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Miraculin, a taste-modifying protein is secreted into intercellular spaces in plant cells

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ABSTRACT

A taste-modifying protein, miraculin, is highly accumulated in ripe fruit of miracle fruit (*Richadella dulcifica*) and the content can reach up to 10% of the total soluble protein in these fruits. Although speculated for decades that miraculin is secreted into intercellular spaces in miracle fruit, no evidence exists of its cellular localization. To study the cellular localization of miraculin in plant cells, using miracle fruit and transgenic tomato that constitutively express miraculin, immunoelectron microscopy, imaging GFP fusion proteins, and immunological detection of secreted proteins in culture medium of transgenic tomato were carried out. Immunoelectron microscopy showed the specific accumulation of miraculin in the intercellular layers of both miracle fruit and transgenic tomato. Imaging GFP fusion protein demonstrated that the miraculin–GFP fusion protein was accumulated in the intercellular spaces of tomato epidermal cells. Immunological detection of secreted proteins in culture medium of transgenic tomato indicated that miraculin was secreted from the roots of transgenic tomato expressing miraculin. This study firstly showed the evidences of the intercellular localization of miraculin, and provided a new insight of biological roles of miraculin in plants.

Introduction

Miracle fruit (*Richadella dulcifica*), a shrub native to tropical West Africa, yields red berries that have the unusual ability to modify a sour taste into a sweet taste. For example, lemons taste like oranges when the pulp of these berries is chewed. Due to this unique property, the berry has been called miracle fruit. The active ingredient in miracle fruit is a taste-modifying protein, miraculin. Miraculin contents can reach up to 10% of the total soluble protein in the miracle fruits (Sun et al., 2006). However, the biological roles are still unknown.

Miraculin was purified and its amino acid sequence has been determined (Theerasilp et al., 1989). It consists of 191 amino acids with two N-glycosylation sites and is a homodimer in the fruit. A cDNA clone corresponding to miraculin has been isolated and sequenced (Masuda et al., 1995). The deduced amino acid sequence suggests that the encoded precursor of miraculin is composed of 220 amino acid residues, including an N-terminal signal sequence of 29 amino acids. Miraculin by itself has no taste, but it can modify a sour taste into a sweet taste, similar to curculin. The maximum sweetness induced by 0.02 M citric acid after tasting 0.4 μM miraculin solution was estimated to be around 3000 times that of sucrose on a weight basis (Kurihara and Beidler, 1969; Theerasilp and Kurihara, 1988; Gibbs et al., 1996; Kurihara and Nirasawa, 1997).

The taste-modifying protein miraculin is obviously an attractive alternative to more traditional sweeteners such as sucrose, but miracle fruit is a tropical plant that is difficult to cultivate outside its natural environment. Therefore, as an alternative to producing miraculin from natural sources, we expressed recombinant miraculin in transgenic lettuce (Sun et al., 2006), transgenic tomato (Sun et al., 2007), and transgenic strawberry (Sugaya et al., 2008), and Ito et al. (2007) also expressed recombinant miraculin in *Aspergillus oryzae*. Among these sources, miraculin is most successfully produced in transgenic tomato plants, although the miraculin content of these plants was at least twice lower than in miracle fruit. Because of the unique feature, taste-modifying activity, of miraculin protein, studies on the practical application have been carried out. However, studies on the biological roles and function in plants are still limited.

Information on the cellular localization of a protein of interest could provide an insight into its biological role. For example, chitinase was shown to be localized in intercellular spaces, indicating its defensive role from the attacking pathogens (Collinge et al., 1993). Although it is speculated that miraculin

Abbreviations: GFP, green fluorescent protein

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has N-terminal signal and is secreted into intercellular spaces in miracle fruit (Masuda et al., 1995), no evidence exists of its cellular localization.

In this study, we obtained information on the cellular localization of miraculin by analyzing miracle fruit and transgenic tomato that constitutively express miraculin using immunoelectron microscopy, imaging GFP fusion proteins, and immunological detection of secreted proteins in culture medium of transgenic tomato. This is the first report demonstrating the subcellular localization of miraculin protein in plants.

Materials and methods

Plant materials for immunoelectron microscopy

Miracle fruit (*Richardella dulcifica* (Schumach. & Thonn.) Baehni) plants were grown in a greenhouse at Inplanta Innovations, Inc. in Hayama town, Kanagawa, Japan. Line 56B of transgenic tomato (*Solanum lycopersicum* L.) highly expressing miraculin was produced in our previous study (Sun et al., 2007) and the non-transgenic tomato cultivar Moneymaker was cultivated in a greenhouse at the Gene Research Centre, University of Tsukuba, Japan. Line 56B of transgenic tomato has a single miraculin gene driven by the 35S promoter from cauliflower mosaic virus and constitutively expresses recombinant miraculin in whole plants. Miracle fruit and tomato fruit were harvested at appropriate intervals to obtain ripe fruits. Tomato leaves were collected from mature plants.

