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Abscisic acid enhances non-photochemical quenching through SnRK2 and ABI3 in *Physcomitrium patens*

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Abstract

The transition of plants in the green lineage from aquatic to terrestrial environments during the bryophyte stage marked a pivotal point in evolution. Successful terrestrialization required evolutionary adaptations to harsh and fluctuating light conditions, where direct irradiation is stronger than in aquatic environments. To cope with these challenges, plants evolved regulatory mechanisms to control cellular activities. One such acclimation is rapidly reversible, energy-dependent non-photochemical quenching (NPQ), which dissipates excess light energy as heat to protect the photosynthetic apparatus. Another critical innovation is abscisic acid (ABA) signaling, believed to have first emerged in bryophytes. Here, we reveal a potential link between these two key acclimations in bryophytes. We demonstrate that exogenous ABA induces NPQ in the moss *Physcomitrium patens*, increasing the levels of LHCSR, a key NPQ regulator, while concurrently decreasing PsbS. Exogenous ABA also enhances the xanthophyll cycle pigments, contributing to NPQ. In mutants deficient in ABA signaling components, including SNF1-related kinase 2 (SnRK2) and the transcription factor, Abscisic Acid-Insensitive 3 (ABI3), ABA-induced NPQ, LHCSR and PsbS expression, and xanthophyll cycle pigment accumulation were significantly reduced. These findings suggest that exogenous ABA enhances NPQ through the SnRK2 and ABI3-mediated signaling pathway by promoting LHCSR expression and xanthophyll cycle pigment production. We propose that the integration of ABA signaling and NPQ represent a critical evolutionary milestone, enabling early land plants to adapt and thrive in terrestrial environments.

Keywords

Abscisic acid signaling, LHCSR, Non-photochemical quenching, *Physcomitrium patens*, Xanthophyll cycle pigments

Statements and Declarations

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Text

Introduction

About 470 million years ago, ancestors of extant land plants advanced from water to land, diverging from charophytes and giving rise to bryophytes, which is one of the most important events in the evolutionary history of plants (Delwiche and Cooper 2015). The transition from the water environments to the terrestrial environments required plants to develop the adaptive mechanisms to cope with the harsh environmental stresses such as drought, high temperature, strong sunlight, and UV irradiation. However, in the process of evolving land plants, how a novel mechanism or network was introduced de novo or rewired from pre-existing forms to withstand the harsh environments remains largely unknown (de Vries and Archibald. 2018; Kumar et al. 2022).

Thermal dissipation of excess energy, non-photochemical quenching (NPQ), in photosystems is one of the primary photo-protective mechanisms in photosynthesis (Müller et al. 2001). Although light is indispensable for photosynthesis, absorption of excessive light energy by chlorophylls induces production of reactive oxygen species (ROS) leading to photo-oxidative damage (Pospíšil 2016). Therefore, photosynthetic organisms possess the mechanism to dissipate the excessive light energy as heat. Because high and fluctuating sunlight conditions are more pronounced on land than underwater, NPQ capacity may have played an important role in supporting the survival of early land plants. So far, LHCSR (Light-Harvesting Complex Stress-Related protein)-dependent (Gretto et al. 2011; Pinnola 2019) and PsbS (Photosystem II Subunit S)-dependent (Correa-Galvis et al. 2016; Gretto et al. 2011) mechanisms for NPQ have been reported in the green plant lineage (*Viridiplantae*). Xanthophyll cycle pigments are also involved in the induction of NPQ. Zeaxanthin, one of the key pigments, acts as a regulator of structural changes in proteins involved in NPQ (such as LHCSR or light harvesting complex II (LHCII)), thereby enabling NPQ to occur (Johnson et al. 2011). LHCSR and PsbS induce NPQ in response to low pH of lumen and zeaxanthin accumulation (Bergantino et al. 2003; Demmig-Adams 1990; Rees et al. 1992). Interestingly, the distribution of LHCSR and PsbS proteins among the green plant lineage is highly variable (Pinnola 2019). In green algae such as *Chlamydomonas reinhardtii*, LHCSR is known to be involved in NPQ. Peers et al. (2009) demonstrated that LHCSR is essential for energy dissipation under high light conditions, thus preventing photodamage to the photosynthetic apparatus. While in bryophytes, both LHCSR and PsbS are known to be involved in NPQ, this involvement of LHCSR and PsbS in NPQ in bryophytes is further distinct as seen in vascular plants; the LHCSR is lost and only the PsbS remains (Gerotto and Morosinotto 2013; Koziol et al. 2007).

Abscisic acid (ABA) is a stress-related plant hormone and plays major roles in abiotic stress response in land plants (Dar et al. 2017). For example, drought, one of the major abiotic stresses in terrestrial environments, triggers to drive the ABA signaling pathway (Rathnayake et al. 2019). Recent studies revealed that the ABA signaling pathway has been gradually established with plant evolution (Sun et al. 2020). All components involved in ABA biosynthesis and signaling, including ABA receptors, PP2C (protein phosphatase 2C), and SNF1-related protein kinase 2 (SnRK2), have been present in a sister group of land plants, streptophyte algae (Sun et al. 2020). However, in streptophyte algae, there was no involvement of ABA in these signaling components (Sun et al. 2020). Thus, the involvement of ABA in activating these signaling elements, such as ABA receptors, PP2C phosphatase, and SnRK2 kinases, collectively known as the core ABA components, likely originated in the ancestors of extant bryophytes (Komatsu et al. 2013; Komatsu et al. 2020). Abscisic Acid-Insensitive 3 (ABI3) is a key transcription factor in plants that regulates gene expression downstream of the core ABA components (Ali et al. 2022; Komatsu et al. 2013; Komatsu et al. 2020) and plays a critical role in stress responses, such as dehydration and cold stress (Tan et al. 2017). As mentioned, bryophytes are known to have acquired ABA dependency for abiotic stress responses (Komatsu et al. 2013), and are believed to be the first land plant group to successfully colonize terrestrial environments (Delwiche and Cooper 2015). To thrive on land, plants needed to adapt to intense light exposure, as terrestrial environments have significantly stronger light intensity compared to aquatic environments (Gjindali et al. 2021; Li et al. 2024), and NPQ plays a crucial role in this process (Müller et al. 2001). However, it remains unclear whether ABA signaling and NPQ operate

independently or if there is any internal signaling connection between these key traits essential for plant terrestrialization. In this study, we used the moss, *Physcomitrium patens*, to investigate the link between the ABA signaling pathway and NPQ. Our results show that SnRK2, a core component of ABA signaling, is involved in enhancing NPQ capacity. Additionally, ABI3, an ABA-dependent transcription factor functioning downstream of ABA signaling, plays a partial role in NPQ capacity, indicating a significant connection between these pathways. Thus, we propose that the establishment of the ABA signaling pathway, coupled with the acquisition of an ABA-NPQ regulatory link, may have been critical for early land plants to survive and adapt to terrestrial environments.

