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BIOSYNTHESIS OF 2,3-DIHYDROXYBENZOIC ACID IN TRANSGENIC *CATHARANTHUS ROSEUS* CELL CULTURES OVEREXPRESSING ISOCHORISMATE SYNTHASE

Retno A. Budi Muljono, Julian R. Talou, Johannes J.C. Scheffer and Robert Verpoorte*

Division of Pharmacognosy, Leiden/Amsterdam Center for Drug Research, Leiden University, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands
Author to whom correspondence should be addressed: fax +31 71 527 4511;
E-mail address: Verpoort@LACDR.leidenuniv.nl (R.Verpoorte)

Abstract - The involvement of isochorismate synthase (EC 5.4.99.6) in the biosynthesis of 2,3-dihydroxybenzoic acid (2,3-DHBA), was confirmed by the incorporation of [1-^{13}C]pyruvate in 2,3-DHBA in a transgenic *C. roseus* cell line constitutively overexpressing the *C. roseus* plant *ics*-gene. The label was mainly in the carboxyl group and at C-3 and C-4, which is consistent with isochorismate as intermediate.

INTRODUCTION

Chorismate is a major intermediate in the biosynthesis of the aromatic amino acids, and from there to a broad range of heterocyclic compounds such as the alkaloids. In *Catharanthus roseus* this leads among others to the terpenoid indole alkaloids, including important antitumor alkaloids such as vinblastine and vincristine. In the past years quite some work has been done to try to produce these alkaloids in cell - cultures of *C. roseus*. An interesting finding in this work was that in response to fungal elicitors, *Catharanthus roseus* cell cultures accumulate 2,3-dihydroxybenzoic acid (2,3-DHBA) which is preceded by the induction of isochorismate synthase (= ICS, EC 5.4.99.6) activity.[1] This enzyme will channel away chorismate from the aromatic amino acids biosynthetic routes, and thus from tryptophan, the precursor of the indole alkaloids in this plant. The induction of ICS suggests the occurrence of a biosynthetic route

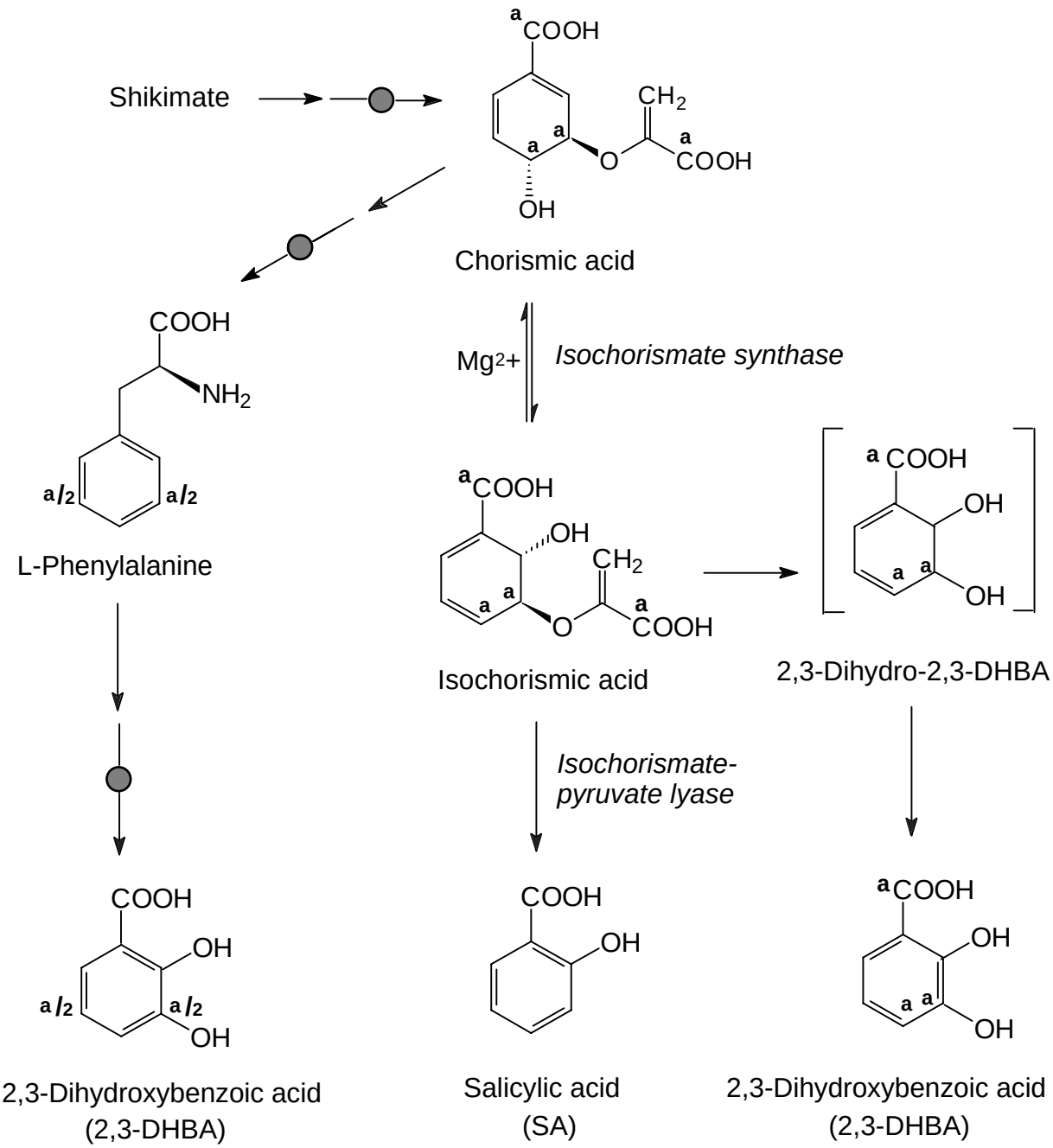
similar as found in microorganisms for the formation of benzoic acid derivatives. In microorganisms salicylic acid (SA) and 2,3-DHBA are precursors for a variety of iron-chelating agents – siderophores –, such as eneterobactin a tricyclic serine-2,3-dihydroxybenzoic acid ester, which are produced under iron-deficient growth conditions.[2]

