Discovery of 2,6-Dihalopurines as Stomata Opening Inhibitors: Implication of an LRX-mediated H$^+$-ATPase Phosphorylation Pathway

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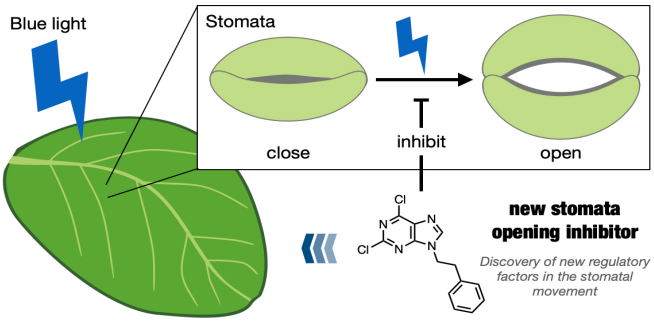
ABSTRACT: Stomata are pores in the leaf epidermis of plants and their opening and closing regulate gas exchange and water transpiration. Stomatal movements play key roles in both plant growth and stress responses. In recent years, small molecules regulating stomatal movements have been used as a powerful tool in mechanistic studies, as well as key players for agricultural applications. Therefore, the development of new molecules regulating stomatal movement and the elucidation of their mechanisms have attracted much attention. We herein describe the discovery of 2,6-dihalopurines, AUs, as a new stomatal opening inhibitor, and their mechanistic study. Based on biological assays, AUs may involve in the pathway related with plasma membrane H$^+$-ATPase phosphorylation. In addition, we identified leucine-rich repeat extensin proteins (LRXs), LRX3, LRX4 and LRX5 as well as RALF, as target protein candidates of AUs by affinity based pull down assay and molecular dynamics simulation. The mechanism of stomatal movement related with the LRXs–RALF is an unexplored pathway, and therefore further studies may lead to the discovery of new signaling pathways and regulatory factors in the stomatal movement.



INTRODUCTION

Plant stomata, consisting of a pair of guard cells, forming pores in leaf epidermis of plants, play a key role in the regulation of gas-water exchange between the plant and the atmosphere to conduct photosynthesis and transpiration.[1] Stomata have been shown to respond to a host of environmental conditions including temperature, water availability, light intensity and CO_2 levels in leaves.[1,2,3] They have also been shown to respond to multiple plant hormones, namely abscisic acid (ABA), auxin, cytokinin, ethylene and jasmonic acid.[3,4,5] Control of stomatal movements (opening/closing) have long been considered to be critical not only to increase biomass and drought tolerance in crops,[6,7,8] but also to decrease global warming by reduction of carbon dioxide.[6,9] The movement of stomata happens quickly, in as little as 10 mins to an hour in response to ABA,[10,11,12] a hormone produced in response to drought,[3] highlighting their significance as a first-line defense against environmental changes around a plant. Furthermore, stomatal movements have central roles in plant growth and other stress responses, attracting much attention, especially for agriculture purposes.

Genetic studies on transgenic plants have indicated that controlling stomatal movement is useful for farming applications. For example, under optimum moisture conditions, genetically altered Rice and Arabidopsis that had increased stomatal aperture, promoted plant growth to increase biomass yield.[6] On the other hand, under moderately dry conditions, genetically altered wheat that had decreased stomatal aperture, reduced transpiration to confer drought tolerance.[7,8] However, such genetic approaches permanently alter the physiological characteristics of plants and are thus not suitable for rapid or temporary treatments. Additionally, the creation of genetically altered plants takes time and is not popular from a societal point. Therefore, in recent years, a small-molecule approach has been used as a complement to genetic studies. The advantage of such an approach requires no prior genetic alteration and can be used against multiple plant species.

Previous small molecule studies for stomatal movements include those by Park *et al.* who synthesized a selective ABA agonist, pyrabactin,[13] which allowed the identification of ABA receptors. Furthermore, the fungal toxin fusicoccin (FC), a plasma membrane (PM) H$^+$-ATPase activator, was employed to reveal a regulatory mechanism of PM H$^+$-ATPase, an enzyme important for the stomatal opening.[14] In addition, tyrphostin 9[15] and tautomycin[16,17], inhibitors of Raf-like protein kinase, blue light-dependent H$^+$-ATPase phosphorylation (BHP), and type 1 protein phosphatase (PP1) respectively, were utilized in the discovery that these proteins are involved in signal transduction from phototropin to PM H$^+$-ATPase. Such studies not only yielded stomatal movement controlling molecules, but were also able to identify novel proteins involved in stomatal

movement pathways. This underpins the power that small molecules have as tools for both research and agriculture.

Recently, inhibitors for agriculture have focused on compounds targeting receptor(s) of the plant hormone ABA, which induces stomatal closing. The ongoing development of ABA agonists indicates that the synthetic ABA agonists have a great potential to improve water use efficiency in plants.[18,19,20,21] However, in this approach, there is a possibility to induce undesired side effects caused by overlap from ABA-mediated processes such as inhibition of seed germination or fruit ripening. Therefore, the development of stomatal movement inhibitors targeting non-ABA receptor/pathways is highly called for.

Recently phenotypic screening in our group successfully identified several new types of stomatal closing compounds (**SCL1**–**SCL9**).[22] While the detailed mechanism of these SCL compounds is not yet clear, it has been shown that they do not affect the ABA signaling pathway. Further phenotype-based screening studies at our institute led to the discovery of non-ABA-targeting stomatal opening inhibitors (PI[23] and SIM[24] compounds).

