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Absciscic acid binds to an *Arabidopsis thaliana* phosphodiesterase and tunes its activity

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Growing evidence suggests that plant proteomes contain numerous proteins that specifically bind absciscic acid (ABA). Many of them are complex multidomain proteins where specific ABA-binding can cause biochemical and physiological changes. Here we show that the *Arabidopsis thaliana* K⁺ transporter AtKUP5 contains both a functional cytoplasmic N-terminal adenylate cyclase (AC) enabling the synthesis of 3',5'-cAMP from ATP and a C-terminal phosphodiesterase (PDE) that hydrolyses 3',5'-cAMP to 5'-AMP. We found that ABA binds in a ligand-specific manner to the catalytic center of the PDE thereby causing a reduction of 3',5'-cAMP hydrolysis *in vitro*. The hydrolytic activity of the PDE is ABA concentration-dependent, biphasic and requires the presence of an intact ABA-binding site similar to the one in the canonical Pyrabactin resistance 1/PYR-like/Absciscic acid receptors, with V_{max} of 1.19 pmole min⁻¹ μg⁻¹ in the absence of ABA, increasing to 1.58 pmole min⁻¹ μg⁻¹ at 2 nM ABA, and decreasing to 0.75 pmole min⁻¹ μg⁻¹ at 50 nM ABA. These findings are therefore consistent with a direct role of ABA in PDE activity modulations and form a functional link between 3',5'-cAMP signaling and K⁺ flux. Furthermore, we predict that a growing number of such receptor-like proteins that specifically and directly interact with ABA will be discovered thereby uncovering complex and ancient layers of signaling and metabolic regulation.

KEYWORDS

absciscic acid, AtKUP5, biphasic effect, molecular tuning, phosphodiesterase

Introduction

Absciscic acid (ABA) is a key regulator of many plant responses including seed maturation, germination, stomatal guard cell movement, dormancy, growth and stress responses (Ng et al., 2014). When an ABA receptor was first identified in 2009, the downstream signaling pathway was recognized as the key to ABA-dependent responses

(Nishimura et al., 2010; Park et al., 2009). The model predicts that ABA operates as ligand for PYR/PYL/RCAR receptors (Pyrabactin resistance 1/PYR-like/Abscisic acid receptor). PYR/PYL/RCAR receptors are members of the family of ATPase transport proteins. Binding of ABA to the receptor inhibits protein phosphatases 2C (PP2C) and triggers ABA-dependent downstream signaling including SNF-related serine/threonine-protein kinase (SnRK2) activation. SnRK2 kinases are the primary kinases implicated in the control of stress responses. Transcription factors and other effector proteins are phosphorylated when SnRK2 kinases are activated. ABF (ABRE-binding factor) and MYB, for instance MYB2, 41, 44 and 96, are transcription factors that are activated by ABA, and they then enable ABA-dependent transcriptional programs (Fujita et al., 2011; Munemasa et al., 2015; Ng et al., 2014; Weiner et al., 2010).

An important question that arises is whether or not the PYR/PYL/RCAR receptor can fully account for all ABA-dependent processes. So far, four examples suggest that they may not. The first notable case is the relationship between ABA and mitochondrial adenine nucleotide translocators (ANT) (Kharenko et al., 2011). Through ATP/ADP exchange across the inner membrane of the mitochondria, ANT helps control the rate of ATP synthesis, an essential cellular process since cellular metabolism is inhibited when ANT activity declines (Neuhaus et al., 1997). Notably ABA can bind to ANT transporters thereby modifying their activity (Kharenko et al., 2011). Both native isolated spinach mitochondria and recombinant *A. thaliana* ANT2-containing proteoliposomes showed ANT-dependent ABA uptake with increasing administered ABA concentrations found to stimulate increased ATPase activity in mitochondrial extracts from spinach (Kharenko et al., 2011). A second example is the ABA interaction with Rubisco (Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase). By determining the dissociation constant at 47 nM, it was possible to validate the high affinity of ABA for Rubisco and to identify possible binding sites for ABA using chemical proteomics methods and structural investigations (Galka et al., 2015). Furthermore, it was also suggested that ABA may function as a negative regulator of Rubisco activation since ABA causes a suppression of Rubisco catalytic activity but a considerably higher inhibition of enzyme activation. These findings are consistent with direct ABA-Rubisco interactions where, under specific conditions; ABA can modulate Rubisco activity presumably to ensure plant homeostasis (Galka et al., 2015). A third example is the direct and specific binding of ABA to the Guard Cell Outward-Rectifying Potassium Channel (GORK) which mediates K⁺ efflux from guard cells thereby causing stomatal closure. Whole-cell current-voltage experiments on HEK293 cells transfected with GORK showed that ABA directly increases K⁺ efflux in a non-homologous system and hence in the absence of the PYR/PYL/RCAR receptor (Ooi et al., 2017). It is noteworthy that alterations of critical amino acids in the GORK ABA-binding site, results in an attenuation of the response to ABA-dependent response. A fourth and most recent example is the nitrate level sensing transceptor NRT1.1 which also interacts with ABA in a specific manner thereby integrating the plant nutrient status with ABA-dependent stress signaling (Ma et al., 2025; Wang and Tsay, 2025).