In the microbial pathway, the formation of these compounds starts with the conversion of chorismate into isochorismate by ICS.[3] Two different pathways from isochorismate lead to SA and 2,3-DHBA respectively (Scheme 1). In case of 2,3-DHBA, the enzyme 2,3-dihydro-2,3-dihydroxybenzoate synthetase converts isochorismate into 2,3-dihydroxy-2,3-dihydrobenzoic acid, which is subsequently oxidized to yield 2,3-DHBA by 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase. The conversion of isochorismate to SA is catalyzed by the enzyme isochorismate pyruvate lyase. Whereas in plants, the benzoic acid derivatives, such as SA and 2,3-DHBA are thought to be derived from phenylalanine, through the intermediacy of *trans*-cinnamic acid and benzoic acid.[4]

In order to investigate the presence of the isochorismate-pathway as an alternative biosynthetic route to benzoic acid derivatives in plants, *C. roseus* cell cultures were chosen as a model. These cell cultures have already been shown to be a suitable system for such an approach after feeding with [1-^{13}C]glucose. The sites of ^{13}C-label-incorporation in secologanin showed clearly that the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway was involved in the biosynthesis of this iridoid.[5]

In the present study [1-^{13}C] pyruvate was chosen as early precursor of chorismate instead of either chorismate or isochorismate as a direct precursor of 2,3-DHBA. Those two compounds are not commercially available with a label in the appropriate position and difficult to synthesize. The fed [1-^{13}C]pyruvate should directly be incorporated in the two carboxyl group of chorismate (Scheme 2). In case of the isochorismate pathway leading to 2,3-DHBA, the labeled carboxyl group would be retained. Whereas in the phenylpropanoid pathway, the carboxyl group of 2,3-DHBA would not carry any labeled as it originates from the unlabeled C-3 of pyruvate.

In our preliminary incorporation experiments, fungal elicited *C. roseus* cell cultures were fed either with [1-^{13}C]pyruvate or [U-^{13}C]phenylalanine. No enrichment at any position of the carbon atoms of 2,3-DHBA was observed (data not shown). Lack of incorporation of the fed compounds into 2,3-DHBA might be caused by inability of those two compounds to reach the right cellular compartment at the right time or they are not intermediates of the biosynthetic pathway. Recently, the gene encoding ICS has been cloned from *C. roseus*.[6]Coupled with the constitutive 35 S -promoter this gene was overexpressed in *C. roseus* cell cultures.[12] These transgenic lines, with constitutively high levels of ICS, and producing considerable amounts of 2,3-DHBA after elicitation, were fed with [1-^{13}C]pyruvate to obtain further proof for the involvement of ICS in the biosynthesis of 2,3-DHBA.



