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Transport of camptothecin in hairy roots of *Ophiorrhiza pumila* ☆

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Abstract

We have investigated the subcellular accumulation and transport of camptothecin (CPT), a monoterpene indole alkaloid, in hairy roots of *Ophiorrhiza pumila*. This hairy root produces high amounts of CPT and excretes it into the culture medium. When the hairy roots were exposed to UV radiation, autofluorescence emitted from CPT showed subcellular localization of CPT in the vacuole. Treatment with several inhibitors suggested that CPT excretion is a transporter-independent passive transport controlled by the concentration gradient of the compound. Interestingly, the hairy roots treated with brefeldin A, a vesicle transport inhibitor, showed increased CPT excretion. This could be explained by an increased transport rate of CPT from the endoplasmic reticulum (ER) to the cytoplasm when transport of CPT to the vacuole is blocked. The much higher concentration of CPT in the cytoplasm resulted in the increased excretion rate. This result indicates that CPT is biosynthesized at the ER and transported to accumulate in the vacuole by the same machinery that is used for vacuolar protein sorting. How *O. pumila* is insensitive to CPT is discussed.

Keywords: *Ophiorrhiza pumila*; Hairy root; Excretion; Transport; Accumulation; Camptothecin

1. Introduction

Plants produce secondary metabolites as a response to both biotic and abiotic interactions with the environment. Of these, alkaloids are a very large group of secondary metabolites. As many alkaloids exhibit pharmacological properties and have been used by humans as medicines, manipulation of the pathway for improved production of these alkaloids is an important objective. To achieve this goal, many researchers have been actively studying their biosynthetic pathways, metabolite transport and accumulation sites.

Camptothecin (CPT), a monoterpene indole alkaloid, has been found in several plant species including *Camptotheca acuminata*, *Nothapodytes foetida* and *Ophiorrhiza pumila* (Lorence and Nessler, 2004). Since it possesses topoisomerase I poisoning properties, its semi-synthetic derivatives, topotecan and irinotecan, have been developed to be clinically used as anticancer drugs. Previously, we have established a hairy root culture of *O. pumila* which has already been shown to be a desirable experimental system to study the biosynthesis of camptothecin, since the culture produces a high level of CPT and excrete it into the culture medium (Saito et al., 2001; Yamazaki et al., 2003a,b, 2004). Because of CPT toxicity, the presence of a species-specific detoxification mechanism has been suggested in CPT-producing plants. It has been known that many secondary metabolites are

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stored in specialized compartment such as vacuoles or secreted to outside of cells after biosynthesis to avoid toxicity. CPT has been reported to be accumulated in glandular trichomes of leaves and stems (Li et al., 2002), and in the vacuole of *C. acuminata* (Pasqua et al., 2004). In addition, CPT is also excreted from hairy root cultures of both *C. acuminata* and *O. pumila* (Lorence et al., 2004; Saito et al., 2001). It has been shown that metabolite excretion by cultured cells is a consequence of their cytotoxicity and the relative degree of their lipophilicity (Guern et al., 1987). Several studies characterizing the root exudation process have been reported. Amino acid efflux in plant roots is driven passively by large differences in concentration between the inside and outside of root cells (Phillips et al., 2004). Recently, a number of studies have shown the involvement of ATP-binding cassette (ABC) transporters in secondary metabolite transport, including anthocyanin (Goodman et al., 2004), sclareol (an antifungal terpenoid) (Jasinski et al., 2001) and berberine (a benzylisoquinoline alkaloid) (Shitan et al., 2003). An ABC transporter is involved in the active transport and uses ATP hydrolysis as a source of energy. In addition, the role of ABC transporters has been reported in the mechanism of resistance to CPT derivatives by reducing drug accumulation in human cancer cells and yeast (Brangi et al., 1999; Hendricks et al., 1992; Lalloo et al., 2004; Leggas et al., 2004; Reid et al., 1997). These reports suggest the possibility of the involvement of ABC transporters in CPT transport in planta.

Despite increasing attention having been paid to the biotechnological production of this alkaloid (Lorence et al., 2004; Saito et al., 2001), the biosynthetic pathway of CPT is still largely unknown. To date, only a few genes in the pathway have been cloned and characterized (Lopez-Meyer and Nessler, 1997; Lu et al., 2005; Lu and McKnight, 1999; Yamazaki et al., 2003a). Because of the lack of information on biosynthetic enzymes, CPT transport remains ill-defined. In the present study, we investigate the accumulation and transport of CPT in the hairy root of *O. pumila*. A pharmacological approach was employed to study the effect of various inhibitors on CPT accumulation and excretion. We also discuss a possible mechanism underlying CPT resistance in *O. pumila*.

