Bioorganic & Medicinal Chemistry Letters 22 (2012) 1761–1765

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Screening and characterization of a chemical regulator for plant disease resistance

Eun-Kyung Seo [a], Hidemitsu Nakamura [a], Masaki Mori [b], Tadao Asami [a,*]

[a] Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Disease Resistance Unit, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan

ARTICLE INFO

Article history:
Received 27 October 2011
Revised 15 December 2011
Accepted 16 December 2011
Available online 23 December 2011

Keywords:
Systemic acquired resistance (SAR)
Salicylic acid (SA)
Inhibitor

ABSTRACT

Plants activate systemic acquired resistance (SAR), a form of long-lasting induced defense, to confer protection against a broad spectrum of pathogens. SAR induction is associated with the salicylic acid (SA)-mediated defense signaling networks. For detailed understandings of the SA-mediated signaling of SAR induction, we screened chemical inhibitors that block SA-mediated signaling from a 9600-compound chemical library. As a result, we identified one candidate chemical, 4-phenyl-2-{[3-(tri-fluoromethyl)anilino]methylidene}cyclohexane-1,3-dione (PAMD), that suppresses the expression of pathogenesis-related (*PR*) gene. PAMD also down-regulates SA-induced gene expression and enhances susceptibility to pathogen.

Plants live in complex environments in which they are continuously threatened by a broad range of harmful pathogens including viruses, bacteria, and fungi. To respond to the attacks of a diverse range of pathogens, plants have developed a multilayered immune system.[1] At the site of infection, plants activate the pathogen-associated molecular pattern (PAMP)-triggered basal resistance and the resistance (R) gene-mediated defense response.[2] Subsequent to these defense responses, a systemic defense response is triggered in distal leaves to protect the uninoculated tissues from subsequent invasion of pathogens. This long-lasting and broad-spectrum induced resistance is termed systemic acquired resistance (SAR).[3]

The induction of SAR requires the accumulation of salicylic acid (SA) and a subset of the pathogenesis-related (*PR*) genes in both local and systemic tissues.[4] The elevated levels of SA after pathogen infection induce PR protein accumulation and resistance to pathogens. Mutants that are impaired in SA-mediated signaling are incapable of SAR development. NahG transgenic plants that encode the bacterial SA-degrading enzyme salicylate hydroxylase fail to express *PR* genes and activate SAR.[5] The enzymatic pathways of SA biosynthesis have been unraveled by studies on several mutants that are defective in SA biosynthesis.[6] Related to the pathway downstream of SA, NPR1 (NONEXPRESSOR OF *PR* GENES1) has been identified and characterized as an important transducer of SA signal.[7] The mechanism of SAR development has been examined in detail, but many of the processes of SAR signaling remain to be revealed.

To clarify the SAR signaling pathway, we used a chemical biology technique that utilizes chemical tools to elucidate biological mechanisms. Various SAR-inducing chemicals such as probenazole and its derivatives, 1,2-benzisothiazol-3(2*H*)-1,1-dioxide (BIT),[8] benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH),[9] and 2,6-dichloroisonicotinic acid (INA),[10] have been identified and utilized for the analysis of SAR signaling, but none of the chemical inhibitors targeted by the SAR mechanism has been identified. As we expected that such types of inhibitors can dissect the SAR signaling and will be useful for identifying new signal components involved in SAR signaling, we focused on part of the SAR-related pathway downstream of SA biosynthesis. To identify such inhibitors, we established a high-throughput system for the easy identification of chemicals that inhibit SA-induced PR protein accumulation. Eventually, we discovered a novel chemical inhibitor.

Firstly, we screened a chemical library of 9600 randomly synthesized compounds.[11] Eight-day-old transgenic *PR1::GUS Arabidopsis* plants grown in 96-well tissue culture plates containing test compounds were treated with 2 mM SA by foliar spraying; three days later, GUS staining assay was performed.[12] Then, candidate inhibitor chemicals (**1–5** shown in Fig. 2) that suppressed SA-induced *GUS* expression were selected. As shown in Figure 1a, GUS activity was exhibited in *PR1::GUS* plants treated with SA; however, SA-induced *GUS* expression was suppressed by the treatment with candidate inhibitor chemicals. Consequently, the inhibitory

Abbreviations: SA, salicylic acid; SAR, systemic acquired resistance; PR gene, pathogenesis-related gene.

* Corresponding author. Tel.: +81 3 5841 5192; fax: +81 3 5841 8025.
E-mail address: asami@pgr1.ch.a.u-tokyo.ac.jp (T. Asami).

