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Short Communication

Stress-Induced Factor Involved in Flower Formation of *Lemna* is an α-Ketol Derivative of Linolenic Acid

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A stress-induced substance(s) (factor C) incubated with norepinephrine (NE) has strong flower-inducing activity in *Lemna paucicostata*. We isolated an essential component (FIF) of factor C, and clarified its chemical structure as 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid, an α-ketol derivative of linolenic acid, which is formed via 9-hydroperoxy linolenic acid. Synthesized FIF showed flower-inducing activity after incubation with NE (factor C activity) equivalent to that formed in the stressed *Lemna*. Jasmonic acid and 13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid (12,13-α-ketol linolenic acid), both of which are formed via 13-hydroperoxide of linolenic acid and all other derivatives of FIF synthesized by chemical and enzymatic processes failed to show the factor C activity. These results suggest that the molecular structure of FIF is very specific for the factor C activity.

Key words: Flowering — *Lemna* — Linolenic acid — Octadecadienoic acid — Oxilipin — Stress.

The water homogenate of many plants, in particular *Lemna paucicostata* and *Pharbitis nil*, has been reported to induce flowering in *L. paucicostata* strain 151 (P151) at very low concentrations (30–100 mg FW liter^{-1}) (Takimoto et al. 1989). Most of the flower-inducing activity was generated during and after the homogenization (Takimoto and Kaihara 1990). At least two factors are involved in this flower-inducing activity; one is norepinephrine (NE) detectable in the supernatant after centrifugation of the plant homogenate, and the other is factor C localized in the pellet, both of which are inactive alone (Takimoto et al. 1991). We also found that *Lemna* plants that had been subjected to drought, heat or osmotic stress, released factor C into the surrounding water (Takimoto et al. 1994). Recently, we isolated an essential component of factor C (flower-inducing factor, FIF). FIF did not induce flowering in P151, but did so after incubation with NE (factor C activity). The factor C activity increased in parallel with the decrease in the FIF concentration in the mixture (data not shown), and we supposed that a flower-inducing substance is produced by the reaction between FIF and NE. However, in a preliminary study, several unstable compounds, each having weak flower-inducing activity were found, and we have not yet been able to identify the active substance. In this paper, only the chemical structure and some characteristics of FIF will be reported.

To isolate FIF, we spread *L. paucicostata* 441 (P441) on dry filter paper for 30 min (drought stress), and immersed the stressed plants in pure water (5 g FW in 100 ml) for 2 h. The water in which the stressed plants had been immersed (WS) was lyophilized and subjected to HPLC (LC 100 system, Yokogawa Electric, Tokyo, Japan) on a CAPCELL PAK C18 column (250×4.6 mm I.D., Shiseido, Tokyo, Japan) at 30°C. The eluent was 50% acetonitrile containing 0.1% trifluoroacetic acid, monitored at 210 nm and flow rate was 1 ml min^{-1}. Each HPLC fraction was lyophilized and the amount equivalent to 6 g FW P441 was dissolved in 400 μl of 2 mM NE at pH 8 (Tris buffer). After incubation for 6 h at 25°C, the solution was added at various concentrations to 10 ml of 1/10 strength E medium containing 1 μM benzyladenine in a flask. P151 was cultured in this medium for 7 d under continuous light, and the percentage of fronds with flowers (% flowering) was examined. This % flowering was regarded as the factor C activity. For details, see our previous paper (Takimoto et al. 1994). In this experiment, however, we used (−)-(R)-norepinephrine hydrogen tartarate (Wako Pure Chemical) as NE.

Most of the factor C activity was concentrated in one peak (FIF). Besides the major activity, two slightly active fractions, one more hydrophilic and one more hydrophobic, were found. However, they were very unstable and could not be concentrated to specific peaks. Because FIF was easily soluble in chloroform, for further analysis of FIF, we first partitioned WS with chloroform; the active fraction was in the chloroform layer. Acetic acid (0.1 ml) was added to the chloroform fraction irrespective of the

Abbreviations: 12,13-α-ketol of linolenic acid, 13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid; FIF, 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid; NE, norepinephrine; WS, the water in which drought-stressed *Lemna* was immersed.

volume, because FIF was relatively stable under an acidic condition. After fractionation by HPLC (the same condition as above, but column size was 250 × 10 mm I.D.), the yield of FIF was 1 to 2 mg per liter of WS.