Materials and methods

Plant material and growth condition

Protonemal tissues of wild-type *Physcomitrium patens* (Hedw.) Bruch & Schimp (Gransden 2004) (Rensing et al. 2008) and *abi3tko* and *snrk2qko* mutants (Khandelwal et al. 2010; Shinozawa et al. 2019) were cultured in cellophane-covered BCDAT medium (1.01 mM $MgSO_4$, 1.84 mM pH6.5 KH_2PO_4, 9.99 mM KNO_3, 44.96 µM $FeSO_4$, 0.34 µM $CuSO_4$, 9.93 µM H_3BO_3, 0.42 µM $CoCl_2$, 0.12 µM Na_2MoO_4, 0.19 µM $ZnSO_4$, 1.97 µM $MnCl_2$, 0.17 µM KI, 5.00 mM Ammonium tartrate, 1.00 mM $CaCl_2$, 0.8% Agar). Culture condition was 25 ℃, continuous light (100 µmol photons/m^2/s) under fluorescent lamps (FHF32EX-W-H, Toshiba, Japan), which supports stable moss growth (Caine et al. 2020; Ortiz-Ramírez et al. 2016). The photosynthetic photon flex density (PPFD) was measured using a photo-radiometer (HD2102.2 with LP471PAR probe, Deltaohm, Italy)

ABA and DMSO treatment

For chlorophyll fluorescence measurement, *P. patens* protonemata were homogenized using polytron homogenizer and subsequently subcultured in BCDAT medium for 5 days. Then, half were transferred to BCDAT medium containing 50 µM ABA to effectively induce the formation of vegetative diaspores (brachycytes or brood cells) (Hiroguchi et al. 2022; Koselski et al. 2019; Perroud et al. 2018), while the other half were transferred to BCDAT medium containing 0.1% DMSO, used as a vehicle control. For HPLC and western blotting, *P. patens* protonemata were cultured in BCDAT medium for 3 days and then treated in the same way as above.

Chlorophyll fluorescence measurement

P. patens treated with ABA or DMSO were taken out of the incubator on days 0, 1, 3, and 5, and chlorophyll fluorescence was measured using a PAM-2000 (WALZ, Germany). Before measuring chlorophyll fluorescence, *P. patens* protonemata were left at room temperature in the dark for 40 min. All measurements were conducted in a state in which external light was blocked as much as possible.

For the measurement of Fo, a red measuring light (with a peak at 655 nm, 5 µmol photons/m^2/s) was irradiated. The measuring light was set to remain on until the end of the measurement. For the measurement of Fm, a saturation pulse was flashed. After waiting for 10 sec, red actinic light (with a peak at 655 nm, 500 µmol photons/m^2/s) was irradiated for 300 sec. After that, the saturation pulse was flashed to measure the value of NPQ.

SDS-PAGE

Prior to sampling, protonemal cells were kept in the dark at room temperature for 30 min, followed by exposure to 500 μmol photons/m^2/s of white light for 5 min. Immediately after the light treatment, the samples were frozen in liquid nitrogen. Frozen protonemal cells were homogenized for 2 min using a Shake Master Neo shaker (Bio Medical Science, Japan) with stainless steel beads. Then, suspension buffer containing 50 mM imidazole/HCl (pH 7.0), 20% (w/v) glycerol, and 1% (w/v) protease inhibitor cocktail (Merck, Germany) was added to the powder and immediately mixed with Shake Master Neo for 30 sec. After collecting 5 μL of each suspension for pigment analysis, the remaining suspensions were centrifuged at 21,600 × *g* for 5 min at 4 ℃. LDS buffer containing 50 mM Tris/HCl (pH 8.0), 2% (w/v) lithium dodecyl sulfate (LDS), 350 mM sucrose, and 100 mM dithiothreitol was added at a concentration of 0.2 mg chlorophyll/mL, and the suspensions were centrifuged at 21,600 × *g* for 5 min at 4 ℃. The supernatants were denatured by heating at 65 ℃ for 10 min and used as loading samples for SDS-PAGE. Equal amounts of chlorophyll-based protein samples (0.01 μg chlorophyll equivalents for a single lane for LHCSR and 1.2 μg chlorophyll equivalents for a single lane for PsbS) were separated on 14% polyacrylamide gels using the Laemmli buffer system (Laemmli 1970). Loading control for the experiments was performed using RBCL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) followed by Coomassie brilliant blue (CBB) staining (Wako, Japan).

Immunoblot Analysis

Immunoblot analysis of LHCSR and PsbS was performed using anti-LHCSR antibodies (AS15 3081, Agrisera, Sweden) and anti-PsbS antibodies (AS09 533, Agrisera, Sweden). Proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride membrane by a conventional wet blotting system using the transfer buffer containing 25 mM Tris and 192 mM glycine with 10% ethanol. The membrane was blocked with the buffer containing 5% (w/v) nonfat dry milk dissolved in PBS buffer containing 0.05% Tween-20. Primary antibodies were diluted (1:15,000 for LHCSR and 1:5,000 for PsbS) with Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo, Japan). Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Merck, Germany) were diluted with Can Get Signal Immunoreaction Enhancer Solution 2 (Toyobo, Japan). Western Lightning Plus-ECL (PerkinElmer, USA) was used to detect PsbS and LHCSR proteins. Immunoblotting result images were quantified using GelAnalyzer 19.1 software (www.gelanalyzer.com).