Immunoelectron microscopy analysis

The method for electron microscopy was based on that of Toyooka et al. (2000). Pulp of miracle fruit and pericarp of tomato fruit and leaves were cut into ~1.0-mm^3 cubes and first fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.4) for 4 h at 4 °C. After dehydration of tissue pieces in a graded methanol series, the dehydrated pieces were embedded in LR White resin, and ultrathin sections were mounted on nickel grids (400 mesh; Electron Microscopy Sciences, Hatfield, PA) and blocked with 10% fetal bovine serum in Tris-buffered saline (25 mM Tris–HCl, pH 7.4, 150 mM NaCl) for 30 min at room temperature. The sections were then labeled with an affinity-purified rabbit polyclonal antibody to miraculin (Sun et al., 2006) in Tris-buffered saline (1:100–1:750). After washing with Tris-buffered saline, the sections were indirectly labeled with 12-nm colloidal gold particles coupled to goat anti-rabbit IgG (1:20). The gold-labeled sections were washed with Tris-buffered saline, rinsed in water, and then stained with 4% aqueous uranyl acetate. The grids were examined under a transmission electron microscope (model 1010EX; JEOL, Tokyo, Japan) at 80 kV.

Imaging miraculin–GFP fusion protein in tomato hypocotyls

To construct the miraculin–GFP fusion gene, the GFP plasmid, a pUC18-based vector containing CaMV-35S-sGFP (S65 T)-NOS3 (Chiu et al., 1996), which was kindly provided by Dr. Yasuo Niwa (University of Shizuoka, Japan), was digested by XbaI and EcoRI. The digested sGFP-NOS3 fragment was put into the pBI221 plasmid between the XbaI and EcoRI restriction sites with substitution of the GUS-NOS fragment. The coding region of the miraculin gene in pUC19 was amplified by PCR to remove the stop codon and to introduce XbaI and BamHI sites. PCR primers used were forward primer: 5′-TTTTCTAGAATGAAGGAATTAACAATGCT-3′ and reverse primer: 5′-TTGGATCCGAAGTATACGGTTTTGTTGA-3′. Underline indicated the restriction site. The amplified miraculin fragments were then digested and cloned into the generated plasmid to fuse in-frame with the N terminus of sGFP cDNA.

Hypocotyls were cut from 2-week-old seedlings grown *in vitro* and placed in Petri dishes containing solid Murashige and Skoog medium (Murashige and Skoog, 1962). Hypocotyl samples were then bombarded with 900-μg gold particles (1 μm) coated with 3 μg of plasmid DNA at a distance of 9 cm using a helium biolistic device (PDS-1000; Bio-Rad, Hercules, CA) at 900 p.s.i. (6.2 MPa) with a He pressure under a vacuum of 700 mm Hg. After incubation at 25 °C in the dark on MS medium for 24 h, the epidermis of tomato hypocotyls was peeled off with forceps and green florescence was examined. Images were taken from epidermal cells under a confocal laser-scanning microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany). Excitation wavelengths were set at 488 nm and images were collected through a fluorescein isothiocyanate filter and processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Detection of miraculin protein in culture medium from liquid cultures of transgenic tomato

Seeds of transgenic tomato line 56B were surface sterilized with 1% (v/v) sodium hypochlorite solution containing two drops of Tween 20 for 20 min and then rinsed with sterilized water three times for 5 min each. The seeds were germinated in a plant box with 30 mL of MS medium containing 15 g L^{-1} sucrose and 3 g L^{-1} Gelrite. The pH was adjusted to 5.8 before autoclaving. Five days after sowing, 10 tomato seedlings per bottle were transferred to 10 mL of 1/4 strength MS liquid medium and cultured in a shaker at 40 rpm. All cultures were maintained at 25 °C in a 16-h light/8-h dark cycle with fluorescent light (irradiance of 60 μmol m^{-2} s^{-1}). After 3, 5, and 7 days of culture, liquid medium and roots were harvested. Harvested liquid medium was filtered to remove cell debris using a 45-μm filter (Dismic; Advantec, Tampa, FL), and filtered liquid medium was concentrated by freeze-drying to a 24-fold concentration. Fifty microliters of concentrated medium was used for protein gel blot analyses. Harvested roots were ground to a fine powder in liquid nitrogen and homogenized in three volumes of extraction buffer consisting of 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 2% polyvinylpolypyrrolidone (PVPP). The extracts were centrifuged at 15,000 rpm for 20 min at 4 °C, and the resulting supernatant was used for protein gel blot analysis. SDS-PAGE and protein gel blotting were performed as described previously (Sun et al., 2006).