Scheme 1. Possible biosynthetic routes to 2,3-Dihydroxybenzoic acid (2,3-DHBA).

In C*atharanthus roseus* cell cultures, we proposed that the isochorismate pathway is present.

In addition, to have better insight on the effect of the overexpression of the ICS on other chorismate utilizing enzymes in *C. roseus* cell cultures, the levels of anthranilate synthase (= AS, EC 4.1.3.27) and chorismate mutase (= CM, EC 5.4.99.5) were also determined.

MATERIAL AND METHODS

Plant material and elicitor preparation

The transgenic *Catharanthus roseus* (L.) G. Don (Madagascar periwinkle) lines were obtained by transformation of line CRPM58 with the ICS cDNA (~2000 bp) gene isolated from *C. roseus* [6] and expressed under the regulatory control of the cauliflower mosaic virus (CaMV) 35S promoter using pMOG22 vector (MOGEN, Leiden, the Netherlands) containing a hygromycine-resistance (HPT) gene.[12]
The cell cultures were grown in 50 mL MS medium (Murashige and Skoog)[8] containing 3% sucrose, and supplemented with 1-naphthaleneacetic acid (2 mg/L), kinetin (0.2 mg/L). The medium had a pH of 5.8. Subculturing was performed weekly by inoculation of about 6 g of cell FW per flask containing 50 mL of medium. The cultures were kept on a gyrotary shaker (115 rpm) at 25°± 1°C under continuous light (2800 lux).
The fungus *Pythium aphanidermatum* (CBS 313-33) was maintained on MS medium with 3% sucrose (without growth regulators) at 27°C in the dark. The fungus was transferred to MS liquid medium and placed on a gyratory shaker (300 rpm) at 30°C in the dark for one week. The resulting mixture was autoclaved. The fungal cells were separated from medium by filtration and subsequently the filtrate was used as elicitor preparation.

Elicitation of *C. roseus* cell suspension cultures

The elicitation was performed by addition of an autoclaved elicitor preparation: 10 mL per culture flask of the fungal elicitor or 3 mL per flask of sterile-filtered yeast extract (DIFCO, 10 %, w/v). As control, 10 mL of the fresh fungal growth medium was added per flask. The cells were harvested 24 h after elicitation, separated from the medium by filtration. The growth medium was used for isolation of 2,3-DHBA and SA.

Quantification of 2,3-DHBA and SA by HPLC

The analyses were made with an isocratic RP-HPLC system consisting of a 515 HPLC pump from Waters (Milford, MA, USA) equipped with a Gilson model 231 autosampler (sample injection volume 20 μL),

and a fluorescence detector RF 535 (Shimadzu) equipped with an 8 µL flow cell operating at an emission wavelength of 437 nm and excitation wavelength of 306 nm.

The analyses were carried out at room temperature using a 4.0 mm (i.d.) x 125 mm LiChrospher 60 RP-select B column (Merck) with a particle size of 5 µm, and a flow rate of 1.0 mL/min. The mobile phase consisted of 20 mM aqueous NaOAc - MeOH (9:1, v/v) at pH 5.0. The peaks were identified by direct comparison with known standards (Sigma).

To quantify 2,3-DHBA and SA in the medium of the *C. roseus* cell cultures, after filtration the medium was centrifuged at 3500 rpm for 10 min and was adjusted to about pH 4 with phosphoric acid. From 5 mL of the resulting solution the phenolic acids were extracted with twice the volume of ethyl acetate. The organic layer was collected and evaporated under vacuum until dryness. The residue was redissolved in the mobile phase and subsequently submitted to HPLC.

Determination of chorismate-utilizing enzymes

The assays for AS and ICS were performed according to the method of Poulsen *et al.*,[9] but with a slight modification using 2 mM barium chorismate as substrate instead of 1 mM and a shorter incubation time of 30 min. Chorismate mutase was assayed according to Bongaerts.[10] Protein content of the crude enzyme preparation was determined according to Bradford [11] using BSA as standard.

