)], majority of the available reports either remained completely silent about this possibility or emphasized only on the photo-oxidative damage of chloroplast-derived ROS on D1 protein (Taiz et al. 2015; Takagi et al. 2017).

In the present article, the hypothesis of Ca^{2+}-homeostasis mediated and ROS-intervened mechanism of chloroplast avoidance movement is discussed and a supporting working model is proposed.

Strong blue irradiation-signal for chloroplast avoidance through ROS production

Light can regulate chloroplast movements both qualitatively (red light/blue light) and quantitatively [low fluence (weak) /high fluence (strong) blue light)]. However, this varies greatly over the range of organisms and depends on the type of movement i.e. accumulation or avoidance (Banaś et al. 2012). While in lower plant groups red light can induce chloroplast movements and red and blue spectral regions are responsible for accumulation movement in aquatic angiosperm, chloroplast avoidance movement in terrestrial angiosperms has been attributed exclusively to BL irradiance and phototropins (PHOTs) are identified as the sole photoreceptor involved (Kasahara et al. 2002; Wen et al. 2008; Banaś et al. 2012; Wada and Kong 2018). Commensurate to this is the observation that the action spectrum responsible for chloroplast relocation exhibits typical three-finger pattern specific for BL responses (Taiz et al. 2015).

As absorption of high intensity light leads to production of O_2-derived free radicals owing to spill over of excess excitation energy and reducing power, chloroplasts essentially function as one of the primary sites of ROS generation in plants (Asada 2006; Derks et al. 2015; Kale et al. 2017). Chloroplastic ROS generation occurs via thylakoid membrane-located large, multi-subunit oxidoreductase protein complexes, namely photosystem I and II (PS I and PS II). Under high light, the rates of energy transfer and electron (e^-) transport through the photosynthetic e^- transport chain (ETC) are much slower than light energy harvesting (Krieger-Liszkay 2004; Krieger-Liszkay et al. 2008;

Ruban et al. 2012; Foyer 2018). Thus, excess photo-energy absorbed by the PS II antenna complex chlorophyll may not be completely converted to electrochemical potential via charge separation and consequently a unique (low-energy) excited state is produced, the chlorophyll triplet state ($^3Chl^*$). The newly formed triplet chlorophylls then transfer their triplet excitation energy to O_2 (readily being produced in close proximity by O_2-evolving complex) forming singlet oxygen ($^1O_2$) (Krieger-Liszkay 2004). PS II reaction centre chlorophyll may also form $^1O_2$ as the β-carotenes (Car_{D1} and Car_{D2}) are located away from chlorophyll dimer P_{D1} and P_{D2} and thus are unable to quench $^3P680^*$ (Pospíšil 2016). Among the major ROS forms viz. $^1O_2$, superoxide ($O_2^{\cdot -}$), H_2O_2 and hydroxyl radical ($OH^{\cdot}$), H_2O_2 specifically mediates chloroplast avoidance response by modulating actin dynamics (Wen et al. 2008; Majumdar and Kar 2016). As $O_2^{\cdot -}$ is readily dismutated to H_2O_2 either spontaneously or by the activity of superoxide dismutase (SOD) enzyme, it practically functions as the precursor of H_2O_2 and is considered equally important for avoidance movement. In contrary to the production of $^1O_2$ (via energy transfer process), $O_2^{\cdot -}$ and H_2O_2 are produced via e^- transfer reactions (Foyer and Noctor, 2000; Laloi et al. 2007). Under high light, $O_2^{\cdot -}$ is produced significantly at the reducing side of photosystem I (PS I) where molecular O_2 competes with $NADP^+$ for receiving e^- from PS I acting as the terminal e^- acceptor and produces $O_2^{\cdot -}$ by Mehler reaction (Kozuleva et al. 2014; Foyer 2018). Thylakoid Cu/Zn SODs efficiently convert $O_2^{\cdot -}$ to H_2O_2 which may function as signalling molecule (Awad et al. 2015; Foyer 2018). In PSII, reduction of O_2 by "spilled over" e^- from pheophytin, plastoquinone, plastosemiquinones and cyt b_{559} results in $O_2^{\cdot -}$ formation whereas H_2O_2 may originate from incomplete oxidation of H_2O or one-electron reduction of $O_2^{\cdot -}$ (Pospíšil et al. 2006; Pospíšil 2012, 2016). Involvement of ETC generated ROS (particularly H_2O_2) in chloroplast avoidance was evident from the effects of treatment with DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. DCMU blocks e^- flow from PS II at plastoquinone level (from Q_A to Q_B) (Moncada 2004) that ultimately blocks the formation of $O_2^{\cdot -}$ and subsequently stops the production of H_2O_2. Induction of partial inhibition of chloroplast avoidance movement under DCMU treatment conformed to the mediatory role played by H_2O_2 (Wen et al. 2008; Majumdar and Kar 2016). Interestingly, augmentation of avoidance upon treatment with another photosynthetic inhibitor MV [methyl viologen (Paraquat); accepts e^- from PSI)] was observed, which may be explained by its ability to form $O_2^{\cdot -}$ after reacting with O_2 thereby essentially producing ROS forms that facilitates avoidance movement i.e. $O_2^{\cdot -}$ and H_2O_2 (Taiz et al. 2015; Majumdar and Kar 2016).