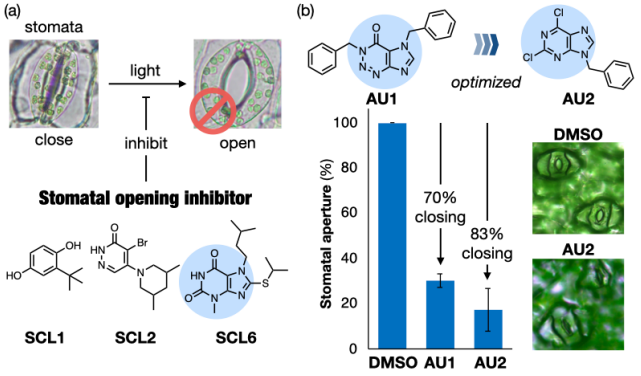


Figure 1. Stomatal opening inhibitors. (a) The first-generation non-ABA-targeting stomatal opening inhibitors (SCLs). (b) The stomatal opening inhibitors (AUs) developed in this work. The inhibitory effect of the compound (50 μM) on light-induced stomatal opening is evaluated relative to the mock (DMSO) treatment in each experiment and is presented as mean ± SD (n = 1; 60 stomata per 4 leaf discs per replicate).

Small molecules regulating the stomatal movement are transformative plant chemical biology tools to help elucidate molecular mechanisms related to stomatal movement, as well as key players in agricultural applications. In parallel to exploring stomata chemical biology,[22,25] our group has developed a series of plant growth stimulants based on 2-azahypoxanthine which are known as "fairy chemicals".[26] Inspired by the structural similarity of **SCL6** with our previous fairy chemicals, we decided to investigate the effect of our fairy chemicals with regards to stomatal movements (Figure S1). As expected, it was discovered that one of the derivatives **AU1**, reported as a plant growth inhibitor, inhibits stomatal opening (Figure 1). In addition, a further second-round structure-activity relationship (SAR) study revealed that 2,6-dichloropurine derivative **AU2**, which is easier to synthesize than 2-azahypoxanthine derivatives, showed activity the same as **AU1** (Figure S2/S3). We herein describe SAR studies and a detailed mechanistic study of **AU2**, as well as a pull-down assay and molecular dynamics (MD) simulation of AUs which identified leucine-rich repeat extensin proteins (LRXs) as well as RALF as candidate target proteins. Our results indicate a potential new role for this class of proteins as well as implicating new signaling pathways and regulatory factors in the stomatal movements of multiple plant species.

RESULTS AND DISCUSSION

SAR study of AU derivatives. To investigate functional sites of **AU2** responsible for the stomatal-opening inhibition effect, we performed a SAR study. As shown in Figure 2a, **AU2** can be easily synthesized by the S_N2 reaction of commercially available 2,6-dichloropurine with benzyl bromide in the presence of potassium carbonate. The alkylation proceeded at the *N*7 and *N*9 positions to afford **AU2'** and **AU2**, respectively (substitution positions were determined by the ^{1}H-NMR chemical shift of hydrogen atom at the C8 position[27]). The activity of **AU2'** was similar to **AU2**. These two compounds (**AU2**/**AU2'**) did not influence the viability of guard cells in *C. benghalensis*, because epidermal fragments that had been incubated

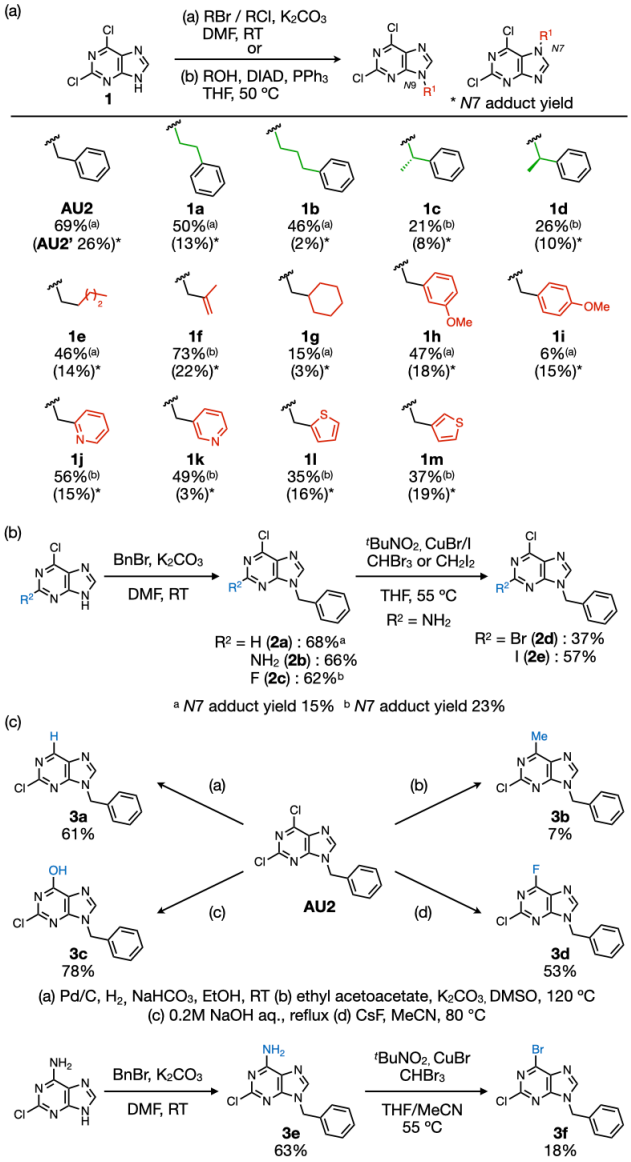


Figure 2. Synthesis of AU2 derivatives. (a) The synthesis of *N*-alkylated derivatives. (b) The synthesis of C2-derivatized compounds. (c) The synthesis of C6-derivatized compounds.

