Daphnetin Methylation by a Novel O-Methyltransferase Is Associated with Cold Acclimation and Photosystem II Excitation Pressure in Rye*

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In plants, O-methylation of phenolic compounds plays an important role in such processes as lignin synthesis, flower pigmentation, chemical defense, and signaling. However, apart from phenylpropanoids and flavonoids, very few enzymes involved in coumarin biosynthesis have been identified. We report here the molecular and biochemical characterization of a gene encoding a novel O-methyltransferase that catalyzes the methylation of 7,8-dihydroxycoumarin, daphnetin. The recombinant protein displayed an exclusive methylation of position 8 of daphnetin. The identity of the methylated product was unambiguously identified as 7-hydroxy-8-methoxycoumarin by co-chromatography on cellulose TLC and coelution from high performance liquid chromatography, with authentic synthetic samples, as well as by UV, mass spectroscopy, 1H NMR spectral analysis, and NOE correlation signals of the relevant protons. Northern blot analysis and enzyme activity assays revealed that the transcript and corresponding enzyme activity are up-regulated by both low temperature and photosystem II excitation pressure. Using various phenylpropanoid and flavonoid substrates, we demonstrate that cold acclimation of rye leaves increases O-methyltransferase activity not only for daphnetin but also for the lignin precursors, caffeic acid, and 5-hydroxyferulic acid. The significance of this novel enzyme and daphnetin O-methylation is discussed in relation to its putative role in modulating cold acclimation and photosystem II excitation pressure.

Low temperature is one of the most important environmental factors limiting the productivity and distribution of plants. Exposure of plants to low, nonfreezing temperatures, a process known as cold acclimation, induces the genetic system required for increased freezing tolerance. Knowledge of the molecular, physiological, and biochemical changes that occur during this process could lead to the improvement of plant productivity. This complex process has been extensively studied and several cold-responsive genes have been isolated from a range of dicotyledonous and monocotyledonous species (1, 2). Although the functions of some of these genes are known (3–6), the details of the processes responsible for their regulation and detection of temperature changes are still incomplete.

Previous studies have shown that development of freezing tolerance in winter cereals, such as wheat and rye, is correlated with an increase in their photosynthetic capacity (7). Thus, growth at low temperature not only induces freezing tolerance, but also results in an increased resistance to low temperature-induced photoinhibition of photosynthesis, and requires adjustment to a combination of light and low temperature. The common photosynthetic response of plants to low temperature and normal light is rationalized in terms of photosystem II (PSII) excitation pressure, which is a measure of the redox state of the first electron acceptor, quinone A (7–9). It has been shown that cold-acclimated rye and wheat grown at 5 °C/250 μmol m−2 s−1 (5/250) (°C temperature/ light intensity μmol m−2 s−1) exhibit a similar tolerance to photoinhibition as plants grown at high light (20/800) because both cold-acclimated and high-light plants are exposed to a comparable high excitation pressure measured as 1-qp (10). Similarly, nonacclimated rye and wheat grown at 20/250 exhibit a similar sensitivity to photoinhibition as plants grown at low temperature but low light (5/50) because both nonacclimated plants and plants grown at 5/50 are exposed to comparable low excitation pressure. Our previous studies have shown that in addition to the traditional role of photosynthesis in energy transduction, the redox state acts as a signal that initiates a transduction pathway coordinating genetic and biochemical responses in wheat and rye plants (8, 9). Genetic analysis revealed that several genes are associated with increased photosystem II excitation pressure (9). Of these genes, one exhibited homology to several plant O-methyltransferases (OMTs).

Methyltransferases are essential enzymes for directing intermediates into specific biosynthetic pathways (11). Enzymatic O-methylation, catalyzed by S-adenosyl-L-methionine-dependent OMTs, is a ubiquitous reaction that takes place in almost all organisms including bacteria, fungi, plants, and mammals. In plants, O-methylation of phenolic compounds such as phenylpropanoids, coumarins, and flavonoids, play an important role in processes such as structural support, flower

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‡ The abbreviations used are: PSII, photosystem II; OMT, O-methyltransferase; HPLC, high performance liquid chromatography; ScOMT1, rye cDNA encoding O-methyltransferase; TLC, thin layer chromatography; qP, photoinhibition. 