Although several different enzymes help in maintenance of cellular ROS homeostasis e.g. superoxide dismutase (SOD), peroxidase (Prx), amine oxidase, oxalate oxidase, quinone reductase etc., in plant cells (both above and below ground) the cascade of ROS production (involved in signalling, growth and development) primarily starts with the generation of $O_2^{\cdot -}$ by one-electron reduction of oxygen by PM NADPH oxidase (NOX) (Fluhr 2009). Superoxide is then converted to H_2O_2 either enzymatically (by SOD) or non-enzymatically (through spontaneous conversion). Being the most stable ROS form, H_2O_2 serves as the signalling molecule which can be utilized in various plant processes ranging from growth and development to resistance against pathogen attacks (Mittler 2017; Qi et al. 2017). Stimulation and maintenance of NOX activity depends on different factors that varies according to the plant parts. Interestingly, NOX has been reported to be induced by BL in both plants and animals. Pagano et al. (2018) reported that continuous exposure for 4 h of monochromatic BL ($\lambda_{peak} = 468$ nm) led to increase in intracellular ROS production in zebra fish (*Danio rerio*). Using specific inhibitors they confirmed the source of ROS to be NADPH oxidase. On the other hand, when subjected to strong BL exposure, rapid production of ROS was observed at *Arabidopsis* root apex which could be detected with NBT (nitro blue tetrazolium chloride) or DAB (3,3′-diaminobenzidine) staining (for $O^{\cdot -}$ and H_2O_2, respectively), or by using fluorescent probes (for intracellular H_2O_2) viz. Peroxy-Yellow1 Methyl Ester (PY-1ME) and 2′,7′-dichlorofluorescein ($DCFH_2$-DA) (Yokawa et al. 2011, 2013). The authors have also reported severe reduction in PY1-ME fluorescence on DPI (Diphenyleneiodonium chloride; specific NOX inhibitor) treatment suggesting NOX as the source of $O^{\cdot -}$ and the enzyme is stimulated by strong BL leading to ROS-burst. Interestingly, BL-induced positioning at the plasma membrane and activation of NOX in wheat coleoptiles have been observed by Chandrakuntal et al. (2010). Therefore, strong BL seems to have the potential to promote NOX and increase cellular ROS production. Treatments with specific NOX inhibitors resulted in partial inhibition of chloroplast avoidance movement, analogous to DCMU effect, demonstrating the involvement of NOX-produced ROS in the process. Concomitantly, coupled treatment with inhibitors of both ETC and NOX abolished avoidance movement completely indicating clearly the necessity of both these two sources for providing ROS required for signalling chloroplast avoidance movement (Majumdar and Kar 2016).

Role of ROS in triggering chloroplast avoidance movement

For a considerable time, ROS have been denoted only as harmful in various ways in both plants and animals causing lipid peroxidation, DNA damage, ageing etc. (Garg and Manchanda 2009). However, apart from their profound

detrimental effects on biological systems, ROS are presently being identified as potent molecules capable of mediating cross-talks among signalling pathways in connection with plant growth, development as well as immunity (Mittler, 2017; Qi et al. 2017). Although ROS-related redox changes, like rise in antioxidant [e.g. Asc (ascorbate) or GSH (glutathione reduced)] content in chloroplasts (Heyneke et al. 2013), have been attributed to be crucial for photoprotection during high irradiance, roles played by ROS itself mostly remain unexplored (Szymańska et al. 2017). Some authors explained the repressing effect of DPI on avoidance movement as being solely incurred through inhibition of photoreceptor, phototropins (Sakurai et al. 2005; Sakai and Takagi 2005). However, DPI is a noted inhibitor of PM NOX and thus the effects could have been linked with reduction in ROS production too. Among the few available reports, Wen et al. (2008) demonstrated the involvement of H_2O_2 in strong BL-induced chloroplast avoidance movement and speculated the underlying mechanism to be the regulation of LOV (*L*ight, *O*xygen and *V*oltage sensing) domains of phototropins (specially PHOT2) by ROS. In our investigation with *Hydrilla verticillata*, we have demonstrated the indispensible involvement of ROS in chloroplast avoidance movement (Majumdar and Kar 2016). Apart from the NOX and ETC inhibitor-induced partial (when used singly) or complete (used in combination) abolition of avoidance movement, treatments with selective scavengers of $O_2^{\cdot -}$ and H_2O_2 inhibited avoidance drastically. Conversely, exogenous application of H_2O_2 resulted in induction of avoidance in leaves incubated even in darkness, whereas it augmented the velocity of avoidance movement in Strong BL-incubated leaves. Concurrent generation of ROS and chloroplasts avoidance movement under Strong BL and transition of such events from lighted to far-off zone of a leaf may be visualized under microscope by staining with ROS specific dyes [NBT, TMB (3,3′,5,5′-tetramethylbenzidine) and DAB]. Such staining was partially reduced when treated with either NOX or ETC inhibitor and abolished completely under combined treatment of these agents or individual treatments with ROS scavengers. Therefore, it was hypothesized that chloroplast avoidance movement involves both NOX and ETC as the source of ROS for signalling (Majumdar and Kar 2016). Moreover, the ROS-intervened mechanism was found to be dependent upon threshold $[Ca^{2+}]_{cyt}$ (being built up from Ca^{2+} influx through plasma membrane) and its functioning through actin filament reorganization.

