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Biochemical complementation of the betalain biosynthetic pathway in *Portulaca grandiflora* by a fungal 3,4-dihydroxyphenylalanine dioxygenase

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Abstract. 3,4-Dihydroxyphenylalanine (DOPA) dioxygenase from *Amanita muscaria* catalyses the key reaction of betalain biosynthesis, namely the conversion of DOPA to betalamic acid by a 4,5-ring-opening reaction. In addition, it catalyses a 2,3 opening which yields the fungal pigment muscaflavin, a compound that has never been found in plants. In this work, a cDNA clone (*DodA*) encoding *A. muscaria* DOPA-dioxygenase was expressed in white *Portulaca grandiflora* petals, using the particle bombardment technique. Transformation resulted in the formation of yellow and violet spots that contained betalain pigments and muscaflavin, indicating that the fungal enzyme was expressed and active in plants, and could complement the plant betalain biosynthetic pathway. The presence of muscaflavin in transformed plants indicates a difference in the specificity of the plant and *A. muscaria* enzymes.

Key words: *Amanita* – Betalain – Dioxygenase – Heterologous expression – Particle gun

Betalain pigments appear exclusively in the plant order Caryophyllales and also in some basidiomycete species, such as *Amanita muscaria* (Musso 1979; Böhm and Rink 1988). From this mushroom, two enzymes involved in betalain synthesis have been characterised (Girod and Zrÿd 1991; Terradas and Wyler 1991; Mueller et al. 1996, 1997). These enzymes, a tyrosinase and a 3,4-dihydroxyphenylalanine (DOPA) dioxygenase, convert the precursor tyrosine to DOPA and to the yellow chromophore betalamic acid. The latter condenses with amino acids or amines to form yellow betaxanthins, or with cyclo-DOPA derivatives to form violet betacyanins (Böhm and Rink 1988).

Knowledge of the enzymology of betalain synthesis in plants is limited. The only enzyme preparations that have been described catalyse the glucosylation of betacyanin aglycones (Heuer and Strack 1992; Heuer et al. 1996). The genetics of betalain synthesis, on the other hand, has been studied extensively in the large-flowered purslane (*Portulaca grandiflora*). A model involving three loci in the biogenesis of betalains has been established (Trezzini and Zryd 1990): *locus C* is responsible for colour, *locus R* for the generation of betacyanins, and *locus I* inhibits the accumulation of betaxanthins. At least under in vitro conditions betalamic acid reacts spontaneously with amino acids to form the corresponding betaxanthins, and it has been proposed that this also happens in the plant vacuole, while the formation of cyclo-DOPA and its reaction with betalamic acid is thought to be cytoplasmic (Trezzini 1990). In this case, *locus I* might act on the level of the transport of the betalamic acid into the vacuole, thereby preventing the condensation with vacuolar amino acids (Trezzini 1990). Despite its simplicity, this genetic model accounts for all *P. grandiflora* phenotypes and fits well with the biochemistry of betalain formation (Trezzini and Zryd 1990).

The *A. muscaria* DOPA-dioxygenase is an extradiol-ring-cleaving dioxygenase (Impellizzeri and Piattelli 1972) that cleaves on either side of the two hydroxyl groups and thus produces both betalamic acid (cleavage between carbons 4 and 5) and muscaflavin (cleavage between carbons 2 and 3; Mueller et al. 1997). The latter pigment is characteristic of fungi belonging to the genus *Hygrocybe* and is also found in some *Amanita* species, e.g. *A. muscaria*, but it has never been found in plants (Reznik 1978). This could indicate a fundamental difference in the enzymology of betalain biosynthesis in plants and *A. muscaria*. However, since the betalain/muscaflavin product ratio of the *A. muscaria* enzyme is pH dependent (Terradas and Wyler 1991), and could depend on other unidentified factors, expression of the

Abbreviations: CaMV = cauliflower mosaic virus; DOPA = 3,4-dihydroxyphenylalanine; Gus = β-glucuronidase

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A. muscaria enzyme in *P. grandiflora* should be tested with regard to the nature of the products formed. The prerequisite for these experiments is the expression of DOPA-dioxygenase in an active form in the correct subcellular compartment.

True-breeding white *P. grandiflora* lines had been established in our laboratory by Trezzini (1990). For the complementation experiments, petals from plants homozygous recessive for *c* (no pigment production) and a background containing either *RI* or *ri* (violet or yellow color) were used. These petals accumulate high concentrations of DOPA and dopamine, making them an ideal system for the expression of fungal DOPA-dioxygenase.