Given that a conserved ABA-binding amino acid motif may identify candidate ABA-binding proteins other than GORK (Wong

et al., 2022), we were interested to see if ABA-binding domains are present in complex regulatory proteins and if so, if we could establish biochemical and physiological functions of such binding. Here we report that the majority of identified plant phosphodiesterases (PDEs) which break down cyclic mononucleotides, notably cAMP and cGMP, to AMP or GMP respectively, harbor ABA-binding motifs in their sequences. This may be indicative for a novel mechanism by which ABA modulates cAMP homeostasis and signaling. We also demonstrate that ABA specifically directly binds to and modulates the hydrolytic activity of a PDE that is part of the *Arabidopsis thaliana* K⁺-Uptake Permease (AtKUP5) and this suggests that AtKUP5 may function as an ABA-responsive protein. Finally, if we accept that PDEs can be considered as an “off” signal in cyclic mononucleotide signaling, then modifying PDE activity with ABA may constitute a novel signaling mode of ABA.

Results

ABA-binding sites are present in plant phosphodiesterases

Previously built databases of *Arabidopsis thaliana* PDEs (Kwiatkowski et al., 2022, 2021) were scanned for the presence of an amino acid ABA-binding motif (Ooi et al., 2017; Wong et al., 2022). This motif consists of four key amino acid residues that form a binding site (Figure 1A) in previously identified and confirmed ABA-binding candidates. Of the 53 plant PDEs, 30 harbor an ABA binding motif often close to or overlapping the catalytic center of the PDE domain (Supplementary Table 1). We selected the *Arabidopsis thaliana* K⁺ uptake permease 5 (AtKUP5; At4g33530) (EC 3.1.4.17) to examine ABA effects on the PDE which is located in the C-terminal cytoplasmic domain. In AtKUP5 the PDE catalytic center (Y669 – E706) overlaps with the ABA-binding site (E657 – K685) (Figures 1A, B). Further analysis of the AtKUP5 structure generated by AlphaFold (Jumper et al., 2021) revealed two possible ABA binding sites as ascertained by the presence of surface grooves or pockets that could spatially accommodate the ligand. Molecular docking showed that both binding sites could dock with ABA, and the ligand assumes binding poses that could potentially form interactions with key amino acids in the ABA motif, notably E657 and Y678, with the former appearing to be in closer distance to the ligand. ABA docked with binding affinities of -4.8 kcal/mol and -4.7 kcal/mol at the respective Y678 and E657 pockets. Such affinities are consistent with other proteins identified by the same ABA motif which are considered to be advantageous as the ligand could be more easily released when concentration decreases and consequently cease allosteric modulation (El-Maslahi et al., 2025; Yu et al., 2026). Since the site of E657 appears to overlap with the putative cAMP binding site of PDE, we performed docking of 3',5'-cAMP at the PDE domain to assess how ABA might interfere with the activity of PDE. 3',5'-cAMP was well accommodated within the PDE catalytic center, with a predicted binding energy of -5.3 kcal/mol. Notably, the presence of ABA at the site of E657 does not seem

