Inactivation of Brassinosteroid Biological Activity by a Salicylate-inducible Steroid Sulphotransferase from *Brassica napus*

(Received for publication, March 15, 1999, and in revised form, April 29, 1999)

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Recent discoveries from brassinosteroid-deficient mutants led to the recognition that plants, like animals, use steroids to regulate their growth and development. We describe the characterization of one member of a *Brassica napus* sulphotransferase gene family coding for an enzyme that catalyzes the O-sulphonation of brassinosteroids and of mammalian estrogenic steroids. The enzyme is specific for the hydroxyl group at position 22 of brassinosteroids with a preference for 24-epicastasterone, an intermediate in the biosynthesis of 24-epibrassinolide. Enzymatic sulphonation of 24-epibrassinolide abolishes its biological activity in the bean second internode bioassay. This mechanism of hormone inactivation by sulphonation is similar to the modulation of estrogren biological activity observed in mammals. Furthermore, the expression of the *B. napus* steroid sulphotransferase genes was found to be induced by salicylic acid, a signal molecule in the plant defense response. This pattern of expression suggests that, in addition to an increased synthesis of proteins having antimicrobial properties, plants respond to pathogen infection by modulating steroid-dependent growth and developmental processes.

Many developmental and physiological processes in organisms ranging from fungi to humans are regulated by a small number of steroid hormones. However, unlike recently the plant kingdom was almost completely excluded from the field of steroid endocrinology. The recent demonstration that the *Arabidopsis* de-etiolated2 (*det2*) and the constitutive photomorphogenesis and dwarfism (*cpd*) mutants are defective in the synthesis of brassinosteroids (*BRs*) focused attention on the physiological similarities with animal steroids, it has been proposed that BRs might interact with a soluble receptor in order to regulate the expression of specific genes (Fig. 1). This hypothesis has recently been challenged with the molecular characterization of the *Brassinosteroid-Insensitive-1* (*BRI1*) gene that encodes a putative BR receptor (4). The *BRI1* protein belongs to a family of plant receptor-like transmembrane kinases. Members of this family have an extracellular leucine-rich repeat domain, a transmembrane domain, and an intracellular serine/threonine protein kinase domain, suggesting that BRs mediate their effects at the cell surface. Despite this important discovery, direct or indirect binding of BRs to the *BRI1* receptor has not been demonstrated, and their mode of action remains to be characterized.

In mammals, it is well recognized that sulphotransferases (STs) play an important role in the modulation of the biological activity of a number of compounds, such as steroid and thyroid hormones, and catecholamine neurotransmitters (10–13). Sulphonate conjugation not only facilitates transport and excretion of hydrophobic molecules by increasing their water solubility, it abolishes the biological activity of hormones, such as estrogens. Considering the important role of mammalian STs in the modulation of the biological activity of hormones, our research interest is to elucidate the function of plant STs in growth and development. In this paper, we describe the characterization of one member of a *Brassica napus* ST gene family coding for an enzyme that sulfonates BRs as well as mammalian estrogenic steroids. We also demonstrate that enzymatic sulphonation of 24-epibrassinolide abolishes its biological activity. In addition, our results indicate that the pattern of expression of the *B. napus* steroid ST gene is similar to other pathogenesis-related (PR) proteins, *i.e.* having very low constitutive expression in control seedlings and being inducible by salicylic acid (SA) (14). This represents the first example where the function of a PR protein can be linked to the modulation of the biological activity of a plant hormone by conjugation.

**EXPERIMENTAL PROCEDURES**

*Isolation of the BNST Genes*—A *B. napus* genomic library constructed in pTZ18R was probed with 32P-labeled *RaR047* cDNA (15) under conditions of high stringency according to standard procedures (16). Mapping analysis of two positive clones resulted in the identifica-
tion of three genes (BNST1, -2, and -3), which were subcloned in pBSK− (Stratagene) and sequenced in an automated system (Applied Biosystems Inc.). The alignment of the deduced amino acid sequences of BNST1, -2, and -3 and RaR047 was created with the PILEUP program of the GCG sequence analysis package (17).