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pigmentation, chemical defense, and signaling (12). Of the large number of plant OMTs that are involved in secondary metabolism (11), only a few involved in coumarin biosynthesis have been enzymatically characterized (13–15). In addition, a cDNA encoding the 5-OMT for bergaptol (a linear furanocoumarin), has been cloned (16).

Simple plant coumarins are the cyclization products of their corresponding o-hydroxycinnamic acids (17). They are widely distributed in plants, although members of the Apaceae, Rutaceae, and Moraceae are particularly rich sources of coumarins. Several members of these families are used as spices and vegetables in human diet, as well as for medicinal purposes (18). Coumarins are considered to be components of the general defense response of plants to abiotic and biotic stresses. In addition, various substituted coumarins exhibit antimicrobial or anti-inflammatory activity and act as inhibitors of numerous enzyme systems (17). Furthermore, coumarins exhibit numerous effects of medicinal value (18).

We report here the molecular and biochemical characterization of a rye cDNA, ScOMT1 encoding a novel enzyme that exclusively methylates the 7,8-dihydroxycoumarin (daphnetin) at position 8 to yield 7-hydroxy-8-methoxycoumarin. This enzyme is regulated by both photosystem II excitation pressure and low temperature. The possible role of this enzyme in the modulation of photosystem II excitation pressure and cold acclimation is discussed.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Seeds of winter rye (Secale cereale L., Gramineae, cv. Muskeater) were germinated in coarse vermiculite and grown at temperatures of 20–22 °C during the growing period and 16-h photoperiod in controlled environment chambers (Conviron, Manitoba, Canada) as described previously (9). Growth irradiance was adjusted to 50 or 250 μmol m⁻² s⁻¹ at 5 °C (5/50 and 5/250, respectively) and 50, 250, or 800 μmol m⁻² s⁻¹ at 20 °C (20/50, 20/250, or 20/800, respectively).

Enzyme Substrates

S-Adenosyl-L-[³H]dimethionine (AdoMet; specific activity 55 Ci/μmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO) and unlabeled AdoMet was from Roche Molecular Biochemicals (Montreal, Quebec). The phenolic substrates and reference compounds used were from our laboratory collection. The 7,8-dihydroxycoumarin (daphnetin) was purchased from Extrasynthese (Lyon, France).

Chemical Synthesis

Daphnetin (7,8-Dihydroxycoumarin)—Daphnetin was synthesized according to the method of Molyneux and Jurd (19), in which a mixture of pyrogallol and malic acid was heated in concentrated sulfuric acid according to the method of Molyneux and Jurd (19), in which a mixture was germinated in coarse vermiculite and grown at temperatures of 20–22 °C during the growing period and 16-h photoperiod in controlled environment chambers (Conviron, Manitoba, Canada) as described previously (9). Growth irradiance was adjusted to 50 or 250 μmol m⁻² s⁻¹ at 5 °C (5/50 and 5/250, respectively) and 50, 250, or 800 μmol m⁻² s⁻¹ at 20 °C (20/50, 20/250, or 20/800, respectively).

O-Methylation of Daphnetin—Because none of the methylated derivatives of daphnetin are available commercially, both methylated isomers were synthesized as follows. A mixture of trimethylsilyldiazomethane in hexane and N,N-dimethylpropyamine in dioxane-MeOH was stirred for 2 h at room temperature. The mixture was poured into 2 N HCl, extracted with EtOAc, and then washed with saturated aqueous NaCl. Solvent evaporation and chromatography of the residue on a silica gel column, using toluene-acetone (10:1, v/v) as eluent, gave rise to 7-hydroxy-8-methoxycoumarin, 8-hydroxy-7-methoxycoumarin, and 7,8-dimethoxycoumarin.

Spectroscopic Data

UV spectra were obtained from the diode array detector during HPLC and are given as λmax nm. 1H NMR spectra were recorded on a 270 MHz JEOL JHM-EX270 spectrometer in acetone-d₆, or in CDCl₃; NOESY by a 500 MHz Bruker AMX500 in acetone-d₆ and coupling constants (J values) in Hz.