2. Results and discussion

2.1. Subcellular localization of CPT in hairy roots of O. pumila

Autofluorescence of CPT provides a direct way to investigate the subcellular localization of CPT in hairy roots without having to perform tissue sectioning. In addition, by comparing the intensity of fluorescence, relative concentrations of CPT in different regions can be examined. The intensity of fluorescence at the root tip was much higher than that of the elongated region (Fig. 1A and B), consistent with the previous report that CPT is highly accumulated in the young parts such as young leaves and flower buds (Lopez-Meyer et al., 1994; Yamazaki et al., 2003b). Based on the morphological appearance of fluorescence, we suggested that CPT is localized in the vacuole (Fig. 1C and D). Pasqua et al. (2004) have also reported the accumulation of CPT in vacuole of *C. acuminata*. Accumulation of CPT in vacuole is considered as a detoxification mechanism.

Comparing the fluorescence pattern between cells in root tip and elongated root, less or no fluorescence was observed from many cells in the elongated area (Fig. 1B). This result suggests the outward transport of CPT from the vacuole. It has been proposed that the lipophilic form of alkaloids is protonated to the hydrophilic form in the acidic conditions of a vacuole (Matile, 1976). As a result, the protonated form cannot move across a tonoplast membrane. However, two distinct pathways mediated by carrier or vesicular transport have been used to explain the transport mechanism of solute from vacuole (for review, see Echeverria, 2000).

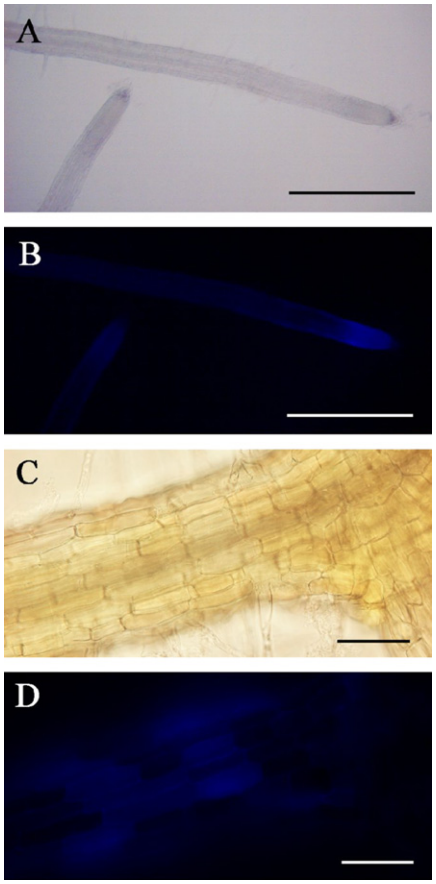

Fig. 1. Accumulation of fluorescence emitted from CPT in hairy roots of *O. pumila*. (A) Light microscopy image of hairy roots. (B) Fluorescence microscopy image of the roots shown in (A). (C) Light microscopy image of a higher resolution optical section of the elongated root. (D) Fluorescence microscopy image of the root shown in (C). Bar = 1 mm in (A) and (B) and 50 μm in (C) and (D).

2.2. CPT excretion is driven by simple diffusion

To examine the involvement of a transporter in CPT excretion, several compounds which are known to inhibit the activity of various transporters were added to the culture medium at various concentrations for 18 h. To confirm that the inhibitors do not cause cell death, the viability of hairy roots was observed under a microscope: no tissue darkening, a sign of cell death, was found. The concentrations of CPT both in medium and root were determined. The involvement of an ABC transporter on CPT excretion was examined by using the following inhibitors: sodium azide, potassium cyanide, sodium vanadate, verapamil and nifedipine. Sodium azide and potassium cyanide are known to inhibit ATP synthesis. Sodium orthovanadate is a membrane ATPase inhibitor. Verapamil and nifedipine are calcium channel blocker and also inhibit ABC transporters. A dose–response experiment with each inhibitor showed that the relative CPT excretion remained unchanged compared with the control (Fig. 2a–c). Potassium cyanide and nifedipine also did not affect CPT excretion (data not shown). Higher concentrations of these inhibitors were also tested but resulted in cell death (data not shown). Therefore, these results indicate that CPT excretion is an energy-independent process and does not involve a membrane-localized transporter.