Expression of Recombinant BNST3 in Escherichia coli—Oligonucleotides (5'-GGGATCCATGTCATCATTAAAGC-3'; 5'-GGGAAGCTTAAACTATCATCATTAAAGC-3') were designed to introduce BamHI and HindIII restriction sites at the respective 5' and 3' ends of the coding region of BNST3 by polymerase chain reaction amplification using Vent DNA polymerase (New England Biolabs). This allowed the directional cloning of the BNST3 coding sequence in the bacterial expression vector pQE30 (Qiagen). The expression of BNST3 in E. coli cultures (A<sub>600</sub> 0.7) was induced with 1 mM isopropyl b-D-thiogalactopyranoside for 9 h at 22°C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, 14 mM b-mercaptoethanol, and lysed by two passages in a French pressure cell (14,000 pounds/square inch). The recombinant BNST3 recovered in the soluble fraction was purified by affinity chromatography onto nickel-nitrilotriacetic acid-agarose matrix as recommended (Qiagen).

Expression of BNSTs and RaR047 in Plants—B. napus cv. Westar and Arabidopsis thaliana ecotype Columbia were grown in vermiculite under continuous white light. The roots of 7-day-old B. napus seedlings and of 5-week-old A. thaliana plants were drenched with an aqueous sodium salicylate solution (10 mM, pH 7.0) and grown for various times. Plants were pulverized in liquid nitrogen and then either extracted in phenol/chloroform as described (18) to isolate total RNA or boiled in reducing SDS sample buffer to obtain total proteins. Northern blot analysis of total RNA was achieved under high stringency conditions according to standard procedures (16) using the 32P-labeled coding region of BNST1 as a probe. Protein extracts were separated by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose, and STs were detected using anti-BNST1 polyclonal antibodies (dilution 1:3000) and goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase (dilution 1:3000; Bio-Rad). Anti-BNST1 polyclonal antibodies were raised in rabbits using purified recombinant BNST1 expressed in E. coli as the antigen. The antibodies were found to cross-react with BNST2 and -3 (data not shown).

Enzymatic Assays—Analysis of substrate specificity was performed by testing enzymatic activity with three different concentrations of acceptor substrate as follows: 200, 5, and 0.1 mM. Reaction mixtures (50 μl) also contained 2 mM 35S-labeled PAPS (NEN Life Science Products) and approximately 5 μg of affinity purified recombinant BNST3 in 50 mM Tris, pH 7.5. For kinetic analysis, a PAPS concentration of 5 mM was used in the same buffer. Concentrations of 24-epicastasterone were 2, 1, 0.5, and 0.2 mM and of 24-epiteasterone were 50, 20, 10, and 5 μM. Stock solutions (1 mM) of acceptor substrates were prepared in dimethyl sulfoxide or methanol. The final concentration of dimethyl sulfoxide or methanol in the assay was 5%. Commercial substrates were obtained from Sigma, and desulfoglucosinolates were kindly provided by D. Reed, Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada. Reactions were allowed to proceed for 10 min at 25°C. Incorporation of the 35S-labeled sulfate was monitored as described (19). Kinetic parameters were deduced from double-reciprocal Lineweaver-Burk plots.

Bean Second Internode Test—24-Epibrassinolide sulfate was produced enzymatically and extracted twice in ethyl acetate as a tetrabutyl ammonium salt (19). Test compounds (10 nmol) solubilized in 2 μl of methanol were applied at the base of the second internode of 7-day-old bean seedlings as described (20). Methanol alone (2 μl) was applied on control bean plants. Bean seedlings were grown at 21°C with a 16-h
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Dots indicate gaps introduced for optimal alignment. Dashes indicate identical amino acids. Regions I and IV conserved in all cytosolic STs are boxed. Residues critical for catalysis and PAPS binding are indicated by arrows (42–44). Domain II involved in the determination of substrate specificity is underlined (26).