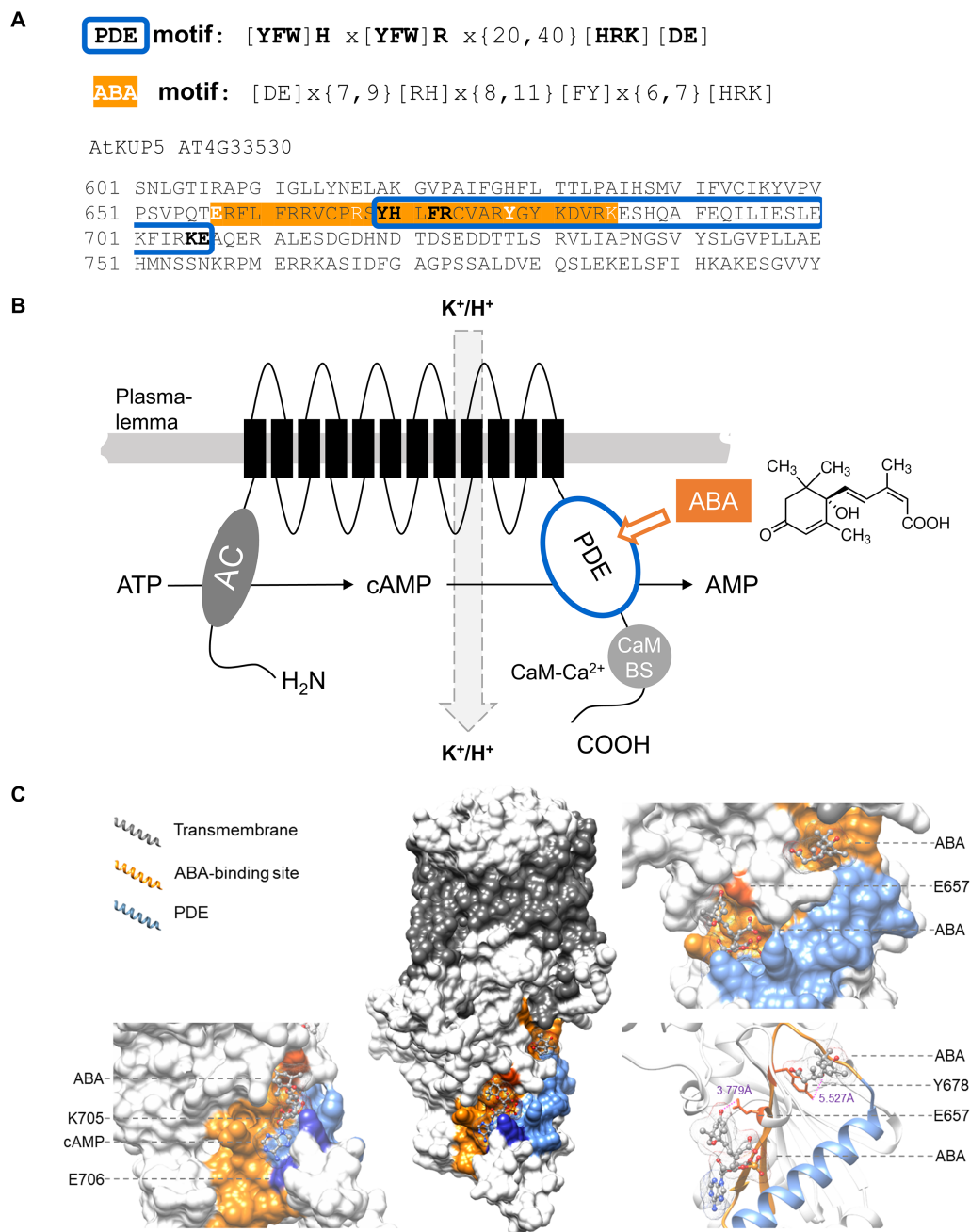


FIGURE 1

Sequence and structural analyses of AtKUP5 ABA binding site. **(A)** Amino acid sequence of AtKUP5, highlighting (orange) the ABA-binding site with the motif key amino acids bolded. Sequence of the PDE motif is framed in blue. **(B)** Architectural schematic of AtKUP5 for the dampening function of PDE in K^+ transport regulation, illustrating the N-terminal AC center that is regulated by K^+ ions to generate cAMP, a substrate for C-terminal PDE that is activated by the Ca^{2+} -calmodulin complex in CaM BS (calmodulin binding site) or altered allosterically by ABA. **(C)** A three-dimensional model of AtKUP5 docked with ABA and cAMP. Molecular docking studies indicate that AtKUP5 could dock with ABA at two sites, forming interactions with key amino acids E657 and Y678 in the motif. The presence of ABA in the pocket of E657, may lead to a tighter fit of cAMP at the PDE domain. Structural assessment and image preparation were performed with AutoDock Vina (version 1.1.2) and UCSF Chimera.

to cause steric hindrance that might prevent the interaction of 3',5'-cAMP at the overlapping PDE domain (Figure 1C).

ABA concentration-dependent effects on PDE activity

PDE catalytic activity was evaluated by enzymatic tests with 3',5'-cAMP as the substrate in the presence or absence ABA. A

sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) approach was used to identify and quantify the AMP products where we observe 5'-AMP formation, indicating hydrolysis of only the 3'5'-cAMP isoform (Figure 2A). The results of the *in vitro* reaction showed that the hydrolytic activity of PDE changes depending on the concentration of ABA in the reaction mix (Figure 2B). The PDE activity alters between concentrations of 2 to 50 nM ABA. The highest amount of cAMP hydrolysis was