To demonstrate that the CPT excretion process depends on a concentration gradient between inside and outside the cell, we examined CPT excretion after co-culturing hairy roots with the polystyrene resin, Diaion HP-20, which adsorbs CPT by hydrophobic interaction. In the presence of this adsorbent resin, CPT in the medium was always maintained at a low level. We have previously reported that CPT excretion, but not biosynthesis, was stimulated by the presence of the resin (Saito et al., 2001). As a result, lower fluorescence intensity and CPT concentration, when compared with the control, was observed in the Diaion HP-20 co-cultured hairy root, suggesting higher CPT excretion (data not shown). This result supports the hypothesis that CPT in root cells is passively excreted due to a large difference in concentration between the inside and outside of the root cells. It is also evident that CPT can freely diffuse in the cell and vesicle-mediated excretion can be neglected as a major transport mechanism for CPT.

2.3. CPT transport to the vacuole is associated with the endoplasmic reticulum (ER) but not the Golgi

It has been reported that benzylisoquinoline alkaloids, such as sanguinarine and berberine, are transported from the ER to the vacuole via vesicles (Alcantara et al., 2005; Bock et al., 2002). Since CPT accumulates in the vacuole,

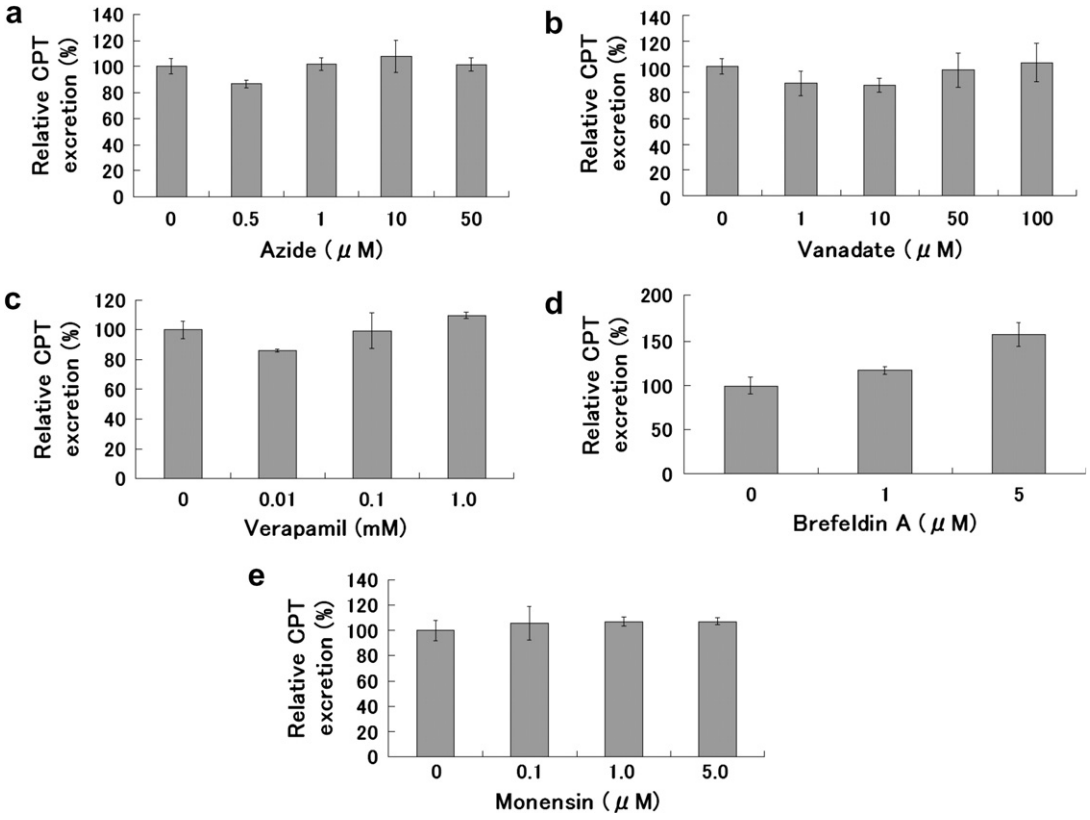

Fig. 2. Effect of various inhibitors on CPT excretion in hairy root of *O. pumila*. Three-week-old hairy roots were washed, transferred to new medium containing each inhibitor and incubated for 18 h. Dose-dependence of azide (a), vanadate (b), verapamil (c), brefeldin A (d), and monensin (e). The relative CPT excretion of the control is given as 100%.