Administration of [1-^{13}C]pyruvate and elicitation of *C. roseus* cell suspension cultures

The elicitation was carried out by addition of an autoclaved elicitor preparation (see above) together with 100 µL 500 mM [1-^{13}C]pyruvate (99%, MSD ISOTOPES, Canada) into each flask of a batch of 20 flask of eight-day old cells grown on the normal growth medium containing already 1.0 mM [1-^{13}C]pyruvate. As controls, one batch of 20 flasks of cells was grown under the same conditions, but containing 1.0 mM unlabeled pyruvate. The cells were harvested 24 h after elicitation. The growth medium was used for the incorporation study.

NMR spectroscopy

^{1}H and ^{13}C-NMR spectra of 2,3-DHBA were recorded in MeOD using a Bruker DPX 300 spectrometer equipped with an indy (silicon graphics) computer. ^{13}C-NMR spectra were measured as follows: 38° pulse (4 µs): repetition time 2 s; spectra width, 18.83 kHz data set, 32 kilo-words; temperature, 20°C; line broadening, 2.5 Hz; ^{1}H decoupling by WALTZ 16 during acquisition and relaxation. All ^{13}C-NMR signals of 2,3-DHBA were assigned unequivocally on the basis of two-dimensional ^{1}H-^{13}C correlation experiments in conjunction with ^{1}H homocorrelation experiments and long range C-H data (HMBC). In

the incorporation experiment, the ^{13}C abundance of the carbon showing the lowest intensity was assumed to have the same intensity as found in the controls. This signal intensity was set at 1, the increments of the other signals were expressed relative to this signal.

Extraction 2,3-DHBA from the medium

The medium (around 800 mL) was adjusted to pH 4.0 with phosphoric acid and extracted gently with twice the volume of ethyl acetate (Baker, Deventer, The Netherlands). After separation of the two layers, the organic layer was evaporated under reduced pressure until dryness. No further purification was performed. The sample was dissolved in MeOD and subsequently analyzed for the incorporation using ^{13}C NMR spectrometry.

RESULTS AND DISCUSSION

Characterization of transgenic *Catharanthus roseus* cell lines

Transgenic *C. roseus* cell culture lines (S1, S4 and S9) which constitutively overexpressed the *C. roseus ics*-gene were available from the previous transformation experiments.[12] In these cell lines the endogenous level of ICS was in the range of about 2.5-4.0 nkat/mg protein. This is similar to previously measured values for these cell lines.[12] These activities are between ten to fifteen times higher than in the control wild type *C. roseus* cell line (*ca.* 0.25 pkat/mL) used for the transformation. Under the normal growth condition (without elicitation) only the cell lines S1 and S4 produced and excreted a trace amount of 2,3-dihydroxybenzoic acid (2,3-DHBA) (*ca.* 2-5 ng/mL) into the medium.

In order to see whether the production of 2,3-DHBA and the induction of ICS could be increased further, these cell lines were elicited with a fungus (*P. aphanidermatum*) and yeast extract. In accordance with previous results,[1] the wild type (WTP) *C. roseus* cell cultures produced 2,3-DHBA and a small amount of salicylic acid (SA) in response to the elicitation (Figure 1A). These products are mainly found in the media.[1]

In fungal-elicited cell line S1, a larger amount of 2,3-DHBA (*ca.* 25µg/mL) was obtained in the medium about 24 h after the treatment, than in cells treated with yeast elicitor (Figure 1B). It was about 25-fold higher than in the non-elicited cell line S1. In contrast, the fungal-elicited cell line S4 produced much less 2,3-DHBA (Figure 1C), despite its high ICS activity. Therefore, the cell line S1 was chosen for a further incorporation study after treatment with the fungal-elicitor.

