Brief Report

Synthesis of fluorescently labeled pyrazole derivative inducing a triple response in Arabidopsis seedlings

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Supplementary material

A fluorescent labeled pyrazole derivative with a dansyl moiety (EH-DF) was synthesized. Design of EH-DF was carried out by using a dansyl moiety to substitute the naphthalene moiety of the parent compound (EH-1). At a concentration of 30 µM, EH-DF displayed biological activity on inducing a triple response in Arabidopsis seedlings. Compared with the non-chemical treated control, the hypocotyl length of EH-DF-treated Arabidopsis seedlings was reduced from approximately 9.2 ± 0.7 mm to 2.4 ± 0.2 mm. The length of the roots was reduced from 1.7 ± 0.1 mm to 1.0 ± 0.1 mm, and the curvature of the hook of Arabidopsis seedlings increased from 60 ± 16 degrees to 245 ± 35 degrees. The maximum excitation wavelength and emission wavelength of EH-DF were 350 and 535 nm, respectively. Data obtained via fluorescent microscope analysis indicated that intensive fluorescent signals of EH-DF were observed in the shoot of Arabidopsis seedlings.

Keywords: plant hormone, ethylene, fluorescent probe.

Introduction

Plant hormones are important signal molecules involved in plant growth and development.1,2 Ethylene is a gaseous plant hormone that displays a variety of biological functions.3–6 Ethylene has been well characterized as a key hormone in the induction of seed dormancy release,7 the formation of the apical hook in dark-grown seedlings,8 flower opening,9 the control of fruit ripening,10 and senescence.11 Ethylene has also been implicated in the defense response to flooding12 and pathogen infection.13 Despite ethylene’s strong potential as a powerful agrochemical, its properties as a flammable gas made it difficult to use directly in many conditions. To meet the demands of developing new chemicals which are non-gaseous at normal atmosphere but with ethylene-like activity, we conducted a chemical screening of the compound library, and we found a pyrazole derivative (EH-1, the chemical structure is shown in Fig. 1) that displays promising activity for inducing a “triple response,” an assay method widely used to determine ethylene activity, in Arabidopsis seedlings.12

Fluorescence-labeled small molecules are powerful tools for biological study. They provide highly sensitive methods for monitoring complex cellular processes for research applications.13 To gain our understanding of the action mechanism of EH-1, we designed and synthesized a fluorescence-labeled EH-1 derivative (EH-DF, the structure is shown in Fig. 1). In a previous work, we carried out a structure–activity relationship study of EH-1, and we found that the pyrazole moiety of EH-1...
displayed an important effect on promoting the biological activity. Based on this observation, the sulfonamide moiety of EH-1 is a good candidate for introducing a fluorophore to EH-1, thereby developing fluorescence-labeled chemicals with ethylene-like biological activity. In the present work, we report the synthesis of N-methyl-N-[(1,3,5-trimethyl-1H-pyrazol-4-yl)methyl]-5-dimethylamino-1-naphthalenesulfonamide (EH-DF); we also determined the biological activity of EH-DF and discussed the application of EH-DF.

Materials and methods

1. General

1H-NMR spectra were recorded with a JEOL ECP-400 spectrometer (Tokyo, Japan), with chemical shifts being expressed in ppm downfield from tetramethylsilane (TMS) as an internal standard. High-resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectra were recorded on an Exact MS system (Thermo Fisher Scientific, Waltham, MA, USA).

2. Reagents

Chemicals for synthesis were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Reagents were of the highest grade commercially available. N-methyl-1-(1,3,5-trimethyl-1H-pyrazol-4-yl)-methanamine (I) was purchased from Sigma-Aldrich.