FIF was a colorless oil, and was considered to be a single substance from the data described below. The molecular formula $C_{18}H_{30}O_4$ was confirmed from the quasimolecular ion peak $(M\text{-}H)^+$ at m/z 311 and $(M\text{-}H)^-$ at m/z 309 in the positive and negative fast atom bombardment mass spectrometry (FAB-MS). The trimethylsilylated product showed a molecular ion peak $(M+3TMS)^+$ at m/z 526 in the electron ionization mass spectrometry (EI-MS). The infrared (IR) spectrum showed absorption bands due to hydroxyl and carbonyl moieties at 3,420 and 1,712 cm^{-1}. The ^1H- and ^{13}C-NMR spectra showed the signals due to a primary methyl {δ 0.98 (t); δc 15.4}, two olefins (δ 5.25, 5.40; δc 133.5, 123.0), (δ 5.55, 5.62; δc 128.4, 134.6), carboxyl carbon (δc 178.5), and ten methylenes, which indicated a fatty acid. Besides the signals described above, the signals of hydroxyl-bearing methine {δ 4,09 (dd); δc 78.6}, and ketone moiety (δc 213.8) were also observed. The proton and carbon signals could be assigned from the 2D-NMR spectrum {correlation spectroscopy (COSY), homonuclear hartmann-hahn spectrum (HOHAHA), homonuclear multi quantum coherence (HMQC)}. The ^1H-detected multiple-bond heteronuclear multiple quantum coherence spectrum (HMBC) showed that keto-carbon has a long-range similarity with the 9-hydroxyl-bearing methine proton and 11-methylene protons, suggesting that FIF includes an α-ketol structure. Furthermore, the differential nuclear Overhauser effect (NOE) experiment showed two olefin groups to have a cis configuration. Based on the evidence described above, the structure of FIF was determined to be 9-hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid (see Fig. 1). This structure has been reported as one of the oxidative products by

