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Discovery of synthetic small molecules that enhance the number of stomata: C–H functionalization chemistry for plant biology†

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The increasing climate changes and global warming are leading to colossal agricultural problems such as abatement of food production and quality. As stomatal development is considered to play a key role in crop plant productivity and water-use efficiency, studying stomatal development is useful for understanding the productivity of plant systems for both natural and agricultural systems. Herein, we report the first-in-class synthetic small molecules enhancing the number of stomata in *Arabidopsis thaliana* that have been discovered by screening of the chemical library and further optimized by the Pd-catalyzed C–H arylation reaction. The present study shows not only huge potential of small molecules to control the cellular and developmental processes of stomata without using genetically modified plants, but also the power of C–H functionalization chemistry to rapidly identify the optimized compounds.

Stomata are epidermal pores essential for the growth and survival of land plants. They are surrounded by two guard cells, which act as gates to balance the gas exchange for photosynthesis against the loss of water vapor.[1] Considering that 40% of the atmospheric CO_2 passes through stomata every year,[2] manipulation of stomatal development and function plays a key role in crop plant productivity and water-use efficiency. Previous reports have shown that the number of stomata is influenced by environmental conditions, such as light[3] and atmospheric CO_2 levels.[4] To this end, plants use endogenous peptide hormones to regulate the number of stomata on the leaf surface.[5] In addition, the plant steroid hormone brassinosteroids modulate stomatal development *via* acting on peptide signaling.[6] As the stomatal density has been considered as a target trait suitable for plant engineering, one idea would be to use such peptides to control the stomatal number. Indeed, genetic modification to increase peptide amounts that are known to alter the numbers of stomata could improve the plant water-use efficiency (when the stomatal numbers are reduced) or photosynthetic capacity (when the numbers of stomata are increased).[5] However, growing genetically-modified crop plants in the field may be subject to restrictions. An alternative approach would be to use synthetic small molecules to manipulate the stomatal number. For example, stomata can be increased or decreased by the application of the synthetic peptide hormones,[5e–h] though it is not realistic to use peptides for agricultural purpose due to the cost of synthesis. Very recently, a small molecule, bubblin, which increases stomata has been reported.[7] However, this compound exerts severe inhibition of seedling growth, thereby excluding its potential use for improving the plant productivity. Therefore, the development of small molecules that increase the stomatal number without side effects is an urgent issue.

Herein, we report the discovery of small molecules that increase the numbers of stomata. Through derivatization of the original hit compound *via* Pd-catalyzed C–H functionalization, we successfully separated the potency for elevating stomatal differentiation from the side effects of growth inhibition.

We began our study by applying our specially curated ITbM chemical library including 80 compounds from the LOPAC® Pfizer chemical library[8] toward plant-based phenotypic screening. We counted the number of stomata on the leaves of *Arabidopsis thaliana* treated with compounds for 9 days and successfully identified compounds **CL1** and **CL2** as stomatal development enhancing molecules (Fig. 1). Interestingly, these molecules have a similar structure to Celecoxib©, which is a non-steroidal anti-inflammatory drug. The target of Celecoxib in humans is cyclooxygenase-2 (COX-2) that produces prostaglandin.[9] However, higher plants including *Arabidopsis thaliana* possess neither orthologs of the COX-2 gene nor prostaglandin. The stomata increasing effect of **CL1** and **CL2** might arise from their interactions with plant-specific proteins. Although **CL1** and **CL2** increased the