ROS generators are located throughout the plant body and function depending on their site of occurrence (Kar 2015). However, production of ROS is regulated spatiotemporally and integration of the ROS signals from different sources (or organelles) is key to the efficient responsiveness of the system towards different environmental stimuli or developmental requirements. Apart from being produced constitutively, apoplastic and chloroplastic ROS production may also be induced by strong blue irradiation (Wen et al. 2008; Majumdar and Kar 2016). It has been suggested that apoplastic signal or cue triggers production of chloroplastic ROS which may regulate nuclear gene expression exhibiting retrograde signalling (Padmanabhan and Dinesh-Kumar 2010; Shapiguzov et al. 2012). In addition to ROS, the chloroplast-to-nucleus retrograde signalling involves Ca^{2+} sensing and effectively regulates explicit gene expressions in response to different environmental conditions (Chan et al. 2016; Kretschmer et al. 2020).

Modulation of actin filament organization by ROS

Chloroplasts are essentially dependent on cytoskeletal dynamics for locomotion and involvement of actin filaments in chloroplast avoidance movement has been reported time and again with recent emphasis on the roles of cp-actin filaments and CHUP1 protein (Oikawa et al. 2008; Wada and Kong 2018, 2019). Treatments with anti-actin drugs like cytochalasin B and D and Latrunculin B showed aberrant chloroplast aggregation as a result of actin filaments disruption (Kandasamy and Meagher 1999; Kong and Wada 2011). On the other hand, TIBA (2,3,5-triiodobenzoic acid), having actin-hyperstabilization activity, retarded both chloroplast avoidance and accumulation movements (Dhonukshe et al. 2008; Majumdar and Kar 2016). Samardakiewicz et al. (2015) labelled actin cytoskeleton with actin-specific fluorescent stain Alexa Fluor 488 Phalloidin and visualized microfilament bundles twisting around chloroplasts in *Lemna trisulca* mesophyll cells. Under strong BL, cp-actin filaments were found to depolymerize at the light-perceiving side of chloroplasts and were polymerized de novo on the opposite side at the leading edge (towards the direction of chloroplasts movement) (Kadota et al. 2009; Kong et al. 2013; Wada and Kong 2018). Therefore, it may be apprehended that ROS must have actin-modulating properties in order to act as signalling component for chloroplast avoidance movement. Interestingly, the role of ROS in actin filament polymerization/stabilization has been well reported in both animal and plant systems. For instance, in neuronal growth cones, depletion of ROS led to reduction in F-actin content, dynamics and contractility (Munnamalai et al. 2014; Wilson and González-Billault, 2015). Source of such ROS was confirmed to be NOX by using specific NOX inhibitors. Disturbed actin organization following loss of NOX function demonstrated the necessity of NOX-derived ROS for F-actin dynamics in neuron (Wilson et al. 2015; Wilson and González-Billault 2015). On the other hand, the translocation of cytosolic components of NADPH oxidase (e.g. $p40^{phox}$) to the plasma membrane and assembly of the

enzyme subunits are reported to require F-actin polymerization (Shao et al. 2010; Stojkov et al. 2017) indicating a correlation between NOX activity and actin microfilaments conditions.