Semi-quantitative PCR

Protonemal cells were kept in the dark at room temperature for 30 min, followed by exposure to 500 μmol photons/m^2/s of white light for 5 min. Immediately after the light treatment, the samples were frozen in liquid nitrogen. Frozen samples were ground using a mortar and pestle. Total RNA was extracted from the pulverized sample using the Qiagen RNeasy Plant Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. cDNA was synthesized from extracted total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) following the manufacturer's instructions. For semi-quantitative PCR, primers for the *PsbS* (Pp3c20_23430V3.1), *LHCSR1* (Pp3c9_3440V3.1), *LHCSR2* (Pp3c15_11070V3.1), and ubiquitin conjugating enzymes E2 (*UBE2*) (Pp3c14_21480V3.1) were prepared. For *PsbS* 5'-CTTTGGGTGACCGTGGAA-3' and 5'-ACACGGGGCCCTTTTCAT-3', for *LHCSR1* 5'-CCCCAGCAGCCTACCAGGTG-3' and 5'-AGGCAGCACGAGCCAACAAA-3', for *LHCSR2* 5'-TTGCAGCTGCACCTCACGCA-3' and 5'-CGTGAGTGATTTCCGACTCC-3', for *UBE2* 5'-ATGGGAGCATTTGCTTGGACAT-3' and 5'-GCAGATTGAGAGCAGCACCTTG-3' were used. cDNA was amplified with a MiniAmp Plus thermal cycler (Applied Biosystems, USA) with GoTaq Green Master Mix (Promega, USA) according to the following steps (98 ℃, 1 min; 98 ℃, 10 sec, 52 ℃, 30 sec, 72 ℃, 1 min, 30 cycles; 72 ℃, 4 min). The amplicon was analyzed by electrophoresis using a 4% agarose gel. Semi-qPCR images were quantified using GelAnalyzer 19.1 software (www.gelanalyzer.com). The relative value was calculated by comparing the intensity of each quantified band with the band intensity of *UBE2* of each sample. The expression level of *LHCSR* was calculated by adding the expression levels of *LHCSR1* and *LHCSR2*.

HPLC

Protonemal cells were kept in the dark at room temperature for 30 min, followed by exposure to 500 μmol photons/m^2/s of white light for 5 min. Immediately after the light treatment, the samples were frozen in liquid nitrogen. Frozen samples were ground to powder using a MultiBeads Shocker (Yasui Kikai Corporation, Japan). Pigments (chlorophyll *a*, *b*, violaxanthin, antheraxanthin, zeaxanthin, and α-, β-carotene) were extracted from 10 mg of frozen leaf by homogenization in pre-cooled acetone with stainless beads (5 mm in diameter) (BioMedical Science, Japan) for 1 min using the ShakeMaster bead shaker (BioMedical Science, Japan). Extracts were centrifuged at 21,600 × *g* for 5 min, and the supernatant was analyzed by high-performance liquid chromatography (HPLC) using a C18 column (250 mm in length, 4.6 mm in column inner diameter) (YMC, Japan). The analysis of carotenes and other pigments was carried out separately. The sample was eluted with an isocratic flow of solvent A (100% methanol) for 17 min, followed by a linear gradient from solvent A to B (60% methanol, 20% ethanol, 20% hexane) in 5 min and with an isocratic flow of solvent B at a flow rate of 0.8 mL/min for chlorophyll and xanthophyll analysis. The sample was eluted with an isocratic flow of solvent B for 15 min for carotenes. The eluates were monitored by an L-2450 photodiode array detector (HITACHI High Technologies Science, Japan) at 450 nm. The pigments were identified and quantified by comparing their absorption spectra with relevant standard pigments

Statistical analysis

Gnu PSPP software (https://www.gnu.org/software/pspp/) was used to analyze the data. An independent *t* test was used to compare values between DMSO and ABA treatments. All ANOVA test were performed with Tukey's HSD posthoc using R (version 4.3.1) in RStudio (version 2023.06.1+524, https://posit.co/download/rstudio-desktop/)

Results

Enhancement of NPQ capacity via SnRK2-dependent ABA signaling pathway

We first examined the effect of ABA treatment on NPQ, an indicator of thermal energy dissipation, and Fv/Fm, the maximum quantum yield of photosystem II (PSII), by measuring chlorophyll fluorescence parameters in 5-day-old protonemata of *P. patens* (Genty et al.1989; Krause and Weis 1984). In the absence of ABA, NPQ increased with the PPFD up to 500 μmol photons/m^2/s, but remained nearly constant beyond this value. In contrast, after a 3-day ABA treatment, NPQ levels were more than twice as high as in the absence of ABA; however, the PPFD-dependent changes in NPQ plateaued beyond 500 μmol photons/m^2/s (Fig. 1a). These results suggest that the maximum NPQ occurs at the PPFD of 500 μmol photons/m^2/s. Therefore, we used actinic light at this PPFD for all subsequent experiments examining changes in NPQ and Fv/Fm. After 1 and 6 hours of 50 μM ABA treatment in the wild type, no significant increase in NPQ was observed (Fig. 1b). On day 1, the NPQ value reached 7.3 and remained stable without significant changes on days 3 and 5, maintaining approximately twice the level observed in the control group (Fig. 1c). In contrast, Fv/Fm remained unchanged throughout 5 days of ABA treatment (Fig. 1d). These results suggest that exogenous ABA significantly enhances NPQ capacity in *P. patens* protonemata, without causing observable functional damage to PSII.

The ABA signaling pathway is mediated by core components, including SnRK2, which phosphorylate downstream targets, driving diverse physiological responses. One key downstream target of SnRK2 is the transcription factor ABI3, which regulates the expression of stress-responsive genes. We selected SnRK2 and ABI3 due to their well-conserved roles and their representative functions in land plants (Nakashima et al. 2009; Shinozawa et al. 2019). To investigate how these components contribute to the regulation of non-photochemical

quenching (NPQ), we tested two mutants: the *abi3* triple-knockout (*abi3tko*) mutant and the *snrk2* quadruple-knockout (*snrk2qko*) mutant, which knocked out *SnRK2* and *ABI3* genes located in the ABA signaling pathway (Khandelwal et al. 2010; Shinozawa et al. 2019). In the *abi3tko* mutant, NPQ increased to 5.0, which was 32% lower than the ABA-treated wild type (7.3 on day 1 in Fig. 1c, e). On the other hand, the Fv/Fm value was not affected by ABA like wild-type, indicating PSII function was maintained in the *abi3tko* mutant (Fig. 1f). We next examined these parameters in the *snrk2qko* mutant, and found no increase in NPQ even after 5 days of exogenous ABA treatment (Fig. 1g), while having no significant effect on Fv/Fm (Fig. 1h). These results indicate that SnRK2 is essential and ABI3 may be partially involved in the ABA-dependent induction of NPQ.