**Fig. 1.** ScOMT1 sequence analysis. A, nucleotide and deduced amino acid sequences of ScOMT1. Shaded amino acids represent conserved residues proposed for plants OMTs. B, amino acid alignment of ScOMT1 with various plant OMTs. C, phylogenetic tree for plant OMTs. Clustal W version 2.2 (www.genome.ad.jp/mafft/clustalw) was used in this analysis are the ones listed in Table 1 above. Bootstrap confidence values (n = 100) are shown at the branches. OMT sequences used in this analysis are the ones listed in

7-Hydroxy-8-methoxycoumarin—m.p. 163–164 °C; UV λmax MeOH nm 255, 324; EIMS (m/z) (rel. int.) 192 [M⁺] 100, 179 (19), 149 (13), 121 (10); 1H NMR (acetone-d₆, 270 MHz, solvent peak at δ 3.04 as int. std.; 3.93 (1H, s), 6.17 (1H, d, J = 8.5 Hz) (weak), 7.20 (1H, d, J = 8.4), and 7.80 (1H, d, J = 9.6). O-Methylation of Daphnetin—Because none of the methylated derivatives of daphnetin are available commercially, both methylated isomers were synthesized as follows. A mixture of trimethylsilyl diazomethane in hexane and N,N-dimethylpropyamine in dioxane-MeOH was stirred for 2 h at room temperature. The mixture was poured into 2 N HCl, extracted with EtOAc, and then washed with saturated aqueous NaCl. Solvent evaporation and chromatography of the residue on a silica gel column, using toluene-acetone (10:1, v/v) as eluent, gave rise to 7-hydroxy-8-methoxycoumarin, 8-hydroxy-7-methoxycoumarin, and 7,8-dimethoxycoumarin.
and between H-4 and H-3 at δ 6.17 (d, J = 9.6 Hz) (medium), whereas in that of 8-hydroxy-7-methoxycoumarin NOE strong correlation signals were observed between 7-OCH₃ at δ 3.94 (s) and H-6 at δ 7.01 (d, J = 8.6 Hz) and medium correlation signals between H-6 and H-5 at δ 7.12 (d, J = 8.6 Hz), H-5 and H-4 at δ 7.84 (d, J = 9.6 Hz), and H-4 and H-3 at δ 6.19 (d, J = 9.6 Hz). These results indicate unequivocally the substituted patterns of the methoxy protons, a hydroxy proton, two aromatic protons, and two olefinic protons in both compounds.

Construction and Screening of the cDNA Library

Poly(A⁺) RNA from plants grown at 20 °C/800 μmol m⁻² s⁻¹ was used to synthesize double stranded cDNA (Amersham kit) and ligated to XhoI-EcoRI adaptors as previously described (9). The library was screened with ³²P-labeled cDNA probes prepared from poly(A⁺) RNA isolated from plants grown at 20 °C/800 μmol m⁻² s⁻¹ and at 20 °C/250 μmol m⁻² s⁻¹ (control plants). The plaques showing a differential...
Protein Extraction and Quantification

Protein was routinely extracted from the plant material at 4 °C using phosphate-buffered saline, pH 7.3. After centrifugation at 10,000 × g, the supernatant was desalted on a PD10 column further. Proteins were quantified by the method of Bradford using the Bio-Rad reagents and bovine serum albumin as the protein standard.