Cyclic conversion between monomeric (G-actin) and polymeric (F-actin) protein subunits is a constitutive cellular process. In plants, ROS-dependent modulation of actin filament may be direct modification of actin amino acids by ROS e.g. oxidation of –SH groups of exposed cysteines (e.g. Cys272, Cys285, Cys374) followed by glutathionylation or via actin-binding proteins. DPI-induced inhibition of NOX (reduction in $O_2^{\cdot -}$ production) resulted in diminished actin polymerization at barbed ends together with reduction in exposed barbed ends in endothelial cells (EC) (Moldovan et al 2000). In addition, DPI treatment also resulted in reduced speed and directionality of EC migration (Moldovan et al. 2006). Secondary pathways like TOR (target of rapamycin) may also be involved in ROS-actin communication (Niles and Powers 2014; Rispal et al. 2015; Yokawa and Baluška 2016). In yeasts, polarization of actin cytoskeleton by TOR complex 2 (TORC2) through downstream protein kinase Ypk1 [TORC2/Ypk1 signalling] is mediated by ROS (Niles and Powers 2014). MAPK phosphorylation cascade is reported to form a positive amplification loop with ROS (mostly originating from NOX) at different physiological conditions (Xia et al. 2015; Liu and He 2017). Since mutual interactions between MAPK and actin filaments have been reported in mammals, yeast and plants (Šamaj et al. 2004), ROS-induced activation of MAPK may regulate actin dynamics and create another signalling nexus. In case of tip growth, modulations of actin-binding proteins that mediate actin reorganization require stimulation of ROS production as well as optimum pH and Ca^{+2} gradient (Vazquez et al., 2014). Presence of G-actin pool in the organs showing tip growth (e.g. root hairs) corroborates this idea (He et al. 2006; Vazquez et al., 2014). Interestingly, some common signalling cues which act as initiator/mediator of both actin formation/polymerization as well as ROS production have been postulated. It has been reported that *R*ho-like GTPases *O*f *P*lant (ROP) proteins, besides activating NADPH oxidases by direct binding (Feiguelman et al. 2018), also promote F-actin assembly/disassembly during several physiological processes viz. leaf morphogenesis and pollen tube growth (Fu et al. 2005; Zhou et al. 2015; Schmidt et al. 2016). As H_2O_2 can cross both plasma membrane and chloroplast membranes through aquaporins (Mubarakshina and Ivanov 2010; Bienert and Chaumont 2014), $O^{\cdot -}$ produced at the chloroplast or apoplast may diffuse into the cytoplasm after being converted to H_2O_2 and modify actin polymerization process.

Wide array of actin-binding proteins (ABPs) viz. villin/gelsolin, ADF/cofilin, profilins play important roles in configuration of actin cytoskeleton by regulating nucleation and polymerization/depolymerization involving cyclic conversion of the F- and G- forms (Li et al. 2015; Qian and Xiang 2019). Oxidation of ABPs by ROS at specific residues alters actin dynamics in both animals and plants (Moldovan et al. 2006; Xu et al., 2017). Inhibition of cofilin by H_2O_2-induced oxidation of Cys139 and Cys147 resulted in obstructed actin association and electrostatic repulsion, respectively and facilitated mesenchymal cell motility (Cameron et al., 2015). It is speculated that increase in the actin severing activity of gelsolin and exposure of barbed ends resulting in enhanced actin polymerization may be mediated by ROS (Moldovan et al. 2006). Involvement of ROS (H_2O_2) in activation of filamin (actin cross-linking protein) and FAK (Focal Adhesion Kinase) is also reported in animals (Hastie et al. 1998; Usatyuk and Natarajan 2005; Moldovan et al. 2006).

Although the regulation of actin cytoskeleton by Ser/Thr protein phosphatase 2A (PP2A) is thoroughly studied, an isoform of the catalytic subunit of PP2A, namely PP2A-2, has only recently been identified to act as a positive regulator of Strong BL-induced PHOT2-mediated chloroplast avoidance movement (Wen et al. 2012). Actin depolymerizing factor (ADF)/cofilin family proteins are reversibly activated by dephosphorylation which, on binding with actin filaments, promotes actin dynamics. Under high fluence BL, ADF/cofilin reorganizes actin filament and facilitates avoidance movement. Using specific inhibitors of PP2A (e.g. okadaic acid and cantharidin) and performing mutant studies, Wen et al. (2012) have confirmed the regulatory role of PP2A-2 in ADF/cofilin activation thereby establishing the positive involvement of PP2A-2 in BL-induced chloroplast avoidance movement. Interestingly, PP2A enzymes have been found to regulate ROS-dependent plant responses/processes under biotic or abiotic stress e.g. pathogen attack, wounding or high light (Rahikainen et al. 2016; Máthé et al. 2019). Thus, chloroplast avoidance movement may also be regulated by the interaction of ROS and PP2A through modulation of ADF/cofilin phosphorylation status, apart from its regulation through ROS-dependent direct modification of actin.

ROS-Ca^{2+}-actin: indispensible signalling pathway

A potent mode of ROS-mediated actin modification involves Ca^{2+}, which, functioning both up- and downstream of actin organization, has a unique regulatory relationship with actin (Chen et al. 2013; Qian and Xiang 2019). Since Ca^{2+} is a prime second messenger, maintenance of critical cytosolic Ca^{2+} concentration [$(Ca^{2+})_{cyt}$] is crucial for several cellular processes including chloroplast movements (Tlałka and Fricker 1999). It is well reported that Ca^{2+} functions as an activator of NOX [by directly binding to the N-terminal EF-hand motif or by phosphorylating through CDPKs or