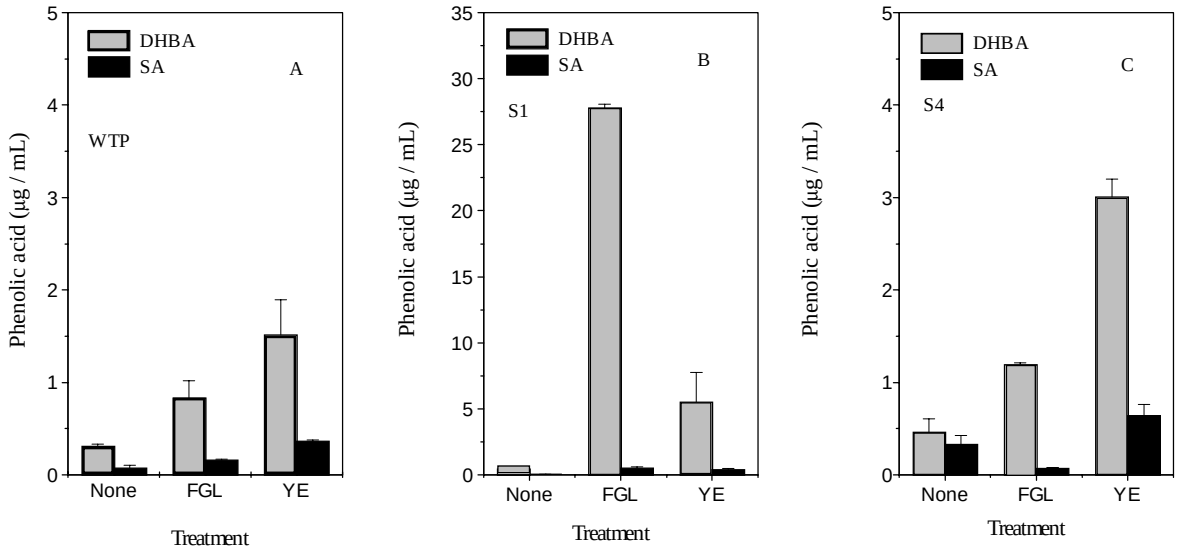


Figure 1. The levels of 2,3-dihydroxybenzoic acid (2,3-DHBA) and salicylic acid (SA) in the media of wild-type (WTP) *Catharanthus roseus* cell line and in the transgenic *C. roseus* cell lines: S1 and S4, 24 h after elicitation with a fungal cell preparation of *Phytium aphanidermatum* (FG) and yeast extract (YE). None = untreated control cells.

With regard to the chorismate-utilizing enzymes, the ICS activity increased about thirty-fold in the wild type *C. roseus* cell line compared to that of the non-elicited cell line, about 24 h after the treatment with fungal or yeast elicitor (Figure 2A). In the elicited, transgenic cell lines S1 and S4 (Figures 2B and 2C) the total ICS activity was almost doubled if compared with the control non-treated transgenic cells. The increase of ICS activity in the transgenic *C. roseus* cell lines can be explained by the induction of the native gene.

Chorismate is an important branching point in primary metabolism. Particularly the enzymes anthranilate synthase (AS) and chorismate mutase (CM), leading to the aromatic amino acids tryptophan and phenylalanine/tyrosine respectively, are important for both primary and secondary metabolism. Under normal growth conditions lines S1 and S4 have similar constitutive levels of AS as the wild-type cell line. However, after elicitor treatment, the transgenic cell lines did not show the strong increase of AS activity as observed in the wild-type control cell line (Figure 3).

No significant difference in the level of CM could be observed between the wild type cells and the transformed cells before and after elicitation (Figure 4).