Enzyme Assay and Product Identification

The ScOMT1 assay was performed as previously described (21) using a final concentration of 200 μM substrate (in 1% dimethyl sulfoxide), 2.5 μM [1-14C]AdoMet (containing 25 nCi), and up to 100 μg of protein in 1× phosphate-buffered saline in a total volume of 100 μL. The reaction was started by the addition of the enzyme, incubated at 30 °C for 30 min, and stopped by the addition of 10 μL of 6 M HCl. The methylated product was extracted in a mixture of benzene-ethyl acetate (1:1, v/v) and an aliquot of the organic phase was counted for radioactivity using a toluene-based scintillation fluid. The remaining sample was chromatographed on either a cellulose TLC plate (20 × 20 cm) using ethyl acetate, acetic acid, H2O (1:3:7, v/v/v) or an Agilent Eclipse C18 silica column (4.6 × 250 mm; particle size, 5 μm; Waters, Milford, MA) using 20% methanol in 1% acetic acid for 2 min followed by a linear gradient to 40% methanol and 1% acetic acid for 30 min; maintained for 5 min, then equilibrated to the original conditions for 15 min. The developed TLC plate was then exposed in a Bio-Rad molecular imager system and the data were analyzed with the software provided.

RESULTS

Sequence Analysis of ScOMT1—The nucleotide sequence of ScOMT1 (Secale cereale OMT1) comprises 1373 base pairs containing 71 nucleotides of 5′-untranslated and 237 nucleotides of 3′-untranslated sequences including the poly(A) tail. The cDNA encodes a polypeptide of 355 amino acid residues with a calculated molecular mass of 38 kDa and a predicted pI value of 7.4 (Fig. 1A). Comparison of the deduced amino acid sequence with other plant OMTs reveals sequence identities ranging from 26 to 40% (Fig. 1B). However, the highest identity (57%) is observed with the maize herbicide Safener-binding protein, SafBP (22). A sequence alignment of these proteins shows five regions in the C-terminal portion (regions I to V, Fig. 1B) that have been reported to be conserved among most plant OMTs (25) and proposed to be involved in the AdoMet binding site for plant OMTs (26). Several amino acids not included in the five conserved regions were found to be conserved between ScOMT1 and other plant OMTs (Fig. 1B). They may correspond to binding sites for phenolic substrates, given that these compounds share some structural similarities.

The phylogenetic relationship of ScOMT1 to other plant OMTs was deduced from the amino acid alignment presented in Fig. 1B. The parsimony tree generated from 13 different OMTs shows a good alignment of their sequences that is supported by high (72–100%) bootstrap frequencies. It also illustrates that the ScOMT1 and maize Safener-binding protein sequences occupy a distinct branch relative to the other plant OMTs (Fig. 1C). The fact that Pinus radiata, Arabidopsis thaliana, and Populus tremuloides OMTs are phylogenetically more distant and highly divergent from ScOMT1 and the other OMTs suggests that they may have evolved independently despite the conservation of their consensus motifs (Fig. 1C).

Expression and Partial Purification of the ScOMT1 Protein—Expression of the recombinant ScOMT1 gave rise to a fusion protein that possessed a six-histidine tag as part of a leader sequence at the N terminus of the protein. The molecular mass of this protein is consistent with the addition of a 3.7-kDa His tag leader sequence fused to the 38-kDa ScOMT1. The purified protein did not exhibit any significant enzyme activity. This may be attributed to the inhibiting effect of Ni2+ ions that may leach from the column and remain in contact with the enzyme protein (26). However, dialysis and the addition of both EDTA and β-mercaptoethanol to the protein extract following affinity chromatography did not prevent the loss of enzyme activity. On
the other hand, ammonium sulfate precipitation followed by DEAE-cellulose chromatography resulted in an enzymatically active ScOMT1.