Rho-type plant GTPase (Fluhr 2009; Gilroy et al. 2014; Kurusu et al. 2015)]; conversely, ROS promotes Ca^{2+} influx across the PM (Pottosin and Zepeda-Jazo 2018). Thus a feed-forward loop exists between ROS and Ca^{2+}. Similarly, roles of Ca^{2+} in regulating actin dynamics also depend on either direct binding to the ABPs or by phosphorylation through the action of CDPKs (Hussey et al. 2006; Helper 2016; Qian and Xiang 2019). Actin severing protein, gelsolin is reported to be stimulated by direct binding of Ca^{2+} to the C-terminal half inducing conformational changes. Actin filament bundling abilities of LILIM1 protein from *Lilium longiflorum* and PLIM2c from *Arabidopsis* are regulated by Ca^{2+} in a dose-dependent manner where high Ca^{2+} concentration inhibits the proteins (Wang et al., 2008; Papuga et al., 2010). Ca^{2+}, at concentration of 1 µM or higher, sequester profilin/G-actin complex thereby checking the actin polymerization process (Kovar et al., 2000). According to Yokota et al. (2003), plant villin proteins are also stimulated by Ca^{2+}. Just contrary to the action of PP2A-2, increased $[Ca^{2+}]_{cyt}$ phosphorylate ADF/cofilin through CDPK and deactivate the protein. However, Ca^{2+}-dependent regulation of actin works downstream to ROP signalling (Hussey et al. 2006). ROP1 is found to mediate pollen tube growth in a Ca^{2+}-dependent manner at the tip-growing cells by regulation of F-actin dynamics being catalysed by counteracting RIC3/4 (ROP-interactive CRIB motif-containing) proteins (Lee et al. 2008; Zhou et al. 2015). On the other hand, RIC1-mediated severing and capping of apical F-actin in the cytosol is Ca^{2+}-dependent (Zhou et al. 2015). Using deletion mutant of *CHUP1*, Schmidt von Braun and Schleiff (2008) have shown that CHUP1 binds with profilin thereby forming a bridge between chloroplasts and actin filaments along with enhancing actin polymerization. Since profilin itself is regulated by Ca^{2+} concentrations, CHUP1-profilin binding and further downstream signalling seems to be Ca^{2+}-dependent. PMI1 and THRUMIN1 are also actin-binding proteins which help in cp-actin organization (Wada and Kong 2018). Having a C2 domain at the N-terminus, PMI1 functions in a Ca^{2+}-dependent manner (Suetsugu et al. 2015) whereas THRUMIN1 may protect actin bundles from Ca^{2+}-induced actin-depolymerization or severing (Takamatsu and Takagi 2011).

As Ca^{2+} influx through PM is directly promoted by ROS that originates mostly from NOX, the downstream Ca^{2+}-actin communication can be postulated to be ROS-regulated. While exogenous Ca^{2+} inflicted avoidance response in leaves incubated even under weak BL demonstrating the pivotal role of Ca^{2+} in signalling for avoidance movement, inhibitor of Ca^{2+} channels ($LaCl_3$) or Ca^{2+}-chelator [EGTA; ethylene glycol bis (2-aminoethyl) N, N, N′, N′-tetra acetic acid] diminished avoidance significantly indicating the importance of Ca^{2+}-influx through PM for building threshold $[Ca^{2+}]_{cyt}$ necessary for avoidance movement (Majumdar and Kar 2016). The involvement of ROS in Ca^{2+}-mediated chloroplast avoidance was confirmed as exogenous H_2O_2 was able to overcome the inhibitory effect of EGTA and induced avoidance despite the presence of EGTA in the incubating medium (Majumdar and Kar 2016).

ROS and phototropins

Among the two distinct BL photoreceptors of plants viz. cryptochromes and phototropins, only phototropins (PHOT1 and PHOT2) are involved in light-induced chloroplast movements (Ohgishi et al. 2004; Banaś et al. 2012; Taiz et al. 2015; Sztatelman et al. 2016). Phototropins typically have a Ser/Thr kinase domain at the C-terminus and two LOV domains (LOV1 and LOV2; ~ 110 amino acids) at the N-terminus which belong to the PAS (Per, ARNT, Sim) superfamily (Sakai et al. 2001; Christie 2007). LOV domains are essential for phototropin functioning as they bind to the cofactor FMN (flavin mononucleotide) and undergo photocycles (LOV_{447} at dark, inactive; LOV_{390} under BL, active) thereby rendering photosensitivity to the protein (Christie 2007). Upon BL illumination, phototropins undergo either auto- or trans-phosphorylation. Although the phosphatase involved in dephosphorylation of PHOT1 C-terminal Ser/Thr kinase domain remains to be identified, PP2A has been found to be responsible for PHOT2 dephosphorylation (Sztatelman et al. 2016). PP2A-2 has been established to convene PHOT2-mediated strong BL-induced chloroplast avoidance movement (Wen et al. 2012). While it was reported by Tseng and Briggs (2010) that A1 subunit of PP2A [ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID 1 (RCN1)] downregulate photoactivated PHOT2, Wen et al. (2012) have suggested that PP2A-2 alters PHOT2 phosphorylation status in a different way than RCN1 and it participates in avoidance movement by dephosphorylating/activating ADF/cofilin.