SnRK2 mediating ABA-dependent regulation of *LHCSR* and *PsbS* gene expression

LHCSR and PsbS are known to be major proteins that regulate PSII photochemistry by facilitating NPQ in green plants. Bryophytes are distinct in that they have both LHCSR and PsbS proteins, and these two proteins are also known to be functional (Gerotto and Morosinotto 2013). We examined whether exogenous ABA and the ABA signaling components regulate LHCSR and PsbS in bryophytes. To determine how ABA treatment affects LHCSR and PsbS proteins, we analyzed their protein levels using western blotting. Protein levels were quantified and compared between day 0 and day 3 for each treatment (Fig. 2a, b). In the wild type, after 3 days of ABA treatment, the LHCSR protein level increased by 56% compared to day 0, while a decrease of 9% was observed when treated with DMSO. A 63% increase was also observed in the *abi3tko* mutant after 3 days of ABA treatment. However, a minimal increase in LHCSR protein level was observed in the *snrk2qko* mutant (Fig. 2c). In contrast to LHCSR, the opposite trend was observed for PsbS. In the wild type and *abi3tko* mutant, the PsbS protein level decreased by 40% and 36%, respectively after 3 days of ABA treatment. On the other hand, little change in the PsbS protein level was observed in the *snrk2qko* mutant (Fig. 2d). These results suggest that ABA treatment affects the protein levels of LHCSR and PsbS in wild type, and this process is mediated by SnRK2, but not by ABI3.

To understand whether the difference in protein levels resulted from the transcriptional control of each gene, we examined the gene expression levels of *LHCSR* and *PsbS* through semi-quantitative PCR after 3 days of exogenous ABA treatment (Fig. 2e, f). In the wild type, a 54% increase in *LHCSR* gene expression was observed after 3 days of exogenous ABA treatment, whereas an 11% increase was observed with DMSO treatment (Fig. 2e). A 35% increase in *LHCSR* gene expression was also observed in the *abi3tko* mutant after ABA treatment. However, in the *snrk2qko* mutant, a 13% decrease in gene expression was observed after ABA treatment (Fig. 2e). The expression of the *PsbS* gene after ABA treatment was opposite to that of *LHCSR* (Fig. 2f). In the wild type, the expression of the *PsbS* gene decreased by 36% after ABA treatment, while it decreased by 8% after DMSO treatment (Fig. 2f). In the *abi3tko* mutant, a 42% decrease in *PsbS* gene expression was observed. However, no decrease in *PsbS* gene expression was observed in the *snrk2qko* mutant (Fig. 2f). Taken together, our data indicate that exogenous ABA affects the amount of LHCSR and PsbS proteins by, at least, regulating the transcription levels of *LHCSR* and *PsbS* genes; exogenous ABA downregulates the *PsbS* gene expression and upregulates the *LHCSR* gene expression in *P. patens*. Furthermore, SnRK2 mediates ABA-dependent regulation of *LHCSR* and *PsbS* gene expression but ABI3 does not.

Induction of xanthophyll cycle pigments by SnRK2-dependent ABA signaling pathway

Since xanthophyll cycle pigments are involved in plant NPQ (indicated by the red dotted square in Fig.3a) (Jahns and Holzwarth 2012), we next examined whether ABA signaling regulates these pigments in *P. patens*, as previous studies have suggested a potential link between ABA and xanthophyll cycle pigments (Zhu et al. 2011). To investigate the effect of exogenous ABA on xanthophyll cycle pigments, changes in the pigments were measured before and after ABA treatment. In the wild type, the amount of all three xanthophyll cycle pigments increased (Fig. 3b-d). Violaxanthin and antheraxanthin levels increased by 69% and 79%, respectively, after ABA treatment (Fig. 3b, c). Zeaxanthin, which has the strongest impact on NPQ, was increased by 178% (Fig.

3d).

Then, we examined if ABI3 or SnRK2 is involved in the levels of these pigments. In the *abi3tko* mutant, the levels of violaxanthin and antheraxanthin increased by 56% and 59%, respectively (Fig. 3b, c). Zeaxanthin was increased only 39% (Fig. 3d). In the *snrk2qko* mutant, no significant change was observed in any of the xanthophyll cycle pigments (Fig. 3b-d). Notably, in the *abi3tko* mutant, whereas the levels of violaxanthin, antheraxanthin, and zeaxanthin increased following ABA treatment, these increases were lower compared to the wild type. This result suggests that ABI3 plays a partial role in the ABA-dependent accumulation of these pigments.

To determine the xanthophyll cycle pigments pool size, the total amount of pigments was measured. In the wild type, the xanthophyll pigment pool was increased by 114% after ABA treatment (Fig. 3e). In the *abi3tko* mutant, an increase of only 50% compared to the wild type was observed. On the other hand, no significant changes were observed in the *snrk2qko* mutant. Thus, our results support that ABA treatment increases the total amount of violaxanthin, antheraxanthin, and zeaxanthin, and this process is SnRK2-dependent and partially ABI3-dependent.

De-epoxidation of xanthophyll cycle pigments by SnRK2-dependent ABA signaling pathway

Among the xanthophyll cycle pigments, zeaxanthin plays an important role in NPQ (Jahns et al. 2009). Therefore, we investigated whether ABA is involved not only in the total amount of xanthophyll cycle pigments but also in their conversion. Specifically, we tested whether ABA treatment affects the conversion of violaxanthin to zeaxanthin and vice versa (Fig. 4a). To assess this, we calculated the de-epoxidation state of xanthophyll cycle pigments using the formula (zeaxanthin + 0.5 × antheraxanthin)/(total amount of xanthophyll cycle pigments) (Johnson et al. 2009). In the wild type, the de-epoxidation state of xanthophyll cycle pigments after ABA treatment was significantly higher than that observed after DMSO treatment ($p = 0.006$). On the other hand, no significant changes in the de-epoxidation state were observed in the *abi3tko* mutant and the *snrk2qko* mutant ($p = 0.205$ and $p = 0.498$, respectively) after ABA treatment compared to that after DMSO treatment (Fig. 4b). These results indicate that ABA controls the de-epoxidation state of xanthophyll cycle pigments and that this process is both ABI3- and SnRK2-dependent.