Substrate Specificity of ScOMT1 and Product Identification—The partially purified recombinant ScOMT1 exhibited exclusive specificity for daphnetin as a substrate for O-methylation. The enzyme did not accept either the 6,7-dihydroxy analog, esculetin, or caffeic acid, 5-hydroxyferulic acid, luteolin (a 3',4'-dihydroxyflavone), quercetin (a 3',4'-dihydroxyflavonol), umbelliferone (7-hydroxycoumarin), naringenin (a 4'-hydroxy-
flavanone), apigenin (a 4′-hydroxyflavone), or kaempferol (a 4′-hydroxyflavonol) among other substrates tested. This result indicates that this novel OMT exhibits a high degree of both substrate and stereospecificity. Kinetic analysis of the partially purified ScOMT1 gave an apparent $K_{m}$ of 152 μM for daphnetin and 19 μM for AdoMet. The $K_{m}$ value for daphnetin is comparable with $K_{m}$ values reported for other OMTs (11). The ScOMT1 reaction product was unambiguously identified as 7-hydroxy-8-methoxycoumarin. It gave a single product when chromatographed in a nonpolar solvent system on cellulose TLC (Fig. 2A) with a higher $R_{f}$ value (0.6) than that of daphnetin (0.25), suggesting the presence of a methyl group and, consequently, the reaction product elutes later ($R_{f}$ 26.0 min) than daphnetin ($R_{f}$ 19.0 min) after HPLC on a C_{18} Agilent column (Fig. 2B). The enzyme reaction product coeluted with an authentic sample of 7-hydroxy-8-methoxycoumarin ($R_{f}$ 25.8 ± 0.5 min) on HPLC, and was well separated from the 7-methoxy-8-hydroxy isomer ($R_{f}$ 27.5 ± 0.4 min) (Fig. 2C). Moreover, the UV absorption maxima of the enzyme reaction product, which were identical to that of an authentic sample of 7-hydroxy-8-methoxycoumarin, is lower than that of daphnetin ($\lambda_{max}$ 265 and 330), whereas its mass is increased by 15 mass units, indicating the introduction of a methyl group into daphnetin. In addition, the chemical structure of the enzyme reaction product was verified by NOESY in comparison with the other methylated isomers, which indicates unequivocally the substitution pattern of the methoxy protons, a hydroxy proton, two aromatic protons, and two olefinic protons in both 7-hydroxy-8-methoxycoumarin and 8-hydroxy-7-methoxycoumarin as described under "Experimental Procedures."

Effect of Growth Temperature and Growth Irradiance on PSII Excitation Pressure—Table I summarizes the estimated kinetic activity of endogenous ScOMT1 protein in rye leaves grown at the indicated temperature/light (°C/μmol m^{-2} s^{-1}) conditions using daphnetin as substrate. B, specific activities of various OMTs in rye leaves grown at low temperature. 20/250 represents rye plants grown at 20 °C and 250 μmol m^{-2} s^{-1}; 5/250, represents rye plants grown at 5 °C and 250 μmol m^{-2} s^{-1}. The phenolic substrates used were: Daph, daphnetin; Esc, esculetin; Api, apigenin; 5-OHF, 5-hydroxyferulic acid; Nar, naringenin; CA, caffeic acid; Quer, queretin; Lat, luteolin.

TABLE I

| Growth regime | 1-qP  |
|---------------|------|
| 20/50         | 0.031 ± 0.009 |
| 20/250        | 0.134 ± 0.057 |
| 20/800        | 0.325 ± 0.062 |
| 5/50          | 0.143 ± 0.025 |
| 5/250         | 0.352 ± 0.023 |

Fig. 3. Expression analysis of ScOMT1. A, Northern blot of ScOMT1 regulated by PSII excitation pressure. 20/800, 20/250, and 20/50 represent rye plants grown at 20 °C and 800, 250, or 50 μmol m^{-2} s^{-1}, respectively. 5/250 and 5/50 represent rye plants grown at 5 °C and 250 or 50 μmol m^{-2} s^{-1}, respectively. B, kinetics of ScOMT1 mRNA accumulation during high-light and cold-temperature exposure: plants grown at 20/250 were transferred to either 20/800 or 5/250 for the indicated times. Equal amounts of total RNA (10 μg) were separated by agarose gel electrophoresis in the presence of formaldehyde and transferred to nitrocellulose membranes. *, represents an ethidium-bromide stained 28 S ribosomal band as a load control.

Fig. 4. OMT activities of cold-acclimated rye leaves. A, specific activity of endogenous ScOMT1 protein in rye leaves that were grown at the indicated temperature/light (°C/μmol m^{-2} s^{-1}) conditions using daphnetin as substrate. B, specific activities of various OMTs in rye leaves grown at low temperature. 20/250 represents rye plants grown at 20 °C and 250 μmol m^{-2} s^{-1}; 5/250, represents rye plants grown at 5 °C and 250 μmol m^{-2} s^{-1}. The phenolic substrates used were: Daph, daphnetin; Esc, esculetin; Api, apigenin; 5-OHF, 5-hydroxyferulic acid; Nar, naringenin; CA, caffeic acid; Quer, queretin; Lat, luteolin.