3. Chemical synthesis

The general procedure for preparing N-methyl-N-[(1,3,5-trimethyl-1H-pyrazol-4-yl)methyl]-5-dimethylamino-1-naphthalenesulfonamide (EH-DF) was carried out by reacting N-methyl-1-(1,3,5-trimethyl-1H-pyrazol-4-yl) methanamine with dansyl chloride in a condition as described previously. (yield: 33%), oil. 1H NMR (400 MHz, CDCl3): δ: 2.01 (s, 3H), 2.07 (s, 3H), 2.63 (s, 3H), 2.89 (s, 6H), 3.67 (s, 3Hs), 4.08 (s, 2H), 7.19 (d, J=7.3 Hz, 1H), 7.52–7.57 (m, 2H), 8.21 (d, d, J1=2.07, J2=7.3 Hz, 1H), 8.45 (d, J=8.6 Hz, 1H), 8.57 (d, J=8.6 Hz, 1H); 13C NMR (100 MHz, CD3OD): δ: 129.9, 148.2, 140.5, 135.1, 134.2 (s2), 131.4, 131.4, 130.8, 128.9, 124.2, 120.7, 116.7, 116.3, 111.5, 51.8, 45.6, 43.8, and 33.5. The HRMS-ESI calculated for C20H26N4O2S [M+H]+ was 386.1776, and we found 386.1759.

4. Plant materials, growth conditions, and triple response assay

Wild-type Arabidopsis (ecotype Columbia) seeds were purchased from Lehle Seeds (Round Rock, TX, USA). A triple response assay was performed in a 24-well plate (Fukae Kasei Co., Ltd., Kobe, Japan). Stock solutions of all chemicals in this study were dissolved in dimethyl sulfoxide (DMSO) in designed growth media at 0.1% (v/v), as we described previously. For each data point, 10 measurements were taken with the excitation slit and emission slit settings at 10 and 10 nm, respectively. Samples dissolved in the H2O containing 0.1% DMSO were used.

5. Determining the fluorescence spectra of EH-DF

The fluorescence spectra of EH-DF were measured using an F-4500 Fluorescence Spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan). For each data point, 10 measurements were taken with the excitation slit and emission slit settings at 10 and 10 nm, respectively. Samples dissolved in the H2O containing 0.1% DMSO were used.

6. Fluorescence microscopy

To image the cells in plant tissues, the plants were placed on slide glasses and mounted with cover slips. Bright and fluorescence images were captured with a BX51 fluorescence microscope (Olympus, Tokyo) equipped with a set of optical filters: WU-type (330–385 nm) and WIB-type (460–490 nm). Digital images were recorded with a DP70 (Olympus, Tokyo) digital camera.

Results

1. Chemistry

We have previously shown that the modifications of the sulfonamide moiety did not significantly influence the biological activity of EH-1. Thus, introducing a dansyl moiety to EH-1 represents a straightforward approach to developing fluorescence-labeled EH-1. Dansyl chloride (5-(dimethylamino)naphthalene-1-sulfonyl chloride) is a reagent that reacts with amines to produce stable blue or blue-green fluorescent sulfonamide adducts that display sufficient fluorescence properties. Moreover, the chemical structure of the target compound N-methyl-N-[(1,3,5-trimethyl-1H-pyrazol-4-yl)methyl]-5-dimethylamino-1-naphthalenesulfonamide (EH-DF) is similar to that of EH-1. Based on these observations, we thus designed the target compound by introducing a dansyl moiety to the EH-1 (Fig. 1). The synthesis of EH-DF was carried out by reacting dansyl chloride with commercially available N-methyl-1-(1,3,5-trimethyl-1H-pyrazol-4-yl)methanamine (Sigma-Aldrich) in a condition we described previously (Scheme 1).
2. Fluorescence properties of EH-DF

The fluorescence spectra of EH-DF are shown in Fig. 2. EH-DF displayed a maximal excitation wavelength at 350 nm and a maximal emission wavelength at 535 nm. At a concentration of 10 μM, EH-DF displayed sufficient fluorescence intensity of approximately 20 000 in our experimental conditions.