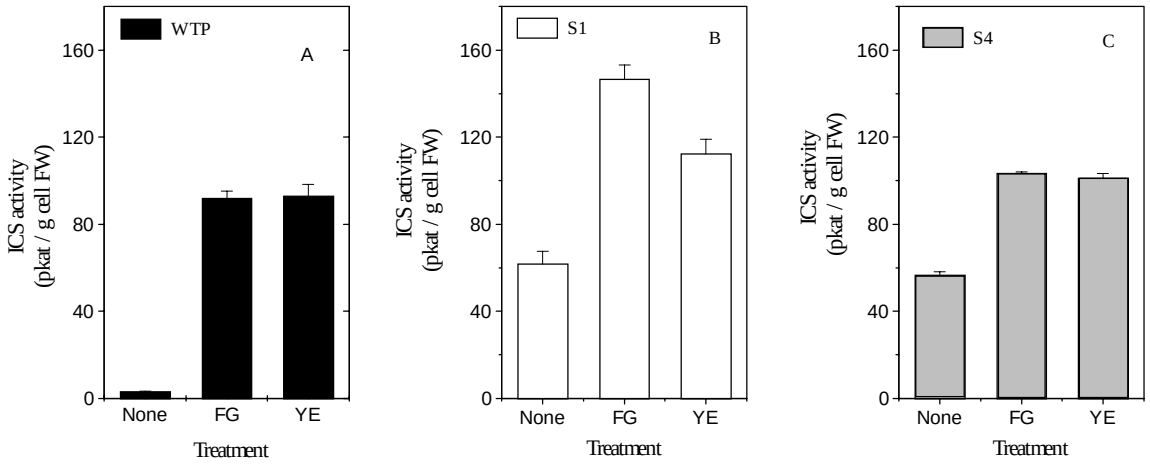


Figure 2. Isochorismate synthase (ICS) activity in the wild-type *Catharanthus roseus* (WTP) cell line and in the transgenic *C.roseus* cell lines: S1 and S4, with and without the addition of elicitor. FG = *Pythium aphanidermatum*-elicited cells, YE = yeast-elicited cells

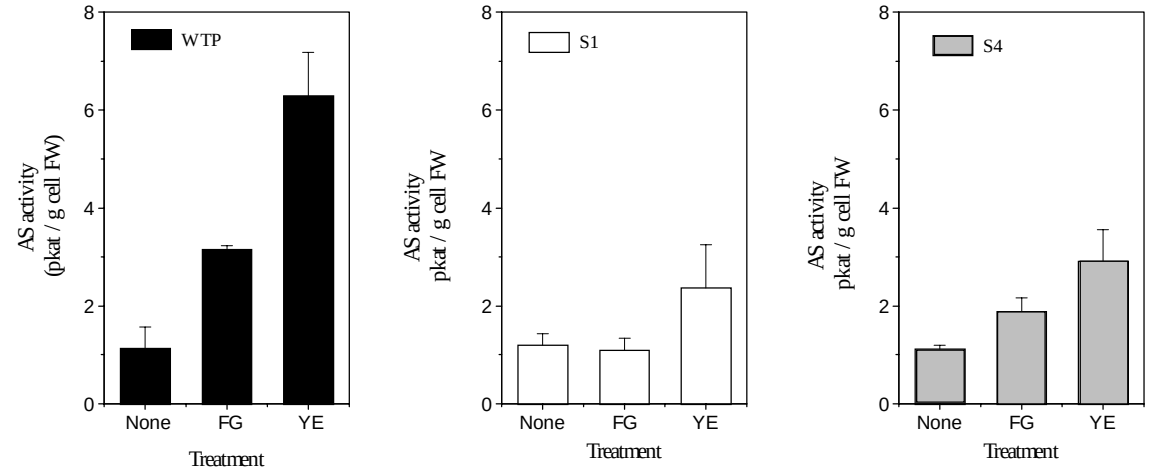


Figure 3. Anthranilate synthase (AS) activity in the wild-type *Catharanthus roseus* cell line (WTP) and in the transgenic *C. roseus* cell lines S1 and S4, with and without the addition of elicitor. FG = *Pythium aphanidermatum*-elicited cells, YE = yeast-elicited cells.