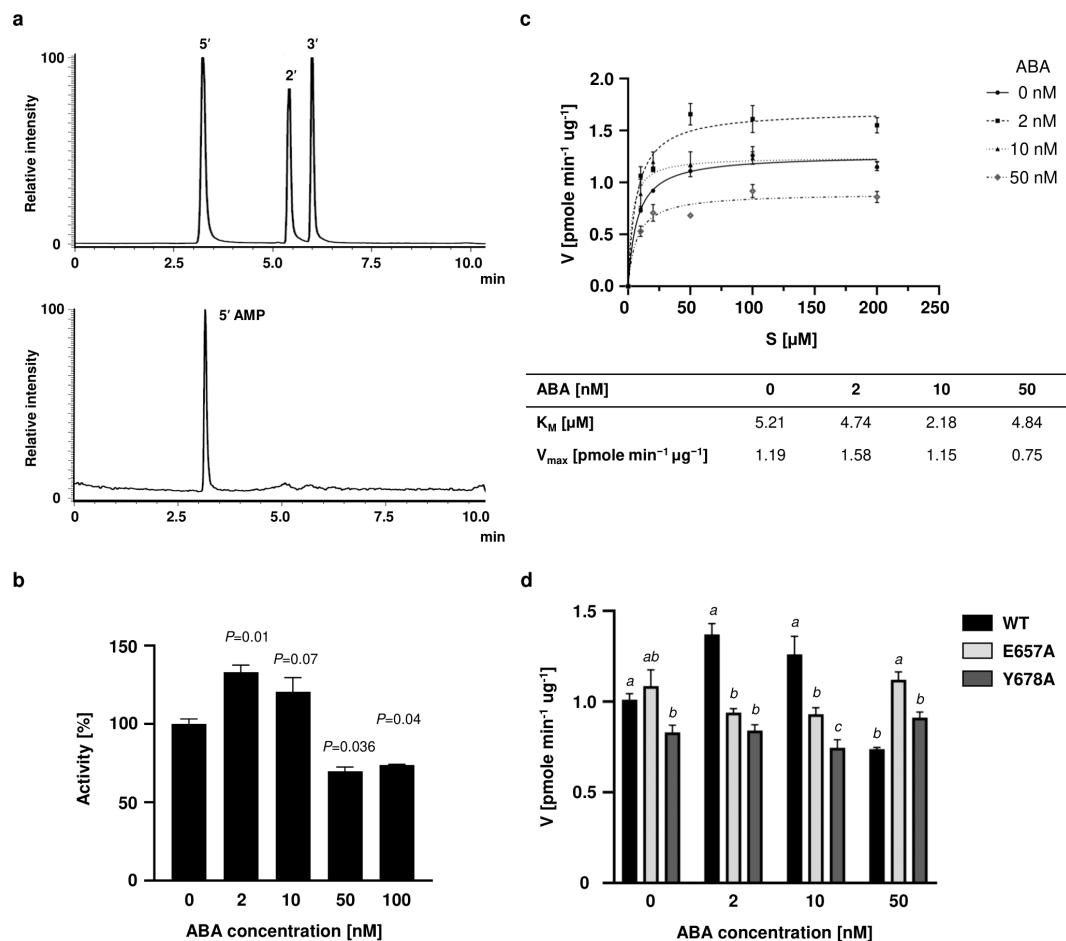


FIGURE 2

Effect of abscisic acid on the modulation of PDE activity. (A) Substrate specificity of AtKUP5 PDE. The upper panel shows the retention time of the separation of a standard of the three AMP isoforms. The lower panel shows the result of the separation of the PDE reaction using the substrates 2'3'-cAMP and 3'5'-cAMP. Only the 5'-AMP isoform is visible in the chromatogram, indicating hydrolysis of only the 3'5'-cAMP isoform. (B) PDE activity of AtKUP5 at different ABA concentrations. Data are mean values ($n = 3$) and error bars show the standard error of the mean. Statistical analysis was performed by Student's *t*-test for independent samples with different variances. $P < 0.05$ indicates that the difference between groups is statistically significant. (C) Michaelis-Menten plot for PDE activity towards cAMP substrate in the presence of different ABA concentration. The inset shows table with a comparison of the kinetic parameters of the reaction with ABA at concentrations ranging from 0 to 50 nM. Data are mean values ($n = 3$) and error bars show the standard error of the mean. (D) Effect of single point mutations E657A and Y678A on PDE activity in the presence of different ABA concentrations. Data are mean values ($n = 3$) and error bars show the standard error of the mean. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test. Significant differences between variants at each ABA concentration are indicated by different letters ($p < 0.05$).

observed at 2 nM ABA, where phosphodiesterase activity - and consequently AMP levels - increased by more than 30% compared to the control (in the absence of ABA) (Figure 2B). Hydrolysis appears inhibited at ABA concentrations of 50 nM and above translating to a decrease in activity of about 30% as compared to the control (Figure 2B). The kinetic parameters of the reaction at the different concentrations of ABA indicates that ABA, even at 50 nM, appears to alter the apparent kinetic parameters of the enzyme compared to the control reactions (K_M of 5.21 μ M) (Figure 2C). As an example, in the presence of 10 nM ABA the calculated K_M value is approximately twofold lower than in the control reactions (K_M of 2.18 μ M). However, given the variability at lower substrate concentrations, this apparent change in K_M should be interpreted cautiously. The maximum reaction rate increases only in the presence of 2 nM ABA. Further increases in ABA concentration

result in a $> 35\%$ reduction in the apparent V_{max} . While Ca^{2+} -calmodulin ($\text{Ca}^{2+}/\text{CaM}$) complexes enhance PDE activity of AtKUP5 as previously reported (Kwiatkowski et al., 2021), the addition of ABA results in the absence of an additive effect upon combined treatment with $\text{Ca}^{2+}/\text{CaM}$ and 2nM ABA, and even a small decrease in the PDE activity is observed (Supplementary Figure 1). This suggests a potential interplay between these modulatory factors and that ABA may modulate PDE activity through an allosteric mechanism.

Since increases in ABA inhibit PDE activity *in vitro*, the level of endogenous 3'5'-cAMP was determined in *E. coli* induced to express AtKUP5 and consequently more PDE is present in cells (Supplementary Figure 2). AtKUP5 expression leads to a decrease in endogenous cAMP levels compared to the control, which is more enhanced in the presence of 2 nM ABA versus 100 nM ABA.

Mutation of amino acids in the ABA-binding domain affect interactions with PDE

The 3D model and docking analyses revealed that ABA may dock in two pockets with varying affinities, potentially forming interactions with key amino acids in the ABA motif, notably E657 and Y678, with the former appearing to be in closer distance to the ligand (Figure 1C). Therefore, we substituted both these amino acids with alanine obtaining AtKUP5^{E657A} and AtKUP5^{Y678A}. The mutants were tested for PDE activity in the presence or absence of ABA (2 to 50 nM). *In vitro* reactions showed that the addition of ABA, unlike in the case of the wild type AtKUP5, did not significantly affect the course of the reaction, consistent with the predicted essential functions of E657 and Y678 in ABA binding (Figure 2D). In contrast to the wild-type protein, ABA treatment of cells expressing AtKUP5^{Y678A} does not result in a statistically significant change in cAMP levels relative to the control variant, although a modest increase in cAMP levels can still be observed (Supplementary Figure 2).

To further characterize the protein-ABA interaction, fluorescence spectroscopy was employed. ABA (0 nM to 16 nM) was added to both the wildtype and the mutated proteins (Figure 3). AtKUP5 exhibits a clear peak of fluorescence emission at 333 nm originating from tryptophan residues upon an excitation at 280 nm. While the maximal emission wavelength remains constant, the intensity of the fluorescence rises (or decreases in the case of the E657A mutant) as the ABA concentration increases (Figures 3A–C). To account for ligand depletion under tight-binding conditions, the binding data were fitted using a quadratic Morrison equation (see Materials and Methods section for details). In the mutants with altered ABA-binding pockets the change in fluorescence intensity is much smaller, as reflected by the estimated binding constants (Figure 3D). The estimated binding constant (K_a) for wild-type AtKUP5 is $19.27 \pm 3.94 \times 10^8 \text{ L} \cdot \text{mol}^{-1}$, while for AtKUP5^{E657A} is $4.63 \pm 1.44 \times 10^8 \text{ L} \cdot \text{mol}^{-1}$ and for AtKUP5^{Y678A} is $2.03 \pm 0.62 \times 10^8 \text{ L} \cdot \text{mol}^{-1}$. These values correspond to estimated K_d values in the sub-nanomolar to low nanomolar range (approx. 0.5–4.9 nM). This suggests that ABA-binding to the AtKUP5^{E657A} mutant is reduced four-fold while the reduction for AtKUP5^{Y678A} is almost ten-fold. While the titration range was partially constrained by fluorescence detection limits at sub-nanomolar concentrations, these constants provide a robust estimate of the relative affinity differences. ABA-specific binding is further supported by the observation that only residual binding with indole acetic acid (IAA) or jasmonic acid (JA) was detected (Supplementary Figure 3). ABA binds to AtKUP5 with a substantially higher affinity ($10^9 \text{ L} \cdot \text{mol}^{-1}$) than JA and IAA, for which association constants in the $10^6 \text{ L} \cdot \text{mol}^{-1}$ range were obtained.