3. Triple response-inducing activities of EH-DF in Arabidopsis seedlings

To determine the effect of EH-DF on inducing triple responses of Arabidopsis seedlings, we recorded the morphological characteristics of Arabidopsis seedlings that were grown in the dark for three days (Fig. 3A). First, we determined the effect of EH-DF on the hypocotyl elongation of Arabidopsis seedlings while using 1-aminocyclopropane-1-carboxylate (ACC, 10 μM) and EH-1 (10 μM) as a positive control. As shown in Fig. 3B, the hypocotyl length of the non-chemically treated control was approximately 9.2 ± 0.7 mm (the white bar), while the hypocotyl lengths of ACC- or EH-1-treated Arabidopsis seedlings were approximately 4.6 ± 0.5 mm (the yellow bar) and 3.5 mm, respectively. This result indicated that ethylene and EH-1 inhibit the hypocotyl elongation of Arabidopsis seedlings in our assay system. In terms of the biological activity of the synthesized EH-DF, we found that EH-DF reduced the hypocotyl length of Arabidopsis seedlings in a dose-dependent manner (Fig. 3B). The lengths of the hypocotyl of Arabidopsis seedlings were 5.2 ± 0.5 mm (5 μM, blue bar), 4.2 ± 0.4 mm (10 μM, green bar), 2.4 ± 0.2 mm (30 μM, red bar), and 1.3 ± 0.1 mm (50 μM, brown bar). Next, we determined the effect of EH-DF on the growth of Arabidopsis seedling roots. As shown in Fig. 3C, the root length of the non-chemically treated control was approximately 1.7 ± 0.1 mm (the white bar), while the hypocotyl lengths of the positive controls of ACC (10 μM) and EH-1 (10 μM)-treated Arabidopsis seedlings were approximately 0.5 ± 0.1 mm (the yellow bar) and 1.0 ± 0.1 mm, respectively. We found that EH-DF reduced the root lengths of Arabidopsis seedlings in a dose-dependent manner (Fig. 3C). The lengths of the roots of Arabidopsis seedlings were 1.8 ± 0.2 mm (5 μM, blue bar), 1.2 ± 0.1 mm (10 μM, green bar), 1.0 ± 0.1 mm (30 μM, red bar), and 0.5 ± 0.1 mm (50 μM, brown bar). Data obtained in the present work indicated that the activity of EH-DF on reducing the root elongation of Arabidopsis was weaker than that of EH-1. Finally, we determined the effect of EH-DF on the apical hook development. As shown in Fig. 3D, the curvature of the hook of the non-chemically treated control was approximately 60 ± 16 degrees (the white bar), while the curvature of the hook of ACC (10 μM, the yellow bar)- and EH-1 (10 μM)-treated Arabidopsis seedlings were approximately 119 ± 19 degrees (the yellow bar) and 205 ± 19 degree (the black bar), respectively. The curvatures of the hooks of EH-DF-treated Arabidopsis seedlings were found to be 150 ± 20 degrees (5 μM, blue bar), 180 ± 25 degrees (10 μM, green bar), 100 ± 24 degrees (30 μM, red bar), and 80 ± 18 degrees (50 μM, brown bar).
grees (10 μM, green bar), 245 ± 35 degrees (30 μM, red bar), and 175 ± 40 degrees (50 μM, brown bar). This result indicated that a high concentration of EH-DF may have a negative effect on inducing the curvature of the hook of Arabidopsis seedlings. The asymmetric growth in the hypocotyl is the driving force for the apical hook curvature. The impairment of the apical hook as induced by treatment of 30 μM EH-DF is assumed to be due to its excessively inhibitory effect on hypocotyl growth, which results in the loss of the cell elongation for asymmetric growth.

Data obtained in the present work clearly indicate that EH-DF displays promising activity for reducing hypocotyl elongation as well as root growth of Arabidopsis seedlings in a dose-dependent manner. However, reducing the root elongation of Arabidopsis seedlings requires a high concentration of EH-DF (Fig. 3B). In addition, EH-DF displays promising activity for inducing the curvature of the Arabidopsis seedlings. Taking the above results together, we have chosen a 30 μM concentration of EH-DF for further fluorescence studies.