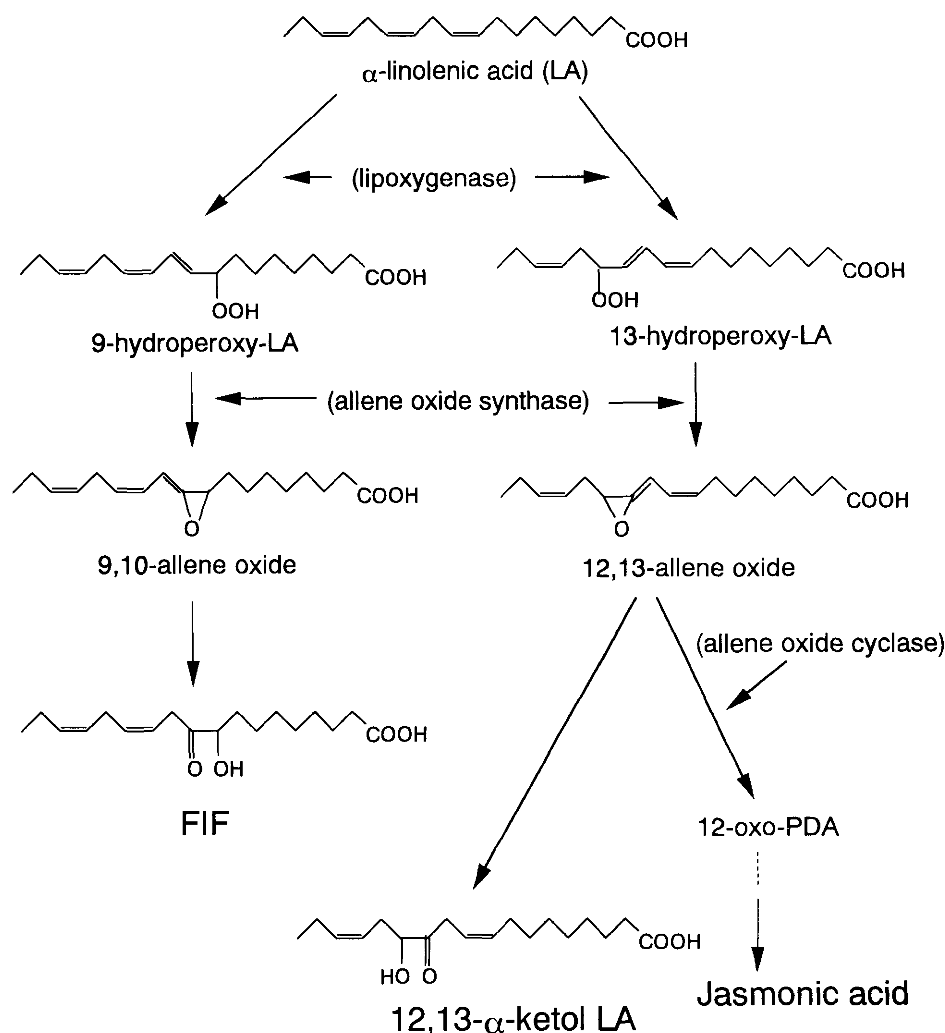

Fig. 1 The putative synthetic route of FIF, 12,13-α-ketol linolenic acid and jasmonic acid.

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Table 1 Factor C activity[a] of drought stress-induced (S) and enzymatically (E) produced FIF

	FIF[b]	Concentration (μM FIF eq)				
		0.01	0.03	0.1	0.3	1
Expt 1	S	—[c]	—	39.4±2.8	47.3±1.7	52.5±3.1
	E	—	—	51.1±4.2	43.6±1.4	54.8±4.5
Expt 2	S	0.7±0.7	21.1±1.0	40.1±7.9	—	—
	E	2.0±2.0	15.5±5.1	31.9±2.7	—	—

[a] Percentage of flowering of P151 cultured on the medium containing the resultant reaction mixture of FIF and NE (2 mM each of FIF and NE had been incubated at pH 8 for 24 h).
[b] For the production of each FIF see text.
[c] Not performed.
Expt 1 and 2 were performed separately at different concentrations.
Data are shown as means±standard errors.

a lipoxygenase of linolenic acid (Graveland 1973). The spectral data were identical with our EI-MS spectral data.

FIF is presumed to be formed by two enzymatic steps; linolenic acid is first oxidized to 9-hydroperoxide by lipoxygenase, and then dehydrated to α-ketol group via allene oxide by allene oxide synthase (Vick and Zimmerman 1987) (Fig. 1). We prepared crude lipoxygenase and allene oxide synthase to synthesize FIF in vitro. Yamamoto and Fujii (1980) found that the treatment of linoleic acid with rice germ lipoxygenase yielded 9- and 13-hydroperoxides at a ratio of 97 : 3. Lipoxygenase of rice germ was fractionated by ammonium sulfate (30 to 70%), followed by heating at 63°C for 5 min and precipitated with ammonium sulfate (70%) according to the method reported by Yamamoto and Fujii (1980). Allene oxide synthase was partially fractionated by ammonium sulfate (45%) in 50 mM phosphate buffer, pH 7.0, using acetone powder of flax seed and dialyzed against phosphate buffer, pH 7.0 (Zimmerman and Vick 1970, Song and Brash 1991). Sodium linolenate was converted to FIF at about a 20% yield when it was treated with the two enzymes. The structure of enzymatically synthesized FIF was identified by IR, ^{1}H-NMR and ^{13}C-NMR (data not shown). This directly suggests that FIF is formed via 9-hydroperoxide and allene oxide. Synthesized FIF showed strong factor C activity at essentially the same level as that isolated from WS, when incubated with NE at pH 8 (Table 1).

