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Research article

Biosynthesis of flavan 3-ols by leucoanthocyanidin 4-reductases and anthocyanidin reductases in leaves of grape (*Vitis vinifera* L.), apple (*Malus x domestica* Borkh.) and other crops

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Abstract

Catechin and epicatechin biosyntheses were studied of grape (*Vitis vinifera* L.), apple (*Malus x domestica* Borkh.) and other crop leaves, since these monomers and the derived proanthocyanidins are important disease resistance factors. Grape and apple leucoanthocyanidin 4-reductase (LAR; EC 1.17.1.3) enzymes were characterized on basis of plant and recombinant enzymes. In case of grape, two LAR cDNAs were cloned by assembling available EST sequences. Grape and apple leaf anthocyanidin reductase (ANR; EC 1.3.1.77) cDNAs were also obtained and the respective plant and recombinant enzymes were characterized. Despite general low substrate specificity, within the respective flavonoid biosyntheses of grape and apple leaves, both enzyme types deliver differently hydroxylated catechins and epicatechins, due to substrate availability in vivo. Furthermore, for LAR enzymes conversion of 3-deoxyleucocyanidin was shown. Beside relevance for plant protection, this restricts the number of possible reaction mechanisms of LAR. ANR enzyme activity was demonstrated for a number of other crop plants and its correlation with (–)-epicatechin and obvious competition with UDP-glycosyl:flavonoid-3-*O*-glycosyltransferases was considered.

Keywords: Apple; Catechin biosynthesis; Epicatechin biosynthesis; Flavonoids; Grape; Plant resistance; Proanthocyanidins

1. Introduction

The elucidation of flavanol ((+)-catechins, (–)-epicatechins and proanthocyanidins) biosynthesis in plants has made progress in two important ways. Firstly, a gene of a leucoanthocyanidin 4-reductase (LAR; (2*R*,3*S*)-catechin:NADP$^+$ 4-oxidoreductase; EC 1.17.1.3) has recently been cloned from a legume [41]. Furthermore, with the anthocyanidin reductase (ANR; flavan-3-ol:NAD(P)$^+$ oxidoreductase; EC 1.3.1.77) reaction, a previously unknown enzymatic reaction was identified with a recombinant enzyme from an *Arabidopsis* gene (*banyuls*) involved in proanthocyanidin biosynthesis [45].

Twenty years ago, LAR was described as an enzymatic reaction from conifers (*Pseudotsuga menziesii*, *Cryptomeria japonica*) [23,38], *Ginkgo biloba* [37], barley (*Hordeum vulgare*) [27], and later from legumes (*Onobrychis viciifolia*) [40,41]. It converts the flavan 3,4-diol products of the dihydroflavonol 4-reductase (DFR) reaction to the corresponding catechins (Fig. 1). The legume LAR shows some substrate specificities with respect to B-ring hydroxylation [41]. The first *lar* gene, recently cloned from the legume *Desmodium uncinatum* [41], was found to belong to the superfamily of isoflavone reductases.

Abbreviations: ANR, anthocyanidin reductase; DFR, dihydroflavonol 4-reductase; DHK, dihydrokaempferol; DHQ, dihydroquercetin; DMACA, dimethylaminocinnamicaldehyde; EGME, ethylenglycolmonomethylether; ERI, eriodictyol; EtOAc, ethylacetate; EtOH, ethanol; HOAc, acetic acid; HPLC, high performance liquid chromatography; LAR, leucoanthocyanidin 4-reductase; MeOH, methanol; NADPH, nicotinamide adenine dinucleotide phosphate; RT-PCR, reverse transcription/polymerase chain reaction; TLC, thin layer chromatography; UFGT, UDP-glycosyl:flavonoid-3-*O*-glycosyltransferase.

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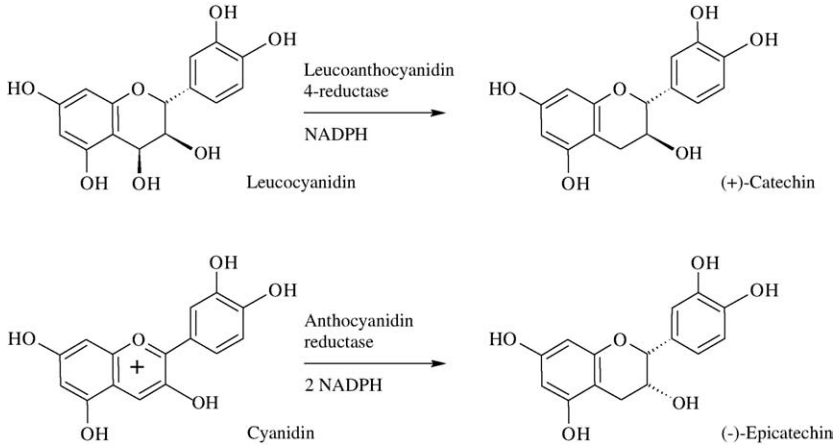

Fig. 1. Enzymatic steps in (+)-catechin (a) and (–)-epicatechin biosynthesis (b) performed by LAR and ANR, respectively.