FIG. 2. Comparison of the deduced amino acid sequences of B. napus ST genes and A. thaliana RaR047 (accession number Z46823). Dots indicate gaps introduced for optimal alignment. Dashes indicate identical amino acids. Regions I and IV conserved in all cytosolic STs are boxed. Residues critical for catalysis and PAPS binding are indicated by arrows (42–44). Domain II involved in the determination of substrate specificity is underlined (26).

FIG. 3. A, Northern analysis of BNST gene expression in B. napus seedlings treated with 10 mM salicylic acid for the indicated times. The blot was hybridized with the coding region of the BNST1 gene. B, same blot as shown in A hybridized with an actin probe. C, Western analysis of ST gene expression in B. napus and A. thaliana. Total proteins were extracted from B. napus seedlings (lanes 1–3) and A. thaliana (lane 4) that have been incubated with 10 mM salicylic acid for the times indicated. The membrane was incubated with anti-BNST1 antibodies. Size of molecular weight markers is indicated on the left.
was dictated by the fact that it is expressed in SA-treated seedlings as determined by reverse transcriptase-polymerase chain reaction and is the most similar to A. thaliana RaR047. Although a variety of acceptor molecules of plant and mammalian origin (e.g. desulfoglucosinolates, gibberellins, phenolic acids, flavonoids, steroids, phytosterols, and terpenoids) were used as substrates, BNST3 catalyzed the transfer of the 35S-labeled sulfonate group from the cosubstrate PAPS to BRs and estrogens only (Fig. 1 and Table I). The enzyme is also sensitive to the level of oxidation of their precursors. The differences observed in their natural occurrence in this plant. The differences observed in their natural occurrence in this plant. The differences observed in their natural occurrence in this plant. The differences observed in their natural occurrence in this plant. The differences observed in their natural occurrence in this plant.

To determine the position of sulfonation and the substrate preference of BNST3, several natural and synthetic BRs were tested (Fig. 1). Of those, BNST3 exhibits the highest affinity for 24-epicastasterone followed by 24-epiteasterone with apparent $K_m$ values of 1.4 and 4.2 $\mu M$, respectively (Tables I and II). The apparent $K_m$ value for PAPS was found to be 1.0 $\mu M$. The catalytic efficiency ($V_{\text{max}}/K_m$) of the enzyme is 13-fold higher for 24-epicastasterone as compared with 24-epiteasterone (Table II). Furthermore, 24-epicastasterone is the only substrate for which a significant enzymatic activity is measured at a concentration of 0.1 $\mu M$ (2.5 pkatal/mg). Although BNST3 also accepts 24-epibrassinolide as a substrate, this activity is only observed at high substrate concentration (Table I). The enzyme is also sensitive to the level of oxidation of ring B, as revealed by the low relative activity values obtained with 6-deoxy-24-epicastasterone and 6-deoxy-24-epicathasterone as substrates. The most striking feature of BNST3 substrate specificity is its efficient use of the natural substrate 24-epibrassinolide and the synthetic enantiomer (22S,23S)-28-homobrassinolide, whereas it is inactive with the natural substrate brassinolate (Table I).

The fact that the enzyme accepts 24-epicastasterone and 24-epiteasterone but does not accept the synthetic substrate 22-deoxy-24-epiteasterone suggests strongly that sulfonation is taking place at position 22 of the steroid side chain (Table I). The specificity of the enzyme for position 22 explains why early BR intermediates and BR precursors lacking a hydroxyl group at this position are not accepted by the enzyme.

