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The selectivity of 6-nor-ABA and 7′-nor-ABA for abscisic acid receptor subtypes

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ABSTRACT

Abscisic acid (ABA), a plant hormone, is involved in many plant development processes and environmental stress responses that are regulated by a Pyrabactin Resistant 1 (PYR)/Pyrabactin Resistant-Like (PYL)/Regulatory Component of ABA Receptor (RCAR) receptor protein-mediated signal transduction pathway. In *Arabidopsis thaliana*, PYL proteins constitute a 14-member family comprising two distinct subclasses: dimeric receptors (PYR1 and PYL1–PYL3) and monomeric receptors (PYL4–PYL13). The individual contributions of PYL subclasses/subtypes with specific physiological actions are still poorly understood; consequently, the development of PYL subclass/subtype-selective agonists should be useful to reveal the different functions of these receptors. In this study, we focused on the ABA analogs 6-nor-ABA and 7′-nor-ABA, which were expected to function as monomeric receptor-selective agonists on the basis of crystal structures of PYL-ABA complexes and sequence alignments of PYL subtypes. In a protein phosphatase 2C (PP2C) assay, the agonist activities of both analogs were lower than those of ABA toward all tested PYL proteins, regardless of subclass/subtype. Nevertheless, we found that 6-nor-ABA acts as a selective agonist at the physiological level: it induced stomatal closure but did not inhibit seed germination and root growth. On the basis of observed inhibitory activity against PP2C among different PYL subtypes, this biological effect of 6-nor-ABA may be attributed to the activity of that agonist on PYL5 and/or PYL6.

The plant hormone abscisic acid (ABA, compound **1**) plays a key role in many physiological processes, such as seed dormancy, root growth, stomatal closure and abiotic stress response.[1,2] The physiological actions of ABA are controlled by a signal transduction process involving the interaction of two types of proteins: a PYR/PYL/RCAR (PYL) receptor and group-A protein phosphatases 2C (PP2Cs)—including HAB1, ABI1 and ABI2—that act as negative regulators of ABA signaling. By binding to PYL proteins, ABA induces a conformational change associated with a mobile loop (gate) closure that enables the receptor to bind and inhibit PP2Cs.[3–6] Arabidopsis PYL proteins, which constitute a 14-member family,[7,8] are divided into two distinct subclasses according to their oligomeric state: dimeric receptors (PYR1 and PYL1–PYL3) and monomeric receptors (PYL4–PYL13).[9–11] Among these proteins, the receptor property of PYL13 remains controversial. Several studies have indicated that PYL13, which lacks the lysine residue crucial for ABA binding, does not bind ABA and inhibits specific PP2Cs independently of ABA.[11,12] In contrast, a recent report has claimed that PYL13 inhibits these PP2Cs in an ABA-dependent manner.[13] Further study is needed to resolve this discrepancy.

Both dimeric and monomeric receptors are involved in ABA-induced physiological responses, with each receptor contributing additively to regulation of ABA responses.[14,15] These receptors differ substantially in function, as evidenced by the contrasting expression patterns of the genes encoding various PYL-subtype members. Although PYL8 is known to play a nonredundant role in root sensitivity to ABA,[15] characterization of the individual PYL subtypes is generally difficult because of the functional redundancy of the receptors. The details of these functional differences are thus largely unknown. A chemical compound capable of selectively activating PYL subclasses/subtypes would be a valuable tool for evaluation of the effect of the specific receptors on the various roles of ABA. Although some selective agonists of dimeric receptors, such as pyrabactin and quinabactin, have been described,[16,17] no reports have appeared of agonists that preferentially activate