4. Fluorescence imaging of EH-DF-treated Arabidopsis seedlings

To determine the effect of EH-DF on the growth of Arabidopsis seedlings, we recorded the fluorescence images of EH-DF-treated Arabidopsis seedlings. As shown in Fig. 4, the blue-green fluorescence signal of EH-DF was significantly observed in the shoots of Arabidopsis seedlings (Fig. 4B). Merging the images captured under bright lights (Fig. 4A) and fluorescence microscopy (Fig. 4B) indicated that the fluorescence signals of EH-DF were intensively found in the upper/or lower parts of cotyledon cells (Fig. 4C). Interestingly, limited fluorescence signals of EH-DF were observed in root cells. To confirm whether these spatial distributions are special to EH-DF, we observed the fluorescence in Arabidopsis seedlings treated with a mock control or 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM), the fluorophore of EH-DF. As shown in Fig. 4D and 4E, the mock control–treated Arabidopsis seedlings displayed dark blue fluorescence (Fig. 4D), while 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)-treated Arabidopsis seedlings displayed green fluorescence (Fig. 4E) in cotyledon cells. Meanwhile, blue and green fluorescence signals were found in stem and root cells of the mock control- and 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)-treated Arabidopsis seedlings, respectively (see supplemental information). In addition, as expected, 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)-treated Arabidopsis seedlings did not show phenotypic characteristic of exaggerated apical hooks (Fig. 4D and 4E). These results indicate that EH-DF has a specific tissue- and cellular-distribution purpose in Arabidopsis seedlings, which could contribute to its physiological effects.

Because the chemical structures of EH-1 and EH-DF are quite similar, it is possible that both compounds display the same mode of action for inducing a triple response in Arabidopsis seedlings. To verify this possibility, we tested the complementary effect of EH-1 on EH-DF-induced triple response by the co-application of EH-1 (15 μM) to EH-DF (15 μM), and the biological activities of the mixed compounds were determined. As shown in Fig. 4F, the co-application of EH-1 to EH-DF enhanced the biological activity of EH-DF, which can be found by
comparing with the control (Fig. 4B, 30 µM EH-DF), while the fluorescence intensities observed in Arabidopsis seedlings were decreased (by comparing Fig. 4C and Fig. 4G). This result indicated that EH-DF may bind to the same target as EH-1.

Discussion

Our previous study identified EH-1 as a non-gaseous chemical inducer of triple response, which is expected to be applied in basic research and agriculture. However, the target and modes of action of EH-1 are still elusive. To provide a strategy for studies on EH-1, we designed and synthesized a derivative (EH-DF) by introducing a fluorescent moiety that could facilitate tracing the chemical in planta (Fig. 1). The fluorescence of EH-DF can be excited and detected at 350 nm and 535 nm (Fig. 2). Morphogenetic observations confirmed that EH-DF displays bioactivities similar to those of EH-1 in triggering a triple response (Fig. 3), indicating their common mode of actions in planta.

We traced EH-DF by observing the fluorescence in Arabidopsis seedlings. Interestingly, we found a tissue-specific accumulation of fluorescence in the cotyledon and hypocotyl but not in the primary root (Fig. 4), while this fluorescence pattern and the exaggerated apical hook were not observed in seedlings treated by the mock control (autofluorescence) or fluorescent moiety of EH-DF (Fig. 4D, 4E, respectively). These results suggest a tissuespecific distribution of EH-DF that could contribute to its physiological effects in hypocotyl elongation and apical hook development. However, the reasons for EH-DF’s inhibition of the primary root growth despite the observance of no fluorescence locally in root remain elusive. For this issue, yet to be determined, we postulate possibilities that EH-DF could interact with a target protein specifically localized in the shoot, or EH-DF could be transported or metabolized in the root.

In addition to tissue specificity, we also noticed a celluarily polar distribution of EH-DF (Fig. 4), which is reminiscent of the distribution pattern of PIN FORMEDs (PINs), the transporters for polar auxin transports (PATs). It has been well characterized that PINs mediate the PAT and are critical for apical hook development, which was also observed in seedlings treated with EH-1 and EH-DF. Thus, it will be interesting to investigate the relationship between EH-DF and PINs or other auxin-related components.

Taken together, although the target protein of EH-1 still must be determined, we have developed EH-DF as a fluorescence tool to dissect the molecular modes of action. Data obtained from the present work clearly confirmed that EH-DF is a biofunctional mimic of EH-1 and indicated a relationship between EH-1 derivatives and auxin. Further investigation of the effects of EH-1 and EH-DF in relationship with auxin may lead us to clarify their modes of action and target proteins.

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Electronic supplementary materials

The online version of this article contains supplementary material (Fig. S1), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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