**Sulfonation of 24-Epibrassinolide Abolishes Its Biological Activity**—It has previously been reported that hydroxylation of brassinosteroid intermediates at position 22 increases considerably their biological activity, suggesting that sulfonation at this position by BNST3 might lead to the loss of their function (24). To test this hypothesis, we compared the ability of 24-epibrassinolide and its sulfonated derivative to promote growth of the bean second internode, a well established biological test for the activity of BRs (29). As expected, application of 10 nmol of epibrassinolide dramatically induced elongation of the internode (mean = 6.5 cm, S.D. = 1.2 cm, n = 5) (Fig. 4A), as compared with control plants (mean = 1.1 cm, S.D. = 0.4 cm, n = 5). However, the addition of 10 nmol of epibrassinolide sulfate did not stimulate the internode elongation (mean = 1.5 cm, S.D. = 0.5 cm, n = 5) (Fig. 4B) as compared with control bean seedlings.

**DISCUSSION**

We have isolated three genes (BNST1, -2, and -3) from B. napus which encode STs. They belong to a gene family comprising at least 12 members having a high level of sequence identity with a previously characterized cDNA clone (RaR047) from A. thaliana (15). According to the guidelines on sulfotransferase nomenclature, RaR047 and the BNSTs fall below the 45% amino acid sequence identity threshold value to be members of the flavonol SULT3 family (30). Therefore, they represent the first characterized members of the new SULT4 family. When expressed in E. coli, the enzyme encoded by the BNST3 gene exhibited specificity for steroid substrates and catalyzed the sulfonation of the plant-specific BRs, and of mammalian estrogens such as 17$\beta$-estradiol (Fig. 1 and Table I). Whereas several studies (31, 32) described the presence in plants of the mammalian steroids estradiol, estrone, and testosterone, as well as their physiological effects on growth, development, and flowering, the functional significance of estrogen sulfonation by a plant enzyme remains to be elucidated.

Despite the lack of information on the accumulation of 24-epimers of BRs in B. napus, our results suggest that they are in fact synthesized in this plant. These results call for a thorough investigation of BRs in B. napus tissues in order to confirm their natural occurrence in this plant. The differences observed in the domain responsible for ST substrate specificity of BNST1, -2, and -3 suggest that they have distinct substrate preferences, and it is possible that other members of the BNST family are involved in the sulfonation of brassinolide and/or its precursors.

### Table I

| Substrate                  | Relative activity In % at 200 $\mu M$ | Relative activity In % at 5 $\mu M$ |
|---------------------------|--------------------------------------|-----------------------------------|
| Brassinolide              | <5                                   | <5                                |
| (22R,23R)-28-Homobrassinolide | <5                                  | <5                                |
| (22S,23S)-28-Homobrassinolide | 100$^a$                              | 15                                |
| 24-Epibrassinolide        | 64                                   | 15                                |
| 24-Epicateasterone         | 27                                   | 5                                 |
| (22S,23S)-28-Homocastasterone | 51                                  | 28                                |
| 24-Epicateasterone inh.$^{b}$ | inhb                                | 30                                |
| 24-Epicateasterone        | inhb.$^{c}$                          | 100$^d$                           |
| 22-Deoxy-24-epicateasterone | <5                                  | <5                                |
| 6-Deoxy-24-epicastasterone | 45                                   | 8                                 |
| 6-Deoxy-24-epicastasterone | 16                                   | 5                                 |
| $\beta$-Estradiol         | 43                                   | 3                                 |
| $\beta$-Estradiol 3-methyl ether | 48                                  | 5                                 |

$^a$ Maximum specific activity equals 11 pkatal/mg.
$^b$ Substrate inhibition (inh.) was observed at concentrations above 50 $\mu M$.
$^c$ Substrate inhibition (inh.) was observed at concentrations above 5 $\mu M$.
$^d$ Maximum specific activity equals 7.0 pkatal/mg.

### Table II

| Substrate                  | $K_m$ | $V_{\text{max}}$ | $V_{\text{max}}/K_m$ |
|---------------------------|-------|------------------|-----------------------|
| Substrate                 | $\mu M$ | pkatal mg$^{-1}$ | pkatal mg$^{-1}$$\mu M^{-1}$ |
| 24-Epicateasterone         | 4.2    | 9.6              | 2.3                   |
| 24-Epibrassinolide         | 1.4    | 42               | 31                    |

$^a$ Kinetic parameters of recombinant BNST3 for the preferred BR substrates.