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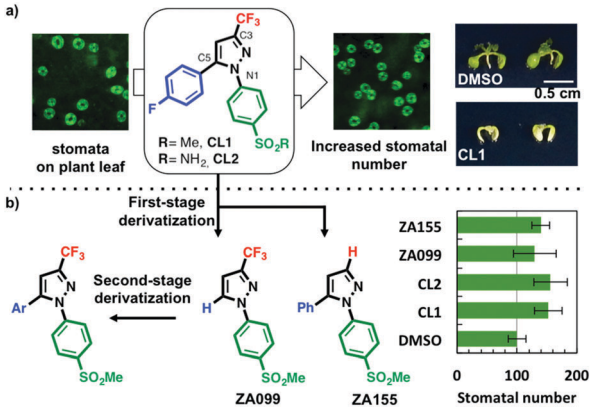
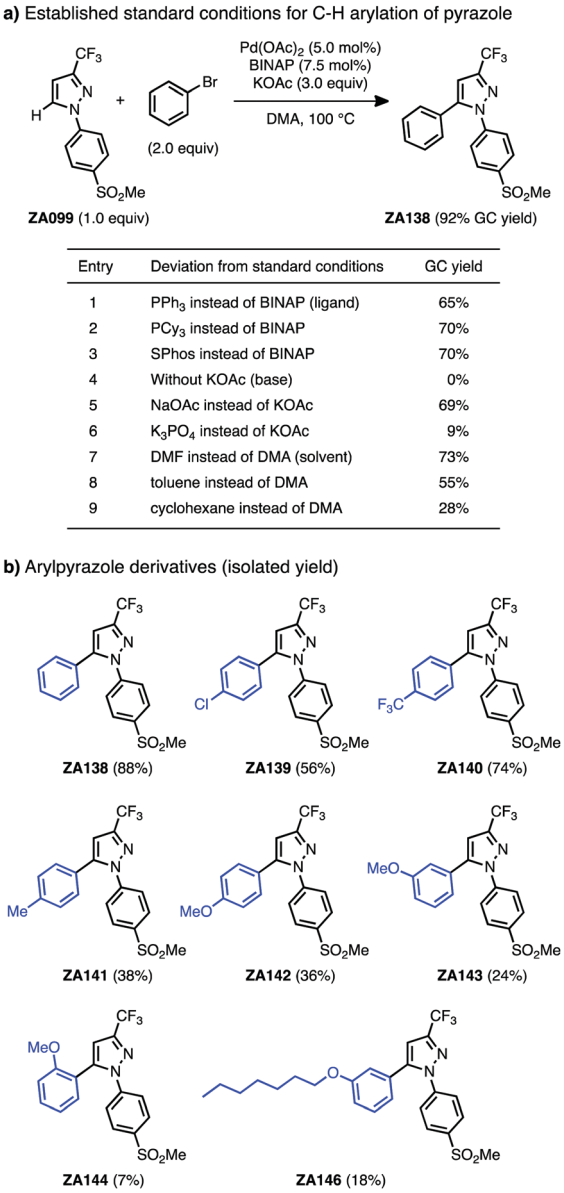


Fig. 1 (a) Library-based discovery of compounds **CL1** and **CL2** that increase the number of stomata, yet toxic. (b) Summary of the initial structure–activity relationship study.

number of stomata on the plant leaves, they conferred adverse effects at high concentrations (Fig. 1a). Seedlings remain smaller with chlorosis when **CL1** was applied at optimal concentrations for the stomata increasing effect (Fig. 1a). We therefore strategized our efforts to develop new compounds that can increase the numbers of stomata while minimizing the toxicity at high concentrations.

We first synthesized the analogues lacking a trifluoromethyl (CF_3) group in the C3-position (Fig. 1b, **ZA155**) or the aryl group in the C5-position of the pyrazole core (Fig. 1b, **ZA099**). In the case of **ZA155**, growth inhibition was observed whereas **ZA099** did not inflict any visual growth inhibition on the plant (see ESI† for details). These results led us to postulate that further derivations on C5-position of the pyrazole while keeping its CF_3 group in the C3-position may achieve increased number of stomata on plant leaves without growth inhibition. With our established background in C–H functionalization chemistry,[10–12] we chose to approach the synthesis of new small molecules with different aryl groups on the C5-position by C–H arylation of **ZA099** rather than relying on time-consuming conventional multi-step synthesis.[13]