Discussion

The discovery of a conserved ABA-binding amino acid signature in many proteins suggested that ABA-signaling might be more complex and intricate than hitherto suspected. The first indication

of widespread ABA-binding to target proteins came when GORK was shown to bind ABA and that this binding is causative for modulations of the K^+ -flux through the channel (Ooi et al., 2017). The amino acid motif appears to be highly conserved and present not just in the canonical ABA receptor but also in > 200 candidate ABA-binding proteins in the *Arabidopsis thaliana* proteome (Wong et al., 2022). Among these are a number of annotated PDEs (Supplementary Table 1) and proteins with dual or multiple activities (e.g. adenylate cyclase (AC) and PDE) (Kwiatkowski et al., 2022; Turek et al., 2024). This may suggest that by affecting either the AC or PDE activity or both, such dual activity proteins can modify 3',5'-cAMP homeostasis and signaling and hence operate as 3',5'-cAMP tuners. Such dual or indeed multifunctional proteins are also referred to as moonlighting proteins (Irving et al., 2012; Wong et al., 2015) that are increasingly recognized as critical signaling modules that can integrate multiple second messengers such as Ca^{2+} and 3',5'-cAMP and 3',5'-cGMP (Irving et al., 2012; Muleya et al., 2014; Świeżawska-Boniecka et al., 2021). Interestingly, sequence analysis indicates that motif derived from the proposed ABA-binding signature can be identified in a substantial fraction of PDE sequences across bacteria, fungi and animals (Supplementary Table 2). However, the presence of such sequence motifs alone does not necessarily imply a conserved functional role. In AtKUP5, the ABA binding motif is positioned within a structurally defined pocket that partially overlaps with the PDE catalytic center and enables ABA-dependent modulation of activity. In contrast, in non-plant PDEs, these motif-containing sequences may not be part of a compatible spatial configuration, may be structurally buried, or may lack the spatial arrangement required for ligand binding. However, given that ABA-binding motifs occur in more than half of the PDEs in non-plant organisms and the widespread occurrence of ABA across bacteria, fungi, and animals (Hartung, 2010; Magnone et al., 2020), it may be speculated that ABA-dependent modulation of enzymatic activity may represent an evolutionarily ancient regulatory mechanism rather than a plant-specific innovation.

Here we show that AtKUP5 which contains functional AC (Al-Younis et al., 2018) and PDE catalytic centers (Kwiatkowski et al., 2021) also harbors an ABA-binding motif close to the PDE center. Structural analysis combined with molecular docking studies revealed that the ABA-binding motif spans multiple pockets within the PDE catalytic center and points to essential roles for E657 and Y678 in ABA-PDE interaction. Since substitution of these residues with alanine resulted in a significant loss of ABA-dependent modulation of PDE activity (Figure 2) as well as interactions (Figure 3), the ABA pockets might therefore confer allosteric regulation or in the case of the pocket at E657 orthosteric regulation, where the presence of ABA causes a tighter fit of cAMP that favors hydrolysis (Figure 1C). High ABA concentrations could potentially interfere with substrate binding or alter the conformation of the catalytic pocket, and this might account for the biphasic effect observed in our study. Taken together, these findings not only validate AtKUP5 as an ABA-responsive protein but also demonstrate that the ABA-binding motif is an effective tool for the discovery of ABA-binding candidates.

In vitro experiments demonstrated that 3',5'-cAMP-specific PDE activity is modulated by ABA in a biphasic manner. At low