A plant expression vector was constructed (pNco DodA) that contained the DOPA-dioxygenase cDNA (Hinz et al. 1997) under the control of the cauliflower mosaic virus (CaMV) 35S promoter, a Kozak translation initiation sequence and a 35S terminator (Fig. 1). The pNco DodA was precipitated on gold particles as previously described (Christou 1994). For ten shots, 100 µl of gold particle suspension (50 $\mu g \cdot \mu l^{-1}$) was added to a mixture containing 20 µl of DNA (1 $\mu g \cdot \mu l^{-1}$) and 80 µl of water followed by vortexing. Then, 120 µl of a 2.5 M solution of $CaCl_2$ was slowly added and vortexed. After a 1-min incubation on ice, 60 µl of spermidine (0.1 M stock) was added and vortexed. Finally, 500 µl of ethanol was added, vortexed, and centrifuged. The pellet was resuspended in 80 µl water and again 20 µl of DNA was added, and thoroughly vortexed.

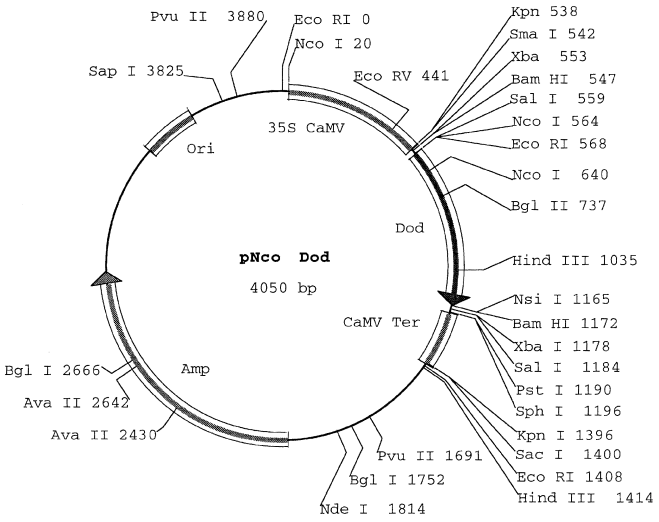

Fig. 1. The plant expression vector pNco DodA expressing DOPA-dioxygenase from *Amanita muscaria*. The DOPA-dioxygenase cDNA (*DodA*) is under the control of a CaMV 35S promoter and terminator, cloned into a pUC18 vector. The plant expression vector was constructed by cloning the *DodA* cDNA (Hinz et al. 1997; Mueller et al. 1997) into pDH51 (Pietrzak et al. 1986). The *DodA* cDNA was expressed as a β-galactosidase fusion protein in a pBluescript construct, pBS DodA. An *Nsi* I-*Cla* I fragment was removed at the 5′ end of the *DodA* cDNA in pBS DodA and replaced with a linker containing a *Bam* HI site. The *Bam* HI-*Eco* RI fragment containing the *DodA* cDNA was cloned into an *Nde* I-*Bam* HI-digested pET 3a vector using an adapter containing an in-frame ATG, resulting in the bacterial expression vector pET DodA. This plasmid was digested with *Xba* I and the 5′ overhang filled with the Klenow enzyme, and then digested with *Bam* HI, and the *Xba* I-(blunt)-*Bam* HI fragment cloned into the pDH51 plant expression vector opened with *Sma* I-*Bam* HI, resulting in pDH DodA. In order to supply a better eukaryotic translation initiation context, a *Sac* I-*Eco* RI fragment from pDH DodA containing the *DodA* cDNA and the CaMV terminator was digested and cloned into pDH 51 that had been previously digested with *Sal* I and *Sac* I. The *Sal* I-*Eco* RI gap was filled using an adaptor containing the ATG within the Kozak sequence. The resulting plasmid was pNco DodA. Molecular biology techniques were performed as described by Sambrook et al. (1989)

For the bombardement of the petals, a particle inflow gun was used as described previously (Vain et al. 1993a). The optimal shooting parameters for *P. grandiflora* petals were established using a β-glucuronidase (Gus) construct, pNco Gus, which was identical to pNco DodA except for the coding region. After incubation for 2 d at 26 °C, petals were stained for Gus activity by incubating the petals in Gus assay buffer [0.2 M Na-phosphate buffer, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6] \cdot 3H_2O$, 10 mM Na_2EDTA (pH 8.0), 0.1 $mg \cdot ml^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GlcA)] in 24-well enzyme-linked immunosorbent assay (ELISA) plates. Blue spots were counted after 24 h of incubation. The following parameters had to be adjusted: (i) the osmolarity of the semi-solid medium, on which the tissue to be bombarded is deposited (Vain et al. 1993b), (ii) helium pressure, (iii) sample stage height, and (iv) the height of a baffle that was placed in the helium stream. The pressure was varied from 5 to 8 bars at 0.5-bar intervals and the stage and filter height were varied from 50 to 100 mm and 100 to 200 mm intervals, respectively, with steps of 10 mm. The optimal conditions were: medium containing 0.6% agar supplemented with 44 $g \cdot l^{-1}$ mannitol (250 mosmol), helium pressure 6.5 bar, 115 mm distance from aperture to sample and 65 mm distance from aperture to baffle. When a vacuum of −0.8 bar (against outside ambient pressure) was reached, the shoot button was pressed twice. Another important factor was the age of the petals. *Portulaca grandiflora* flowers are in bloom for only 1 d and then wilt. Accumulation of DOPA is highest just before the flower opens (Trezzini 1990), and therefore young petals isolated from flower buds were the preferred material. The petals were mounted in the center of a petri dish.