It is interesting to note that in spite of having nearly similar amino acid sequences, PHOT1 and PHOT2 have differential participation in chloroplast movements. While both PHOT1 and PHOT2 are involved in weak BL-dependent chloroplast accumulation movement, only PHOT2 has been reported to mediate strong BL-induced avoidance response (Jarillo et al. 2001; Sakai et al. 2001; Kong et al. 2013; Wada and Kong, 2018). This conforms to the differential sensitivities of the two receptors towards BL fluence rates; PHOT1 is activated in a wide range of BL intensities starting from as low as 0.01 $\mu mol \cdot m^{-2} \cdot s^{-1}$ whereas PHOT2 specifically requires strong BL for activation i.e. > 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Sakai et al. 2001; Goh 2009). Sub-cellular localization of PHOT1 and PHOT2 is widely studied and it is generally considered that both the proteins are localized to the plasma membrane in dark but their distribution under BL varies (Sakamoto and

Briggs 2002; Kong and Wada 2014). According to Wada and Kong (2018) both PHOT1 and PHOT2, which are necessary for accumulation, are localized at the plasma membrane (absorbing weak BL) whereas only PHOT2, which is solely involved in avoidance, is localized on chloroplast outer envelope (absorbing strong BL). In a recent study, Sakata et al. (2019) have demonstrated that relocation of MpPHOT (*Marchantia polymorpha* phototropin; analogous to *Arabidopsis* PHOT2 but not PHOT1) to chloroplast outer periphery is essential for BL-induced avoidance movement.

Phototropin mediated increase in cytosolic Ca^{2+} under BL illumination is well documented; while both PHOT1 and PHOT2 mediate Ca^{2+} influx into the cytosol through PM, only PHOT 2 is involved in release of Ca^{2+} from endosomes (Harada et al. 2003). Depending on their respective sensitivities towards different ranges of BL fluence rates, PHOT1 increases $[Ca^{2+}]_{cyt}$ at 0.1–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluence while PHOT2 increases $[Ca^{2+}]_{cyt}$ at 1–250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluence (Harada et al. 2003). Strong BL-induced and phototropin-mediated Ca^{2+} increase and formation of threshold $[Ca^{2+}]_{cyt}$ is involved in many plant processes e.g. chloroplast movements (Wen et al. 2008), phototropism of *Arabidopsis* hypocotyls (Zhao et al., 2013). Involvement of PHOT 2 in ROS signalling was evident as *phot 2* mutant reduced H_2O_2 generation significantly under high fluence BL (Wen et al. 2008). Considering the repressing effects of NOX inhibitor (DPI) and *phot 2* mutant on avoidance, Wen et al. (2008) speculated that the increase in $[Ca^{2+}]_{cyt}$ induced by PHOT 2 under Strong BL irradiation promoted ROS (H_2O_2) production by activating NOX and H_2O_2 may directly act upon PHOT2 to stimulate avoidance movement. Moreover, both Ca^{2+} and H_2O_2 promote PM H^+-ATPase (Majumdar and Kar 2018), which, in turn, may activate HACC channels (hyper polarization activated Ca^{2+} channels) further enabling Ca^{2+} entry into cytosol facilitating the ROS-Ca^{2+}-actin communication leading to chloroplast avoidance movement.

Role of PM H^+-ATPase in BL-induced chloroplast avoidance movement

Being involved in diverse plant processes, ranging from growth and development to defence against biotic or abiotic stresses, PM H^+-ATPase functions as one of the key enzymes to sustain plant life (Falhof et al. 2016). It is well reported that BL-induced phosphorylation of phototropins precedes the phosphorylation (activation) of PM H^+-ATPase and both are inhibited by DPI, a potent NOX inhibitor (Kinoshita et al. 2003; Takemiya and Shimazaki 2016). On the other hand, the process of targeting onto plasma membrane as well as activation of NOX has also been reported to be positively regulated by BL exposure in wheat coleoptiles (Chandrakuntal et al. 2010). Involvement of BL as stimulus for activation of both the enzymes hints that the Ca^{2+}-mediated positive feed-forward loop between NOX and PM H^+-ATPase, as already identified during root growth of *Vigna radiata* (Majumdar and Kar 2018), may be operational also during strong BL-induced chloroplast avoidance movement. Inhibition of such movement by Vanadate (PM H^+-ATPase inhibitor) treatment (Supplementary Fig. S1) corroborates the hypothesis that blocking one enzyme of the loop results in down regulation of the other one. Indeed, interplay between PM H^+-ATPase and NOX has been demonstrated under different physiological conditions (Zhang et al. 2007; Li et al. 2011; Zhao et al. 2015). It is reported that exogenous treatment with 5 mM H_2O_2 increases the expression of PM H^+-ATPase genes in *Cucumis sativus* (Janicka-Russak et al. 2012). Activity of PM H^+-ATPase has also been found to be promoted by exogenous H_2O_2 treatment, while the enzyme was significantly inhibited under treatment with different ROS scavengers and NOX inhibitor (Majumdar and Kar 2018). The functional synchronization between NOX and PM H^+-ATPase has been extended further to downstream located superoxide dismutase (SOD) and class III Peroxidase (Prx) which function in orchestration with NOX-PM H^+-ATPase loop (Majumdar and Kar 2019). With established effects as activator/regulator of NOX and PM H^+-ATPase, Ca^{2+} was found to play pivotal role of linking the enzymes. The stimulating effect of H_2O_2 on chloroplast avoidance movement (Wen et al. 2008; Majumdar and Kar 2016) is likely to be dependent upon the said enzymatic synchronization which includes $O_2^{\cdot -}$ generation by NOX and membrane polarity maintenance by PM H^+-ATPase enabling Ca^{2+} influx through PM [by hyperpolarization-activated Ca^{2+} channels (HACC)] required for generation of critical $[Ca^{2+}]_{cyt}$.