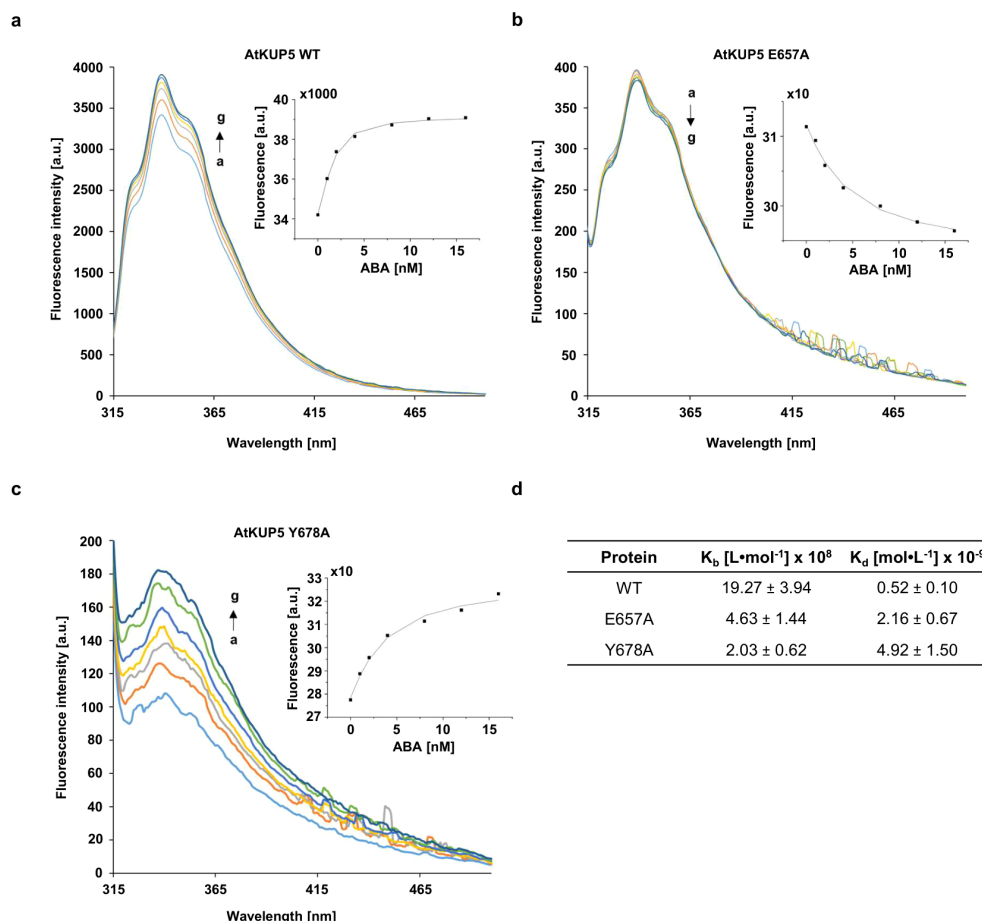


FIGURE 3

The fluorescence spectra of AtKUP5 wildtype and mutants in the presence of ABA. (A) The fluorescence enhancement spectra of AtKUP5. (B) The fluorescence enhancement spectra of AtKUP5^{Y678A}. (C) The fluorescence quenching spectra of AtKUP5^{E657A}. The concentration of recombinant protein was 2 nM and treatments were at 22°C. The concentration of ABA ranged from a to g were 0, 1, 2, 4, 8, 12, and 16 nM, respectively. (D) Comparison of kinetic parameters of interactions between ABA and AtKUP5 wildtype and mutants, K_b – binding constant, K_d – dissociation constant.

ABA concentrations (2–10 nM), there is a notable increase in hydrolytic activity, whereas higher concentrations (50 nM and above) there is inhibition (Figure 2A). It is conceivable that ABA can prevent signal attenuation at higher concentrations to maintain cellular sensitivity to stimuli possibly by preventing cAMP binding to the active site. Alternatively, ABA may promote cAMP hydrolysis at low concentrations to tune downstream reactions. This pattern is consistent with known hormone dose–response relationships, where low concentrations promote signaling or activity, while higher concentrations trigger negative feedback or receptor saturation effects (Umezawa et al., 2010). At low nanomolar concentrations (2–10 nM), ABA likely promotes the active conformation of the PDE complex. Such concentrations are within the physiological range of ABA reported in well-watered plants (Wilkinson and Davies, 1997), and may serve as a signal to modulate enzyme activity under basal conditions. The decline in V_{max} observed at higher ABA concentrations reflects a shift toward an inhibitory mechanism which may result from allosteric inhibition or conformational destabilization of the catalytic domain. High ABA levels are known to trigger feedback loops and downstream signaling cascades that modulate the sensitivity and output of ABA-responsive proteins (Fujita et al., 2011). Interestingly, the K_M values

obtained in this study are lower than those reported for PDE3 enzymes from non-plant systems. Several factors may contribute to this discrepancy, which may influence the biological significance of ABA binding. First, the kinetic parameters were determined using a truncated recombinant form of AtKUP5 (cytosolic C-terminus) expressed in *E. coli*, which lacks post-translational modifications and the full-length structural context that may influence substrate binding. Second, plant PDEs such as AtKUP5 appear to differ structurally from classical PDE families described in animals. Canonical PDE3 enzymes are characterized by a conserved catalytic core containing a well-defined metal-binding pocket, typically coordinating metal ions, as well as invariant residues involved in cyclic nucleotide recognition and stabilization (Maurice et al., 2014). In contrast, sequence and structural predictions for AtKUP5 suggest that, although a functional catalytic motif is present, several residues typically involved in substrate coordination in PDE3 enzymes are not strictly conserved or may adopt different spatial arrangements. In addition, the PDE activity in AtKUP5 is embedded within a multidomain membrane protein, rather than forming a dedicated soluble catalytic domain. This architectural difference may impose distinct conformational constraints on the catalytic pocket, potentially affecting substrate accessibility and

binding geometry (Al-Younis et al., 2018; Kwiatkowski et al., 2021). These structural differences may contribute to the altered substrate affinity observed in our assays.

Biphasic responses are not entirely uncommon (Agathokleous et al., 2019; Caraty et al., 1989; Li et al., 2022, 2017). Characteristics of dose responses have recently been reviewed and reevaluated (Agathokleous et al., 2019). It appears that perhaps contrary to expectations, linear dose-responses are not the default response to hormones but the biphasic dose-response is. This phenomenon is termed hormesis and may point the beneficial effects of basal level stress for optimum health. Hormesis has been shown to occur in many species and it appears that the low stress responses are a precondition or priming point which in turn is essential to survive otherwise lethal massive stresses (Ling et al., 2018).

