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Intercellular Localization of Cysteine Synthase and Alliinase in Bundle Sheaths of *Allium* Plants

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Abstract

Cysteine synthase and alliinase (alliin-lyase) are terminal enzymes responsible for synthesis of cysteine and degradation of alliin (*S*-alk(en)yl-cysteine sulfoxide), respectively, in *Allium* plants. We determined the intercellular localization of cysteine synthase and alliinase using antibodies against these proteins. Cysteine synthase was predominantly localized in bundle sheath and phloem cells of three *Allium* plants, *A. tuberosum*, *A. cepa* and *A. sativum*. Lower amounts could be detected in mesophyll cells. Alliinase was almost exclusively localized in bundle sheath cells of the three *Allium* species. These results suggest the importance of bundle sheath cells for both synthesis and degradation of sulfur-containing compounds in *Allium* plants.

Keywords: alliin, alliinase, *Allium cepa*, *Allium sativum*, *Allium tuberosum*, bundle sheath, cysteine synthase, phloem, *S*-alk(en)yl-cysteine sulfoxide, sulfur-containing compounds.

Abbreviation

FAA, formaldehyde-ethanol-acetic acid.

Introduction

Certain plant species such as garlic and onion, belonging to the genus *Allium*, accumulate large amounts of sulfur-containing secondary compounds. These sulfur metabolites, *S*-alk(en)yl-cysteine sulfoxides (ACS), are synthesized from L-cysteine *via* a pathway which has not been completely elucidated yet (Lancaster and Boland, 1990) (**Fig. 1**). Upon rupture of *Allium* plant tissues by predators or pathogens, an enzyme alliinase (alliin-lyase, ACS-lyase) degrades ACS to form the reactive product, sulfenic acid, which spontaneously can be converted into a variety of sulfur compounds such as allicin, thiosulfinates, thiols etc. characteristic for different *Allium* plants (Block, 1992) (**Fig. 1**). These degraded compounds from ACS are the chemical principles for characteristic flavor, odor and pharmacological actions of *Allium* plants.

The intercellular localization of the enzymes involved in synthesis and degradation of sulfur-containing metabolites is an interesting issue with respect to metabolic and tissue cross-talks in plant cells. However, until recently this issue has been poorly documented in the literature. Maize ATP sulfurylase and APS reductase, the first two enzymes in cysteine biosynthesis, have been found exclusively in bundle sheath cells; however, sulfite reductase and cysteine synthase have been shown to be localized in bundle sheath and mesophyll cells in maize (Schmutz and Brunold, 1984). Few papers describe the intercellular localization of enzymes involved in the degradation of sulfur-containing secondary compounds. Alliinase has been preliminarily reported to be localized in bundle sheath cells of garlic clove (*A. sativum*) (Ellmore and Feldberg, 1994) and foliage leaves of *A. tuberosum* (Manabe *et al.*, 1998). Myrosinase, an enzyme degrading glucosinolates, has been shown to be distributed in the epidermis of radish plants (Hara *et al.*, 2000). However, no information concerning the intercellular localization of enzymes for both synthesis and degradation of sulfur-containing metabolites in a

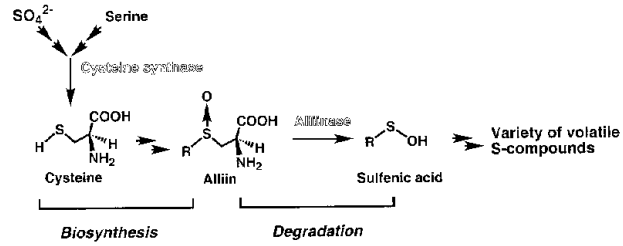

Fig. 1 Synthesis and degradation of sulfur-containing metabolites in *Allium* plants.

single plant is currently available. In the present communication we describe the tissue localization of cysteine synthase and alliinase involved in synthesis and degradation of sulfur-containing compounds, respectively, in three *Allium* species, e.g., *A. tuberosum* (Chinese chive), *A. cepa* (onion) and *A. sativum* (garlic).

Materials and Methods

Tissue preparation for immunostaining

Immunohistochemical staining was carried out as described by Marrison and Leech (1992). Plant tissues, green foliage leaves of *A. tuberosum*, bulbs (storage leaves) of *A. cepa*, and bulbs and inflorescence axes of *A. sativum*, were cut into 2-3 mm cubes with a razor blade in FAA fixative (3% *p*-formaldehyde, 50% ethanol and 5% acetic acid). The tissue pieces were fixed overnight in the dark at room temperature in fresh FAA prior to dehydration by aqueous ethanol series (50%, 70%, 90% and 100%). The samples were embedded in molten polyethylene glycol (PEG) 1540 at 56 ℃. Transverse tissue sections (8 μm thick) were mounted on dampened slide glass coated with 3-aminopropyltriethoxysilane and left to dry at 40 ℃ overnight. To remove PEG, the slides were soaked in distilled water and air-dried. Subsequently, the sections were re-hydrated by aqueous ethanol series (100%, 90%, 70% and 50%) and equilibrated with phosphate-buffered saline (PBS) (0.16 M NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, 1.5 mM KH_2PO_4).

