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ORIGINAL PAPER

Synthesis of a ^{13}C-labelled seed-germination inhibitor (3,4,5-trimethylfuran-2(5*H*)-one) for the mode of action elucidation

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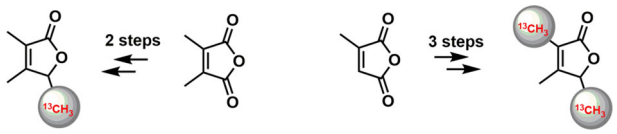
Abstract

It has been found that the butenolide 3,4,5-trimethylfuran-2(5*H*)-one, isolated from plant-derived smoke, efficiently inhibits seed germination and significantly reduces the effect of the highly active germination promotor karrikinolide (KAR_1, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one), another smoke-derived compound. This paper reports the synthesis of stable isotope-labelled 3,4,5-trimethylfuran-2(5*H*)-ones containing one and two ^{13}C atoms for the identification of metabolic degradation products in order to provide insight into the mechanism of action.

Graphical abstract



Keywords Butenolide · Seed-germination inhibitor · Carbon-13 · *Lactuca sativa* · *Lactuca serriola* · Smoke

Introduction

The composition of smoke released by natural fires is very complex with nearly 5000 chemical species [1]. Some of them exhibit a biological activity in the regulation of seed germination. In 2004, a new class of butenolides (karrikins) significantly promoting seed germination was identified in the smoke of burning vegetation [2, 3]. Namely, an extremely efficient germination stimulant, 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (**1**) (KAR_1), was isolated and characterised independently by Flematti et al. [4] and Van Staden et al. [5]. In 2010, a seed-germination inhibitor active at micromolar concentrations was isolated from the smoke and its structure was determined as 3,4,5-trimethylfuran-2(5*H*)-one (TMB, **2**) [6]. Moreover, this compound, antagonistic to the KAR_1, was found to be able to suppress significantly the seed germination promoting effect of KAR_1, as was demonstrated on lettuce seeds (*Lactuca sativa*, cv. 'Grand Rapids') [6].

These two types of smoke-derived regulators, karrikins and TMB, constitute a new class of plant-growth regulators that not only control seed germination [7] but also play an important role in the development of young plants [8] (Fig. 1).

Whereas the germination stimulants karrikins have been a focus of research for their application in agriculture and their mode of action is intensively being investigated [9–16], hardly anything is known about the mode of action of TMB. Despite the structural similarity between **1** and **2**,

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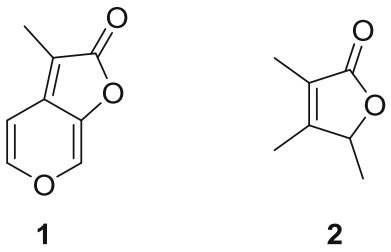


Fig. 1 The structures of **1** and **2**

Soós et al. [17] have demonstrated that these two compounds do not compete for the same receptor. Therefore, the identification of potential TMB-binding proteins and the elucidation of the importance of enzymatic butenolide hydrolysis for biological activity require further investigation.

In order to fill this gap, we have initiated a research project aimed at the identification of the protein-binding site using various types of molecular probes [18]. We have also prepared a series of TMB analogues for a structure–activity relationship study [19, 20] in order to reveal the factors influencing activity and to provide insight into the mode of action of TMB.

In this paper, we report the synthesis of a ^{13}C-labelled inhibitor for the detailed identification of TMB metabolic products and the determination of the metabolic pathway occurring in this process. For this purpose, we have synthesised TMB labelled with one ^{13}C atom (**5**) following a literature procedure [21] and we have also developed a synthetic protocol for the synthesis of TMB labelled with two ^{13}C atoms (**9**).

Results and discussion

Synthesis

Retrosynthetic analysis has revealed that positions 3 and 5 in the TMB molecule are the only accessible sites for stable-isotope labelling using relatively cheap $^{13}CH_3I$. This synthetic protocol has made it possible to introduce two ^{13}C atoms into the molecule, which produces a desirable difference of 2 mass units between the molecular weights of the isotopically labelled and unlabelled TMB and makes it easy to distinguish between metabolites using mass spectrometry. Another increase in molecular mass is possible via deuterium labelling of the TMB molecule, but we have avoided this method due to difficulties in the NMR analysis of the isolated metabolites.