We initiated our methodology development with systematic screening of the different experimental variables to achieve efficient C–H arylation of **ZA099** with aryl bromides under palladium catalysis.[14] After extensive screening of the ligand, base, solvent and temperature, the optimized conditions using a $Pd(OAc)_2$/BINAP system were identified (Fig. 2a, Tables S1–S3, ESI†). Thus, when **ZA099** (1.0 equiv.) was treated with bromobenzene (2.0 equiv.), KOAc (3.0 equiv.), $Pd(OAc)_2$ (5.0 mol%), and BINAP (7.5 mol%) in DMA at 100 °C, the target phenylated pyrazole **ZA138** was obtained in 92% GC yield (Fig. 2a). Listed in Fig. 2a are the effects of reaction parameters. The use of ligands other than BINAP (PPh_3, PCy_3, SPhos) resulted in lower yields (entries 1–3). The base exerted a profound influence on the reaction, since the absence of it inhibited the reaction (entry 4). KOAc was preferred over K_3PO_4 (entries 5 and 6) possibly indicating a concerted metalation deprotonation (CMD) type mechanism.[15,16] The use of polar solvents (DMA or DMF) is also important; less polar solvents such as toluene and cyclohexane resulted in much lower yields (entries 7–9).



Entry	Deviation from standard conditions	GC yield
1	PPh_3 instead of BINAP (ligand)	65%
2	PCy_3 instead of BINAP	70%
3	SPhos instead of BINAP	70%
4	Without KOAc (base)	0%
5	NaOAc instead of KOAc	69%
6	K_3PO_4 instead of KOAc	9%
7	DMF instead of DMA (solvent)	73%
8	toluene instead of DMA	55%
9	cyclohexane instead of DMA	28%

Fig. 2 (a) Optimized conditions for the C–H arylation of **ZA099** with bromobenzene and the effect of reaction parameters. (b) Arylpyrazole derivatives synthesized under standard conditions.

Having established the optimized reaction conditions, we synthesized a range of arylpyrazole derivatives from **ZA099** and the corresponding aryl bromides. The conditions are not optimized for each derivative and the representative analogues that are useful in the following structure–activity relationship study are shown in Fig. 2b. Both electron-withdrawing (compound **ZA140**) and electron-donating (compounds **ZA141**, **ZA142**, **ZA144** and **ZA146**) substituents were well tolerated under the reaction conditions and were obtained in up to 88% isolated yield. Interestingly, even 4-chlorobromobenzene was tolerated and gave access to **ZA139** without losing the C–Cl moiety. Sterically hindered **ZA144** could also be formed, albeit in low yield.

With these analogues in hand, we examined their bioactivity on stomatal numbers using Arabidopsis seedlings expressing