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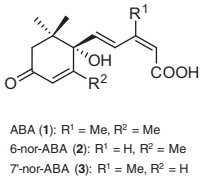
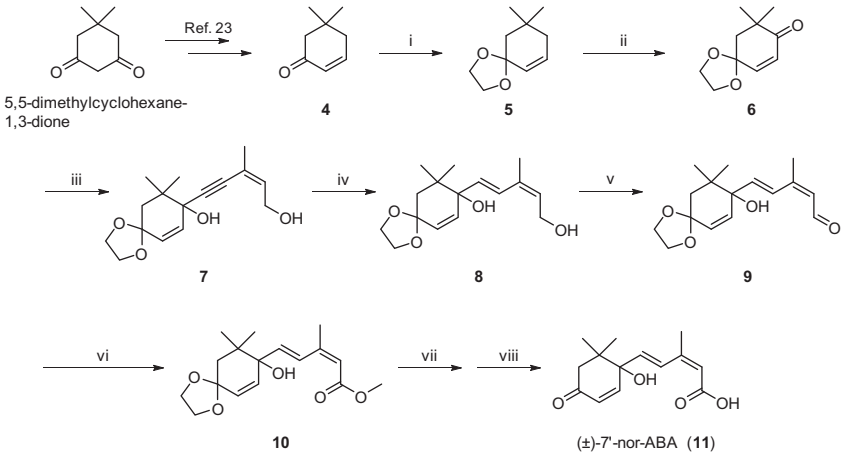


Figure 1. Structures of abscisic acid (ABA) and its analogs.

monomeric receptors. In the present study, we explored the possibility that a structural analog of ABA could function as a monomeric receptor-selective agonist, a role suggested by an examination of the crystal structures of several PYL-ABA complexes[18,19] and sequence alignments of PYL subclasses/subtypes. Although numerous ABA analogs have been designed and synthesized,[20] few have been evaluated from this perspective (Fig. 1).

In PYL–ABA complexes, ABA establishes a hydrophobic network with amino acid residues at the α3 helix and the gate loop to stabilize the gate-closed form of PYL proteins. A comparison of PYL subtypes reveals only one residue differing between dimeric and monomeric receptors, namely, a valine (Val) present in dimeric receptors that is replaced by leucine (Leu) (PYL7-10) or isoleucine (Ile) (PYL4-6, PYL11 and PYL12) in monomeric receptors (Supplementary Fig. S1). Because of its location in the α3 helix, this residue should be involved in the hydrophobic interaction with the C6 and/or C7′ methyl group of the ABA molecule. A consideration of steric bulkiness suggests that hydrophobic interactions should be induced most easily by Leu, followed by Ile and then Val. We therefore predicted that monomeric receptors can adopt the gate-closed form; this should be true even in ABA analogs lacking the C6 or C7′ methyl group (6-nor-ABA, compound **2**, and 7′-nor-ABA, compound **3**, respectively) because of their bulky residues, which likely compensate for the weakening of the hydrophobic interaction due to elimination of the C6 or C7′ methyl group. These two compounds have been reported to be weak ABA agonists,[21] but their activities toward PYL proteins have not been investigated. We therefore investigated whether 6-nor-ABA and 7′-nor-ABA can function as monomeric receptor-selective agonists.

(±)-6-Nor-ABA was prepared according to the method of Ueno et al., while the synthesis of (±)-7′-nor-ABA was carried out following a modification of the route of Nanzyo et al., as shown in Scheme 1.[22] In particular, the carbonyl group of compound **4** (prepared as reported previously)[23] was protected by treating with ethylene glycol in the presence of pyridinium *p*-toluenesulfonate to afford the ketal **5**.[24] Allylic oxidation with manganese(III) acetate and *tert*-butyl hydroperoxide afforded ketone **6**.[25] The side chain was introduced by direct addition of (*Z*)-3-methyl-2-penten-4-yn-1-ol using *n*-butyllithium, generating the alcohol **7**. Reduction (generating compound **8**), oxidation (generating compound **9**) and esterification of **7** resulted in the formation of the



Scheme 1. Synthesis of (±)-7′-nor-ABA. Reagents: (i) ethylene glycol, PPTS; (ii) $Mn_3O(OAc)_9$, $tBuO_2H$, O_2, EtOAc; (iii) (*Z*)-3-methylpent-2-en-4-yn-1-ol, *n*-BuLi, THF; (iv) SMEAH, THF; (v) MnO_2, acetone; (vi) MnO_2, NaCN, AcOH, MeOH; (vii) 1 M HCl, acetone; (viii) 1 M NaOH, MeOH.