1-qP values for PSII excitation pressure for rye plants that were grown at either 20 or 5 °C under increasing irradiance. The data demonstrate that increasing irradiance results in an increased 1-qP at both 20 and 5 °C. It is significant to note that plants grown at either 20/800 or 5/250 not only exhibited comparable 1-qP values but also displayed the highest values, thus, considered to be grown under high excitation pressure. The phenolic substrates used were: Daph, daphnetin; Esc, esculetin; Api, apigenin; 5-OHF, 5-hydroxyferulic acid; Nar, naringenin; CA, caffeic acid; Quer, queretin; Lat, luteolin. Accumulation of ScOMT1 mRNA—Northern blot analysis (Fig. 3A) shows that the ScOMT1 transcript exhibits a high level of expression in plants grown at 5/250 or 20/800 (°C temperature/light intensity μmol m^{-2} s^{-1}) as compared with those grown at 20/250 or 5/50. However, the transcript level is higher in cold-acclimated plants (5/250) than in 20/800. This could possibly be because of an increase in mRNA stability at low temperature. The levels of expression exhibited by ScOMT1 under the various growth conditions cannot be ex-
explained as responses to either growth temperature or growth irradiance. For example, if we compare plants grown at either 20/250 or 5/250 we may conclude that the expression of ScOMT1 is regulated by low temperature. This is clearly not the case for two reasons. First, rye plants grown at 20/800 and 5/250 exhibited a higher level of ScOMT1 mRNA than plants grown at 20/250 and 5/50. Second, plants grown at 5/50 exhibited a lower expression of ScOMT1 mRNA than plants grown at 5/250, indicating that the ScOMT1 transcript is regulated by PSII excitation pressure. The ScOMT1 cDNA insert detects a transcript of ~1000 bases, which is within the expected range for a cDNA clone of that length. This is corroborated by the kinetics of ScOMT1 transcript accumulation during exposure to low temperature (5/250) and high light (20/800) (Fig. 3B). When rye plants grown at 20/250 are transferred to high excitation conditions, the ScOMT1 transcript gradually accumulates and was highest at 12 days exposure to 20/800 and at 40 days to 5/250 (Fig. 3B). Other treatments such as wounding, ABA application, heat shock, or salt stress did not reveal any effect on the expression of ScOMT1 (result not shown). To our knowledge, these results represent the first report of a plant OMT transcript that is regulated by PSII excitation pressure and not by either low temperature or high light per se.

**OMT Activities of Cold-acclimated Rye Leaves**—The endogenous ScOMT1 activity of both cold-acclimated and high-light grown rye leaves show that ScOMT1 exhibits the highest enzyme activity when rye plants are cold acclimated (5/250), as compared with those exposed to high light (20/800) or the control plants (20/250) (Fig. 4A). These results are consistent with the higher accumulation of ScOMT1 mRNA at 5/250 as compared with 20/800 (Fig. 3A).

Moreover, using various phenylpropanoid and flavonoid substrates we show that cold acclimation increases not only ScOMT1 activity against daphnetin, but also for other OMTs that utilize the lignin precursors, caffeic acid and 5-OH-ferulic acid as substrates (Fig. 4B) (27). It is well known that lignin contributes to the strength of plant cell walls, facilitates water transport, and impedes the degradation of wall polysaccharides (28). Therefore, an increase in the activity of OMTs involved in lignin biosynthesis suggests a participation in the plant defense response not only against pathogens, insects, and other herbivores but also against cold stress.