When the pNco Gus construct was used for bombardment, 50–100 blue spots were counted per petal after staining for Gus activity. Since the product of the Gus assay is water-insoluble and precipitates, the spots formed did not exceed the size of a single cell (Fig. 2A). Petals bombarded with gold particles devoid of DNA and stained for Gus activity did not exhibit blue spots (data not shown). A slight browning of tissue was observed in some samples due to wounding.

Microballistic bombardment of petals using the pNco DodA construct was carried out using conditions identical to those for the Gus transformations. Formation of yellow and violet spots (depending on the background genotype) was observed 12–24 h after transformation (Fig. 2B–E). Coloured spots did not form in controls; as negative controls we used transformation with the Gus construct and mock transformation without DNA as an alternative to using an antisense Dod construct. The number and distribution of spots on the petals (Fig. 2E) were comparable to the spots obtained with pNco Gus (data not shown). About 18 h after bombardment small spots appeared that were the size of a single cell (Fig. 2B). The size of the spots increased with incubation time and became visible to the naked eye, probably owing to the fact that the water-soluble pigments diffused through plasmodesmata to neighbouring cells (Fig. 2D). Apparently, a fraction of the pigments was not stored in the vacuole of the transformed cell, where betalains are normally located. One possible explanation is that saturation of the tonoplast membrane transport system occurred, due to