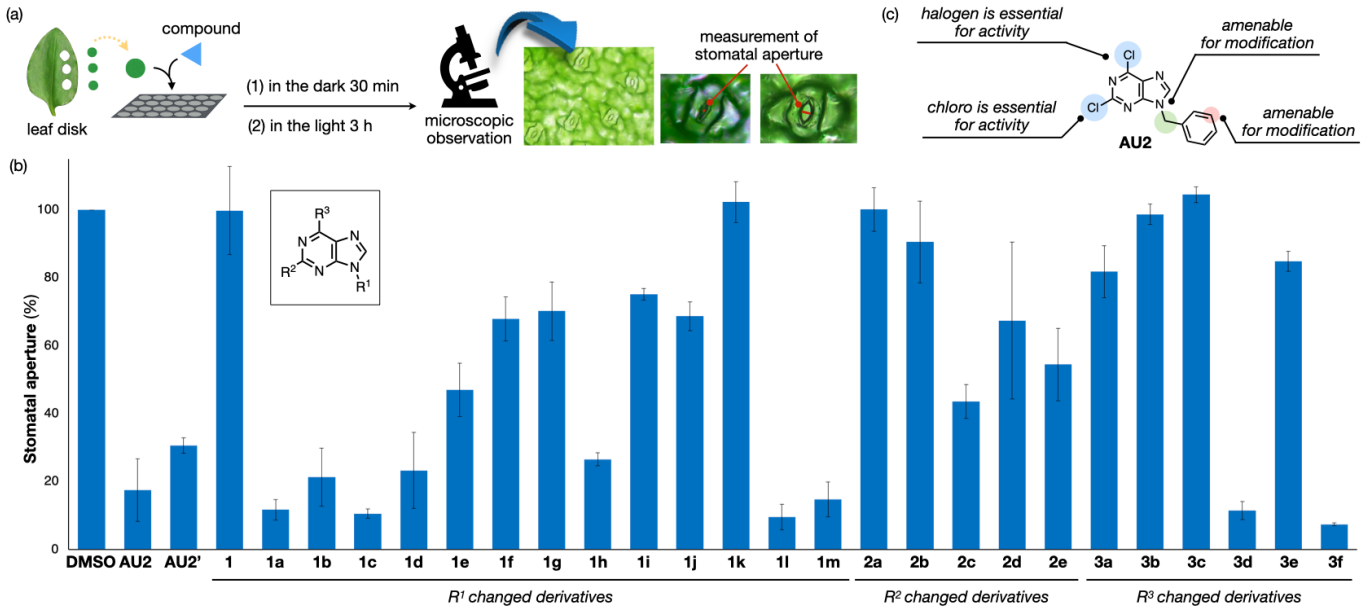


Figure 3. Evaluation on the effect of **AU2** derivatives in stomatal aperture. (a) The method of the stomatal opening assay using *C. benghalensis*. (b) The activity of **AU2** derivatives. The inhibitory effect of the compound (50 μM) on light-induced stomatal opening is evaluated relative to the mock (DMSO) treatment in each experiment and is presented as mean ± SD (n = 3; 60 stomata per 4 leaf discs per replicate). (c) Summary of SAR study of **AU2** derivatives.

with and without compounds for 4 h were stained similarly by fluorescein diacetate (FDA) (Figure S4). As depicted in Figure 2a, by altering alkylation reagents, several *N*9-alkylated compounds **1a**, **1b**, **1e**, **1g**–**1i** were synthesized (method a), and **1c**, **1d**, **1f**, **1j**–**1m** were prepared by Mitsunobu reaction[28] of 2,6-dichloropurine and the corresponding alcohol reagent (method b). Modification of the C2 position commenced with the alkylation of the corresponding starting materials to obtain **2a**–**2c** in moderate to high yields (Figure 2b). The thus-obtained **2b** was converted to **2d** and **2e** in 37% and 57%, respectively, under Sandmeyer reaction conditions.[29] The C6-modified compounds **3a**–**d** were synthesized by derivatization of **AU2** (Figure 2c). For example, Pd-catalyzed dechlorination of **AU2** proceeded to afford **3a** in 61% yield.[30] Introduction of ethyl acetoacetate to **AU2** in an S_NAr manner followed by decarboxylation and deacetoxylation furnished a methylated compound **3b** in low yield.[31] Hydroxylation of **AU2** proceeded to afford **3c** in 78% yield.[32] **3d** was synthesized by halogen exchange using CsF in 53% yield.[33] In addition, 6-chloroadenine was reacted with benzyl bromide to obtain **3e** in 63% yield. The thus-obtained **3e** was converted to a brominated compound **3f** under the Sandmeyer reaction condition.