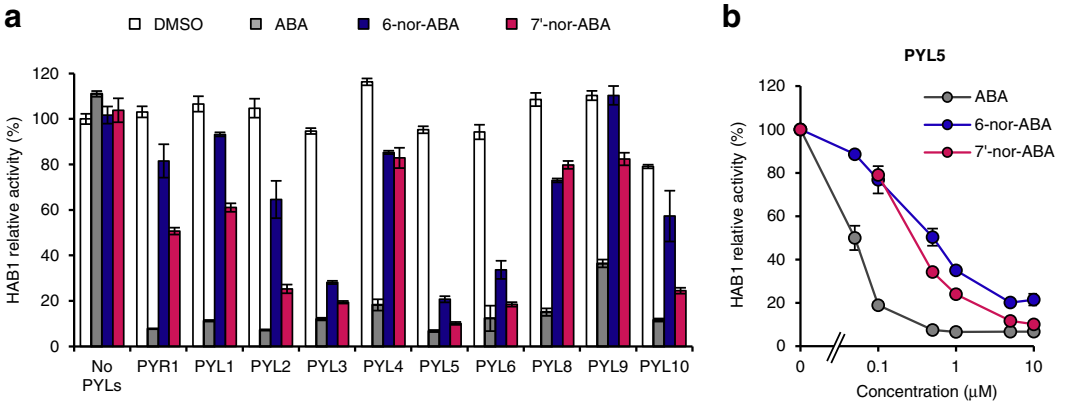


Figure 2. The comparative effects of 6-nor-ABA, 7′-nor-ABA and ABA on inhibition of HAB1 by ABA receptors. Chemical inhibition of HAB1 by (a) various ABA receptors in the presence of 10 μM of each test compound or (b) PYL5 in the presence of various concentrations (0.05, 0.1, 0.5, 1, 5 and 10 μM) of 6-nor-ABA or 7′-nor-ABA.