Discussion

SnRK2 mediates ABA-dependent NPQ by regulating LHCSR protein and xanthophyll cycle pigments

In this study, we investigated the regulation of NPQ under abiotic stress conditions. To this end, we applied exogenous ABA, which is known to induce various abiotic stress responses to *P. patens* (Sakata et al. 2009). Our results demonstrate that ABA treatment enhances the capacity of NPQ in *P. patens* (Fig. 1). The absence of a decrease in Fv/Fm following ABA treatment indicates that ABA did not adversely affect the maximal efficiency or functional stability of PSII. This suggests that the ABA-induced increase in capacity of NPQ is most likely attributable to energy-dependent quenching, rather than photoinhibitory quenching (Murchie and Ruban 2020). In Fig. 1b and c, the enhancement of NPQ capacity following ABA treatment shows a brief lag, with a significant increase observed on day 1, reaching its maximum level. This lag likely reflects the time required for ABA to trigger downstream molecular and physiological acclimation processes. ABA application affected both transcript and protein levels of LHCSR and PsbS, which are essential for thermal dissipation during NPQ and increased the capacity of NPQ. Moreover, ABA treatment also modulates the levels and the de-epoxidation state of xanthophyll cycle pigments, which is another key component of NPQ (Fig. 2) (Murchie and Ruban 2020). Crucially, we identified the core ABA signaling kinase, SnRK2, as being essential for these responses, whereas the downstream ABA-responsive transcription factor ABI3 did not influence *LHCSR* or *PsbS* expression, but

was partially involved in xanthophyll cycle pigments regulation. Thus, our findings revealed a link between exogenous ABA and the enhancement of NPQ capacity in *P. patens*. The observed effects of ABA include an increase in LHCSR and the regulation of xanthophyll cycle pigments, both of which likely contribute to the enhanced NPQ capacity. Additionally, other ABA-induced pleiotropic effects, such as changes in the Calvin cycle, thylakoid architecture, or growth patterns, may also influence the increase in NPQ. Further studies will be required to clarify these ABA-driven mechanisms, including the interplay between LHCSR expression, xanthophyll cycle regulation, and other physiological processes.

The moss, *P. patens* occupies an evolutionary intermediate position in terms of NPQ regulation, as it retains both LHCSR and PsbS proteins. In contrast, green algae rely on LHCSR, while vascular plants depend on PsbS for NPQ. LHCSR and PsbS differ significantly in function: LHCSR binds chlorophyll and xanthophyll pigments such as zeaxanthin, enabling it to directly catalyze energy quenching reactions upon sensing low lumenal pH (Pinnola 2019). By contrast, PsbS does not bind to pigments and instead relies on interactions with pigment-binding proteins to induce quenching (Pinnola 2019). These differences emphasize LHCSR's role in pigment binding and quenching, compared to PsbS's specialized function in pH sensing and structural reorganization. Our findings that ABA treatment enhanced *LHCSR* expression while suppressing *PsbS* suggest that LHCSR plays a predominant role in NPQ regulation under stress conditions in bryophytes. In contrast, vascular plants, which have lost *LHCSR* during evolution, rely on *PsbS* for NPQ. This evolutionary divergence might reflect changes in the promoter architecture of *PsbS*, altering its responsiveness to stress signals. A comparative analysis of *PsbS* promoter sequences between bryophytes and vascular plants could provide valuable insights into this transition and the broader evolutionary dynamics of NPQ regulation.

A potential nexus between ABA signaling and NPQ in terrestrial acclimation

While green algae exhibit NPQ in response to intense light, ABA signaling is thought to have first emerged in bryophytes during the transition to land (Delwiche and Cooper 2015; Komatsu et al. 2013). Our study identifies a connection between exogenous ABA and NPQ regulation, suggesting that this relationship may have facilitated plant acclimation to terrestrial environments. In land habitats, plants face intense and fluctuating light conditions (Gjindali et al. 2021), and ABA-mediated enhancement of NPQ capacity may have played a crucial role in mitigating photodamage under these challenging conditions. This enhanced NPQ capacity, combined with ABA's broader involvement in stress responses, likely contributed to the survival and adaptation of early land plants.

In terrestrial environments, rapid protective responses, such as chloroplast relocation, are essential for preventing photodamage during sudden high-light exposure (Kasahara et al. 2002). However, beyond these immediate mechanisms, ABA's ability to enhance NPQ capacity may have been key to long-term acclimation under prolonged high-light stress. Additionally, ABA's well-established roles in managing drought, heat, and freezing stress provided plants with an integrated strategy to handle multiple environmental challenges (Mahapatra et al. 2024; Mo et al. 2024; Vishwakarma et al. 2017). Since high-light conditions frequently coincide with drought and heat stress, ABA signaling likely supported the ability of early land plants to thrive under complex terrestrial conditions, ultimately aiding their successful colonization of land (Fig. 5).

Evolutionary implications and future directions

Our findings suggest that ABA modulates LHCSR and PsbS levels and regulates xanthophyll cycle pigment composition to enhance NPQ, providing *P. patens* with increased high-light tolerance. This mechanism might have supported the adaptation of early land plants to terrestrial environments. However, whether ABA-dependent NPQ regulation extends to vascular plants remains unclear. Interestingly, exogenous ABA application induces NPQ in the vascular plant, *Zea mays* (Jia and Lu 2003), and this effect is amplified when ABA is combined with drought treatments in *Hordeum vulgare* L. (Skowron and Trojak 2021). These observations suggest that the interplay between ABA signaling and NPQ might constitute a conserved adaptation across land

plants. Understanding the evolutionary conservation of this pathway could shed light on the broader role of ABA in coordinating plant responses to the complex challenges of terrestrial life. Intriguingly, studies in the green algae, *C. reinhardtii* have shown that blue-light and UV-B photoreceptors regulate LHCSR and PsbS, protecting the photosynthetic machinery under stress (Allorent et al. 2016; Petroutsos et al. 2016). Investigating whether ABA signaling interacts with or overlaps with the blue-light or UV-B photoreceptor pathways in *P. patens* could provide deeper insights into the regulatory networks governing NPQ.

In conclusion, our study identifies ABA as a critical regulator of NPQ in *P. patens*, unveiling a previously unrecognized role for ABA in plant acclimation to high light and stress conditions. These findings provide a foundation for further exploration of ABA's influence on photosynthetic efficiency and stress resilience in terrestrial plants, with potential implications for advancing crop resilience and productivity in a rapidly changing climate.

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Competing Interests

The authors declare no competing interests.

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Figure legends

Fig. 1 Effect of exogenous ABA treatment to chlorophyll fluorescence

(a) Changes in non-photochemical quenching (NPQ) under varying PAR intensities following exogenous ABA and DMSO treatment in wild-type *Physcomitrium patens*. **(b)** Change of NPQ in wild-type for 1, 6, 24 hours after ABA treatment. (**c, e, g**) Change of NPQ in wild-type, *abi3tko*, *snrk2qko* for 1, 3, 5 days after ABA or DMSO treatment to 5-day-old *P. patens* protonemata. (**d, f, h**) Change of maximum quantum yield for photochemical reactions (Fv/Fm) after ABA or DMSO treatment to 5-day-old *P. patens* protonemata. Closed circle, triangle, square: ABA treated; open circle, triangle, square: DMSO treated (control). Different letters indicate statistically significant differences as determined by ANOVA with Tukey's HSD post hoc test. n=3 for each experiment. If there are no significant differences in any data points, no indication on the graph.