Following our previously reported AI-based screening method,[22] we analyzed the effect of synthesized **AU2** analogues on inhibition of stomatal opening in *C. benghalensis* (Figure 3a). First, we excised leaf discs from dark-adapted *C. benghalensis* leaves, and incubated the samples with basal buffer containing the test compounds (50 μM) in a multi-well plate under light for 3 h. Then, we qualitatively assessed the stomatal aperture under a microscope and quantified stomatal aperture diameter by capturing images. When subjecting *N*-unprotected 2,6-dichloropurine (**1**), there was no inhibition effect in the stomatal opening, which clearly indicates that *N*-substituent is required for the activity. Analogues having phenethyl group (**1a**, also referred as **AU3**), phenylpropyl group (**1b**), or isopropylbenzyl groups (**1d** and **1e**), instead of benzyl group were also found to be active. The non-aromatic *N*-substituted compounds having pentyl group (**1e**), methallyl group (**1f**), and methylcyclohexyl group (**1g**) were found to be less active. The addition of a methoxy group to the phenyl group on the benzyl moiety at *para* position decreased activity (**1i**), while the addition at the *meta* position (**1h**) had a moderate effect. While *N*-pyridylmethyl substituted compounds (**1j** and **1k**) have low or no activity, the addition of thienylmethyl moiety to the *N*9 position (**1l** and **1m**) induced comparable activity to **AU2**. With regard to a substituent at the C2 position, removal of a chlorine atom caused a complete loss of activity (**2d**). Addition of amine or other halogen atoms led to a decrease in activity (**2a**–**2c**, **2e**). C6-methyl, hydroxy or amino substituted analogues **3d**–**3f** had low or no activity, while fluoro or bromo substituted ones were found to be highly active (**3d** and **3f**). Together, the SAR analysis indicated that the C2-chloro and C6-halogen are essential for activity, and the *meta* position of the phenyl group is amenable for modification (Figure 3c). In addition, we confirmed the activity of **AU3**, one of the derivatives with highest activity, in model plant *A. thaliana* leaf (Figure S5).

Mechanistic study. The mechanism of stomatal movement has only recently begun to become clear, yet there are still many unknowns. One of the mechanisms induced by blue light is shown in Figure 4.[1,3] Signaling of stomatal opening begins with exposing blue light to guard cells to activate phototropin, a photoreceptor.[34,35,36] A protein kinase BLUS1 is phosphorylated through binding with the activated phototropin.[37] The signal is then transmitted to a Raf-like kinase BHP, type 1 protein phosphatase (PP1) and its regulatory subunit PRSL1,[15,16,17] resulting in the phosphorylation of the penultimate residue, threonine, at the C-terminus of the PM H^+-ATPase.[38] The phosphorylated PM H^+-ATPase is activated by the binding of a 14-3-3 protein to the C-terminus of the PM H^+-ATPase, causing a

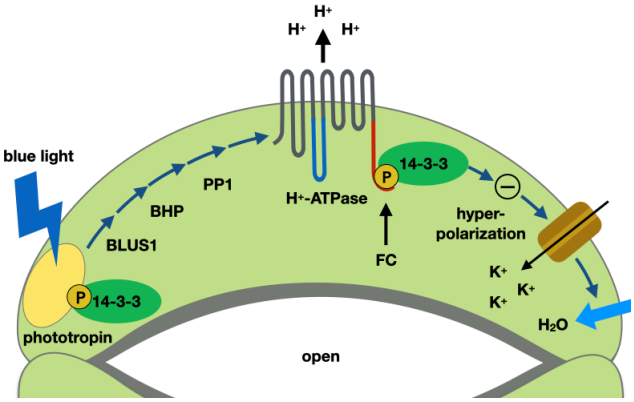


Figure 4. The mechanism of stomatal opening induced by blue light/FC.

negative intracellular potential. To counteract the potential, the concentration of potassium ions in guard cells increases via inward-rectifying potassium channels,[39] and subsequently, the influx of water to the guard cells for regulating their osmotic pressure causes increasing the volume of the cells. This H$^+$-ATPase-mediated cascade induces stomatal opening, because the guard cells have thin outer walls and thick inner walls, expanding outward at their thin walls.

To clarify the mode of action of AUs in guard cells, we first investigated the effect of **AU3**, on fusicoccin (FC)-induced stomatal opening (Figure 5). FC is a natural product that irreversibly stabilizes the interaction between a H$^+$-ATPase and a 14-3-3 protein, maintaining the activity of the downstream potassium channel.[40] Therefore, the addition of FC can promote stomatal opening even under dark condition. **AU3** did not inhibit FC-driven stomatal opening, indicating that **AU3** does not affect the pathway downstream of the PM H$^+$-ATPase.

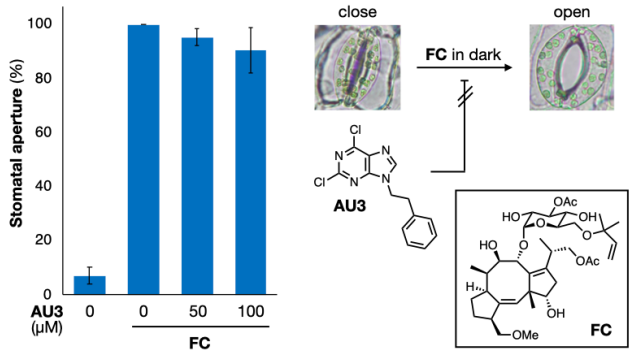


Figure 5. The effect of **AU3** on FC-induced stomatal opening. The activity of **AU3** was evaluated as in Figure. 3b.