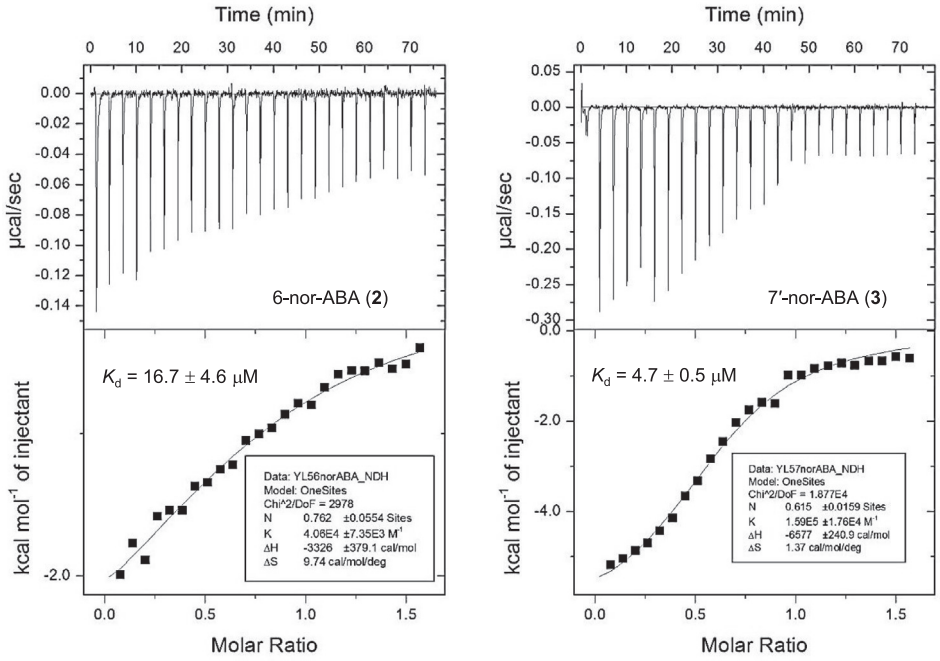


Figure 3. Isothermal titration calorimetric analysis of the binding of 6-nor-ABA and 7′-nor-ABA to PYL5.

ester **10**, followed by acidic and basic hydrolysis of the ester to give (±)-7′-nor-ABA (**11**). (±)-6-Nor-ABA and (±)-7′-nor-ABA were optically resolved by HPLC on a chiral column to obtain the corresponding (+)-isomers with the same exciton chirality as that of *S*-(+)-ABA.[21] Since the unnatural type of ABA analogs showed weaker activities than the natural type of them (Supplementary Fig. S2), we used analogs with the same exciton chirality of the natural type of ABA for all assays.

To examine the agonist activities of 6-nor-ABA and 7′-nor-ABA toward different subclasses/subtypes of PYL proteins, we assessed receptor-mediated PP2C inhibition using nine Arabidopsis PYL proteins (dimeric subclass: PYR1 and PYL1–PYL3; monomeric subclass: PYL4–PYL6, PYL8 and PYL10) and the PP2C HAB1. 6-Nor-ABA induced PYL3, PYL5 and PYL6 to inhibit HAB1, while 7′-nor-ABA caused activation of PYL2, PYL3, PYL5, PYL6 and PYL10. The activities of both analogs were weaker than those of ABA (Fig. 2a). Dose–response analysis revealed that the IC_{50} values of 6-nor-ABA and 7′-nor-ABA for PYL5, the PYL subtype most sensitive to both compounds, were 510 and 360 nM, respectively (Fig. 2b). These values were approximately 10-fold higher than that of ABA (50 nM). Additionally, isothermal titration calorimetry (ITC) was used to measure the binding affinity of 6-nor-ABA and 7′-nor-ABA for PYL5 (Fig. 3). The K_d values of 6-nor-ABA and 7′-nor-ABA were 16.7 and 4.7 µM, respectively, which were 5- to 20-fold higher than that of ABA (0.88 µM).[26] Thus, 6-nor-ABA and 7′-nor-ABA were less effective than ABA toward all PYL proteins tested, implying that the C6 and C7′ methyl groups of ABA play an important role in receptor binding regardless of PYL subclass/subtype. These results suggest that the PYL-subtype selectivity of ABA analogs is not solely determined by the amino acid residues involved in the gate closure.

To analyze the plant physiological effects of ABA and its analogs, we focused on 6-nor-ABA, a more narrow-spectrum agonist for PYL subtypes (PYL3, PYL5 and PYL6). As PYL3 expression is extremely low throughout plant development,[14] 6-nor-ABA may act as a weak PYL5- and PYL6-selective agonist in plants. With respect to its potency, the PP2C assay and the ITC experiment revealed that

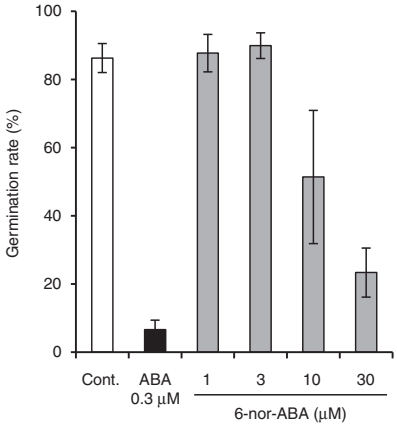


Figure 4. The comparative effects of 6-nor-ABA and ABA on Arabidopsis seed germination. The seed germination rate in response to ABA or 6-nor-ABA was measured at 48 h after stratification (n = 3; error bars represent SD).