Results

Immunolocalization of miraculin protein in miracle fruits

Two different stages (green fruit 5–7 weeks after pollination and red-ripening fruit more than 9 weeks after pollination) of developing fruit were investigated for localization of miraculin protein under immunoelectron microscopy using a polyclonal antibody raised against miraculin protein and recognized the mature miraculin (Fig. 1). In the green fruit stage (Fig. 1a), gold particles were found mostly in the Golgi apparatus, cell wall, electron-dense intercellular layers, and electron-dense granules (DGs) of approximately 0.2–1.0 μm in diameter (Fig. 1c–f). In particular, the DGs containing miraculin were observed between the plasma membrane and the cell wall during the secretory period (Fig. 1c, d). These results indicate that miraculin is moved from the Golgi apparatus to the cell exterior. Moreover, many gold particles were localized in electron-dense structures on

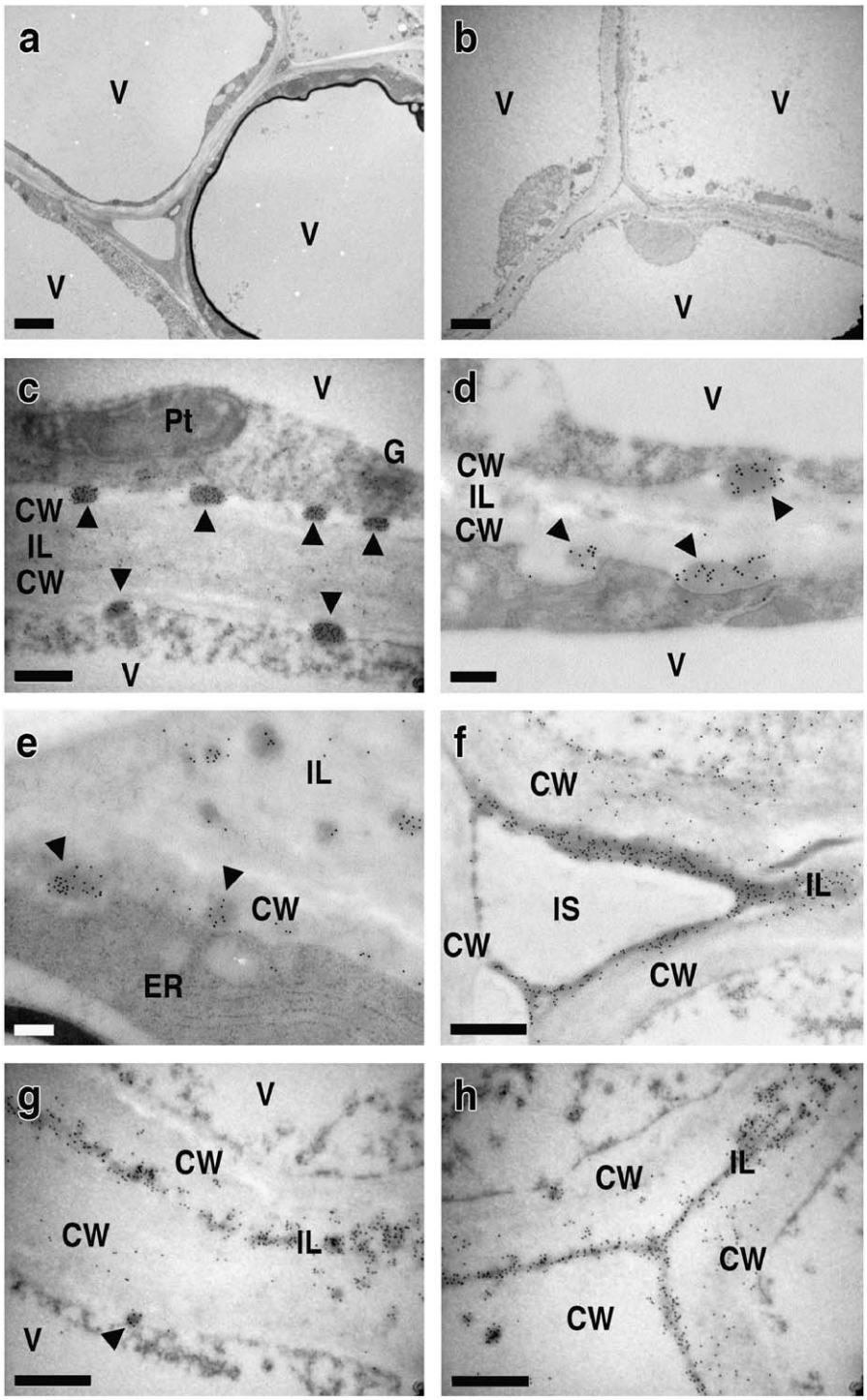

Fig. 1. Immunoelectron microscopy of miraculin protein in miracle fruit pulp. Miraculin protein is secreted into the intercellular layers. (a, c–f) Miracle fruit at the green fruit stage; (b, g, h) Miracle fruits at the red fruit stage. V, vacuole; CW, cell wall; IL, intercellular layer; IS, intercellular space; Pt, plastid; ER, endoplasmic reticulum; G, Golgi apparatus. Arrowheads show electron-dense granules. Scale bars = 2 μm (a, b), 500 nm (c, f–h) and 200 nm (d, e).

