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Isolation and characterization of genes encoding leucoanthocyanidin reductase (*FeLAR*) and anthocyanidin reductase (*FeANR*) in buckwheat (*Fagopyrum esculentum*)

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ABSTRACT

Proanthocyanidins (PAs) are a major group of flavonoids synthesized via the phenylpropanoid biosynthesis pathway, however the pathway has not been fully characterized in buckwheat. Anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) are involved in the last steps of PA biosynthesis. To isolate the genes for these enzymes from buckwheat we performed PCR using degenerate primers and obtained cDNAs of *ANR* and *LAR*, which we designated *FeANR* and *FeLAR1*. A search for homologs in a buckwheat genome database with both sequences returned two more *LAR* sequences, designated *FeLAR2* and *FeLAR3*. Linkage analysis with an F_2 segregating population indicated that the three *LAR* loci were not genetically linked. We detected high levels of PAs in roots and cotyledons of buckwheat seedlings and in buds and flowers of mature plants. *FeANR* and *FeLAR1–3* were expressed in most organs but had different expression patterns. Our findings would be useful for breeding and further analysis of PA synthesis and its regulation in buckwheat.

1. Introduction

Buckwheat (*Fagopyrum esculentum*) is an annual crop grown widely around the world. The flour, made from the seed, is used for noodles and pancakes, and the sprouts are eaten as vegetables. Buckwheat seeds, leaves, and stems contain flavonoids such the flavonol rutin and proanthocyanidins (PAs).

Flavonoids (PAs, flavonols, and anthocyanins) are synthesized and accumulate in a variety of tissues in many plant species and can help to protect against various biotic and abiotic stresses (Winkel-Shirley, 2001, 2002). As part of a balanced diet, flavonoids have beneficial effects on human health as they reduce inflammation, enhance immunity, and lower the risks of cardiovascular diseases and certain cancers (Santos-Buelga and Scalbert, 2000; Yildizogluari et al., 1991). PAs are important in the mouthfeel and astringency of many fruits and in the quality of wine and tea (Aron and Kennedy, 2008). On the other hand, PAs cause undesirable colloidal haze in beer (Gramshaw, 1970) and discoloration of cooked barley grains (Tonooka et al., 2010). In buckwheat, PA oxidation may lead to dark, mottled noodles. Many PA-free mutants have been detected in barley (Wettstein et al., 1977). To prevent the discoloration of cooked grains, a PA-free barley cultivar was bred from a line that cannot produce PAs in the seeds owing to the recessive homozygous state of *ant28* (Himi et al., 2012; Tonooka et al., 2010). It would be desirable to produce a PA-free buckwheat cultivar. The first step towards this goal is to identify the genes related to PA synthesis and their regulation in buckwheat.

On the basis of studies in other plant species, it has been suggested that flavonoids in buckwheat are synthesized via a flavonoid biosynthetic pathway similar to that in other plants (Fig. 1) (Gupta et al., 2011; Li et al., 2010; Matsui et al., 2008b). Flavonols and flavonol glycosides are synthesized from dihydroflavonols

Abbreviations: ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; BGDB, buckwheat genome database; bHLH, basic Helix-Loop-Helix; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; DMACA, 4-dimethylaminocinnamaldehyde; DW, dry weight; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; GT, glucosyl transferase; LAR, leucoanthocyanidin reductase; PA, proanthocyanidin; UFGT, glucose-flavonoid glucosyl transferase.

