**Arabidopsis UDP-glycosyltransferase 78D1-overexpressing plants accumulate higher levels of kaempferol 3-O-β-D-glucopyranoside than wild-type plants**

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**Abstract** Flavonols are a class of flavonoids that are found in most plants. Certain flavonols exhibit anticancer, antioxidant, and antimicrobial functions. An array of genes plays a role in the regulation of flavonoid biosynthetic pathways, including the MYB–bHLH (basic helix-loop-helix-WD40 transcription factor complex. Flavonoids often display altered bioactivities after being glycosylated by the action of glycosyltransferases. These enzymes catalyze the transfer of sugars from a donor to various acceptors. In this study, we generated several transgenic lines of *Arabidopsis* that overexpress UDP-glycosyltransferase 78D1 (*UGT78D1*), which are hereafter referred to as *UGT78D1*-OX, to address three questions: (1) Can *UGT78D1*-OX seedlings accumulate more flavonols? (2) Can *UGT78D1*-OX seedlings accumulate more flavonols in the presence of sucrose? and (3) Will *UGT78D1*-OX be more sensitive to abiotic stresses? We observed that *UGT78D1*-OX seedlings accumulated specific types of kaempferol, while they had a decreased content of flavonols in the presence of sucrose. Contrary to our expectation, more anthocyanins accumulated in the *UGT78D1*-OX lines, although the expression of production of anthocyanin pigment 1 was slightly reduced in *UGT78D1*-OX seedlings compared with that in wild-type seedlings. It appeared that the overexpression of *UGT78D1* did not interfere with abiotic stress tolerance in the mutant plants.

**Keywords** Anthocyanin · *Arabidopsis* · Flavonoid · Flavonol · UDP-glycosyltransferase 78D1

**Introduction**

Flavonoids, including anthocyanins and flavonols, are compounds that are well established to protect plants against pathogen attacks, ultraviolet irradiation, and free radicals [1–4]. The MYB–bHLH (basic helix-loop-helix)–WD40 complex plays crucial roles in the activation of flavonol biosynthesis by regulating several flavonoid biosynthesis genes [5]. Among those genes, the expression of *Arabidopsis thaliana* production of anthocyanin pigment 1 can result in a high level of anthocyanin production in various plants [6, 7]. Thus, metabolic engineering strategies aimed at enhancing the contents of specific types of flavonoids in plants may involve the manipulation of certain genes in the flavonoid biosynthesis pathways.

Flavonoid biosynthesis routes diverge into different pathway branches that lead to the synthesis of specific classes of molecules such as proanthocyanins, flavonols, and lignols. One of these branches converts dihydrokaempferol either into flavonols via the action of flavonol synthase (FLS) or into anthocyanins via the action of dihydroflavonol 4-reductase (DFR). Many flavonoids are glycosylated by glycosyltransferases, which often leads to altered bioactivities following glycosylation [8]. Glycosyltransferases catalyze the transfer of sugars from a donor...
to various acceptors. Glycosylated flavonols can be sequestered in the vacuole [9], which results in a reduced flavonol content in the cytoplasm. If they are not sequestered in the vacuole, they can inhibit flavonol synthesis via a negative feedback mechanism. In our previous study, we observed that the overexpression of flavonol synthase, which synthesizes flavonols, did not lead to the overaccumulation of flavonols in Arabidopsis. Therefore, in the present study, we wanted to determine whether we could increase flavonol synthesis specifically in Arabidopsis by overexpressing a glycosyltransferase alone. If AtUGT78D1-OX would accumulate more glycosylated flavonols, then we were also curious about whether the increased flavonols may confer an enhanced tolerance toward abiotic stresses in Arabidopsis. To address this question, we generated several Arabidopsis lines of overexpressing UGT78D1, which are hereafter referred to as UGT78D1-OX. We observed that UGT78D1-OX accumulated specific types of kaempferol under control conditions and also accumulated kaempferol in sucrose-treated conditions, under which anthocyanins typically accumulate at high levels because of a shift in the utilization of metabolic pathways. We also examined the performance of UGT78D1-OX seedlings in response to osmotic stress.

Materials and methods

Plant materials and growth conditions

UGT78D1-OX transgenic plants were grown with Col-0 [wild-type (WT) plants] in a growth chamber under the following conditions: 16/8-h light/dark period; 23 ± 1 °C, 50–55 μmol photons m–2 s–1, and approximately 70% humidity. Sucrose (2%) with 1/2 Murashige–Skoog (MS) medium containing phytoagar (pH 5.8) was used for plant culture. All seedlings were grown in 1/2 MS medium supplemented with NaCl and mannitol.