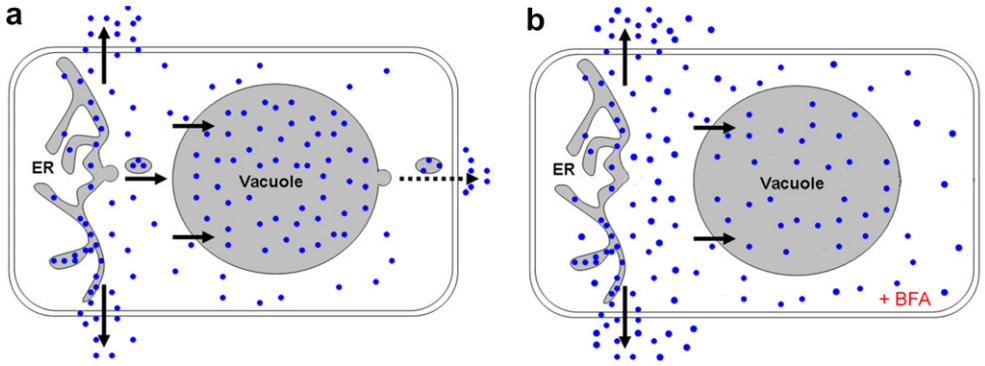

Fig. 3. Proposed models for CPT transport, accumulation, and excretion in the absence (a), and in the presence of brefeldin A (BFA) (b). Blue dots represent CPT molecules. Black arrows indicate CPT trafficking pathways. Blue dots in circles represent vesicle-mediated CPT transport. Dashed arrow indicates possible outward transport from the vacuole.

we assessed the possible involvement of ER and vesicles by treating the hairy roots with brefeldin A (BFA), a well-known inhibitor of secretion and vacuolar protein transport (for review, see Nebenfuhr et al., 2002). A dose–response experiment showed that CPT excretion was affected by BFA (Fig. 2d). Interestingly, the relative CPT excretion was significantly increased in the BFA-treated hairy roots. This can be explained by a higher CPT concentration in the cytoplasm, when CPT transport to the vacuole was blocked and, consequently, an increased rate of CPT excretion to the outside of the cells. This again indicates an equilibrium between the level of CPT inside and outside of the cells. Although vesicle formation was blocked by BFA treatment, the increase in CPT excretion implies that CPT biosynthesis was not affected. Thus, it is likely that CPT could form in the ER as previously reported in sanguinarine biosynthesis (Alcantara et al., 2005). This suggestion is supported by the presence in the deduced amino acid sequence for the cDNA encoding strictosidine synthase, which is involved in CPT biosynthesis from *O. pumila*, of a signal peptide that may direct a protein to the ER (Yamazaki et al., 2003a).

A direct pathway from the ER to the vacuole without the involvement of the Golgi complex has been demonstrated (Jiang and Rogers, 1998). Therefore, we investigated whether CPT is transported via the Golgi complex by testing the effect of monensin, an inhibitor that interferes with secretory function of Golgi complex, on CPT excretion. The result clearly showed that treatment with monensin did not affect CPT excretion, indicating that the Golgi complex is not associated with transport to the vacuole (Fig. 2e).

Taking this evidence together, we propose the following mechanism for CPT transport in the cells of *O. pumila* (Fig. 3a). CPT can either leave the ER or enter the vesicular pathway to be fused to the vacuole. In the cytoplasm, non-vesicular CPT is excreted to the medium. The excretion rate depends on the concentration difference between the inside and the outside of the cell. It is also possible that lipophilic CPT in the cytoplasm can enter and accumulate in the vacuole. An outward transport from the vacuole might be mediated by vesicles, as previously mentioned. In the presence of BFA (Fig. 3b), the concentration of CPT in the cytoplasm is increased because of the inhibition of the vesicular transport and, consequently, CPT excretion to the medium is increased. Our results, based on inhibitor experiments, support the general hypothesis that alkaloid metabolism occurs entirely in the ER (Alcantara et al., 2005; Facchini and St-Pierre, 2005).