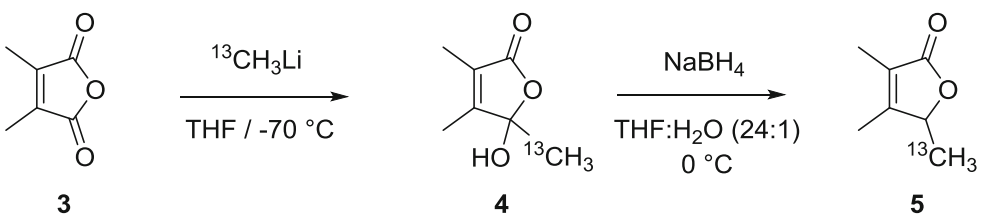
Compound **5**, a molecule bearing one ^{13}C methyl group, was prepared following the two-step procedure reported by Surmont et al. [21] (see Scheme 1), starting from the commercially available 2,3-dimethylmaleic anhydride (**3**). The alkylation of the anhydride with freshly prepared ^{13}C methyllithium gave hemiacetal **4** in a moderate yield of 54% due to the inefficient conversion of $^{13}CH_3I$ to the corresponding organolithium reagent. The target racemic butenolide **5** was then obtained by the reduction of **4** with sodium borohydride in 74% yield. The individual enantiomers were not separated because it had been demonstrated that both forms were equally active as germination inhibitors [6].

Whereas **5** was synthesised according to a published procedure [21], the new three-step synthetic protocol outlined in Scheme 2 was developed for the synthesis of germination inhibitor **9**, labelled with two ^{13}C atoms.

The reaction sequence consists of the known reduction of anhydride to the corresponding 5-hydroxylactone, followed by the introduction of the methyl substituent at C-5 as reported by Canonne et al. [22]. Our synthesis started with the treatment of citraconic anhydride (**6**) with 1.4 equivalents of the reducing agent LiAlH(*t*-BuO)$_3$, which provided hemiacetal **7a** after the separation of a regioisomer mixture of **7a** and **7b**, formed in the ratio 9:1. In the second step, the alkylation of **7a** with freshly prepared $^{13}CH_3MgI$ provided the desired butenolide **8** in a moderate yield of 57%. Finally, methylation in position 3 was accomplished by treating **8** with a strong base followed by the addition of $^{13}CH_3I$ to the preformed enolate. First, the enolate-forming conditions reported by Hinrichs and Margaretha [23] were tested for the synthesis of thiophenones.

However, the heating of **8** with sodium hydride in DMSO did not give the desired product **9** after the

Scheme 1



Scheme 2

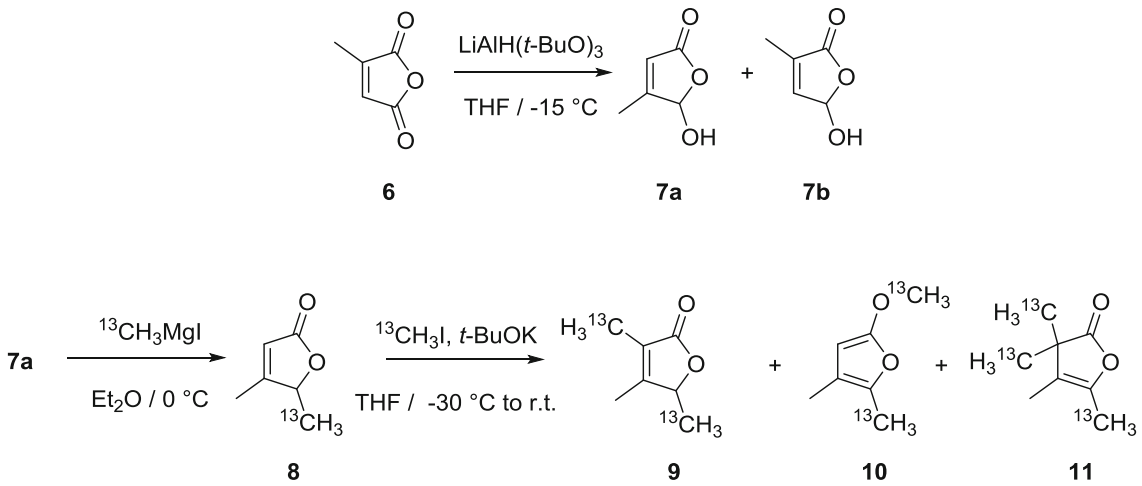


Table 1 The optimisation of the synthesis of **9** (the experiments were carried out with CH_3I prior to the use of $^{13}CH_3I$)