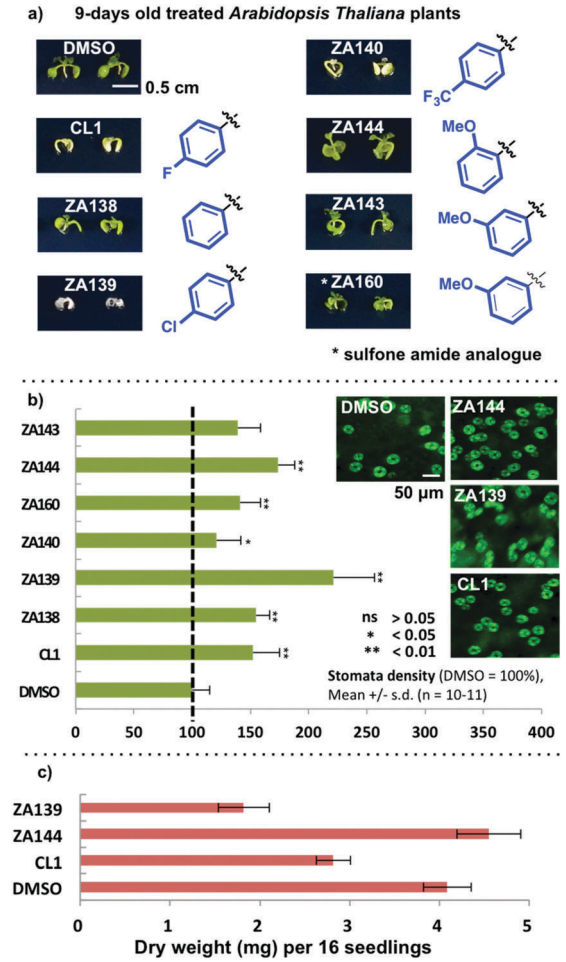


Fig. 3 The effect of the different compounds on *Arabidopsis thaliana* in terms of (a) toxicity and (b) stomatal numbers. (c) The sum for the dry weight of 16 seedlings treated with **CL1**, **ZA144**, or **ZA139** are represented. Dashes lines in (b) indicate the average values of DMSO-treatment and **ZA099**-treatment. Asterisks indicate significant differences by Student's *t*-test (two-tailed) compared to **ZA099**-treatment in (b) and DMSO-treatment in (c): *ns* for *P* value >0.05, * for *P* value >0.05, and ** for *P* value <0.01.

guard-cell specific green fluorescent protein GFP (Fig. 3).[17] After allowing the seeds to grow for 9 days in the presence of these small molecules, the youngest leaves of the newly grown plants were observed under a fluorescence microscope (Fig. 3b). Compound **ZA139** generated the highest stomatal density (number of stomata per mm^{-2}) on cotyledons compared to the other compounds tested (Fig. 3b). It was, however, extremely toxic to the plant with abnormal stomata shape (Fig. 3b and ESI†). The lower dry weight of seedlings cultured in the presence of **ZA139** shows the severe effect of **ZA139** on plant growth (Fig. 3a and c). Another compound worth mentioning is **ZA143** (Fig. 1), which gave a small increase in stomata numbers and was not severely toxic to the plant (ESI†). In order to increase the stomata numbers, we thought that perhaps the sulfonamide analogue (**ZA160**, Fig. 3a) would perform better. The compound, however, did not increase the number of stomata on plant leaves and led to growth inhibition (Fig. 3b). We, therefore, turned our attention towards synthesizing and testing different anisole substituted compounds (**ZA142** and **ZA144**, Fig. 2b). *p*-Anisyl substituted **ZA142** did not show promising results (ESI†), but *o*-anisyl substituted **ZA144**, on the other hand, elicited the highest stomata-increasing activity and did not show any toxicity.

Furthermore, when comparing the dry mass (biomass) of **ZA144** to **ZA139** and **CL1**, we were able to confirm that, in a similar fashion to DMSO, **ZA144** does not confer growth inhibition on the plant (Fig. 3c). Altogether, we identified **ZA144** as the most effective molecule in increasing the number of stomata without severe toxicity and could be used to manipulate plant growth and productivity. While the mode of action of these novel small molecules is yet to be uncovered, this campaign clearly demonstrates the power of synthetic small molecules in exploring plant biology.

In summary, a rapid C–H functionalization methodology made it possible to identify a new bioactive small molecule that can enhance the number of stomata on plant leaves without inhibiting the plant growth in up to 25 µM concentration. In the new $Pd(OAc)_2$/BINAP catalytic system, we were able to easily access a series of derivatized novel small molecules and separate the desirable bioactivity of promoting stomatal development from the side effects of growth inhibition. Future identification of target(s) of **ZA144** may provide the molecular basis of pyrazole-mediated stomatal differentiation and increase in biomass. Our work emphasizes that, with the recent advent of a number of game-changing C–H functionalization reactions, there are significant opportunities to use these technologies to accelerate plant biology and agricultural science.

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Conflicts of interest

There are no conflicts to declare.

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