Fig. 2 Protein and mRNA level change of PsbS and LHCSR

(**a, b**) Immunoblotting and SDS-PAGE of PsbS and LHCSR. RBCL was used as a loading control. 0d indicates just before ABA or DMSO treatment, and 3d indicates days after the treatment. (**c, d**) Quantification of changes in the expression of PsbS and LHCSR proteins. After quantifying the intensity of each band using GelAnalyzer 19.1 software (www.gelanalyzer.com), the difference between 3d and 0d was calculated. (**e, f**) Changes in mRNA levels of *PsbS* and *LHCSR* measured by semi-quantitative PCR after ABA treatment. Different letters indicate statistically significant differences as determined by ANOVA with Tukey's HSD post hoc test Error bar: S.D. n=3 for each experiment.

Fig. 3 Correlation between ABA and xanthophyll cycle pigments

(**a**) Carotenoid biosynthesis pathway from lycopene of *P. patens*. The red dotted rectangle indicates the xanthophyll cycle pigments. (**b-e**) Amount of xanthophyll cycle pigments (zeaxanthin, antheraxanthin, violaxanthin) measured by HPLC after 3 days ABA or DMSO treatment to 3-days-old *P. patens* protonemata. Amount of each pigment were normalized by amount of chlorophyll *a* and chlorophyll *b*. Xanthophyll cycle pigments pool was calculated as violaxanthin + antheraxanthin + zeaxanthin. Different letters indicate statistically significant differences as determined by ANOVA with Tukey's HSD post hoc test. n=3 for each experiment.

Fig. 4 Relation between ABA and de-epoxidation of xanthophyll cycle pigments

(**a**) Epoxidation and De-epoxidation of xanthophyll cycle pigments. (**b**) Proportion of xanthophyll cycle pigments. Cross marks indicate calculated de-epoxidation state of xanthophyll cycle pigments ((Zeaxanthin + 0.5 * Antheraxanthin)/(Violaxanthin + Antheraxanthin + Zeaxanthin)). *p<0.05 at t test, ns: no-significance. n=3 for each experiment.

Fig. 5 Schematic model of NPQ induction started from ABA signaling pathway

ABA synthesis is induced by abiotic stresses in terrestrial environments. The activated ABA signaling pathway induces an increase in NPQ and increases high-light resistance through a complex pathway. In addition, the ABA signaling pathway induces acclimation to environmental stresses such as cold and drought. A combination of these factors may have resulted in the successful terrestrialization of plants. Green arrow represents pathway from results of this research, magenta arrow represents pathway from previous research. ABA: Abscisic acid, PYL: Pyrabactin Resistance 1-Like, PP2C: Protein phosphatase type 2C, SnRK2: SNF1-related protein kinase 2, ABI3: Abscisic acid insensitive 3. [1)]Unknown transcription factors that act downstream of SnRK2 on NPQ induction.