Entry	Conditions	Yield of **9**/%[a]	Yield of **10**/%[a]	Yield of **11**/%[a]
1	NaH, DMSO, 60 °C	0	6	0
2	NaH, DMSO, r.t.	0	9	0
3	NaH, THF, r.t.	2	8	3
4	LDA, THF, − 70 °C to r.t.	8	13	21
5	*t*-BuOK, DMSO, 60 °C	9	15	12
6	*t*-BuOK, DMSO, r.t.	14	11	16
7	*t*-BuOK, THF, r.t.	18	10	14
8	*t*-BuOK, THF, − 30 °C to r.t.	27	8	11

[a]Isolated yield

treatment with CH_3I. The reaction mixture contained mainly polar compounds, most likely resulting from the opening of the butenolide ring under very basic conditions, and traces of product **10**, which was isolated. After the reaction conditions were modified (Table 1), *t*-BuOK was identified as a suitable base for enolate formation (Table 1, entry 8). The target compound **9** was isolated in 24% yield after the addition of $^{13}CH_3I$ (27% yield using CH_3I). Sideproducts **10** and **11** were also isolated and characterised.

The evaluation of the biological activity of TMB (2)

To identify the most suitable model plant for a metabolomics study of the ^{13}C-labelled TMB, we have tested three lettuce accessions. Our tests have demonstrated that cultivated lettuce, *Lactuca sativa* 'Salinas', is highly insensitive to TMB and exhibits only a small delay in germination. The second type, the photoblastic lettuce cultivar *Lactuca sativa* 'Grand Rapids', recently used as a model for the investigation of TMB applied either alone [6] or in combination with karrikins [14], is only sensitive when the achenes are germinated in the dark. The last type, the highly TMB-sensitive wild *Lactuca serriola*, has been found to be the most suitable to study TMB, as its achenes have exhibited more delayed germination (Fig. 2).

Therefore, the sensitive and insensitive accessions and genetic screens based on *Lactuca sativa* 'Salinas' x *Lactuca serriola* inbred populations could provide a useful tool for research into the effect of TMB on germination. Along with stable isotope-labelled derivatives, these systems can shed light on the proposed metabolism of TMB as well as its mode of action.

Conclusion

In conclusion, two types of seed-germination inhibitor (TMB) labelled with one or two ^{13}C atoms have been synthesised. Compound **5** with one ^{13}C atom has been synthesised following the procedure reported in the

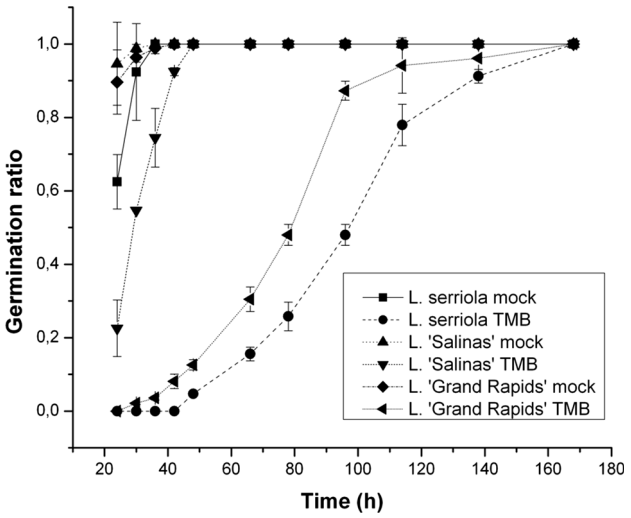


Fig. 2 The germination inhibitory activity of TMB (**2**) and the germination characteristics of *Lactuca serriola* and *Lactuca sativa* 'Salinas' achenes. Achenes (seeds) were germinated in Petri dishes, imbibed in 3 cm^3 of a 20-μM solution of **2**. Germinated achenes were scored for 7 days and the data were analysed with the OriginPro software

literature, while a new synthetic protocol has been developed and optimised for the preparation of **9** with two ^{13}C atoms in the molecule. These stable isotope-labelled molecules may be useful for research into the physiological processes involved in smoke-induced seed-germination inhibition as well as in capturing TMB metabolites and for the assessment their structures and functions. The results from such studies will be reported in due course.