Immunohistochemical staining

The resultant sections were incubated with rabbit antibodies raised against alliinase from *A. sativum* (Manabe *et al.*, 1998) or rabbit antibodies raised against the recombinant protein of spinach cysteine synthase A expressed in *Escherichia coli* (Saito *et al.*, 1992). Antibodies were diluted 1:1500-1:4000 with 0.5% bovine serum albumin (BSA) in PBS. The plant sections on a slide were incubated with 150 μl of antibody dilutions overnight at 4 ℃ in a moist environment.

For negative controls, sections were also incubated with pre-immune sera and pre-absorbed antibodies with the purified antigen proteins, e.g. alliinase from *A. tuberosum* (Manabe *et al.*, 1998) and recombinant spinach cysteine synthase A expressed in *E. coli* (Saito *et al.*, 1992). For the absorption of antibodies with antigens, 80 μg ml^{-1} alliinase or 40 μg ml^{-1} recombinant cysteine synthase were added to the corresponding antibody dilutions and incubated overnight at 4 ℃. After centrifugation of the antibody-antigen mixture at 17,700 g for 10 min, the supernatant was used as the pre-absorbed antibodies.

After incubation with antibodies, sections were rinsed with PBS and serially washed with 0.5% BSA in PBS, 0.01% Tween in PBS and PBS for 15 min respectively. The washed sections were the incubated with goat IgG anti-rabbit IgG-fluorecein-isothiocyanate (FITC) conjugate for 1-2 h at room temperature. The sections were washed again and mounted with Vectashield (Vector Laboratories, Burlingame, CA) and green fluorescence emitted by FITC was monitored under a fluorescence microscope (Olympus BX50) with a filter set (U-DM-FI/TR) for dual exiation (470-490 nm and 545-570 nm) and dual emission (505-535 nm and 580-620 nm). The tissue sections were also stained with safranin and fast green FCF for better visualization.

Gel electrophoresis and western blotting

SDS-Polyacrylamide gel electrophoresis (PAGE) and subsequent immunoblot analysis were carried out as described previously (Manabe *et al.*, 1998).

Results

Cross-reactivity of antibodies

To confirm the cross-reactivity of used antibodies against cysteine synthase and alliinase in *Allium* plants, immunoblot analysis was carried out as shown in **Fig. 2**. In the leaves of *A. tuberosum*, an immunoreactive protein with a molecular weight of approximately 35 kDa was detected by immunoblot analysis using antibody raised against spinach cysteine synthase A (**Fig. 2A**). The reacting protein is most likely the cytosolic cysteine synthase, of which cDNA was isolated recently from *A. tuberosum* (Urano *et al.*, 2000). **Fig. 2B** indicates that the antibodies against *A. sativum* alliinase are also reactive against alliinase in protein extracts of *A. tuberosum*. From these results, we concluded that the antibodies raised against cysteine synthase and alliinase can be used for immunohistochemical studies as judged by their cross-reactivities towards the each protein from different plant species.

Immunohistochemistry of cysteine synthase

Immunohistochemical analysis was carried out for the sections of green foliage leaves, inflorescence axes and bulbs of *A. tuberosum*, *A. sativum* and *A. cepa* by using anti-cysteine synthase A antibodies (**Fig. 3**). With all sections analyzed, immunostaining was most apparent in bundle sheath cells and phloem cells (**Fig.3a**, **3b**, **3c**, **3d**). Guard cells were also often stained. In mesophyll cells of inflorescence axes, weaker signals were observed.