The LAR enzymatic reaction was recently found as a subsequent step in DFR reactions with enzyme preparations from some major crop plants, such as tea (*Camellia sinensis*) [31], rose (*Rosa x hybrida*) [26], and strawberry (*Fragaria x ananassa*) [unpublished results].

The *banyuls* gene of *Arabidopsis,* whose *loss of function*—mutation leads to a proanthocyanidin deficient seed testa, was first supposed to represent an *lar* gene [11], it is like isoflavone reductases distantly related to the DFR. However, upon heterologous expression of the respective *Arabidopsis* and *Medicago truncatula* cDNAs, the yet unknown reaction was identified for the enzymes, leading from anthocyanidins to the respective epicatechins by NADPH-dependent reduction [44,45]. This anthocyanidin reductase (ANR) seems to be the enzyme responsible for (–)-epicatechin biosynthesis in many plants, among them tea as an outstanding example [31].

Flavan 3-ols (catechins and epicatechins) are precursors of the polymeric proanthocyanidins [12, and citations therein]; there are several features that make the monomers and the proanthocyanidins relevant as plant secondary metabolites: the protein precipitation by them influences the brewery process as well as quality of pasture. In fruits they cause astringency [12, and citations therein]. Furthermore, catechins and proanthocyanidins are also important antioxidants and also have other beneficial effects on human health [2,24]. In leaves catechins, epicatechins and the polymeric proanthocyanidins are especially important as plant resistance factors [8,14,34]. For the analysis of the abovementioned functions of the flavanols, in particular in delivering plant resistance factors, the plant and the respective recombinant LAR and ANR enzymes were obtained from grape and apple leaves and studied with respect to their enzyme characteristics. The respective LAR and ANR enzymes from grape and apple possess similar characteristics and substrate specificities, but considering the quite different flavonoid biosyntheses in both plants, they perform partly different biosynthetic reactions. Additionally, a screening for ANR enzyme activity and its correlation with (–)-epicatechin content was performed in a number of crops, to examine prevalence of this biosynthetic pathway.

2. Results

Enzyme activities of both, LAR and ANR, were found to be present in crude enzyme preparations of young grape and apple leaves accumulating catechins and epicatechins. Sequences of cloned genes and available database sequence information were used to amplify the *lar* and *anr* cDNAs from grape and apple leaves by RT-PCR. Heterologous expression in yeast was performed to obtain the recombinant enzymes. The enzymes prepared from leaves and the recombinant enzymes were used for characterization of the respective reactions.

2.1. Biosynthesis of grape catechins and epicatechins

2.1.1. Leucoanthocyanidin 4-reductase

An LAR enzyme assay with enzyme extract from young grape (*Vitis vinifera*) leaves of cultivar Regent confirmed LAR enzyme activity in leaves. Such leaves were then used for cDNA preparation and subsequent cloning of *lar* cDNAs. The cloning of *lar* genes from grape was based on EST database information of various *Vitis* species identified by BLAST N searches. One *lar* cDNA sequence (*v-lar1*) could be completely reconstructed by overlapping EST sequences from *Vitis shuttleworthii*, *Vitis riparia* and *Vitis vinifera* and was subsequently RT-PCR-amplified from grape leaf cDNA. For a second *lar* cDNA (*v-lar2*) only the sequence of the 3′ region could be assembled from three *Vitis vinifera* EST sequences, the 5′ region was obtained by a RACE protocol, subsequently the whole coding region was RT-PCR-amplified. Grape leaf *lar* sequences showed 57% (*v-lar1*) and 54% (*v-lar2*) identity at the nucleotide level and 55% (*v-lar1*) and 52% (*v-lar2*) identity at the amino acid level to the *lar* of *Desmodium*. To each other the grape leaf *lar* sequences showed 65% (nucleotide) and 58% (amino acid) identity. The recombinant enzymes produced by heterologous yeast expression were both active with ^{14}C-labelled leucocyanidin as a substrate. Both recombinant enzymes have flat temperature optima between 30 °C and 37 °C and pH optima of 6.5 ± 0.5. With respect to substrate specificity, v-LAR1 and v-LAR2 of grape are both accepting the three leucoanthocyanidins leucopelargonidin, leucocyani-