Experimental

^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured using a Bruker Avance I 400 spectrometer (400 and 100 MHz), and in some cases a Bruker Avance I 500 spectrometer (500 and 125 MHz), in acetone-d_6 or in $CDCl_3$ with the residual solvent signal acting as the internal standard (acetone-d_6: ^1H 2.05, ^{13}C 29.84 ppm; $CDCl_3$: ^1H 7.26, ^{13}C 77.16 ppm). The data are reported as follows: the chemical shift (δ) in parts per million (ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, m = multiplet), coupling constant (Hertz), integration, and interpretation. High-resolution mass spectra were recorded using a Waters GCT Premier bench-top orthogonal acceleration time-of-flight (OA-TOF) mass spectrometer with chemical ionisation. TLC analysis was carried out using commercial Sigma-Aldrich aluminium plates coated with silica gel with fluorescent indicator F254s. Spots were visualised under UV and developed in permanganate stain.

All syntheses were carried out under an argon or nitrogen atmosphere in anhydrous THF or diethyl ether, which were dried with sodium and benzophenone and distilled prior to use. Commercially available 2,3-dimethylmaleic anhydride, citraconic anhydride. $LiAlH(t\text{-}BuO)_3$, $NaBH_4$, and $^{13}CH_3I$ were purchased from Sigma-Aldrich and used for the synthesis without further purification.

5-Hydroxy-3,4-dimethyl-5-(methyl-^{13}C)furan-2(5*H*)-one (4) [21] Lithium (0.5 g, 72.00 mmol) was stirred and heated to 210 °C in silicone oil under an argon atmosphere until it melted and very small particles were formed. The mixture was allowed to cool slowly under vigorous stirring and then the oil was removed by a syringe and the lithium particles were washed with anhydrous Et_2O (5 × 10 cm^3). The lithium was suspended in 4 cm^3 Et_2O and a solution of 1.0 g $^{13}CH_3I$ (7.05 mmol) in 2 cm^3 Et_2O was added dropwise. The reaction mixture was left to boil spontaneously, after which it was stirred for further 30 min at 45 °C. After cooling, the solution was filtered and used for the alkylation step.

A solution of 630.0 mg **3** (5.00 mmol) in 20 cm^3 anhydrous Et_2O under an argon atmosphere was cooled to − 70 °C. Subsequently, the above solution of $^{13}CH_3Li$ was added dropwise, the mixture was stirred for 15 min and quenched with 20 cm^3 saturated aqueous solution of NH_4Cl. The mixture was extracted with AcOEt (3 × 30 cm^3) and the combined organic fraction was dried with $MgSO_4$. The solvent was removed in vacuo. Purification by column chromatography (SiO_2, 30% AcOEt in petroleum ether) afforded pure **4** (384 mg, 54%) as a white solid. TLC: R_f = 0.22 (30% AcOEt in petroleum ether); ^1H NMR (400 MHz, acetone-d_6): δ = 6.03 (d, J = 5.2 Hz, 1H), 1.97 (q, J = 1.2 Hz, 3H), 1.72 (q, J = 1.2 Hz, 3H), 1.55 (d, J = 128.3 Hz, 3H) ppm; ^{13}C NMR (100 MHz, acetone-d_6): δ = 172.00 (>C<), 159.45 (d, J = 1.9 Hz, >C<), 124.24 (>C<), 106.01 (d, J = 45.7 Hz, >C<), 23.92 (CH_3), 10.45 (CH_3), 8.37 (CH_3) ppm; MS (EI): *m/z* (%) = 143.0 ([M^+], 3), 127.1 (100), 115.1 (10), 99.1 (87), 83.1 (16), 54.1 (53), 44.1 (61).