6-nor-ABA was able to activate PYL5 as strongly as ABA when present in a 20-fold excess over ABA. This result suggested that 6-nor-ABA could be used to examine the effect of PYL5 and PYL6 on the various physiological actions of ABA. To investigate the relationship between the activation of PYL5/PYL6 and ABA-induced physiological responses, we tested the effect of 6-nor-ABA in three different physiological assays. In an Arabidopsis seed germination assay, the inhibitory activity of 6-nor-ABA was at least 100-fold lower than that of ABA (Fig. 4). A similar trend was observed in root growth inhibition of 5-day-old seedlings (Fig. 5). In contrast, 6-nor-ABA showed relatively potent agonistic activity in the induction of stomatal closure and drought tolerance. Because transpiration lowers leaf temperatures through evaporative cooling,[27] we tested the effect of 6-nor-ABA on stomatal apertures using thermal-imaging methods. We observed that ABA-treated seedlings showed increased leaf surface temperatures by reducing transpiration.

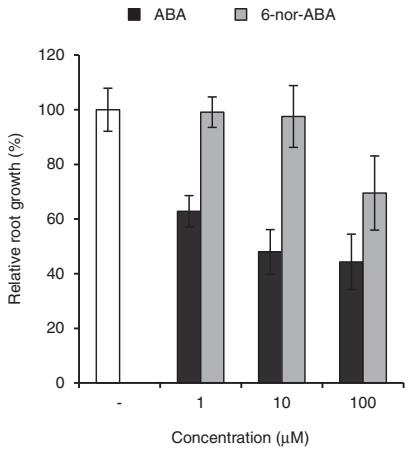


Figure 5. The comparative effects of 6-nor-ABA and ABA on Arabidopsis seedling root growth. Data are averages from three independent experiments (n = 12 each; error bars represent SD).

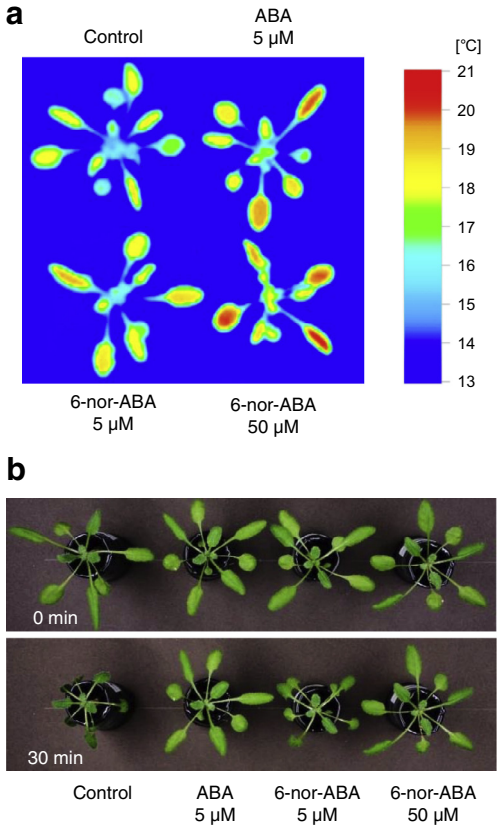


Figure 6. The comparative effects of 6-nor-ABA and ABA on Arabidopsis (a) leaf surface temperature and (b) drought tolerance.