Fig. 1

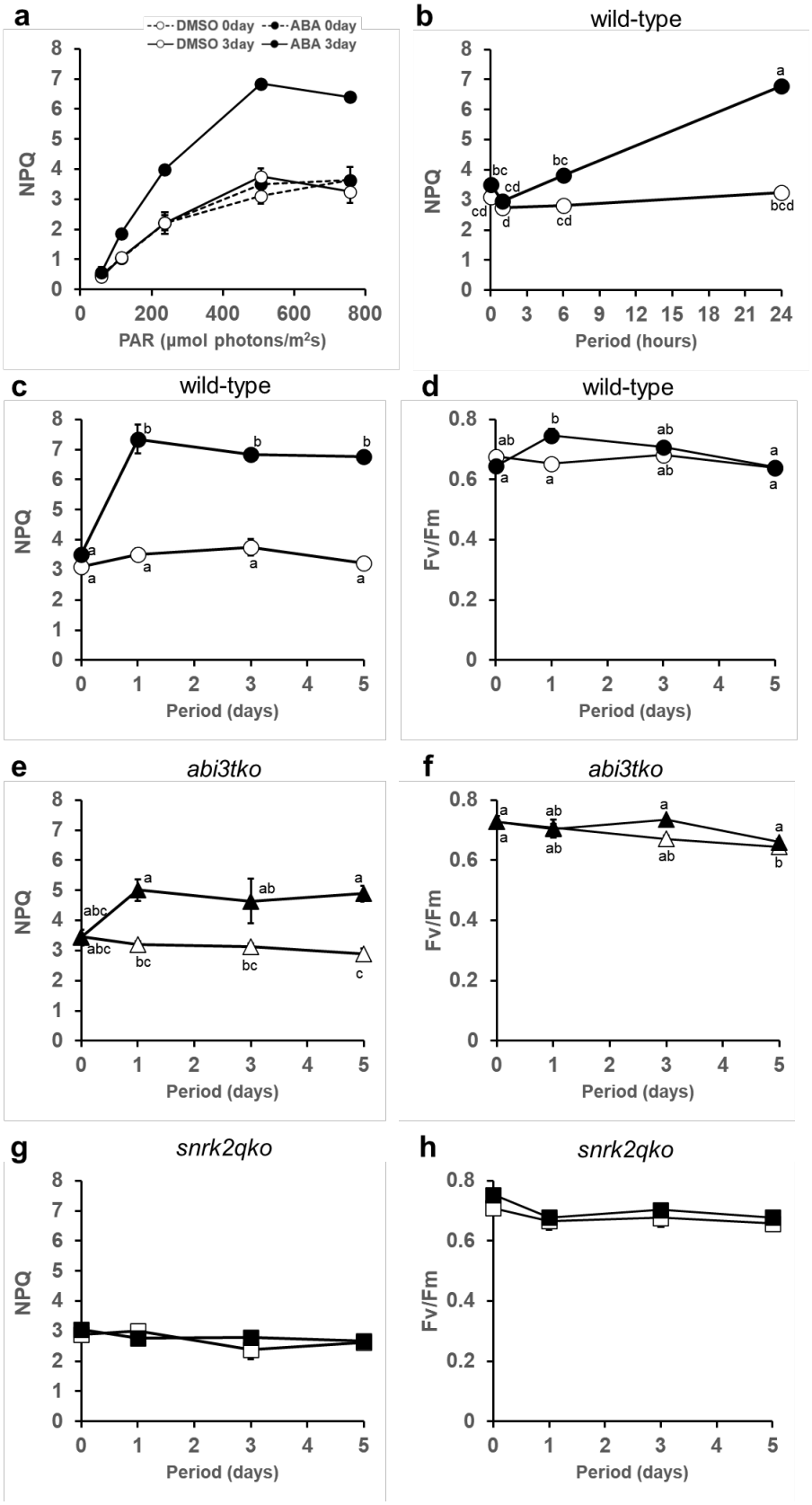
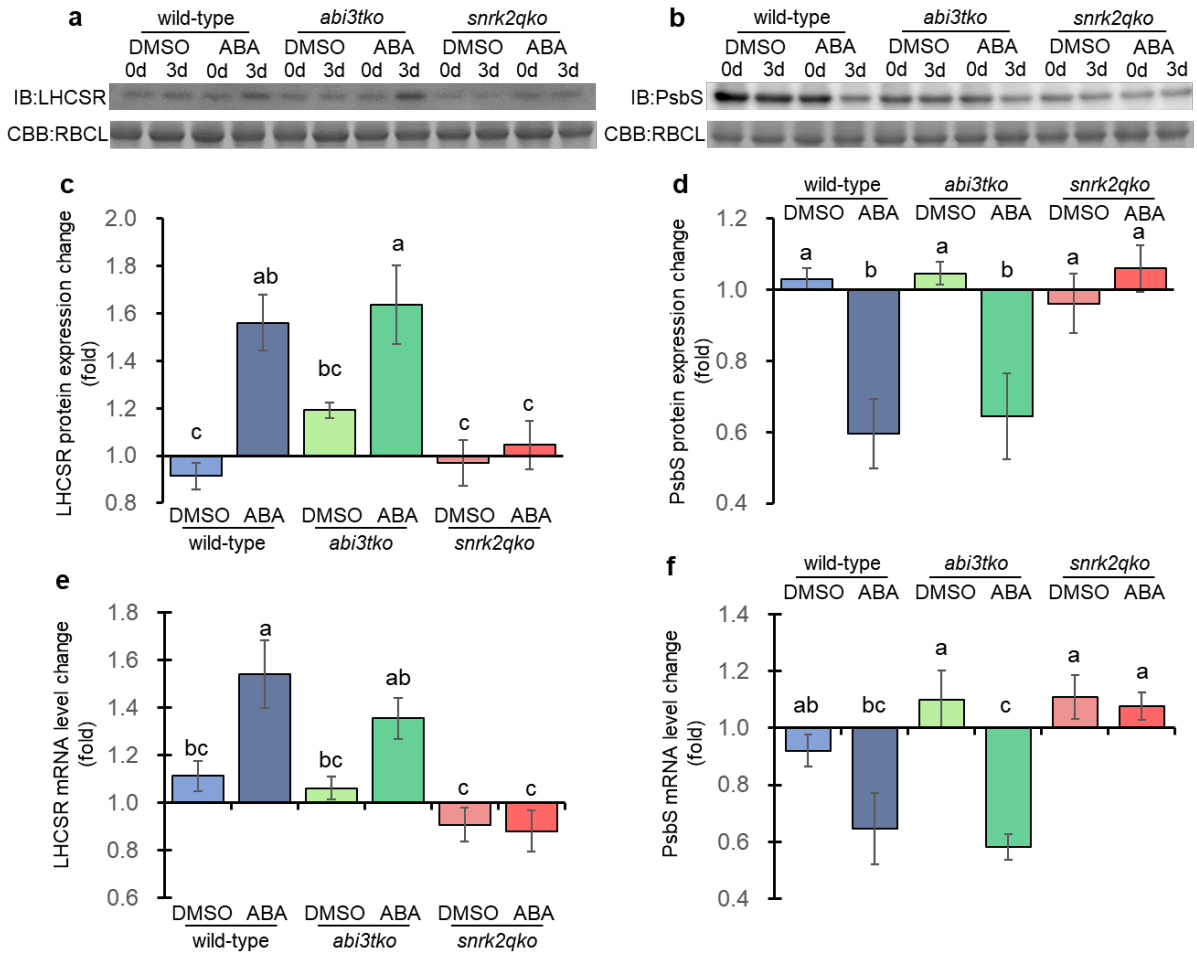

a
DMSO 0day
ABA 0day
DMSO 3day
ABA 3day
NPQ
PAR (μmol photons/m²s)
b
wild-type
NPQ
Period (hours)
c
wild-type
NPQ
Period (days)
d
wild-type
Fv/Fm
Period (days)
e
abi3tko
NPQ
Period (days)
f
abi3tko
Fv/Fm
Period (days)
g
snrk2qko
NPQ
Period (days)
h
snrk2qko
Fv/Fm
Period (days)