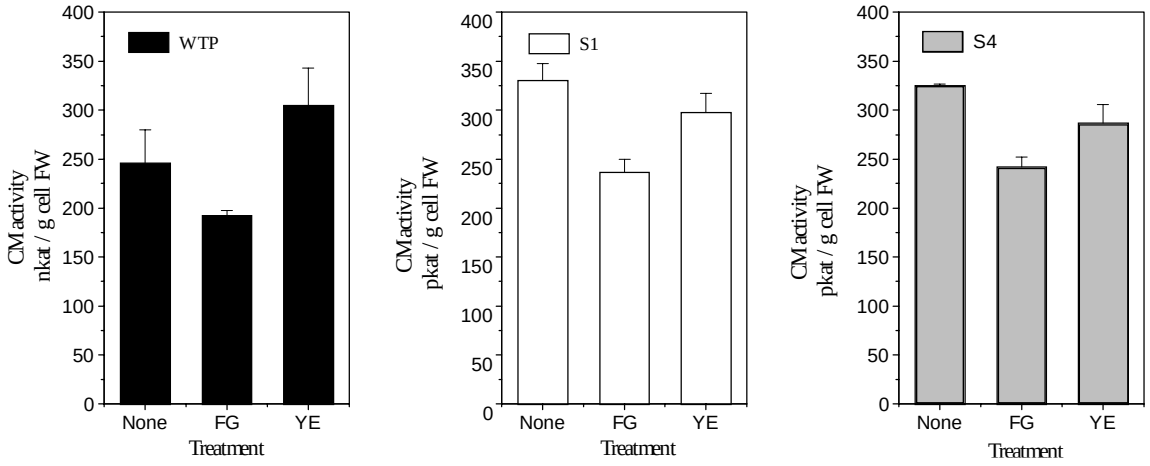


Figure 4. Chorismate mutas (CM) activity in the wild-type *Catharanthus roseus* cell line (WTP) and in the transgenic *C. roseus* cell lines: S1 and S4, with and without the addition of elicitor. FG = *Pythium aphanidermatum*-elicited cells, YE = yeast-elicited cells.

Incorporation [1-^{13}C]pyruvate in 2,3-DHBA in an *ics*-gene overexpressing *Catharanthus roseus* cell line

In an attempt to prove the intermediacy of isochorismate synthase in 2,3-DHBA biosynthesis, the transgenic *C. roseus* cell line (S1) was grown on a medium containing [1-^{13}C]pyruvate, and subsequently treated after 8 days with the fungal elicitor to which further [1-^{13}C]pyruvate was added. The 2,3-DHBA excreted into the medium was isolated and analyzed by ^{13}C-NMR spectrometry. The results of the incorporation experiment are summarized in Table 1.

Table 1. ^{13}C-NMR spectral data of 2,3-DHBA from fungal elicited transgenic *Catharanthus roseus* cells fed with [1-^{13}C]pyruvate

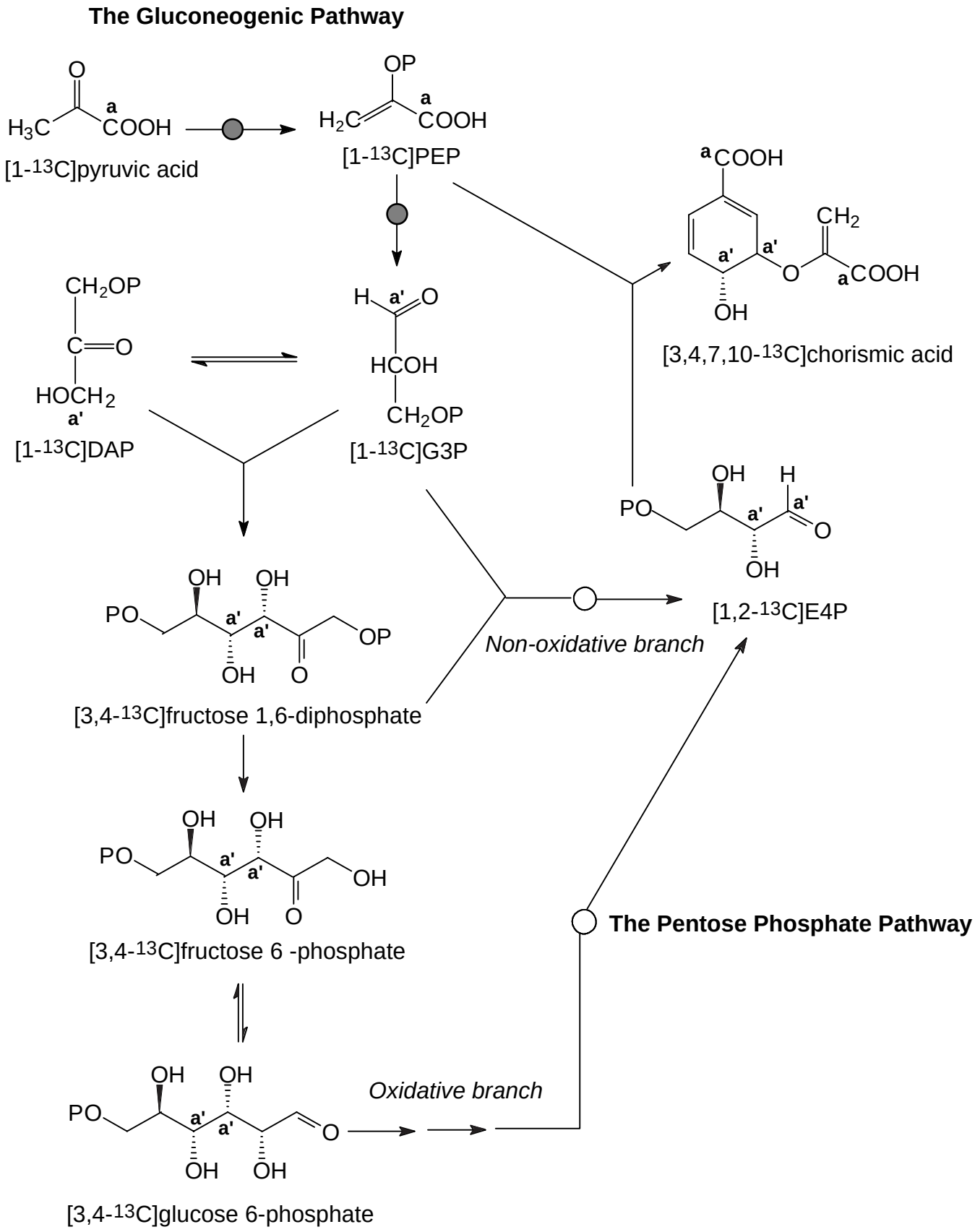
Carbon Atom	Chemical shift [ppm]	Relative Intensity C[a]	Relative Intensity I.E.[b]	Relative increment[c]
1	146.0	2.035	1.605	1.00
2	151.7	1.000	1.000	1.27
3	114.1	1.239	1.613	1.65
4	121.5	6.906	7.879	1.45
5	119.7	7.459	7.249	1.23
6	121.8	7.529	7.123	1.20
7	179.9	1.218	1.631	1.98