Next, we investigated the effect of **AU3** on blue light-induced phosphorylation of PM H$^+$-ATPase in guard cells of *A. thaliana* by immunohistochemistry (Figure 6a). The phosphorylation of PM H$^+$-ATPase was assayed using an antibody against its phosphorylated penultimate threonine.[41] Interestingly, **AU3** inhibited blue light-induced phosphorylation of PM H$^+$-ATPase. This result clearly supports that **AU3** suppresses light-induced stomatal opening by inhibiting blue light-induced activation of the PM H$^+$-ATPase in the guard cells.

The activating pathway of PM H$^+$-ATPase is found not only in guard cells but also in mesophyll cells.[42] Therefore, we also investigated the effect of **AU3** on the activation of H$^+$-ATPase in the mesophyll cells by Western blotting analysis using antibodies against the conserved catalytic domain of AHA2 (anti-H$^+$-ATPase) and phosphorylated penultimate threonine (anti-pThr) for evaluation of phosphorylation level of PM H$^+$-ATPase (Figure 6b).[41,43] Although the phosphorylation of the PM H$^+$-ATPase in the mesophyll cells is induced by white light irradiation or the addition of sucrose,[42,44,45,46,47] **AU3** suppressed their phosphorylation level. To summarize these results, **AU3** may inhibit the phosphorylation of PM H$^+$-ATPase through a common signaling pathway in guard and mesophyll cells (Figure 6c).

It was reported that ABA inhibits light-induced stomatal opening and blue light-dependent phosphorylation of the penultimate residue in PM H$^+$-ATPase.[6,41] Therefore, it is possible that **AU3** suppresses light-induced stomatal opening by affecting the ABA signaling pathway. We investigated the effect of **AU3** on ABA-receptor, such as germination of *A. thaliana* seeds.[48] ABA completely suppressed germination, but **AU3** at a concentration of 50 μM did not (Figure S6).

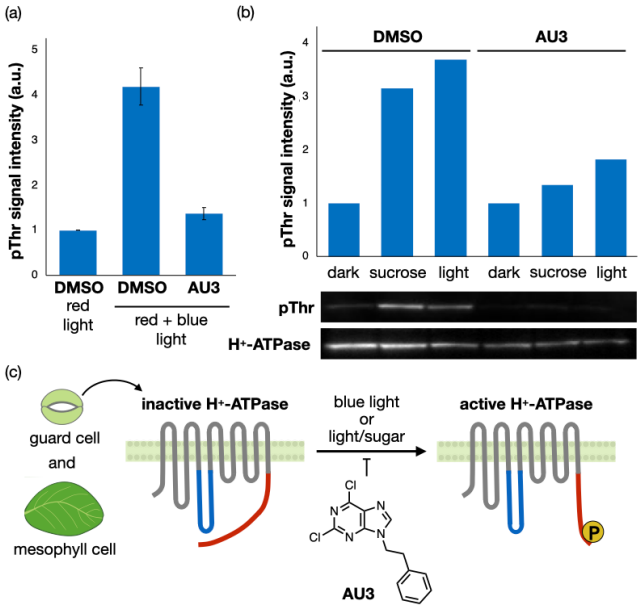


Figure 6. The effect of **AU3** on the phosphorylation of PM H$^+$-ATPase. (a) The immunohistochemical assay by using guard cells of *A. thaliana*. Values are presented as mean ± SD (n = 3; 50 stomata in each experiment). (b) The Western blotting by using the mesophyll cells of *A. thaliana*. The analysis using antibodies against the conserved catalytic domain of AHA2 (anti-H$^+$-ATPase) and phosphorylated penultimate threonine (anti-pThr) Representative data from an experiment replicated three times with different biological samples is shown. (c) The activity of **AU3** to inhibit the phosphorylation of PM H$^+$-ATPase indicated by the results shown in (a) and (b).

Pull-down assay. As the point of action of AUs could be narrowed down, we next set out to identify the target proteins of **AU3** for understanding a more detailed mechanism by using an affinity-based pull-down assay. On the basis of the SAR data (Figure 3), we decided to attach a triethylene glycol linker to the *meta* position of the phenyl group on **AU2** to synthesize azide-containing **AU4** (see SI for the synthesis of **AU4**). Fortunately, **AU4** retained significant stomatal opening inhibition activity at 100 μM and therefore was used as a precursor of the molecular probe (Figure 7a). To prepare an agarose