Construction of UGT78D1-OX and transformation

The PCR™/GW/TOPO® TA Cloning® Kit (Invitrogen, Carlsbad, CA, USA) was used to clone the PCR product from the AtUGT78D1 cDNA. AtUGT78D1 was then cloned into the pFLAG vector (E. coli, kanamycin resistance plant, Basta® resistance) by producing the construct 35Spro: AtUGT78D1, following a previously reported protocol [10]. Cells were centrifuged and suspended in liquid containing 1/2 MS media and 2% sucrose at an OD650 = 0.5. Col-0 plants that were one month old and contained flower buds were soaked in Agrobacterium media for approximately 40–60 s and were then maintained in the dark for one night.

Anthocyanin measurement and ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry analysis

Anthocyanin measurements were taken following a previously described detection method [11]. Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry analysis was conducted following a previously described method for flavonol detection [10].

qRT-PCR and gene accession numbers

Nine-day-old seedlings were collected for RNA extraction [11]. cDNA synthesis and qRT-PCR were performed according to the manufacturer’s protocols (Applied Biological Materials, Richmond, Canada). ACTIN2 was used as an internal control. The gene sequences were obtained from the Arabidopsis Information Resource (www.arabidopsis.org) with the following accession numbers: DFR (AT5G42800), FLS1 (AT5G08640), PAP1 (AT1G56650). Two independent experimented data were performed and subjected to statistical analysis by Tukey’s test (p < 0.001). The asterisks above the columns denote significant differences. The error bars represent standard error.

Results and discussion

Kaempferol 3-O-β-D-glucopyranoside is specifically increased by UGT78D1 overexpression in Arabidopsis

Flavonoids serve multifarious roles in the plant life cycle. Thus, their regulation is controlled via diverse transcription factors (TFs) that can be manipulated by scientists in the field of plant metabolic engineering. For instance, PAP1 overexpression increased the contents of various volatile compounds in rose [12]. Moreover, the interruption of the flavonol synthesis pathway directed the production of high levels of anthocyanins in Arabidopsis [13]. These reports prompted us to examine whether we could manipulate flavonol content by increasing flavonol glycosylation via the overexpression of Arabidopsis UGT78D1. Moreover, we investigated the type of flavonols that would be produced. To address these questions, several UGT78D1-OX lines were generated.

First, we attempted to determine the glycosylated flavonol contents of the UGT78D1-OX lines and the WT plants. As shown in Fig. 1(A), five different types of glycosylated flavonols were detected as follows: K1, kaempferol 3-O-α-L-rhamnopyranosyl-(1->2)-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside; K2, kaempferol 3-O-β-D-glucopyranoside 7-O-α-L-rhamnopyranoside; K3, kaempferol 3,7-di-
Flavonol content and anthocyanin accumulation in UGT78D1-overexpressing (UGT78D1-OX) and wild-type Arabidopsis plants. (A) For flavonol measurement by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry, 14-day-old transgenic plants were gathered. The contents of the flavonols K1 (kaempferol 3-O-α-L-rhamnopyranosyl-[1→2]-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside), K2: (kaempferol 3-O-β-D-glucopyranosyl 7-O-α-L-rhamnopyranoside), 3 K (kaempferol 3,7-di-O-α-L-rhamnopyranoside), Q1 (quercetin 3-O-β-D-glucopyranosyl 7-O-α-L-rhamnopyranoside), Q2 (quercetin 3,7-di-O-α-L-rhamnopyranoside) were confirmed after treatment with sucrose (200 and 300 mM) and naringenin (200 and 400 mM) for 24 h. (B) Four-day-old wild-type and UGT78D1-OX #3, #7, and #12 plants were collected (50 mg samples) after being exposed to liquid sucrose medium (0, 200, and 300 mM sucrose) for 24 h. The treated seedlings were analyzed for their anthocyanin contents by measuring the optical absorbance at 535 and 650 nm by spectrophotometry. Two independent experimental replicates were performed and subjected to statistical analysis by Tukey’s test (p < 0.001). The asterisks above the columns denote significant differences. The error bars represent standard error.