Factor C is produced not only by drought stress, but also by osmotic and heat stresses (Takimoto et al. 1994). Therefore, the concentration of FIF in WS obtained from P441 immersed in 0.5 M mannitol solution for 5 min (osmotic stress) and that obtained from P441 immersed in hot water (55°C) for 5 min (heat stress) was examined, together with that obtained from drought stressed P441 (Table 2). The factor C activity in each WS was also determined. The factor C activity was well correlated with the concentration

Table 2 Factor C activity and FIF content in WS prepared by drought, heat, and osmotic stresses

Treatment	Factor C activity (% flowering)		Concentration of FIF in WS (μM)
	0.1 μM NE eq	1 μM NE eq	
Control	0	0	0
Drought stress	3.2±3.2	27.1±5.4	2.90
Heat stress	0	26.4±4.1	1.42
Osmotic stress	34.9±7.1	58.6±1.1	4.78

Five g FW of P441 was spread on filter paper for 15 min (drought stress), immersed in hot water at 55°C for 5 min (heat stress) or immersed in 0.5 M mannitol for 5 min (osmotic stress). The stressed plants were immersed in 100 ml pure water to obtain WS. One ml of each WS with 5 mM NE added and adjusted to pH 8 was incubated at 25°C for 2 h, and the incubated mixture was bioassayed. The remaining WS was extracted with chloroform and FIF was analyzed by HPLC.
Data are shown as means±standard errors (n=3).

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Table 3 Factor C activity of FIF and its derivatives

FIF and its derivatives	Concentration (μM derivative eq)		
	0.1	1.0	10
9-Hydroxy-10-oxo-12(*Z*),15(*Z*)-octadecadienoic acid (FIF)	48.1±7.2	54.5±0.2	—[a]
13-Hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid	0	0	—
9-Hydroxy-10-oxo-12(*Z*)-octadecaenoic acid	0	0	—
9-Hydroxy-10-oxo-12-phenyl-dodecanoic acid	0	0	—
9-Hydroxy-10-oxo-octadecanoic acid	0	0	—
FIF methyl ester	0	0	—
9,10-Dihydroxy-12(*Z*),15(*Z*)-octadecadienoic acid (a)[b]	0	0	—
9,10-Dihydroxy-12(*Z*),15(*Z*)-octadecadienoic acid (b)[b]	0	0.6±0.6	—
9-Hydroxy-10-oxo-11(E),15(*Z*)-octadecadienoic acid	1.3±0.7	6.8±2.3	—
9,10:12,13:15,16-Triepoxy octadecanoic acid	0	0.6±0.6	—
Azelaic acid	0	0	—
Oleic acid	0	0	0
α-Linolenic acid	0	0	2.3±1.4

Each value in the table is the factor C activity expressed as % flowering. FIF and each derivative (2 mM), was incubated with NE (2 mM) at pH 8 for 24 h, and assayed at the concentrations indicated.
[a] Not examined.
[b] Two diastereomer, syn and anti-type diols.
Data are shown as means±standard errors (n=3).

of FIF in the WS obtained by all three stresses. Thus, FIF is considered to be an essential component of factor C that induces flowering after the reaction with NE.

Jasmonic acid, a well-known biological modulator, is synthesized from linolenic acid via 13-hydroperoxide of linolenic acid (see Fig. 1), but showed no factor C activity, neither did 13-hydroxy-12-oxo-9(*Z*),15(*Z*)-octadecadienoic acid (12,13-α-ketol linolenic acid) (Table 3), which is also formed via 13-hydroperoxide of linolenic acid (see Fig. 1). We also examined the factor C activity of various derivatives of FIF. The synthetic methods of these derivatives are not shown here, but each structure was confirmed by NMR. None of the derivatives showed clear factor C activity, although some compounds showed weak activity (Table 3). These results suggested that the total structure of FIF including α-ketol, two olefin and one carbonyl group, is necessary to elicit the factor C activity.

The physiological significance of FIF remains to be examined further, because FIF was not detectable in fresh *Lemna* plants. We are pursuing studies to identify the flower-inducing substances produced by the reaction between FIF and NE, and to determine whether they are present in the plants under flower-inductive conditions.

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