Treatment with 6-nor-ABA induced stomatal closure (Fig. 6a) and drought tolerance (Fig. 6b) as effectively as did ABA when the former was present in 10-fold excess over ABA; this result is consistent with the observations of agonist potency toward PYL5. Additionally, the profile of this ligand was similar to that of 1′-*O*-methyl-ABA, which has been found to selectively activate PYL5 in a PP2C assay as well.[28] These results suggest that PYL5 plays an important role in the induction of stomatal closure, which is in agreement with the phenotype of the PYL5 over-expression.[6] The selectivity of 6-nor-ABA for PYL subtypes in plants should be investigated in detail at the genetic levels; however, this selectivity has potential use, both as a chemical probe and as a practical application to induce drought tolerance in plants without inhibiting growth.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.06.088.

References and notes

1. Finkelstein, R. *Arabidopsis Book* **2013**, *11*, e0166.
2. Cutler, S. R.; Rodriguez, P. L.; Finkelstein, R. R.; Abrams, S. R. *Annu. Rev. Plant Biol.* **2010**, *61*, 651.
3. Melcher, K.; Ng, L.-M.; Zhou, X. E.; Soon, F.-F.; Xu, Y.; Suino-Powell, K. M.; Park, S.-Y.; Weiner, J. J.; Fujii, H.; Chinnusamy, V.; Kovach, A.; Li, J.; Wang, Y.; Li, J.; Peterson, F. C.; Jensen, D. R.; Yong, E.-L.; Volkman, B. F.; Cutler, S. R.; Zhu, J.-K.; Xu, H. E. *Nature* **2009**, *462*, 602.
4. Miyazono, K.-I.; Miyakawa, T.; Sawano, Y.; Kubota, K.; Kang, H.-J.; Asano, A.; Miyauchi, Y.; Takahashi, M.; Zhi, Y.; Fujita, Y.; Yoshida, T.; Kodaira, K.-S.; Yamaguchi-Shinozaki, K.; Tanokura, M. *Nature* **2009**, *462*, 609.
5. Yin, P.; Fan, H.; Hao, Q.; Yuan, X.; Wu, D.; Pang, Y.; Yan, C.; Li, W.; Wang, J.; Yan, N. *Nat. Struct. Mol. Biol.* **2009**, *16*, 1230.
6. Santiago, J.; Rodrigues, A.; Saez, A.; Rubio, S.; Antoni, R.; Dupeux, F.; Park, S.-Y.; Márquez, J. A.; Cutler, S. R.; Rodriguez, P. L. *Plant J.* **2009**, *60*, 575.
7. Ma, Y.; Szostkiewicz, I.; Korte, A.; Moes, D.; Yang, Y.; Christmann, A.; Grill, E. *Science* **2009**, *324*, 1064.
8. Park, S.-Y.; Fung, P.; Nishimura, N.; Jensen, D. R.; Fujii, H.; Zhao, Y.; Lumba, S.; Santiago, J.; Rodrigues, A.; Chow, T.-F. F.; Alfred, S. E.; Bonetta, D.; Finkelstein, R.; Provart, N. J.; Desveaux, D.; Rodriguez, P. L.; McCourt, P.; Zhu, J.-K.; Schroeder, J. I.; Volkman, B. F.; Cutler, S. R. *Science* **2009**, *324*, 1068.
9. Dupeux, F.; Santiago, J.; Betz, K.; Twycross, J.; Park, S.-Y.; Rodriguez, L.; Gonzalez-Guzman, M.; Jensen, M. R.; Krasnogor, N.; Blackledge, M.; Holdsworth, M.; Cutler, S. R.; Rodriguez, P. L.; Márquez, J. A. *EMBO J.* **2011**, *30*, 4171.