**DISCUSSION**

We report here the molecular and biochemical characterization of a cDNA clone (ScOMT1) that encodes a novel OMT in rye. This novel gene exhibits 26 to 40% amino acid sequence identity to a number of plant OMTs and is most closely related to SafBP, a Safener-binding protein that may protect maize against injury from chloroacetanilide and thiocarbamate herbicides (22), although it did not exhibit any enzymatic activity. This is not surprising because ScOMT1, but not SafBP, possesses in the first motif, the conserved aspartic acid residue proposed to be involved in the binding to AdoMet (29). ScOMT1 exhibited an exclusive specificity for the methylation of the 7,8-dihydroxycoumarin, daphnetin. The fact that it did not accept the 6,7-dihydroxycoumarin analog, esculetin, implies that meta-directed methylation of esculetin to 7-hydroxy-6-methoxycoumarin (scopoletin), an ubiquitous coumarin derivative, is catalyzed by another distinct OMT (Fig. 5), which has yet to be isolated and characterized at the molecular level. The ScOMT1 reaction product was unambiguously characterized as...
7-hydroxy-8-methoxycoumarin by chromatographic and spectroscopic methods in comparison with chemically synthesized compounds. 7-Hydroxy-8-methoxycoumarin was first isolated and partially characterized in 1961 from *Hydrangea macrophylla* and given the trivial name, hydrangetin (30). Since then both the 7-hydroxy-8-methoxy and 7-methoxy-8-hydroxy isomers have been identified by spectroscopic methods from *Daphne tangutica* (31) and *Daphne geraldii* (32), respectively, as the main constituents used as analgesic herbal medicines. This also suggests the existence of a position-specific daphnetin 7-OMT that has not been yet isolated.

Several substituted coumarins have been shown to effectively remove superoxide anions (33). This result was confirmed with fraxetin, a 6,7,8-trisubstituted coumarin that is capable of scavenging superoxide anion radicals, presumably to protect sites of human cytokine activation during the inflammation process (34). These results suggest that coumarins are potent scavengers of peroxyl radicals and are potential candidates for evaluation as protective agents against disorders in which oxidative reactions are implicated. We have demonstrated that the *ScOMT1* transcript level is up-regulated in response to PSII excitation pressure created by either low temperature or high light. Maximum activity of *ScOMT1* was reached after ~40 days of low temperature acclimation or 12 days at high light exposure. In plants, these two conditions result in increased oxidative stress during prolonged exposure (35, 36). Thus, the enhancement of the active oxygen scavenging system that was induced by low temperature or high light could result in a combined increase of *ScOMT1* enzyme activity and accumulation of coumarin derivatives. This suggests a possible role for *ScOMT1* methylation of coumarins as a general defense response against oxidative stress.

The exclusive *O*-methylation of daphnetin by *ScOMT1* is quite significant considering the fact that this coumarin has recently been reported as a protein kinase inhibitor (37). It is well known that the process of protein phosphorylation is governed by the complementary activities of protein kinases and phosphatases, both being important for the regulation of cell functions (37, 38). Low temperature treatment has been shown to increase kinase activity and stimulate protein phosphorylation in wheat (39). Therefore, the methylation of daphnetin by *ScOMT1* may be considered as a means of modulating the effect of daphnetin on protein kinases, allowing them to function during exposure to high PSII excitation pressure and cold acclimation. It may be possible that daphnetin *O*-methylation could shift the phosphorylation state of the protein kinase, thus activating the expression of specific genes involved in the modulation of PSII excitation pressure and cold acclimation.

It has recently been reported that daphnetin exhibits a potent inhibitory activity on inflammatory cytokines, because it can be used to treat rheumatoid arthritis, lumbago, and reduce fever in Turkish folk medicine (40). Daphnetin is also being used in China for the treatment of coagulation disorders (41), and may block the action of the various protein kinases involved in the development of these diseases. Taken together, these observations support the potential role of daphnetin methylation in the modulation of protein kinases.

In summary, we have cloned and characterized a novel OMT that catalyzes the exclusive *O*-methylation of daphnetin to 7-hydroxy-8-methoxycoumarin. *ScOMT1* expression is up-regulated by PSII excitation pressure and low temperature. Because daphnetin is known to act as a protein kinase inhibitor, we suggest that its methylation may be involved in low-temperature signaling. Further studies are required to confirm the importance of daphnetin methylation in signal transduction involving protein kinases and/or in the defense mechanism of the plant against oxidative stress.