3,4-Dimethyl-5-(methyl-^{13}C)furan-2(5*H*)-one (5) [21] A solution of 179 mg **4** (1.25 mmol) in 48 cm^3 H_2O and 2 cm^3 THF was cooled in an ice bath and 237 mg $NaBH_4$ (6.25 mmol) was added in portions. The reaction mixture was stirred for 5 h, after which it was quenched by the addition of 50 cm^3 1 M HCl. The product was extracted with AcOEt (3 × 30 cm^3) and the combined organic fraction was dried with $MgSO_4$. The solvent was removed in vacuo. Purification by column chromatography (SiO_2, 30% AcOEt in petroleum ether) afforded pure **5** (118 mg, 74%) as a colourless oil. TLC: R_f = 0.45 (30% AcOEt in petroleum ether); ^1H NMR (500 MHz, $CDCl_3$): δ = 4.74 − 4.72 (m, 1H), 1.89 (m, 3H), 1.73 − 1.72 (m,

3H), 1.32 (dd, J = 128.8, 6.8 Hz, 3H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.45 (>C<), 160.52 (>C<), 122.74 (d, J = 1.3 Hz, >C<), 79.53 (d, J = 38.5 Hz, CH), 18.11 ($^{13}CH_3$), 11.74 (CH_3), 8.39 (CH_3) ppm; MS (EI): *m/z* (%) = 127.1 ([M^+], 36), 111.1 (12), 83.1 (69), 55.1 (100), 54.1 (16), 44.1 (19).

5-Hydroxy-4-methylfuran-2(5*H*)-one (7a) and 5-hydroxy-3-methylfuran-2(5*H*)-one (7b) A solution of 5.34 g LiAlH(*t*-BuO)$_3$ (21.00 mmol) in 40 cm^3 anhydrous THF was added dropwise over a 30-min period to a solution of 1.68 g citraconic anhydride (**6**, 15.00 mmol) in 50 cm^3 anhydrous THF under a nitrogen atmosphere at − 30 °C. The temperature was maintained at − 15 °C for 3 h and then the reaction mixture was warmed to ambient temperature. The reaction was quenched with 50 cm^3 1 M HCl, the solution was saturated with NaCl, the crude product was extracted with EtOAc (3 × 50 cm^3), and the combined organic fraction was dried over $MgSO_4$. The solvent was removed in vacuo. Purification by column chromatography (SiO_2, 20% AcOEt in petroleum ether) afforded **7a** (1.023 g, 60%) and **7b** (116 mg, 7%) as yellow oils. TLC: R_f = 0.16 (for **7a**), 0.15 (for **7b**) (20% AcOEt in petroleum ether).

7a: ^1H NMR (400 MHz, acetone-d_6): δ = 6.67 (bs, 1H), 6.02 (bs, 1H), 5.87 (p, J = 1.5 Hz, 1H), 2.08 (d, J = 1.5 Hz, 3H) ppm; ^{13}C NMR (100 MHz, acetone-d_6): δ = 171.30 (>C<), 166.65 (>C<), 118.68 (CH), 100.25 (CH), 13.15 (CH_3) ppm; MS (EI): *m/z* (%) = 114.0 ([M^+], 2), 113.0 (7), 86.0 (61), 85.0 (13), 69.0 (100), 68.0 (82), 41.1 (50), 40.1 (65), 39.1 (93).

4-Methyl-5-(methyl-^{13}C)furan-2(5*H*)-one (8) A solution of 2.29 g $^{13}CH_3I$ (16.02 mmol) in 2 cm^3 anhydrous Et_2O was added dropwise at room temperature to a stirred suspension of 600 mg Mg turnings (24.70 mmol) in 5 cm^3 anhydrous Et_2O under nitrogen atmosphere in a two necked flask fitted with a septum and a condenser. The reaction mixture was allowed to spontaneously boil and then was stirred for further 1 h at 40 °C.