There is now a growing body of evidence for ABA-protein interactions that function outside of the classic PYR/PYL/RCAR-PP2C-SnRK2 signaling pathway and the direct ABA effect on AtKUP5 PDE function shown here is a point in case. Direct effects of ABA on protein function was also previously reported in other proteins, namely Rubisco (Galka et al., 2015) and GORK (Ooi et al., 2017). In AtKUP5 and GORK the ABA effect could be attributed to the presence of conserved and specific ABA-binding amino acid motif. In contrast, the effect of ABA on Rubisco in *Pisum sativum* did not appear to be dependent on the presence of the strict [DE].[7,9][RH].[8,11][FY].[6,7][HRK] motif. Crystallographic analysis suggested that ABA might interact with the large subunit of Rubisco and at a site distinct from the active site, a classic example of allosteric modulation. Interestingly, it was also suggested that a possible interaction could occur with arginine, glutamic acid, and tyrosine, hence the residues that are part of the ABA-binding motif described here. Although it has been shown that ABA binds to the non-activated form of the enzyme, ABA might still act as an inhibitor of Rubisco activation. However, only mutagenesis of the key amino acids indicated in the two sites might eventually elucidate the physical nature of these ABA effects. Incidentally, the ABA-binding motif [DE].[7,9][RH].[8,11][FY].[6,7][HRK] is present in the Rubisco activase (At2g39730, Supplementary Figure 4). The Rubisco activase removes the inhibition of phosphorylated compounds that bind to both carbamylated and non-carbamylated Rubisco active sites thereby modulating Rubisco activity in response to temperature and light variations. These inhibitory substances, such as RuBP, are removed by Rubisco activase using energy from ATP hydrolysis, restoring Rubisco catalytic activity (Andersson, 2008). The ABA-binding motif in this enzyme is located in the region spanning the Box VII' and Sensor 2 domains at position 343–368, i.e. in the region responsible for substrate recognition and conformational remodeling of Rubisco (Portis et al., 2008). Given that ABA has been demonstrated to interact with Rubisco, it is conceivable that Rubisco activase and ABA cooperate and that ABA functions as an effector molecule that can either stabilize or destabilize the interaction between Rubisco and Rubisco activase. Additionally, ABA and Rubisco activase may interact to decrease Rubisco activity when photosynthetic efficiency is impaired, directing resources and energy toward stress responses.

The role of cyclic nucleotide monophosphates (cNMPs) in ABA signaling is well established (Al-Younis et al., 2021; Liang et al., 2022), however, most attention is paid to the processes in which ABA participates in signaling rather than to the description of specific signaling components and their mechanisms of action (Świeżawska-Boniecka and Szmidt-Jaworska, 2023). There are known ABA- downstream responses that require cAMP or cGMP e.g. stomatal movement, seed germination or the production of isoflavones under UV-B stress in soybean (Jiao and Gu, 2019; Jin and Wu, 1999; Liu et al., 2023). It is noteworthy that ABA and the recently identified nucleotide cyclases are in some instances closely linked. One such case is the essential chloroplast enzyme involved in ABA production, the *A. thaliana* 9-cis-epoxycarotenoid dioxygenase 3 (AtNCED3). AtNCED3 is both essential for the synthesis of ABA in the carotenoid pathway and synthesis of cAMP since it functions as an AC (Al-Younis et al., 2021). The maize ZmRPP13-like protein 3 (ZmRPP13-LK3) is a heat-induced potential disease-resistance protein found in mitochondria that also functions as an AC (Yang et al., 2021). Under heat stress, ABA increases both cAMP levels and the transcriptional activity of the ZmRPP13-LK3. *In vivo* investigations revealed that cAMP may bind to the ZmABC2 that operates as a potential cAMP exporter. It appears that cAMP contributes to the resilience of maize to heat stress and functions as a downstream component of the ABA-mediated stress response. Direct interactions between ABA and the K⁺ transporter AtKUP5 are consistent with mechanisms that link ABA and cNMPs and ion homeostasis. The control of cellular ion balance is influenced by ABA (MacRobbie, 2000) and AtKUP5 might well function as cAMP-dependent K⁺-flux sensor (Al-Younis et al., 2018) where ABA modulates cAMP generation which in turn is directly dependent on K⁺ flux. Moreover, members of the KT/HAK/KUP potassium transporter family show stress-regulated expression and are implicated in abiotic stress responses, including via modulation by ABA (Yang et al., 2020). ABA, as a central stress hormone, triggers cytosolic Ca²⁺ elevations, which, together with calmodulin, is an activator of AtKUP5 (Kwiatkowski et al., 2021). The observed effects of ABA on PDE activity are concentration-dependent and suggest a more complex mode of regulation than simple independent action from Ca²⁺/CaM. The non-additive behavior observed upon combined treatment with low ABA concentration is consistent with a model in which ABA acts as an allosteric modulator, potentially influencing a regulatory or conformational step that is also targeted by Ca²⁺/CaM (Supplementary Figure 1). This suggests that ABA functions as an upstream stress signal that suppresses cAMP degradation, thereby modulating cyclic mononucleotide signaling under stress conditions. In addition, AtKUP5 function *in planta* has been studied in knockout lines and overexpression lines where it was shown that deletion of AtKUP5 causes both decreased K⁺ uptake and reduced levels of cellular 3',5'-cAMP as well significantly impaired root elongation (Al-Younis, 2018). To elucidate the specific effects of ABA on AtKUP5-dependent K⁺ uptake and its dependence on the AtKUP5-PDE, mutants with altered ABA-binding sites will have to be tested. Such mutant analyses will also allow testing of the hypothesis that (stress-induced) ABA surges inhibit PDE activity