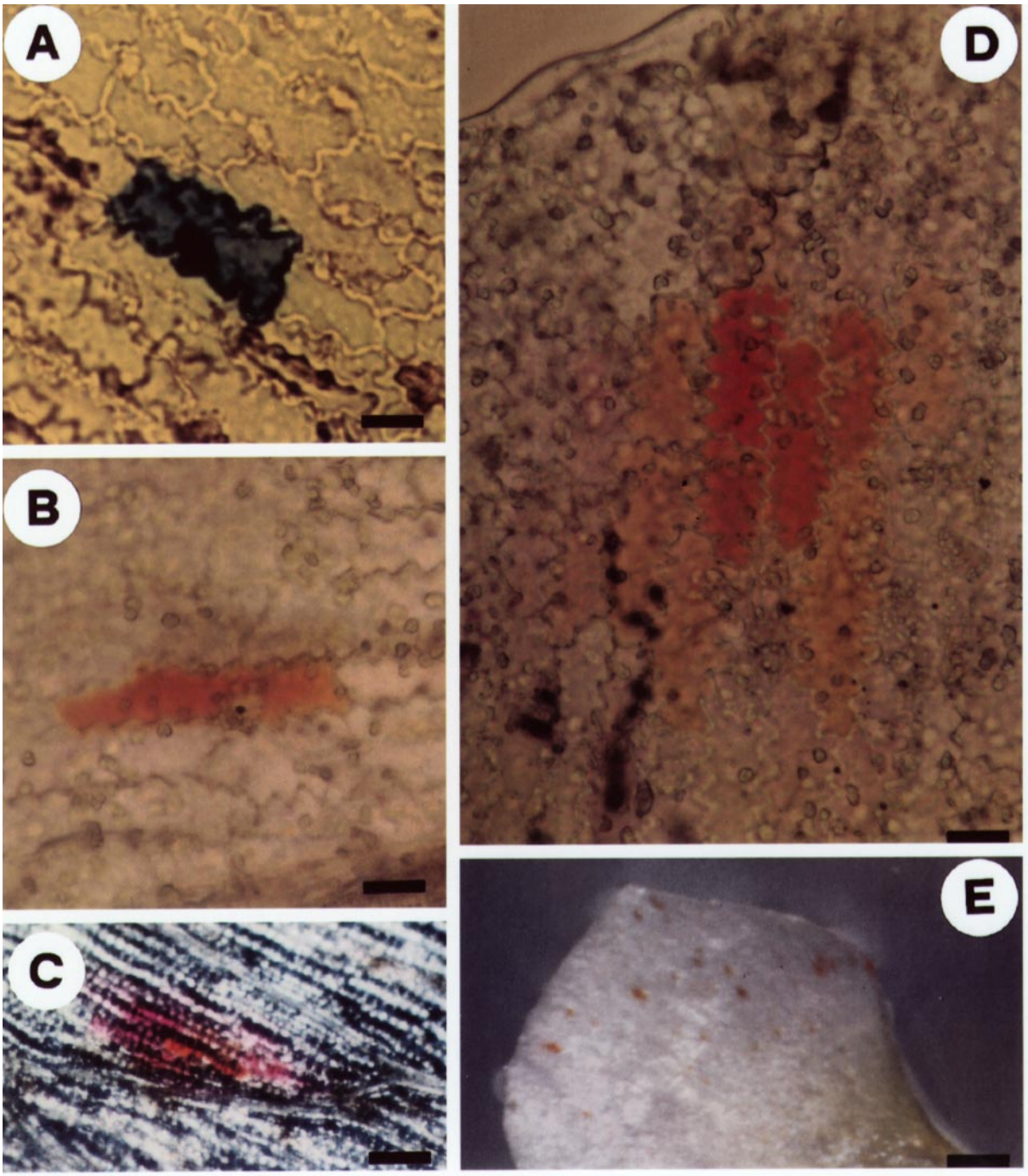

Fig. 2. **A** *Portulaca grandiflora* petals transformed using pNco Gus and the particle bombardment technique. After staining, microscopic blue spots appear. **B** A cell transformed with pNco DodA early after transformation. Transformed cells appear orange. **C** A cell transformed with pNco DodA on petals from plants with a genetic background yielding violet betacyanin pigments. **D** Closeup of spots on pNco DodA-transformed petals, ca. 48 h after transformation. The pigments can be seen to leak into neighbouring cells. **E** Transformed *Portulaca grandiflora* petals using pNco DodA and the particle bombardment technique. Aspect of a petal as seen through a stereomicroscope. Bars = 20 μm (**A**, **B**, **D**), 100 μm (**C**) and 500 μm (**E**)

the high accumulation of betalamic acid in the cytoplasm through DOPA-dioxygenase activity.

The DOPA-dioxygenase is localized in the cytoplasm in *A. muscaria* (data not shown), and it can be assumed that the enzyme was equally localized in the cytosol in the transformed petals. Since the K_m for DOPA is relatively high (5 mM), the high production of pigment confirms that the concentration of DOPA is high in the cytoplasm of *P. grandiflora* petals (Trezzini 1990).

The colour of the spots revealed the genetic background of the plants. Yellow spots were observed when

the genetic background was *rrii* and violet spots were observed when the genetic background was *RRIi* or *RRII*. The particle bombardment method could provide a simple means for the identification of the genetic background in white flowering plants without the need for lengthy crossing experiments.

The *DodA*-transformed petals were extracted with acidified methanol and analysed by HPLC (Waters, Mont sur Lausanne, Switzerland) using a Hypersil column (Bischoff, Leonberg, Germany) as described previously (Trezzini and Zryd 1991). The pigments in the transformed petals were compared with naturally occurring betalain pigments from *P. grandiflora*. The main pigments found in the pNco DodA-transformed petals, identified by comparison with standards, were dopaxanthine (476 nm, 13.2 min), betanine (536 nm, 13.9 min), and miraxanthine V (472 nm, 18.1 min); these betalains occur naturally in the coloured varieties of *P. grandiflora*. A pale-yellow phenotype of *Portulaca* (genotype *CCrrII*) did not accumulate miraxanthine V and accumulated only trace amounts of dopaxanthine (Trezzini 1990); however, these were the main pigments observed in the transformed spots. It is therefore unlikely that all of the pigments were localized in the vacuoles of the transformed and surrounding cells.

In addition to betalain pigments, the transformed petals contained muscaflavin, a natural pigment in the fungus *A. muscaria* that has never been found in plants (Reznik 1978; data not shown). Under in vitro conditions, the *A. muscaria* DOPA-dioxygenase has been shown to produce both betalamic acid and muscaflavin (Mueller et al. 1997). The fact that the spots contain muscaflavin in addition to betalain pigments suggests that the enzyme from *A. muscaria* is exhibiting the same behaviour in vivo.

These results indicate that fungal DOPA-dioxygenase is expressed and active in *P. grandiflora* petals, and complements betalain biosynthesis. The product of *locus C* is therefore a plant enzyme with the properties of the fungal enzyme, except for the capacity to produce muscaflavin. The reaction product of the fungal enzyme expressed in *P. grandiflora* can be turned over in the subsequent betalain biosynthetic steps in the plant (condensation to betaxanthins and betacyanins, glycosylation of betacyanins). The observation that muscaflavin is also formed in the transformed petals indicates a fundamental difference between the enzymology of betalains in *A. muscaria* and in plants of the order Caryophyllales. The fact that, based on sequence homology to the fungal DOPA-dioxygenase, no plant gene has been isolated so far, seems to confirm our point of view (results not shown).

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