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BNST3 has the highest affinity for 24-epicathasterone, of the substrates tested. The 13-fold lower $V_{\text{max}}/K_m$ value observed for 24-epicatase as compared with 24-epicathasterone suggests that the latter might be the only BR sulfated in vivo by BNST3. The preference of BNST3 for an early precursor, as compared with the final product 24-epibrassinolide, is quite surprising considering the fact that the latter is about 1000-fold more active (33). However, cathasterone is considered to be the first BR precursor with significant biological activity, and it has been proposed that its synthesis is the rate-limiting step in the formation of the more potent BRs (5, 29). The substrate preference of BNST3 for 24-epicatase may be an efficient mechanism to block its intrinsic activity and at the same time interfere with the biosynthesis of the more potent final product 24-epibrassinolide.

The fact that the currently used techniques for the analysis of BRs are not suitable for the preservation of the labile sulfate moiety may explain the lack of reports on the characterization of sulfated BRs in plant extracts. However, it has been shown that rice seedlings administered labeled cathasterone produce non-glycosidic polar BR derivatives that may in fact be sulfate esters (34), based on their chromatographic behavior and their susceptibility to solvolysis.

Our results also demonstrate that sulfonation of 24-epibrassinolide abolishes its biological activity in the bean second internode bioassay suggesting that plants, like mammals, may modulate the biological activity of steroids by sulfonation. The recent finding that BRII codes for a potential transmembrane leucine-rich repeat-receptor kinase, which is required for BR perception, suggests that BRs mediate their biological activity on the external surface of the cell membrane (4). Furthermore, the fact that the expression of the CPD gene is confined to specific cell types of leaves, other than those involved in elongation, suggests that BRs must be transported from source to target tissues (35). Therefore, the sulfonation of BRs may interfere with their excretion, their transport, or their binding to the BRI1 receptor. The availability of cloned BNST3 and RaR047 cDNAs provides the tools for further study of the function of these enzymes in relation to BR action. Transgenic A. thaliana overexpressing BNST3 are presently being produced in our laboratory.

Another interesting feature of the BR STs is the induction of their expression following SA treatment in both B. napus and A. thaliana (15), suggesting that the modulation of steroid activity may be part of the defense response of plants to pathogens. The results of several experiments suggest that BRs stimulate plant growth by increasing the plasticity of the cell wall (36). In the context of a pathogen infection, this growth mechanism is undesirable since it reduces the protection offered by the cell wall. The sulfonation of BRs by the SA- and pathogen-inducible steroid STs provides a mechanism through which BR-dependent cell wall hydrolysis might be stopped. Additional mechanisms are likely to parallel steroid ST activity to reduce the levels of biologically active BRs during pathogen infection. A reduced rate of synthesis of BRs is also expected, since the expression of the $\Delta^{24}$-sterol C-methyltransferase, which catalyzes the first committed step in phytosterol biosynthesis, was reported to decrease following elicitation of soybean cells (37). Furthermore, suppression of phytosterol biosynthesis following elicitor treatment has been reported in parsley, potato, tobacco, and Tabernaemontana divaricata (38–41).

The widespread occurrence of STs and sulfatases in mammalian tissues has led to the hypothesis that the concerted action of both groups of enzymes may regulate the level of active steroids (10). Considering the structural, functional, and metabolic conservation of steroids between plants and mammals, it is likely that a common mechanism of modulation of steroid activity has also been conserved through evolution. In support of this hypothesis, we demonstrate the occurrence of steroid STs in plants and that sulfonation of 24-epibrassinolide leads to the loss of its biological activity.

Acknowledgments—We thank Dr. D. Roby for the A. thaliana RaR047 cDNA clone and Dr. G. Bellemare for the B. napus genomic library.

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