Fig. 2


a
wild-type
abi3tko
snrk2qko
DMSO ABA DMSO ABA DMSO ABA
0d 3d 0d 3d 0d 3d 0d 3d 0d 3d 0d 3d
IB:LHCSR
CBB:RBCL
b
wild-type
abi3tko
snrk2qko
DMSO ABA DMSO ABA DMSO ABA
0d 3d 0d 3d 0d 3d 0d 3d 0d 3d 0d 3d
IB:PsbS
CBB:RBCL
c
LHCSR protein expression change (fold)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
c ab bc a c c
DMSO ABA DMSO ABA DMSO ABA
wild-type abi3tko snrk2qko
d
PsbS protein expression change (fold)
1.2
1.0
0.8
0.6
0.4
wild-type abi3tko snrk2qko
DMSO ABA DMSO ABA DMSO ABA
a b a b a a
e
LHCSR mRNA level change (fold)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
bc a bc ab c c
DMSO ABA DMSO ABA DMSO ABA
wild-type abi3tko snrk2qko
f
PsbS mRNA level change (fold)
1.4
1.2
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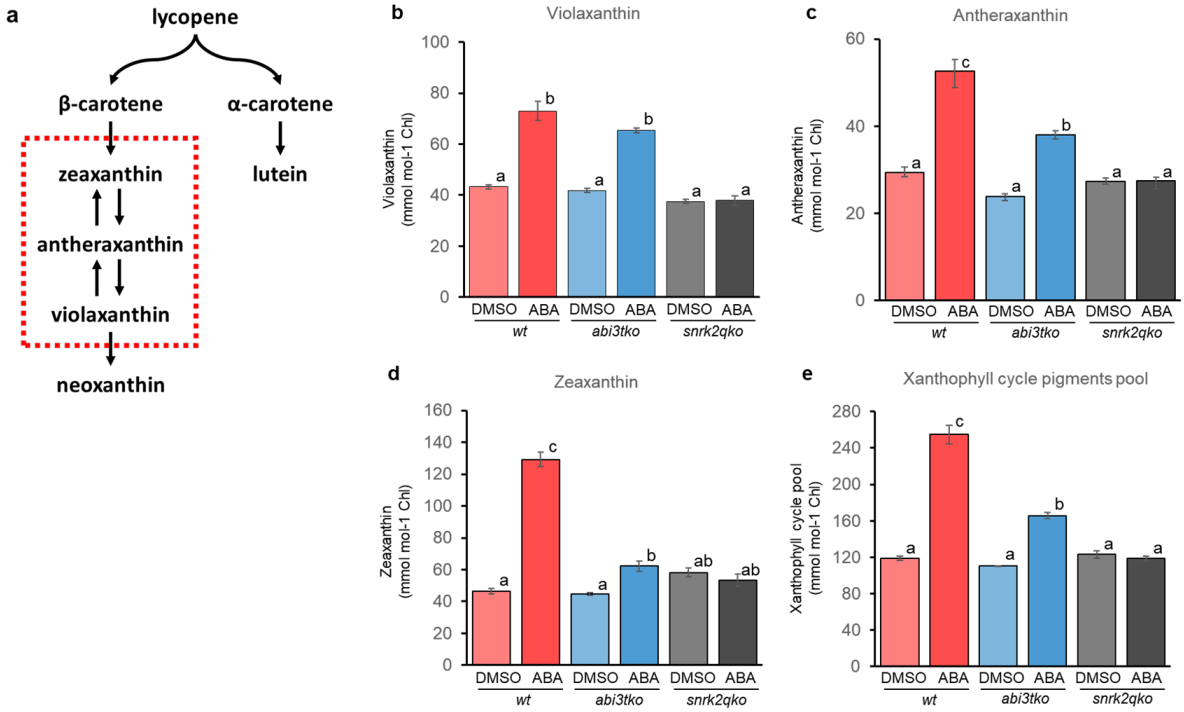
wild-type abi3tko snrk2qko
DMSO ABA DMSO ABA DMSO ABA
ab bc a c a a

603 **Fig. 3**



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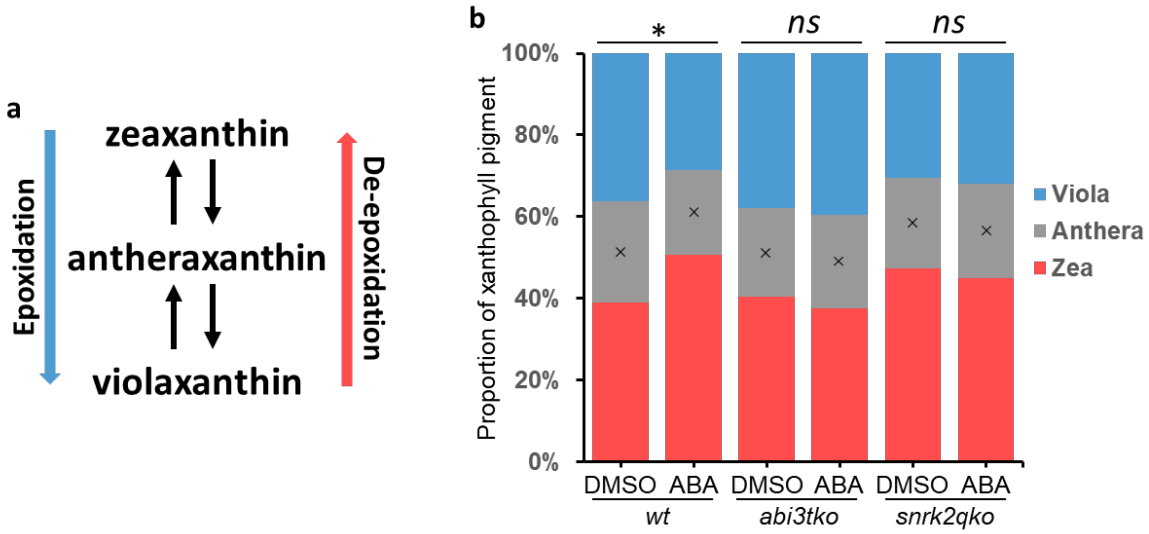
606 **Fig. 4**



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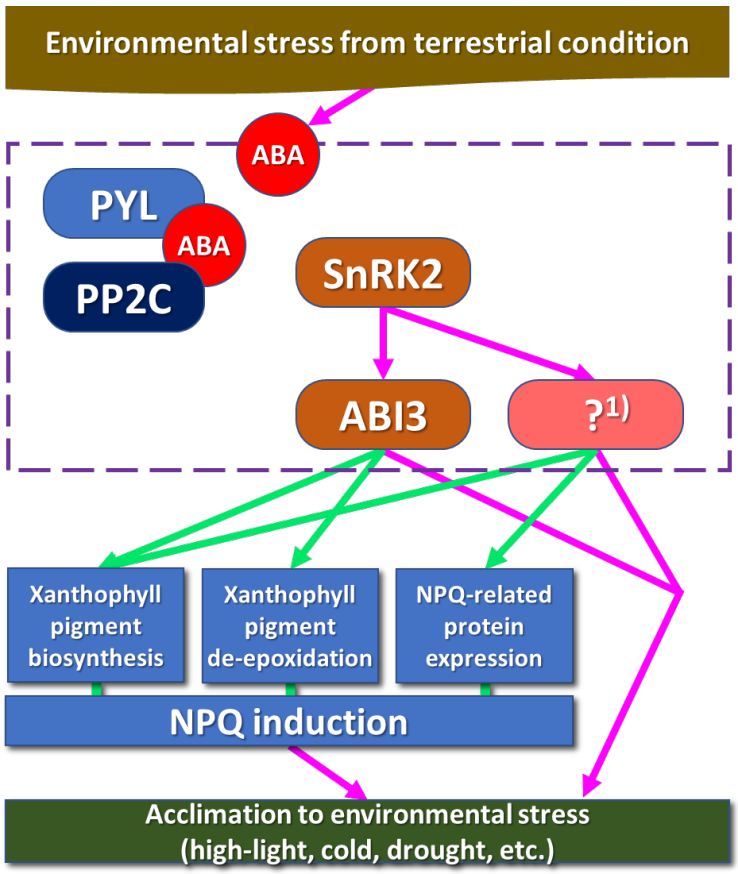
609 **Fig. 5**



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