A new type of plant dioxygenase involved in betalain biosynthesis

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Betalains are violet and yellow pigments specific of plants from the order Caryophyllales where they substitute for anthocyanins. Betalains are also found in some fungi from the genus *Amanita* and *Hygrocybe.* The key enzyme in betalain biosynthesis is a 4,5-dioxygenase which catalyze the transformation of L-DOPA into the chromophore betalamic acid. A dodA gene coding for a specific dioxygenase (AmDOD) has been characterized in the fungus Amanita muscaria; no homologue of this gene has ever been found in plants. Subtractive cDNA libraries were therefore built with total RNA from immature petals of white, yellow and respectively violet flower inbred lines of *Portulaca grandiflora* (Pg). Specific clones for a putative Pgdod gene were first detected by slot blot analysis and then confirmed by Northern blot analysis using RNA from petals of the white and colored flower genotypes (figure 1)

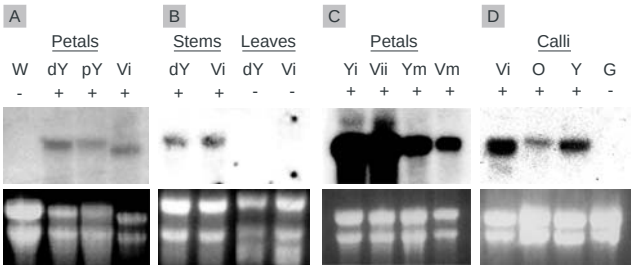
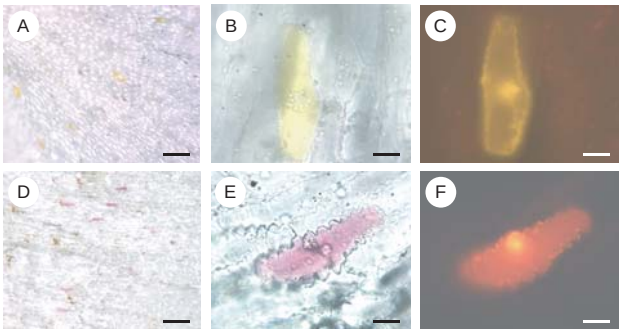
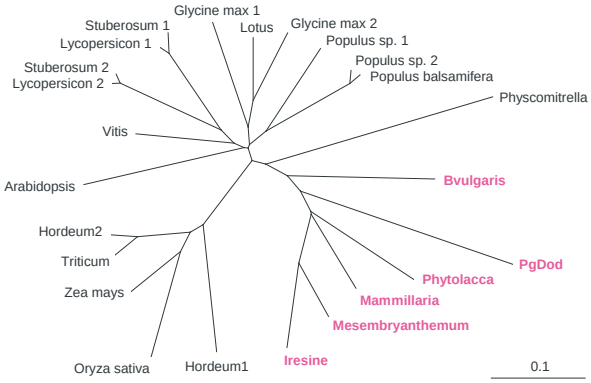
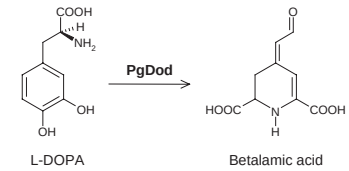


FIGURE 1: Northern blot analysis of ***Pgdod*** gene expression in different tissues from *Portulaca grandiflora* (Pg) flowers and *Beta vulgaris* (Bv) tissue culture. Presence or absence of betalain pigments (no PGDOD) in plant tissues is indicated with (+) or (-) signs. (A) Pg petals of different colored genotypes (W, white; dY, dark yellow; pY, pale yellow; Vi, violet). (B) Pg stems and leaves. (C) Expression at two different stages of Pg flower bud development (Yi, young immature; Vii, violet immature; Ym, yellow mature; Vim, violet mature). (D) Bv calli of different color (Vi, violet; O, orange; Y, yellow; G, green).



(FIGURE 2 : The specificity of the ***Pgdod*** gene is demonstrated by biolistic complementation of the betalain pathway in the white petals of *Portulaca* plants deficient in ***Pgdod***. A pNcoPgDOD expression vector containing full length ***Pgdod*** gene sequence under the control of CaMV promoter has been used with pDsRed2 vector as a positive control. (A) Yellow spots revealed after biolistic transformation of a white petal from a plant with yellow genetic background. (B) Close-up of a cell accumulating betaxanthins in its vacuole. (C) The same cell displaying the DsRed2 fluorescent protein. (D) Violet spots revealed in a white petal from a plant with a violet genetic background. (E) Close-up of a cell accumulating betacyanins. (F) The same cell displaying the DsRed2 fluorescent protein. Bars = 200 um (A,D), 20 um (B,C,E,F)





Biolistic complementation of *Portulaca grandiflora* deficient in the biosynthesis of betalamic acid revealed that PgDOD is a key enzyme the transformation of DOPA into betalamic acid (figure 2). HPLC analysis of the colored spot further confirmed the specificity of this enzyme (figure 3).

In silico analysis revealed the homology of the PgDOD translated protein (30 KDa) with the LigB domain (pfam02900) from the beta subunit from *Sphingomonas paucimobilis* LigAB protein (extradiol 4,5 dioxygenase - AAA17728). Structural analysis revealed that the catalytic amino acids and the iron-binding amino acids are highly conserved in the **PgDOD** from P. grandiflora (figure 4). The **PgDOD** protein is a member of a new group of plant proteins (figure 5).

FIGURE 3: HPLC analysis of the betalains extracted from the violet transformed cells (A) and from the yellow transformed cells (B) in the white *Portulaca* background. These pigments were identified by comparing their elution profile with elution profile of the pigments extracted from violet and deep yellow *Portulaca* petals respectively. Arrows indicate the major peaks of the violet betanin (A) and of the yellow dopaxanthin (B).

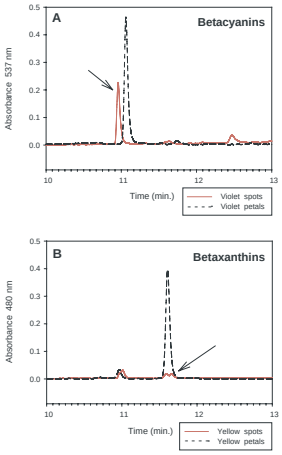


FIGURE 4: Identification of one conserved pattern specific to PgDOD proteins from betalain producing plants by alignment of the PgDOD homologous sequences from different kingdoms. The conserved catalytic amino acid His195 is followed by the pattern P-(S,A)-(N,D)-x-T-P in all homolog of betalain producing plants, whereas at the same place a H-N-L pattern is conserved in all archea, bacteria and a H-N-L-R pattern is conserved in all plant homolog not producing betalains.

FIGURE 5: Phylogenetic analysis of PgDOD homolog in plants. Multiple alignments were done with ClustalW and the tree created with PHYLIP. The moss (bryophyte) *Physcomitrella patens* correspond to the root. Betalains producing species (Caryophyllales) clearly form a cluster distinct from other plants.