10. Hao, Q.; Yin, P.; Li, W.; Wang, L.; Yan, C.; Lin, Z.; Wu, J. Z.; Wang, J.; Yan, S. F.; Yan, N. *Mol. Cell* **2011**, *42*, 662.
11. Li, W.; Wang, L.; Sheng, X.; Yan, C.; Zhou, R.; Hang, J.; Yin, P.; Yan, N. *Cell Res.* **2013**, *23*, 1369.
12. Zhao, Y.; Chan, Z.; Xing, L.; Liu, X.; Hou, Y.-J.; Chinnusamy, V.; Wang, P.; Duan, C.; Zhu, J.-K. *Cell Res.* **2013**, *23*, 1380.
13. Fuchs, S.; Tischer, S. V.; Wunschel, C.; Christmann, A.; Grill, E. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 5741.
14. Gonzalez-Guzman, M.; Pizzio, G. A.; Antoni, R.; Vera-Sirera, F.; Merilo, E.; Bassel, G. W.; Fernández, M. A.; Holdsworth, M. J.; Perez-Amador, M. A.; Kollist, H.; Rodriguez, P. L. *Plant Cell* **2012**, *24*, 2483.
15. Antoni, R.; Gonzalez-Guzman, M.; Rodriguez, L.; Peirats-Llobet, M.; Pizzio, G. A.; Fernandez, M.; De Winne, N.; De Jaeger, G.; Dietrich, D.; Bennett, M. J.; Rodriguez, P. L. *Plant Physiol.* **2012**, *161*, 931.
16. Okamoto, M.; Peterson, F. C.; Defries, A.; Park, S.-Y.; Endo, A.; Nambara, E.; Volkman, B. F.; Cutler, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 12132.
17. Cao, M.; Liu, X.; Zhang, Y.; Xue, X.; Zhou, X. E.; Melcher, K.; Gao, P.; Wang, F.; Zeng, L.; Zhao, Y.; Deng, P.; Zhong, D.; Zhu, J.-K.; Xu, H. E.; Xu, Y. *Cell Res.* **2013**, *23*, 1043.
18. Santiago, J.; Dupeux, F.; Betz, K.; Antoni, R.; Gonzalez-Guzman, M.; Rodriguez, L.; Márquez, J. A.; Rodriguez, P. L. *Plant Sci.* **2012**, *182*, 3.
19. Sun, D.; Wang, H.; Wu, M.; Zang, J.; Wu, F.; Tian, C. *Biochem. Biophys. Res. Commun.* **2012**, *418*, 122.
20. Todoroki, Y.; Hirai, N. *Stud. Nat. Prod. Chem.* **2002**, *27*, 321.
21. Ueno, K.; Araki, Y.; Hirai, N.; Saito, S.; Mizutani, M.; Sakata, K.; Todoroki, Y. *Bioorg. Med. Chem.* **2005**, *13*, 3359.
22. Nanzyo, M.; Oritani, T.; Yamashita, K. *Agric. Biol. Chem.* **1977**, *41*, 1711.
23. Wińska, K.; Grudniewska, A.; Chojnacka, A.; Białońska, A.; Wawrzeńczyk, C. *Tetrahedron: Asymmetry* **2010**, *21*, 670.
24. Bellosta, Â.; Ranaivosata, J.; Gille, B.; Cossy, J. *Tetrahedron* **2001**, *57*, 5173.
25. Shing, T. K. M.; Yeung, Y.-Y.; Su, P. L. *Org. Lett.* **2006**, *8*, 3149.
26. Takeuchi, J.; Okamoto, M.; Akiyama, T.; Muto, T.; Yajima, S.; Sue, M.; Seo, M.; Kanno, Y.; Kamo, T.; Endo, A.; Nambara, E.; Hirai, N.; Ohnishi, T.; Cutler, S. R.; Todoroki, Y. *Nat. Chem. Biol.* **2014**, *10*, 477.
27. Merlot, S.; Mustilli, A.-C.; Genty, B.; North, H.; Lefebvre, V.; Sotta, B.; Vavasseur, A.; Giraudat, J. *Plant J.* **2002**, *30*, 601.
28. Benson, C. L.; Kepka, M.; Wunschel, C.; Rajagopalan, N.; Nelson, K. M.; Christmann, A.; Abrams, S. R.; Grill, E.; Loewen, M. C. *Phytochemistry* **2014**, *113*, 96.