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Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*

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ABSTRACT

Chemical or biological synthesis of plant secondary metabolites has attracted increasing interest due to their proven or assumed beneficial properties and health promoting effects. Resveratrol, a stilbenoid, naringenin, a flavanone, genistein, an isoflavone, and the flavonols kaempferol and quercetin have been shown to possess high nutritional and agricultural value. Four metabolically engineered yeast strains harboring plasmids with heterologous genes for enzymes involved in the biosynthesis of these compounds from phenylalanine have been constructed. Time course analyses of precursor utilization and end-product accumulation were carried out establishing the production of 0.29–0.31 mg/L of *trans*-resveratrol, 8.9–15.6 mg/L of naringenin, 0.1–7.7 mg/L of genistein, 0.9–4.6 mg/L of kaempferol and 0.26–0.38 mg/L of quercetin in defined media under optimal growth conditions. The recombinant yeast strains can be used further for the construction of improved flavonoid- and stilbenoid-overproducers.

1. Introduction

Phenylpropanoid synthesis in plants constitutes a major anabolic process where the amino acid phenylalanine serves as a precursor molecule for the biosynthesis of lignins, stilbenoids, flavonoids and condensed tannins. Among the phenylpropanoid-derived groups, flavonoids and stilbenoids have diverse functions in plants, acting as antimicrobial agents in plant defense (Dixon et al., 1996; Jeandet et al., 1997; Hammerschmidt, 1999), photoreceptors, visual attractors (Winkel-Shirley, 2001), UV protectants (Stafford, 1991), feeding repellants (insect and herbivore protectants), signals in the early steps of rhizobia–legume symbiosis (Dixon et al., 1996), regulators of auxin transport (Havsteen, 2002) and stimulators of pollen germination (Mascarenhas, 1993). In addition, these substances have a range of recorded effects on human health (Manach et al., 2004), involving antioxidant (Pietta, 2000; Williams et al., 2004; Noel et al., 2005), antiallergic (Nowakowska, 2007), anti-inflammatory, antithrombotic and antioncogenic activities (Kris-Etherton et al., 2002; Kampa et al., 2008).

In a recent review (Ververidis et al., 2007a), we have amended the grouping terminology amongst the distinct biosynthetic pathways of phenylpropanoid metabolism leading to stilbenoids, major flavonoids and isoflavonoids synthesis. The diverse physiological properties of members of these groups of metabolites have stimulated scientists to search and identify new member compounds belonging to those groups, with over 8000 known substances on record in year 2000 (Harborne and Williams, 2000; Pietta, 2000) and growing (NAPRALERT database at http://www.napralert.org). The structural multiplicity of these compounds derives from modifications of the basic flavonoid backbone that give the diversity of flavonoids and flavonoid derivatives and are carried out by a variety of isoforms of enzymes participating in the respective metabolic pathways. The enzymes include members of the cytochrome P450 hydroxylase requiring the activity of an NADPH-cytochrome P450 reductase (CPR), NADPH-dependent reductase, 2-oxoglutarate-dependent dioxygenase (ODDs), O-methyltransferase (OMT), acyl and glycosyltransferase

Abbreviations: PAL, phenylalanine-ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid: CoA ligase; RS, resveratrol synthase; CPR, cytochrome P450 reductase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′ hydroxylase; FLS, flavonol synthase

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(UGT) families, which appear to have been recruited into new functions during the rapid evolution that accompanied the domination of plants in moist terrestrial environments (Stafford, 1991; Ververidis et al., 2007a). Furthermore, recent data has shown that despite such diversity in secondary plant products, metabolic channeling and metabolon formation enables plants to effectively synthesize specific natural products and hinder enzymes from accessing unwanted substrates (Winkel, 2004; Jorgensen et al., 2005). These evolutionary effects were further empowered by the ability of several enzymes of the same metabolic step to catalyze the formation of different intermediates or end-products using different starting substrates or precursors. This has been shown from metabolic engineering studies with several enzymes, such as 4-coumaric acid ligase (4CL), chalcone isomerase (CHI), isoflavone synthase (IFS) (Liu et al., 2007) and many others that are able to utilize different substrates within the same dissected metabolic pathway, converting them into different products, when expressed in microbial model (Kang and Back, 2009).

Different phenylpropanoid acids delivered to 4CL provide flavanone-chalcone as well as 5-deoxyflavanone-chalcone to CHI which converts them to the corresponding flavanone or 5-deoxyflavanone (Allina et al., 1998; Hwang et al., 2003; Yan et al., 2005a). Likewise, IFS enzymes convert flavanones or 5-deoxyflavanones to the corresponding isoflavones (Steele et al., 1999). These examples suggest that the cyclization of chalcones and the aryl migration from C-2 position to C-4 as well as aromatization, hydroxylation, glycosylation, acylation, prenylation, sulfation, and methylation are regio-specific reactions (Noel et al., 2005).