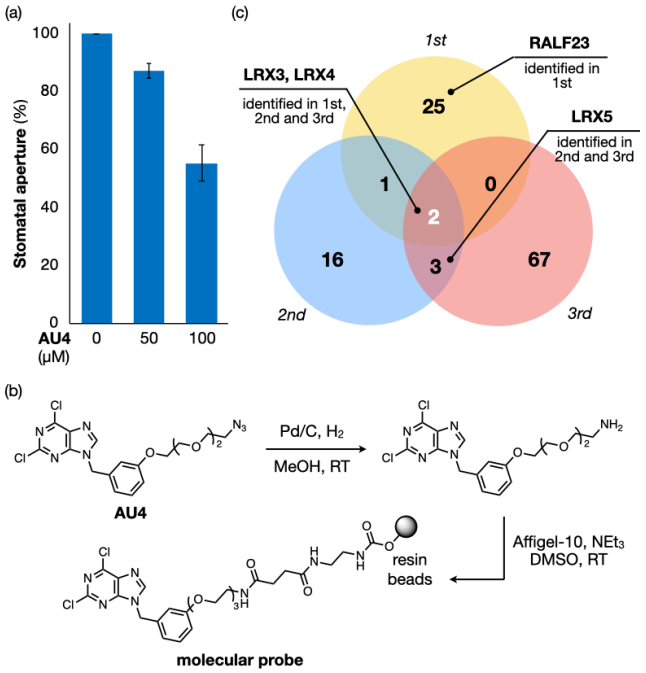


Figure 7. Pull-down assay by using molecular probe. (a) The effect of a precursor of affinity probe, **AU4** on light-induced stomatal opening. The assay was performed as described in Figure. 3b. (b) The synthesis of affinity probe. (c) The result of pull-down assay. The number of identified proteins in the Venn diagrams.

conjugate of **AU4**, reduction of the azide group of **AU4** by Pd/C under a hydrogen atmosphere followed by condensation with Affigel10 (BioRad) was conducted to synthesize the molecular probe (Figure 7b). Proteins from *A. thaliana* mesophyll cell lysates interacting with the agarose conjugate of **AU4** were affinity-purified and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The pull-down assays were performed three times in a competitive manner using **AU3** as a competitor (Figure S7), showing the number of identified proteins in the Venn diagrams (Figure 7c). Leucine-rich repeat extensin protein (LRX) 3 and 4 were identified as target protein candidates in each of the three pull-down assays, and other LRX family protein LRX5 was also identified twice. The LRX proteins (LRX1–LRX11) are known as extracellular regulators of cell wall formation attached to the cell wall.[49,50,51] In particular, LRX3–LRX5 are expressed mainly in main root and shoot, and are found to be involved in the regulation of plant growth and salt stress tolerance.[52,53] Although their detailed functions and interactions with other proteins have been uncovered,[54,55] their involvement in the stomatal movement mechanism has not been reported. To prove that LRX3–LRX5 are involved in the stomatal movement mechanism, we searched for expression of LRX3–LRX5 in guard cell in a public microarray database eFP browser[56] (Figure S8). It was found that LRX3–LRX5 are more expressed in guard cells than other LRX family proteins. Thus, we performed a stomatal opening assay using an Arabidopsis lrx3 lrx4 lrx5 triple knockout mutant (lrx345)[52], and found that stomatal aperture of the knockout mutant is reduced compared to the wild type (Figure S9). The effect of **AU3** was not abolished in the lrx345 mutant, but rather was slightly enhanced compared to the wild type. This result implies that the redundancy of LRX family proteins possibly compensated for their function. In fact, there are reports that overexpression of LRX1 in the lrx345 mutants compensated for the function of the LRX3–LRX5.[57]

Molecular dynamics simulations. To support that LRX3–LRX5 are the target proteins of AUs, we employed a computational approach, namely molecular docking and molecular dynamics (MD) simulations. This approach first predicts the interaction site of the protein with **AU3** by molecular docking simulations, and then checks whether the resulting complex is stable by MD simulation. Since the crystal structures of the LRX3–LRX5 are not available, the simulations were performed using the co-crystal structure of rapid alkalinization factor 4 (RALF4) with a substructure of LRX2, a family protein with high homology to LRX3–LRX5 (PDB ID: 6QXP)[58]. The MD simulations showed that **AU3** did not form a stable complex against LRX2 alone (SI movie1) or RALF4 alone (SI movie2), but surprisingly, it could continuously bind at the same site against the LRX2-RALF4 complex during a long MD simulation (1 μs) (SI movie3). These results suggest that the **AU3** interacts at the boundary of the LRX2-RALF4 complex. Indeed, the resulting structure clearly indicate that **AU3** binds to the pocket formed by RALF4 (blue) and LRX2 (red) (Figure 8a). Figure 8b shows the binding conformation of **AU3** at 500 ns and 1 μs obtained from the MD simulation, where the purine moiety of **AU3** is oriented like a π-π stacking interaction with the side chain of Tyr1066 of RALF4. This