Establishment of proton-gradient across the plasma membrane by PM H^+-ATPase is essential for different plant processes. Such proton (H^+) gradient, in addition to Ca^{2+}, also has been ascribed for regulation of actin cytoskeleton (Helper 2016). According to Chen et al. (2002), pH above 7.0 promotes ADF function leading to actin depolymerisation at the minus end and growth at plus end. As higher activity of PM H^+-ATPase is primarily responsible for maintaining cystoplasmic pH at 7.0 or more, it can be speculated that ADF promotion is dependent upon PM H^+-ATPase and considering the evidences mentioned above, the NOX-ATPase functional loop is involved in the process.

Interestingly, Tlałka and Fricker (1999) reported that excess Ca^{2+} treatment led to slowing of chloroplast movements under Strong BL. The observation can be justified by the differential responses of CDPKs towards a range of Ca^{2+} concentrations (Aldon et al. 2018) as well as differential regulation of PM H^+-ATPse by Ca^{2+}. PM H^+-ATPase activity is influenced by phosphorylation of different amino acids e.g. Thr955, Thr947, Ser938, Ser931

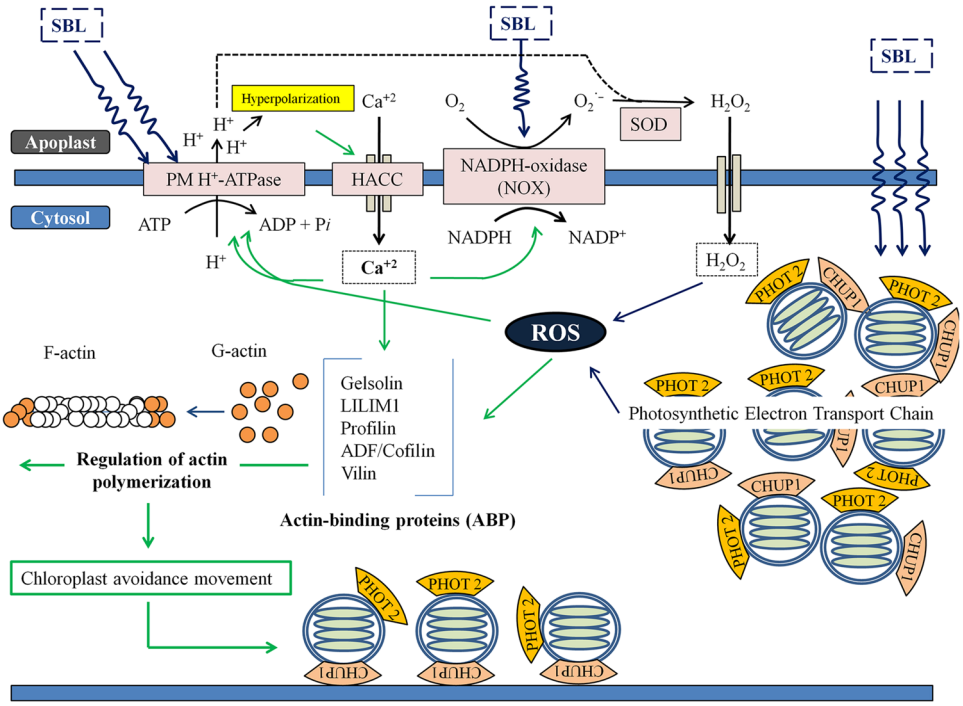

Fig. 1 Possible working model demonstrating the ROS-dependent signaling cascade involved in strong BL-induced chloroplast avoidance movement. Exposure to Strong BL leads to the production of different forms of ROS by the chloroplasts due to electron (e^-) spill over at photosynthetic e^- transport chain (ETC). After being converted to H_2O_2 and diffusing across chloroplast membranes, chloroplast-derived ROS accumulate at the cytosol. On the other hand, Strong BL stimulates both NADPH oxidase (NOX) and PM H^+-ATPase thereby augmenting the rate of $O^{\cdot-}$ production and H^+ transport across plasma membrane. Apart from spontaneous reactions, the NOX-generated $O^{\cdot-}$ is converted to H_2O_2 by SOD which utilizes the H^+ available at the apoplastic space due to PM H^+-ATPase activity. The de novo synthesized H_2O_2 diffuses across the plasma membrane and, together with chloroplast-derived ROS, creates a cytosolic ROS pool. The accumulated ROS activates Ca^{2+}-channels which allows Ca^{2+} influx through plasma membrane. In addition, the membrane hyperpolarization resulting from induced PM H^+-ATPase activity stimulates HACCs (hyperpolarization-activated Ca^{2+} channels) and facilitates Ca^{2+}-entry into cytosol. Consequently, a threshold $[Ca^{2+}]$cyt is built up which also includes Ca^{2+} being released from endosomes. Binding to EF-hand motifs and affecting phosphorylation of different amino acids, $[Ca^{2+}]$cyt regulates the activity of NOX and PM H^+-ATPase and a positive feedback loop is created. The Strong BL-induced accumulation of ROS within cytosol and threshold $[Ca^{2+}]$cyt then modulates actin polymerization process either by directly modifying actin amino acids or by regulating the activities of actin-binding proteins (ABPs). The altered polymerization/depolymerization allows chloroplasts to move along the plasma membrane (linked together by CHUP1 protein) to minimize high light absorption

through different CDPKs (Ookura et al. 2005; Yu et al. 2006; Janicka-Russak 2011).While phosphorylation of some residues is positive for the enzyme's activity e.g. Thr947, negative regulation is observed when other amino acids are phosphorylated e.g. Ser931 (Fugslang et al. 2007; Janicka-Russak 2011). Thus at a threshold concentration, Ca^{2+} promotes the enzyme while supra-optimal concentrations may result in inhibition of the enzyme. Inhibition of PM H^+-ATPase would eventually result in decreased ROS production by NOX and thus avoidance would be hindered. In addition, Ca^{2+}-dependent phosphorylation (deactivation) of ADF/cofilin leading to reduced actin binding may also be linked with excess Ca^{2+}-induced slowing of chloroplast movements.