O-α-L-rhamnopyranoside; Q1, quercetin 3-O-β-D-glucopyranosyl 7-O-α-L-rhamnopyranoside; Q2, quercetin 3,7-di-O-α-L-rhamnopyranoside. The seedlings of UGT78D1-OX line #7 produced 20% more K3 than those of WTs or other the UGT78D1-OX lines (Fig. 1(A)). This increase became more prominent in the presence of sucrose or naringenin. Among the compounds that can elicit flavonoid biosynthesis, sugars are known to be one of the most potent inducers of flavonoid biosynthesis [14]. Therefore, we expected that supplying sucrose would reduce the flavonol content in the UGT78D1-OX lines. On the contrary, we observed that the content of K3 in UGT78D1-OX #7 remained the same regardless of the presence of the absorbance of sucrose (Fig. 1(A)). This indicates that an enhanced glycosylation activity directs the metabolic flux toward the accumulation of flavonols even in the presence of a strong inducer of anthocyanin synthesis such as sucrose. In addition, we examined whether the application...
of naringenin, which is the precursor of kaempferol and quercetin, could alter the flavonol contents of the \textit{UGT78D1-OX} lines. The supply of a high concentration of exogenous naringenin caused an overall decrease in flavonol contents. However, K3 remained the most abundant flavonol in the \textit{UGT78D1-OX #7} line (Fig. 1(A)).

Next, we determined the anthocyanin contents of the WT and \textit{UGT78D1-OX} seedlings in response to sucrose. As shown in Fig. 1(B), all three \textit{UGT78D1-OX} lines accumulated more anthocyanins in the presence of a high concentration of sucrose. This observation suggests that as long as flavonols are glycosylated, sucrose can increase the contents of anthocyanins and flavonols. It would be plausible to expect that glycosylated flavonols are taken up by the vacuoles, thereby reducing the actual flavonol concentration available in the cytoplasm.

The expression of \textit{PAP1}, \textit{FLS1}, and \textit{DFR} was lower in \textit{UGT78D1-OX} seedlings than in WT seedlings

Since we observed different responses in terms of flavonol accumulation among the three \textit{UGT78D1-OX} lines, we were curious about whether the transcript level of \textit{UGT78D1} may vary among those transgenic lines. In accordance with our expectation, \textit{UGT78D1-OX #7} produced almost 10 times more \textit{UGT78D1} transcript (Fig. 2(A)) and its expression level further increased in response to 300 mM sucrose. As the overexpression of the cloned \textit{UGT78D1} cDNA was driven by the 35S CAMV promoter, the further increase in the expression of the \textit{UGT78D1} transcript upon the addition of sucrose indicates that posttranscriptional regulation plays a significant role in determining the transcript level (Fig. 2(A)). The posttranscriptional regulation of gene expression is often observed. For example, when a strong promoter was used to drive the overexpression of \textit{PAP1} in a previous study, the transcript level of \textit{PAP1} still altered when seedlings were exposed to sucrose [15]. Next, we examined whether the elevated levels of anthocyanins and certain flavonols in \textit{UGT78D1-OX} seedlings were due to enhanced transcript levels of flavonoid biosynthesis genes. The transcripts of \textit{PAP1}, \textit{FLS1}, and \textit{DFR} accumulated much less in \textit{UGT78D1-OX} seedlings than in WT seedlings when the plants were exposed to 0, 200, and 300 mM sucrose for 6 h (Fig. 2(B)).

Many flavonoid biosynthesis genes are known to respond to sucrose [16]. Because \textit{UGT78D1} is not a transcription factor, the reduced transcript levels of \textit{PAP1}, \textit{FLS1}, and \textit{DFR} are most likely to result from the induction of feedback mechanisms by the altered flavonoid levels in \textit{UGT78D1-OX} seedlings compared with those in WT plants.

\textit{UGT78D1} is highly induced by sucrose and high salt, but \textit{UGT78D1-OX} seedlings did not exhibit a similar abiotic stress tolerance in comparison with WT seedlings

The high responsiveness of \textit{UGT78D1} to sucrose led us to examine the transcript levels of \textit{UGT78D1} in response to
other plant growth stimulators and inhibitors. As shown in Fig. 3(A), the transcript level of UGT78D1 was highly increased in response to sucrose, high salt, and jasmonic acid. Plants that are metabolically engineered to obtain high levels of certain flavonoids sometimes exhibit an enhanced tolerance to abiotic stresses [17]. However, when the growth and performance of the WT and UGT78D1-OX seedlings were tested in various abiotic stress media, we did not observe any differences between them (Fig. 3B, C). These results imply that the enhanced levels of certain flavonols may not enough to increase the overall rate of plant growth or the degree of abiotic stress tolerance.

Flavonoids are well known for having antioxidant activities, and there have been many attempts to synthesize flavonoids in microbial systems [4, 17, 18]. Recently, K3 was revealed to confer anti-hyperalgesic properties through the activation of cholinergic receptors [19]. Taken together, our results suggest that various pathways involved in the secondary metabolism of plants can be altered to manipulate specifically targeted metabolites. Thus, it should be possible to produce crops that accumulate a high content of flavonoids for human use.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interests.

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