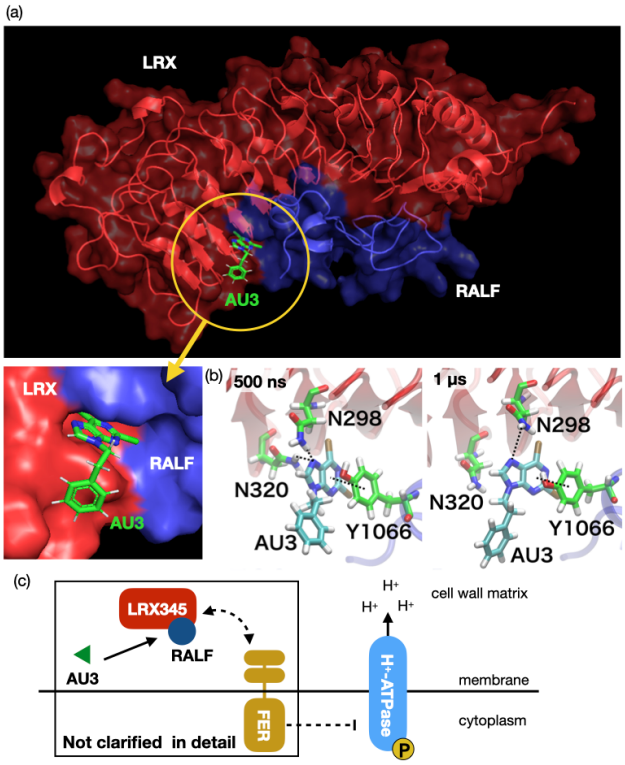


Figure 8. The **AU3** binding site in LRX–RALF by MD simulations and possible mechanism. (a) The **AU3** binding site in LRX–RALF visualized by PyMol. (b) The binding conformation of **AU3** at 500 ns and 1 μs visualized by VMD. (c) The proposed mechanism.

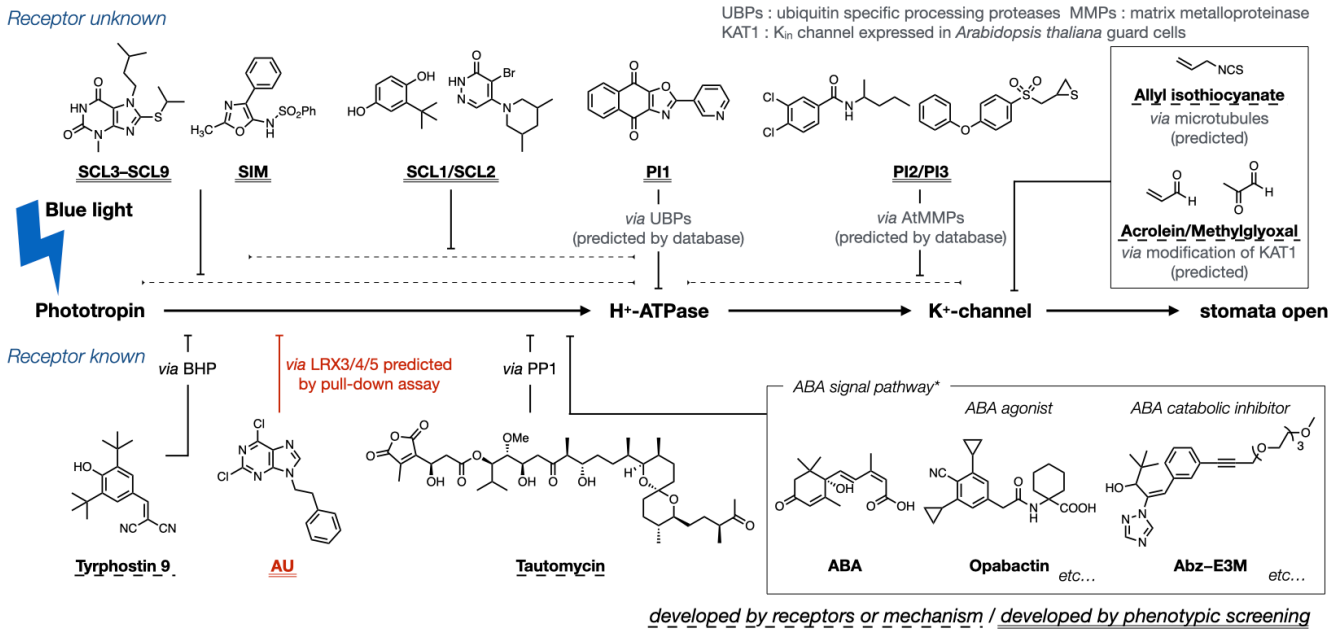


Figure 9. Summary of the inhibitors of blue light-induced stomatal opening and working models for the mode of action of these compounds. Arrows and T-bars indicate positive and negative regulation, respectively.

interaction continued to form throughout the 1 μs MD simulation performed here. In addition, **AU3** was found to form hydrogen bonds with the side chains of Asn298 and Asn320 of LRX2 during the MD simulation. From these results, it is understood that **AU3** can bind in this boundary region due to its strong interaction with Tyr1066 of RALF4 and Asn298 and Asn320 of LRX2. It should be noted that the amino acid sequences around the binding site of the LRX2 and RALF4 are highly conserved among members of the LRX and RALF families, respectively.[58] In addition, looking back at the results of the pull-down assay, it was revealed that RALF23 was actually detected as a candidate target protein in the first pull-down assay. Furthermore, these results were consistent with a previous report that RALF23 interacts with LRX3.[53]

The calculated complex formation was consistent with the experimental results. The phenethyl group, amenable for modification as indicated by the SAR study, is not interfering with the complexation during the MD simulation. The C2 and C6 positions of **AU3**, essential sites for the biological activity as indicated by the SAR study, were positioned close to the interaction surface, implying that these sites are important for the complex formation. To confirm the importance of C2/C6-halogen atoms, similar simulations were performed using compounds in which the C2 position was replaced by an amino group or hydrogen atom and the C6 position by a methyl group. The results showed that during the 100-300 ns MD simulations, these molecules were out of the boundary region of the LRX2-RALF4 complex and no stable complex was formed (SI movies 4–6). The molecular dipole moments of the compounds substituted at the C2 and C6 positions of **AU3** were calculated to be 5.5–6.4 Debye, which are smaller than the dipole of **AU3** (7.0 Debye). The magnitude of the dipole moment represents the polarity of the molecule. Thus, these compounds with smaller dipoles are expected to interact less with the surrounding amino acids than **AU3**, which may be one of the reasons for the unfavorable formation of the complex. These calculations indicate that the C2 and C6 positions of **AU3** are crucial for binding to the LRX-RALF complex.