The significance of plant secondary metabolism products is reflected in the numerous plant-based medicines containing phenylpropanoid-derived active components that have long been used by humans. The benefits of specific flavonoids and other phenylpropanoid-derived compounds to human health and their potential long-term health profits have been recognized relatively recently (Ververidis et al., 2007a, 2007b). Phenylalanine is the connecting metabolite between primary metabolism and a vast array of secondary metabolites. Initially, phenylalanine is deaminated to *trans*-cinnamic acid by the action of phenylalanine-ammonia lyase (PAL). Synthesis of p-coumaric acid follows through the hydroxylation of trans-cinnamic acid at the para-position of the aromatic ring, by cinnamic acid 4-hydroxylase (C4H) (Vannelli et al., 2007). The activity of 4CL leads to the 4-coumaroyl-coenzyme A, a nodal compound of phenylpropanoid metabolism which leads to either stilbenoids or flavonoids (including anthocyanins and catechins) or lignins. With the action of resveratrol synthase (RS) and 3 molecules of malonyl-coenzyme A the cascade enters the pathway of stilbenoids with *trans*-resveratrol to be the first but one of the most interesting compounds of the group. On the alternative metabolic route, the action of chalcone synthase (CHS) with the aid of 3 molecules of malonyl-coenzyme A guides the cascade to the general flavonoid pathway with the production of naringenin-chalcone (referred as chalcone in many cases). The establishing of the main flavonoid chassis is created through a cyclization reaction of flavanone-chalcones (e.g. naringenin-chalcone) to give rise to flavanones by the action of CHI enzyme. Flavanones are also nodal compounds because they are precursors for the synthesis, among other, of isoflavones (e.g. genistein) and of flavonols (e.g. kaempferol, quercetin) (Ververidis et al., 2007a, 2007b).

In this paper, we present the construction of a series of metabolically engineered *Saccharomyces cerevisiae* strains and discuss the results from the heterologous complete reconstitution of the various biosynthetic pathways utilizing phenylalanine as the initial precursor, leading to formation of various pathway end-products such as the stilbenoid resveratrol, the flavanone naringenin, the isoflavone genistein and the flavonols kaempferol and quercetin. Although some of these compounds have been previously produced in *Escherichia coli*, we report a different approach for their synthesis in *S. cerevisiae* by engineering the complete pathway for each plant end-product and thus testing the effect of the number of genes involved as well as the substrate fluxes at certain enzymatic steps in the metabolon formation in relation to the precursor molecule used.

2. Materials and methods

2.1. Culture media and microbial growth conditions

Bacterial cell cultures were grown in Luria-Bertani broth (10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L sodium chloride) with pH adjusted to 7 while yeast cell suspensions were cultured either in Yeast Peptone Dextrose medium (YPD, consisted of 10 g/L yeast extract, 20 g/L bacto-peptone, 20 g/L glucose) or in auxotrophic Complete Minimal medium (CM, consisted of 6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, 10 mg/L isoleucine, 150 mg/L valine, 20 mg/L adenine hemisulfate, 20 mg/L arginine-HCl, 30 mg/L lycine-HCl, 20 mg/L methionine, 200 mg/L threonine, 30 mg/L tyrosine, 50 mg/L phenylalanine and optionally supplemented with leucine, histidine, uracile and tryptophane at concentrations of 100, 20, 20 and 20 mg/L respectively) with pH adjusted to 5.8. Bacteria were cultivated at 37 °C and yeast at 28–30 °C.

2.2. Genetic material, microbial hosts and cloning vectors

Total plant RNA of *Glycine max* and *Solanum tuberosum* was extracted with standard isolation procedures (Ausubel et al., 1994) using the TRI® Reagent RNA Isolation Reagent (Sigma). Total plant RNA of *Vitis vinifera* cv. Sultanina was isolated according to a specific protocol for isolation of functional RNA from grapevine tissues (Skopelitis et al., 2006). Total RNA extracted from each plant source was treated with DNaseI which was then removed with a phenol–chloroform extraction step. Reverse transcriptase reaction (MMLV enzyme) for cDNA synthesis was performed using 1 μg of total RNA with 2 μM 18-mer oligo-dT, 0.5 mM dNTPs, 1 × MMLV buffer and 200 units MMLV. One-tenth volume of the cDNA reaction mixture (cDNA 0.5 μg, 1 × buffer GC, 150 μM dNTPs, 0.2 μM forward and reverse primers and 2U *Phusion*® High-Fidelity DNA Polymerases (Finnzymes)) was used further in the presence of the appropriate PCR primers (Table 1) to amplify the appropriate plant gene clone of interest. PCR was programmed to run the first cycle at 94 °C for 30 s, the next 35 cycles with 3 steps each starting at 94 °C for 10 s, followed by an appropriate annealing temperature for each primer for 30 s and 72 °C for 1.5 min, and a 5-min final cycle at 72 °C.

The bacterial strain used for accomplishing the cloning strategy was the JM83 (F^-, *ara*, Δ(*lac-proAB*), *rpsL* (*Str*^{r}), [Φ*80*, *lacZΔM15*] thi) while the yeast strain was the YPH499 (*mat a*, *ura3–52*, *lys2–801*^{amber}, *ade2–101*^{ochre}, *trp1–Δ63*, *his3–Δ200*, *leu2–Δ1*).

All subsequent cloning and subcloning steps were done using pGEM T-Easy vector (Promega), pBluescriptII KS+ (Stratagene) and pT20, a vector that resulted from the subcloning of the *Eco*RI-*Hind*III multicloning site of pT_3T_7lac into the pSPTBM20 vector (Boehringer). A series of four epitope-tagging (pESC, Stratagene) vectors carrying *GAL1* and *GAL10* yeast promoters, were used for expression and functional analysis of the plant genes in the *S. cerevisiae* strains. These pESC vectors feature an extensive