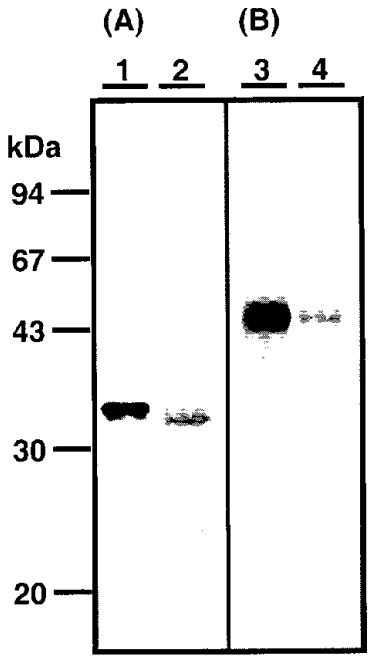

Fig. 2 Immunoblot analysis of cysteine synthase and alliinase. Proteins were separated in 12% SDS-PAGE, transferred onto Immobilon P membrane (Millipore) and reacted with the antibodies (1:500 dilution). Staining was carried out using phosphatase-labeled goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and nitroblue tetrazolium chloride (Life Technologies, Inc.). **(A)** Antibodies against spinach cysteine synthase A was used. *Lane 1*, 5 μg of protein extract of *E. coli* NK3 transformed with a vector pKM1 expressing the recombinant spinach cysteine synthase A (Saito *et al.*, 1992); *lane 2*, 5 μg of protein extract of foliage leaves of *A. tuberosum*. **(B)** Antibodies against garlic alliinase was used. *Lane 3*, 4 μg of protein extract of garlic bulbs; *lane 4*, 4 μg of protein extract of foliage leaves of *A. tuberosum*.

Upon pre-absorbing the antibodies to recombinant cysteine synthase, these signals were completely diminished (**Fig. 3e**, **3f**, **3g**, **3h**); these results indicate the authenticity of the observed signals due to the presence of cysteine synthase in those cells.

Immunohistochemistry of alliinase

For intercellular localization of alliinase, the antibodies raised against garlic alliinase were used for immunohistochemical analysis of green leaves, inflorescence axes and bulbs of three *Allium* plants (**Fig. 4**). Signals were detected in bundle sheath cells in all sections examined (**Fig. 4a**, **4b**, **4c**, **4d**). Pronounced signals were detected in the bundle sheath cells around the phloem, with weaker signals in the cells around the xylem. Sometimes weaker signals were observed in phloem cells. These signals could not be detected with the pre-absorbed antibodies (**Fig. 4e**, **4f**, **4g**, **4h**), indicating the specificity of the signals for alliinase. However, the signals detected in guard cells did not diminish when the immuno-reaction was performed using pre-absorbed antibodies with the purified alliinase, suggesting that these signals were not specific for alliinase.

Discussion

The present investigation clarified that both cysteine synthase and alliinase, responsible for the synthesis and degradation of sulfur-containing metabolites, respectively, are localized predominantly in bundle sheath cells of three *Allium* plants. It is interesting that biosynthesis of cysteine and degradation of ASC derived from cysteine are co-localized. This suggests the indispensable roles of bundle sheath cells in sulfur metabolism in *Allium* plants. The importance of bundle sheath cells in sulfur assimilation was described in a C4 plant maize (Schmutz and Brunold, 1984; Burgener *et al.*, 1998). Bundle sheath cells are the sites primarily responsible for *de novo* cysteine synthesis from sulfate. The first two enzymes of cysteine synthesis, ATP sulfurylase and APS reductase, are almost exclusively localized in the bundle sheath (Schmutz and Brunold, 1984). Cysteine or cystine is the transport metabolite from bundle sheath cells to mesophyll cells (Burgener *et al.*, 1998). Hence, in *Allium* plants as well as in maize, cysteine synthesis takes place predominately in bundle sheath cells.

From the present study, alliinase was also shown to be localized in bundle sheath cells of different organs of three *Allium* plants. So far, alliinase localization has only preliminarily been described for cloves of garlic (Ellmore and Feldberg, 1994) and foliage leaves of *A. tuberosum* (Manabe *et al.*, 1998). Localization in bundle sheath cells is not only in these tissues but also in leaves, inflorescence axes and bulbs of *A. tuberosum*, garlic and onion. Since alliinase is widely believed to contribute to chemical defense through producing bioactive sulfur-containing compounds from ACS (Städler, 2000), the specific localization in bundle sheath cells may be involved in protecting against pathogens and herbivores present in phloem and xylem tracks. By placing alliinase in bundle sheath cells, invasion of microorganisms and small herbivores through vascular bundle tracks to mesophyll cells can be most efficiently protected, because of the surrounding position of bundle sheath to the vascular bundle. Alternatively, it may indicate the close

connection of degradation metabolism catalyzed by alliinase to cysteine formation. Given that cysteine synthesis takes place in bundle sheath cells as suggested from the localization of cysteine synthase, it is assumed that ACS formation and accumulation occur most efficiently in the same tissues; and consequently alliinase localization in the same sites may contribute to minimizing the labor of transport. In literature, it has been reported that alliinase activity was found in isolated vacuoles and ACS compounds were detected in cytoplasm of onion cells (Lancaster and Collin, 1981). Although the intra-cellular localization of cysteine synthase and alliinase is not clarified in this paper, it is supposed that the apparatuses for synthesis and degradation of ACS compounds exist separately in vivo and ACS degradation occur on rupture or wounding of the cells. It is of interest to pursue further studies on the topics of ACS synthesis and accumulation in order to clarify the metabolic network of sulfur-containing compounds.

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