A solution of 625 mg **7a** (5.48 mmol) in 5 cm^3 anhydrous Et_2O was added dropwise to a stirred solution of $^{13}CH_3MgI$ at 0 °C. The reaction mixture was cooled in an ice bath for 1 h, after which it was allowed to warm to ambient temperature and stirred overnight. The reaction was quenched by the addition of 50 cm^3 1 M HCl dropwise, the solution was saturated with NaCl and the crude product was extracted with AcOEt. The combined organic fraction was washed with saturated aqueous solutions of $NaHCO_3$, $Na_2S_2O_3$, and brine and then dried over $MgSO_4$. The solvent was removed in vacuo. Purification by column chromatography (SiO_2, 30% AcOEt in petroleum ether) afforded **8** (352 mg, 57%) as a yellow oil. TLC: R_f = 0.25 (30% AcOEt in petroleum ether); ^1H NMR (500 MHz, acetone-d_6): δ = 5.78 (hept, J = 1.3 Hz, 1H), 5.01 − 4.94 (m, 1H), 2.09 (ddd, J = 1.5, 0.8, 0.4 Hz, 3H), 1.38 (ddd, J = 128.8, 6.8, 0.4 Hz, 3H) ppm; ^{13}C NMR (125 MHz, acetone-d_6): δ = 172.98 (>C<), 171.60 (>C<), 116.35 (CH), 81.38 (d, J = 38.2 Hz, CH), 18.40 ($^{13}CH_3$), 13.52 (CH_3) ppm; MS (EI): *m/z* (%) = 113.1 ([M^+], 26), 97.0 (23), 69.0 (100), 68.0 (19), 44.0 (21), 41.1 (56), 40.1 (25), 39.1 (44).

4-Methyl-3,5-di(methyl-^{13}C)furan-2(5*H*)-one (9) A suspension of 348 mg *t*-BuOK (3.1 mmol) in 6 cm^3 anhydrous THF was cooled to − 30 °C under a nitrogen atmosphere and then a solution of 338 mg **8** (3.0 mmol) in 2 cm^3 THF was added dropwise. The reaction mixture was stirred for 30 min, after which 916 mg $^{13}CH_3I$ (6.4 mmol) in 2 cm^3 anhydrous THF was added dropwise. The mixture was allowed to warm to room temperature, stirred overnight and poured into 100 cm^3 water. The pH was adjusted to 4 with 1 M HCl and saturated with NaCl. The crude product was extracted with AcOEt and the combined organic fraction was washed with water, saturated aqueous solutions of $NaHCO_3$, $Na_2S_2O_3$, and brine and then dried over $MgSO_4$. The solvent was removed in vacuo. Purification by column chromatography (SiO_2, 30% AcOEt in petroleum ether) afforded **9** (92 mg, 24%) as a colourless oil. TLC: R_f = 0.45 (30% AcOEt in petroleum ether); ^1H NMR (500 MHz, $CDCl_3$): δ = 4.78 − 4.72 (m, 1H), 1.92 (s, 3H), 1.76 (ddp, J = 128.8, 2.1, 1.1 Hz, 3H), 1.36 (dd, J = 128.8, 6.8 Hz, 3H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ = 174.56 (d, J = 4.1 Hz, >C<), 160.50 (>C<), 122.92 (dd, J = 48.7, 1.3 Hz, >C<), 79.62 (dd, J = 38.5, 3.6 Hz, CH), 18.21 ($^{13}CH_3$), 11.84 (CH_3), 8.50 ($^{13}CH_3$) ppm; MS (EI): *m/z* (%) = 128.1 ([M^+], 47), 112.1 (15), 84.1 (76), 56.1 (100), 55.1 (18), 44.1 (21).

Germination bioassay

The germination activity of TMB was checked in three lettuce accessions: *Lactuca sativa*'Salinas' cultivated lettuce, the wild-type *Lactuca serriola* lettuce, and the photoblastic *Lactuca sativa* 'Grand Rapids'. Freshly collected (i.e. stored for < 2 month in a seed vault) achenes were germinated on two layers of filter paper placed in Petri dishes. The dishes were dampened with either distilled water (mock) or 3 cm^3 of a 20 μM solution of TMB (**2**). Sealed dishes were placed in a controlled environmental chamber (22 °C; continuous low-light regime, 8 μE or in the dark). The germination for the three biological replicates (50 achenes each) was assayed for 7 days.

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