and hence cause increases of cAMP, and this in the wildtype only. This hypothesis seems to be valid for *in vivo* tests using *E. coli*, where in the presence of various ABA concentrations we determined the level of endogenous cAMP in lines overexpressing AtKUP5 and the ABA-binding mutant AtKUP5^{Y678A} (Supplementary Figure 2). In the case of AtKUP5 protein overexpression, and consequently an increase in the amount of PDE in cells, a decrease in the endogenous cAMP level is observed compared to the control, which is even more enhanced when ABA is added at a concentration of 2 nM. Simulation of stress by incubating cells with 100 nM ABA inhibits PDE activity, equalizing the cAMP level to the initial conditions. In turn, overexpression of the ABA-binding PDE mutant results in a decrease in the level of endogenous cAMP during induction, but we did not observe fluctuations in the level of cAMP hydrolysis after the addition of different concentrations of ABA, as was the case with the AtKUP5 protein, thus reflecting the performed *in vitro* assays.

In summary, we present further support for the notion that, first, specific ABA-binding can occur in a number of proteins and that this binding can exert distinctive biochemical and physiological effects on the target proteins. Given that ABA does occur in lower plant, fungi and animals (Gharib et al., 2024; Ng et al., 2014), it makes this hormone an ancient regulator of numerous biological responses and processes.

Materials and methods

Structural analysis of ABA binding site in AtKUP5

The structural prediction and docking were essentially conducted as detailed in (Zhou et al., 2021). The AtKUP5 model was obtained from AlphaFold (Jumper et al., 2021), available at <https://alphafold.ebi.ac.uk/entry/Q8LPL8>. The ABA and PDE binding sites were evaluated based on the presence of surface grooves or pockets that could spatially accommodate the ligand. Docking studies were conducted with KUP5 as the “receptor” and ABA or cAMP as the “ligand”. The generated binding affinities, ligand binding poses, molecular distances and interactions with key amino acids at the ABA binding sites were assessed. Molecular docking studies were conducted using AutoDock Vina (version 1.1.2) (Trott and Olson, 2010) and structural visualization and image preparation were performed using UCSF Chimera (Pettersen et al., 2004).

PDE biochemical assay and LC-MS/MS analysis

Recombinant PDE domain of AtKUP5 was prepared as described in the Supplementary methods. PDE *in vitro* activity was determined by using LC-MS/MS to determine the rate of AMP formation. The reaction mixture contained: 3 mM Tris-HCl (pH 7.4), 0.01 to 0.2 mM cAMP, 0.1% (v/v) 2-mercaptoethanol, 5 µg of recombinant protein (Supplementary Methods), 0.5 mM MgCl₂ and MnCl₂, and 2 to 100

nM ABA. Samples were incubated at 37 °C for 20 min. The enzyme reaction was terminated by incubation at 100 °C for 5 min and the samples were centrifuged at 13,200 × g for 10 min.

LC-MS/MS experiments were performed using the Nexera UHPLC and LCMS-8045 integrated systems (Shimadzu Corporation, Kyoto, Japan). The ionization source parameters were optimized in positive ESI mode using pure AMP dissolved in HPLC grade water (Sigma, St. Louis, MO, USA). The samples were separated using a Discovery HS C18 column (100 × 2.1 mm, 5 µm, Sigma, St. Louis, MO, USA). A gradient of solvent A (0.05% (v/v) formic acid with 5-mM ammonium formate) and solvent B (100% (v/v) acetonitrile) was applied over 3 min: B: 0–5%, followed by washing and conditioning of the column with a flow rate of 0.4 mL/min. The interface voltage was set at 4.0 kV for positive (ES+) electrospray. Data acquisition and analysis were made with the LabSolutions workstation for LCMS-8045.

Fluorescence and interaction studies

The fluorescence spectra of proteins in the absence and presence of ABA were performed on a JASCO FP-8300 spectrofluorometer with a 10 mm quartz cell (Hellma Analytics, Müllheim, Germany). Measurements were recorded in the range of 300–600 nm after excitation at λ = 280 nm at 22 °C. The final solutions of ABA, IAA and JA were prepared in phosphate buffered saline (PBS). The samples were prepared in 1.5 mL Eppendorf tubes, and they contained proteins (wild type, E657A, and Y678A) at a concentration of 2 nM without or with ABA at the following concentrations: 1, 2, 4, 8, 12, and 16 nM, and PBS at pH 7.4. The fluorescence data were fitted by applying a nonlinear least-squares regression using OriginPro software Version 2016 (OriginLab Corporation, Northampton, MA, USA). To analyze the changes in fluorescence intensity upon the formation of the protein-ligand complex, we used the following quadratic equation to account for ligand depletion under tight-binding conditions:

$$\frac{[PL]}{[P]_t} = \frac{[P]_t + [L]_a + K_d - \sqrt{([P]_t + [L]_a + K_d)^2 - 4[P]_t [L]_a}}{2[P]_t}$$

where K_d is the dissociation constant, $[P]_t$ is the concentration of protein, and $[L]_a$ is the total concentration of the ligand.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

MK: Formal Analysis, Investigation, Methodology, Writing – original draft. AK-P: Methodology, Writing – review & editing. CB:

Methodology, Writing – review & editing. AW: Formal Analysis, Methodology, Writing – review & editing. KJ: Writing – review & editing. HI: Writing – original draft. CG: Project administration, Supervision, Writing – original draft.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author AW declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2026.1809056/full#supplementary-material>

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