[a]C : a relative intensity control experiment with non-labeled pyruvate

[b]I.E.: incorporation enrichment with label [1-^{13}C]pyruvate

[c]Relative increment calculated by normalizing I.E./C on carbon with lowest incorporation

Comparison of the intensity of the signals of each carbon of the isolated 2,3-DHBA with those of the control compound (Table 1) shows that the ^{13}C label is observed mainly in the C-7 (carboxyl group) and to a lesser extent in the C-3 and the C-4 of 2,3-DHBA. The strong labeling in the carboxyl group of 2,3-DHBA occurs only in the isochorismate pathway. In the isochorismate pathway the carboxyl group is retained, and this group is directly labeled from C-1 in [1-^{13}C]pyruvate (Scheme 2). In case of the phenylpropanoid pathway, the carboxyl group of chorismate is lost and replaced by the non-labeled C-3 of the enolpyruvate substituent of chorismate.



Scheme 2. The introduction of labeled [1-^{13}C]pyruvic acid into chorismic acid

The carboxyl group in the benzoic acid derivatives formed along this pathway would thus not carry any label. The isotope-distribution data in Table 1 further show incorporation at the position of the C-3 and the C-4 of 2,3-DHBA. These labels derived from C-1 and C-2 of erythrose 4-phosphate [E4P] formed from the fed [1-^{13}C]pyruvate via the pentose phosphate pathway (Scheme 2).[13] The carbons C-3 and C-5 in 2,3-DHBA have quite different labeling, this is also in agreement with isochorismate as intermediate. In case of phenylalanine as intermediate, these two carbons would be identical, and thus have the same labeling percentage.

CONCLUSIONS

Overexpression of the *ics*-gene in the *C. roseus* cell lines had little effect on 2,3-DHBA production under normal growth conditions. Apparently other enzymes are limiting the flux into the pathway leading to 2,3-DHBA, or rapid catabolism of this compound occurs. However, after elicitation, the transgenic *C. roseus* plant cells overexpressing the endogenous *ics*-gene are able to synthesize 2,3-DHBA through the isochorismate pathway, as can be deduced from the labeling pattern after feeding labeled pyruvate.

Unfortunately the levels of SA were too low to allow measurements of incorporation. Moreover, it cannot be excluded that SA is also formed from isochorismate in the transgenic cells, as also in tobacco overexpressing a microbial ICS encoding gene (entC-gene) produce small amounts of SA, probably formed by chemical degradation of isochorismate.[14]

As feeding experiments with labeled pyruvate and phenylalanine in wild type cells did not result in any incorporation, further studies are needed with other early precursors to confirm unequivocally the intermediacy of isochorismate in the biosynthesis of 2,3-DHBA the non-transgenic cells.

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