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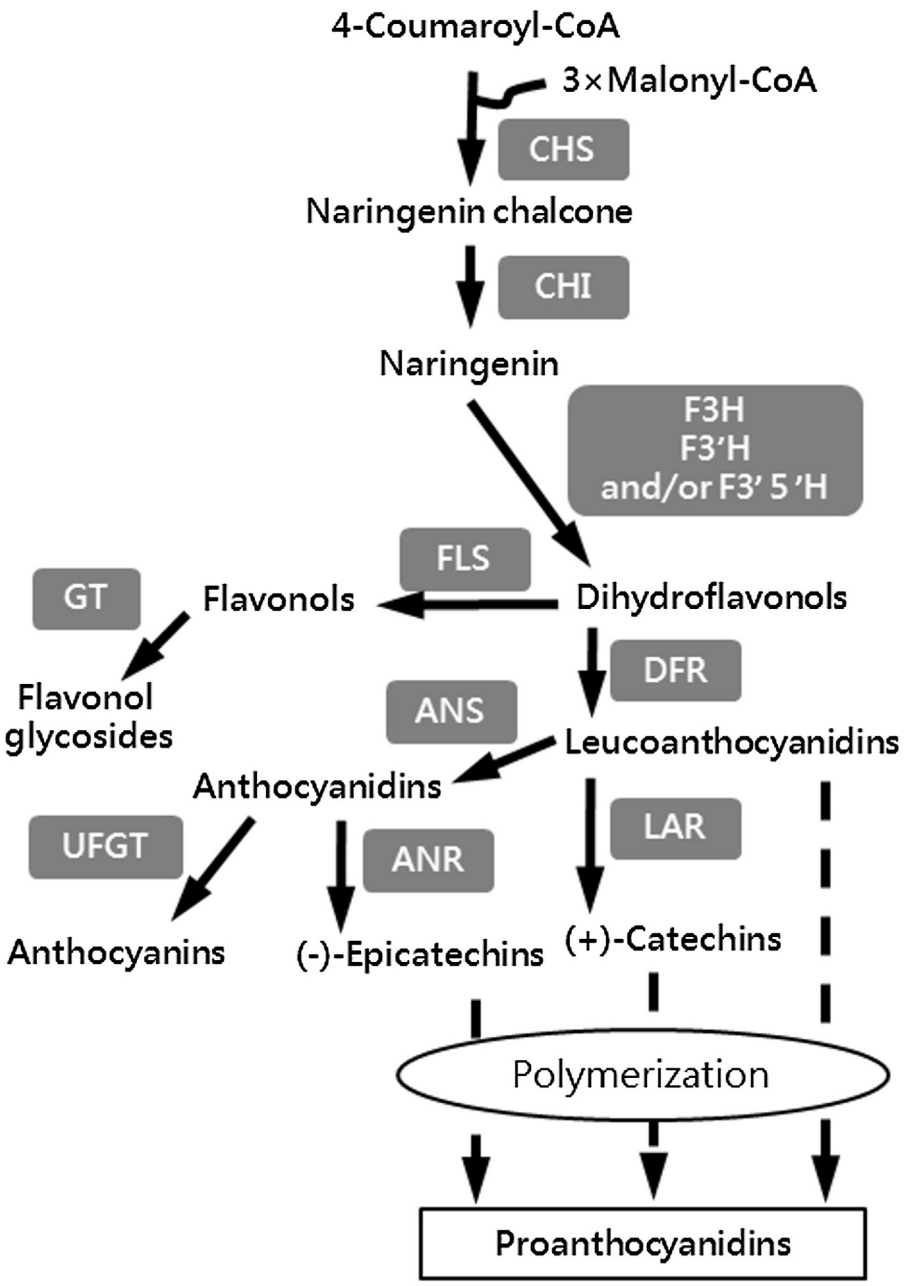

Fig. 1. Putative PA biosynthesis pathway in buckwheat. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; FLS, flavonol synthase; GT, glucosyl transferase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, glucose-flavonoid glucosyl transferase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase.

by flavonol synthase (FLS) and flavonol glycosyltransferase (GT). PAs and anthocyanins are synthesized from the same point by dihydroflavonol 4-reductase (DFR), which produces leucoanthocyanidins (Fig. 1). These can be converted either to (+)-catechins by leucoanthocyanidin reductase (LAR) or to anthocyanidins by anthocyanidin synthase (ANS) and thence to (−)-epicatechins by anthocyanidin reductase (ANR). PAs are then synthesized by the polymerization of (+)-catechins and/or (−)-epicatechins. The accumulation of PAs is positively correlated with the expression levels of genes related to PA biosynthesis in plants such as grapes (Bogs et al., 2005).

In buckwheat, genes encoding FLS, DFR, and ANS have been characterized (Li et al., 2010); however, genes involved in the last steps of PA synthesis (*ANR* and *LAR*) have not yet been isolated. The aim of this study was to identify the buckwheat genes encoding ANR and LAR, which could be used to develop buckwheat cultivars with altered PA levels. To this end, we isolated the *FeLAR* and *FeANR* genes and investigated the relationships between their expression and PA content in several organs of both seedlings and mature plants.

2. Materials and methods

2.1. Plant materials

'Sachiizumi', a leading buckwheat cultivar grown in warm areas of Japan (Matsui et al., 2013), was used for RNA isolation to identify cDNAs encoding ANR and LAR. 'Sachiizumi' was also used to investigate *ANR* and *LAR* expression in different organs of flowering plants and in seeds at different maturity stages, and to relate gene expression levels and PA content. Five cultivars ('Cobalt no Chikara',