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2011.12.082

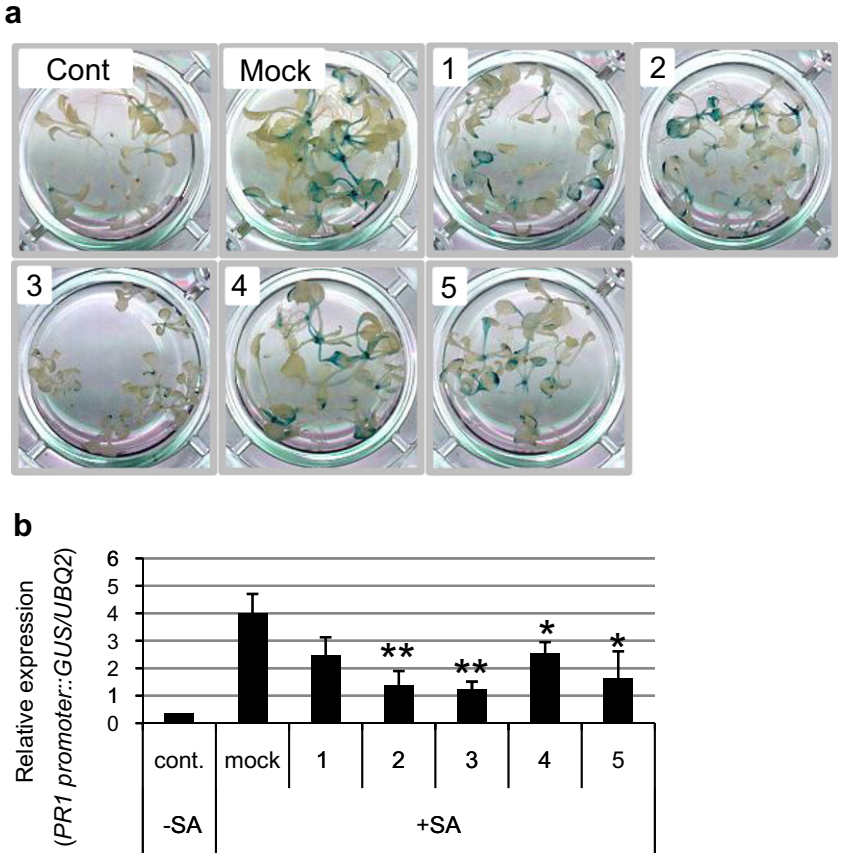

Figure 1. Inhibitory effects of five candidate chemicals identified from chemical library screening. *PR1::GUS* plants were grown for eight days with the indicated chemicals at a final concentration of 20–50 μM, or without chemicals (Mock), and were treated with 2 mM SA by foliar spraying. As a control (Cont.), seedlings grown for eight days without chemicals were sprayed with 0.5% DMSO in water. Then, the GUS staining assay was performed three days after treatment. (a) The histochemical GUS assay. This experiment was repeated three times with similar results. (b) SA-induced gene expression analysis was examined by qRT-PCR. Transcript levels of *GUS* were normalized against *UBQ2* expression. Error bars indicate means ± SD of three independent experiments. $*$ indicates p <0.05, and $**$ indicates p <0.01 compared with mock treatment by the Z-test.

effect of candidate chemicals was confirmed by quantitative real-time PCR (qRT-PCR) analysis.[13] As we expected, they down-regulated SA-induced *GUS* gene expression (Fig. 1b).

Among the chemicals tested in this screening, 4-phenyl-2-{[3-(trifluoromethyl)anilino]methylidene}cyclohexane-1,3-dione (PAMD, **3** shown in Fig. 2) showed the highest inhibitory activity. PAMD also inhibited SA-induced gene expressions of other *PR* genes, *PR2* and *PR5* (Fig. S1). Therefore, in addition to **3**, some PAMD derivatives (**6**–**9** shown in Fig. 2) were subjected to structure–activity relationship studies, in the expectation that such studies could provide a clue to the chemical modification of the candidate inhibitors to find novel chemicals that show more potent inhibitory activity. As shown in Figure 3a, the treatment with PAMD and derivatives reduced SA-induced GUS expression in *PR1::GUS* plants. The inhibitory effect of PAMD derivatives on SA-induced *GUS* gene expression was also observed in qRT-PCR analysis (Fig. 3b). PAMD derivatives showed similar activity to PAMD in terms of down-regulation of SA-induced *GUS* gene expression. As the structure that is shared between PAMD and its derivatives is the 2-substituted enamine moiety conjugated with 1,3-cyclohexadione, this structure may be essential for the SA-signal inhibition activity. A trifluoromethylphenyl ring attached to the nitrogen atom in the enamine moiety is likely to be important for the activity because **3** and **6** are more active than **8** and **9**, in which a thiophene ring binds to the nitrogen atom in the enamine moiety. On the other hand, a phenyl group on the cyclohexanedione ring is not likely to be so important for the activity because **6**, in which a cyclohexane ring is substituted with a dimethyl group instead of a phenyl group, is as active as PAMD.

For further investigation of whether PAMD functions as a negative regulator in plant disease resistance signaling, we performed pathogen infection assay with *Colletotrichum higginsianum*, a fungal pathogen that initially feed on living tissues and continue feeding on the nutrients released from dead tissues.[14,15] The result showed that PAMD-treated plants were more susceptible to *C. higginsianum* than untreated plants. To quantify the levels of *C. higginsianum* in infected plants, we estimated the level of actin mRNA of the pathogen (*Ch-ACT*) using qRT-PCR. The *Arabidopsis CBP20* gene (*At-CBP20*), which is constitutively expressed in *Arabidopsis*, was used for normalization.[13] PAMD treatment increased *Ch-ACT* levels in a dose-dependent manner and down-regulated the transcript levels of SAR marker gene, *PR1*, at the same time (Fig. 4). These data indicated that PAMD negatively affects SA-signal induction in *Arabidopsis* and allows the infection of *Arabidopsis* with *C. higginsianum*.

The plant hormone abscisic acid (ABA) plays important roles in plant development and in response to abiotic stresses such as drought and high salinity.[16] In addition, a recent report has shown that ABA is also involved in the suppression of SAR induction.[17] In this context, we examined whether PAMD functions as an agonist of abscisic acid and suppresses SA-signal induction. The expression levels of several ABA-inducible genes were tested by RT-PCR, but the results clearly demonstrated that PAMD has no effect on the transcription of ABA-inducible genes (data not shown); therefore, PAMD is not an agonist of ABA. The treatment of PAMD induced