Taken together, the pull-down assays and simulation studies strongly indicate that the target protein of **AU3** is the LRX-RALF complex. **AU3** interacts with the complex at the boundary of LRX and RALF, implying that **AU3** may stabilize the LRX-RALF complex as a molecular glue. At this stage, it is unclear how the LRX-RALF complex affects stomatal opening inhibition via PM H^+-ATPase activation. However, some reports indicate that RALF might be involved in the H^+-ATPase and stomatal opening mechanism. For example, it was shown that RALF interacts with its receptor FERONIA (FER) to regulate the activity of H^+-ATPase,[59] and stomatal opening is controlled in a RALF-dependent manner (Figure 8c).[60] Furthermore, LRX3/4/5-RALF-FER modulator has been shown to be associated with plant growth and stress responses.[54,55] These facts suggest that **AU3** may be involved in the stomatal movement mechanism by regulating the dynamics of the LRX-RALF complex.

DISCUSSIONS

While further detailed studies are needed to fully uncover the LRX-mediated PM H^+-ATPase phosphorylation pathway, the uniqueness of AUs as stomatal opening inhibitors is obvious. The blue light-induced stomatal opening inhibitors discovered thus far are summarized in Figure 9.[15,16,20,22,23,24,61,62,63] These inhibitors can be broadly divided into two categories: those targeting known receptor or mechanism (dashed line), and those discovered through phenotype-based chemical screening (double line). The former corresponds to inhibitors of mammalian homologues such as mammalian protein kinases inhibitor, whereas the latter mainly involves the discovery of first-in-class molecules. These novel molecules are not only had potential for agricultural applications but are also powerful tools for discovering new molecular mechanisms and new roles of proteins. While in many cases, their mechanisms of action were only

narrowed down and their target proteins were not identified, AUs have a brand-new structure as a stomatal opening inhibitor and have even identified LRXs as target candidate proteins. In the future, we expect that the LRXs will be used as a starting point to develop new stomatal opening inhibitors.

CONCLUSIONS

In summary, we have discovered 2,6-dihalopurines (AUs) as a new class of stomatal opening inhibitors in plants. Based on biological assays, **AU3** may be involved in a pathway related to PM H^+-ATPase phosphorylation in stomatal guard and mesophyll cells. In addition, we identified LRX3–LRX5 and RALF complex as target protein candidates of **AU3** by pull-down assay. Moreover, MD simulations showed a strong interaction between the LRX–RALF complex and **AU3**, supporting the pull-down assay result that LRX3–LRX5 are the target protein of **AU3**. In addition, the MD simulation results are consistent with our SAR studies. The mechanism of stomatal movement related with LRXs–RALF is an unexplored pathway, and therefore further studies may lead to the discovery of new signaling pathways and regulatory factors in the stomatal movement. Indeed, while most target proteins for stomatal opening inhibitors are intracellular proteins, LRXs are extracellular proteins, indicating that inhibition of stomatal opening may be through a different approach. In addition, to the best of our knowledge, **AU3** is the first small molecule that interacts with LRXs. Therefore, the use of AUs may accelerate research on the function of the LRX family proteins with regards to stomata opening inhibition and beyond. The development of new molecules through the phenotypic screening and identification of their target proteins will become a major trend in the future, because it will lead to the proposal of new mechanisms in stomatal movement, application to agrochemicals, and application to target protein-related research beyond the stomatal research.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the Publications website.

Synthetic procedures, procedures of biological experiments, procedures of calculations, Supporting figure (Figure S1–S8, Table S1–S3) and ^{1}H and ^{13}C NMR chart (PDF)

The MS raw data and analysis files have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the jPOST partner repository (http://jpostdb.org)[64] with data set identifier PXD036922.

MD simulation data (MPG)

SI movie1: MD simulation of **AU3** and LRX2

SI movie2: MD simulation of **AU3** and RALF4

SI movie3: MD simulation of **AU3** and LRX2-RALF4 complex

SI movie4: MD simulation of the compound replaced chloro group at the C4 position of **AU3** by a methyl group and LRX2-RALF4 complex

SI movie5: MD simulation of the compound replaced chloro group at the C2 position of **AU3** by hydrogen atom and LRX2-RALF4 complex

SI movie6: MD simulation of the compound replaced chloro group at the C2 position of **AU3** by an amino group and LRX2-RALF4 complex

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A.U. conceived the concept and conducted the synthesis and biological experiments. Y.A. conducted the immunohistochemically assay. K.J.F. and T.Y. conducted MD simulations and analyzed the results. S.S., K.K. and E.M.-S. conducted LC-MS/MS analysis. H.K., K.A., A.S., and K.I. supervised and helped analyzing the synthesis experiments. Y.A. and T.K. supervised and helped analyzing the biological experiments. A.U., K.A., and K.I prepared the manuscript with feedback from others. All authors have given approval to the final version of the manuscript. K.I. directed the project.

Notes

The authors declare no competing financial interest.

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