Conclusion

The necessity of chloroplast movements for plants' thriving becomes evident from its adoption or retention through evolution in almost every eukaryotic plant groups ranging from algae to aquatic and terrestrial angiosperms (Suetsugu and Wada 2016). Among the several different photo-protective mechanisms (e.g. non-photochemical quenching, cyclic electron flow and photorespiration), induction of antioxidant defence system and chloroplast avoidance movement are the most resilient plant responses towards supra-optimal (or high) light-induced oxidative stress (Mullineaux et al. 2000; Kasahara et al. 2002). However, based on the accumulated evidences it may be argued that at physiologically suitable threshold concentrations ROS itself most likely

function as signalling agent to trigger chloroplast avoidance movement. Strong BL-induced avoidance movement is mediated by ROS (being generated by NOX and ETC as a result of incident high fluence) through modulation of actin filament polymerization/depolymerisation. Threshold $[Ca^{2+}]_{cyt}$ attained through PHOT 2 activation by BL as well as cross-PM Ca^{2+} influx by HACC channels (stimulated by PM H^+-ATPase-induced membrane hyperpolarization) play a pivotal regulatory role. In addition to promoting both NOX and PM H^+-ATPase in a positive feedback loop, threshold $[Ca^{2+}]_{cyt}$ also regulates actin by direct binding or aiding phosphorylation of different ABPs. Ca^{2+}-dependent direct/indirect regulation of CHUP1, PMI1 and THRUMIN1 protein establishes the signalling bridge between chloroplasts and actin, thereby connecting the organelles with the BL-induced upstream signalling cascade convened by ROS. A working model demonstrating the Strong BL-induced ROS-mediated chloroplast avoidance movement has been proposed (Fig. 1).

Future prospect

Success of a plant is mostly determined by its adaptability to the prevailing or changing conditions including the level of irradiance. Chloroplast movement is such an adaptive response and has been an area of interest since long. A plethora of research, from BL perception mechanisms to molecular details of the chloroplast movement along with actin dynamics, has established the intricacy of the process. However, role of ROS in chloroplast avoidance movement has only recently been recognized, which has essentially been trailed by certain questions regarding sites of ROS generation (plasmamembrane vs thylakoids) and their possible cooperation, if any. At the same time, question also remains about the role of phototropins (particularly PHOT2 having possibility of dual location—plasmamembrane and chloroplast membrane) in generating ROS that ultimately participate in chloroplast avoidance movement. Further experimentation involving molecular techniques and mutants may unravel the mystery.

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Author contributions RKK envisaged the study. AM and RKK designed the work. AM performed the experiments. AM and RKK wrote the article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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