Our results could not explain how *O. pumila* detoxifies CPT because CPT diffuses freely inside the cytoplasm. However, the most likely explanation for the self-resistance mechanism is that DNA topoisomerase I, which is the target of CPT, might be different in this plant from in other organisms. Several mutations in topoisomerase I have been found that impact on the efficacy of CPT in human cancer-cell lines and yeast (Fertala et al., 2000; Saleem et al., 1997; Tamura et al., 1991). Current experiments characterizing the DNA topoisomerase I from *O. pumila* are compatible with our hypothesis that the self-resistance mechanism is related to small structural changes (Sirikantaramas et al., in preparation). It will be of interest to study the self-resistance mechanism in other CPT-producing plants.

3. Conclusions

CPT is partially accumulated in the vacuole and partially excreted to the medium. Although little information is available regarding the CPT biosynthetic enzymes, we have shown by inhibitor experiments that CPT is excreted from hairy roots by passive diffusion. No evidence was found for the involvement of a specific transporter in CPT transport. Our results suggest that ER is the site for CPT biosynthesis, and that, after CPT is formed, it can either diffuse into the medium or enter the vesicular pathway to accumulate directly in the vacuole. However, cytoplasmic CPT might be expected to interfere with topoisomerase I. No previously proposed detoxification mechanism has successfully explained CPT resistance but

a mutation of topoisomerase I in *O. pumila* might confer CPT resistance in this plant. Further work is in progress to test this hypothesis.

4. Experimental

4.1. Plant material

Hairy roots of *O. pumila* were cultured as described previously (Saito et al., 2001; Yamazaki et al., 2003a). For inhibitor experiments, hairy roots were cultured in six-well culture plates (Greiner Bio-One) each holding 5 ml of liquid Gamborg B5 medium (Gamborg et al., 1968) and 2% sucrose, at 25 °C on a rotary shaker (60 rpm) in the dark.

4.2. Chemicals

All chemicals used in this study were purchased from Wako Pure Chemicals (Osaka, Japan) or Sigma Chemical Co. (St. Louis, MO, USA).

4.3. CPT analysis

CPT fluorescence in intact hairy roots was observed under an Olympus BX50 microscope (Tokyo, Japan) with a NUA filter set (excitation filter, 360–370 nm; barrier filter, 420–460 nm).

For quantification of CPT, a 10-fold dilution of medium with methanol or a methanolic extract of hairy root tissue were analyzed by reverse phase HPLC using a TSK gel ODS-80TM (4.6 mm × 150 mm) column with a solvent system of methanol:H_2O (7:3); monitoring was by fluorescence (excitation at 365 nm and emission at 428 nm) (Saito et al., 2001).

4.4. Inhibitor experiments

Stock solutions in sterilized water (100 mM) for sodium azide, potassium chloride, sodium orthovanadate, and verapamil hydrochloride, in DMSO for brefeldin A (30 mM) and nifedipine (100 mM), and in methanol for monensin sodium salt (30 mM) were prepared. Three-week-old hairy root cultures (~0.05 g fresh weight) in six-well culture plates were washed and transferred into new plates each holding 5 ml of medium that contains each inhibitor at different concentrations. In the control, water, DMSO or methanol was added to the same concentration as in the treatments. The cultures were incubated for 18 h on a rotary shaker in the dark, as described in Section 4.1. After incubation, both hairy roots and medium were collected for determination of CPT. Experiments were done in triplicate. The effect of inhibitors on CPT excretion was evaluated from the relative CPT excretion which is defined as a ratio between excreted CPT and total CPT. Since the rate of CPT excretion depends on the concentration difference between inside and outside cell, the quantity of hairy root in each experiment affects the values of relative CPT excretion. Hairy roots with approximately equal weight were used when performing each experiment. A control was included in each independent experiment. Relative CPT excretion of the control was given as 100%.

4.5. Absorption to the polystyrene resin

Hairy roots in B5 liquid medium with 2% sucrose (250 ml per 500 ml flask) were co-cultured with the polystyrene resin (Diaion HP-20, Mitsubishi Chemical, Tokyo) for 4 weeks as described previously (Saito et al., 2001). The resin was packaged with nylon mesh (200 mg resin per packet). The packets were changed every week. After 4 weeks, the hairy roots were harvested and extracted for determination of CPT.

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