intercellular layers, but not in the intercellular airspaces (Fig. 1e, f), by the end of secretion. In the red-ripening fruit stage, cells of the mature tissue lost their shape and the cytoplasm degenerated (Fig. 1b). Subcellular localization indicated the presence of a much higher density of immunogold particles in the intercellular layer. In contrast, DGs were hardly observed in cells (Fig. 1g, h). These results indicate that the secreted miraculin moved through the cell wall matrix and accumulated specifically in the intercellular layers and junctions of mature miracle fruits.

Immunolocalization of miraculin protein in transgenic tomato (Figs. 2 and 3)

Leaves (Fig. 2) and two different stages (green and red-ripening) of developing tomato fruit (Fig. 3) were investigated for localization of recombinant miraculin protein under immunoelectron microscopy as in miracle fruits. No immunogold particles were detected in leaves of non-transgenic tomato (Fig. 2a–c), whereas immunogold particles were observed

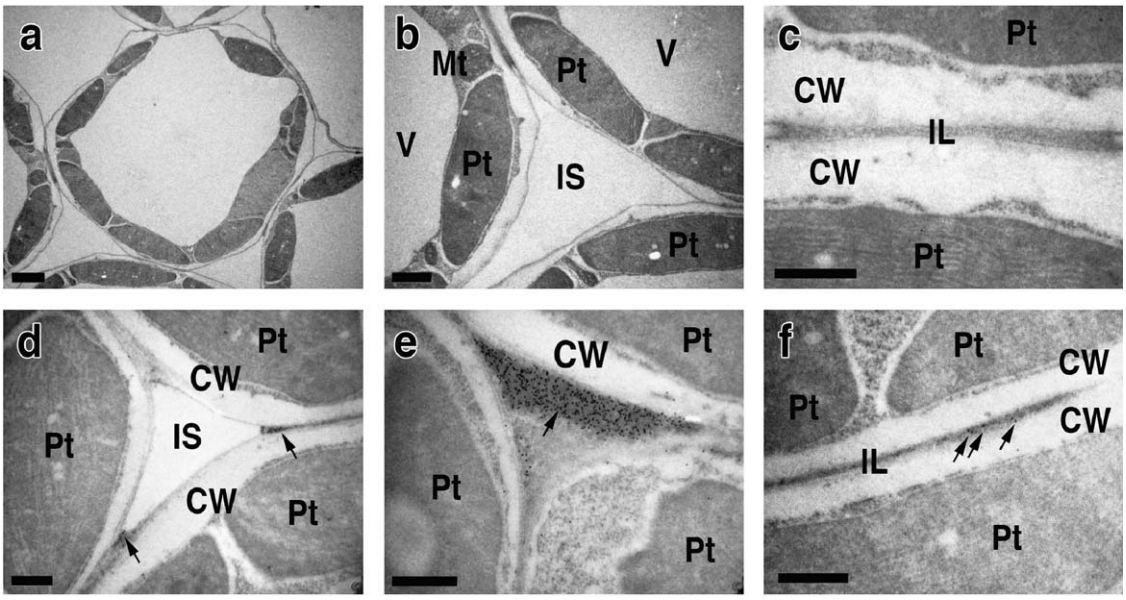

Fig. 2. Immunoelectron microscopy of miraculin protein in leaves of transgenic tomato. (a–c) Transverse sections of mesophyll of a non-transgenic tomato; (d–f) Transverse sections of mesophyll of a transgenic tomato. V, vacuole; CW, cell wall; IL, intercellular layer; IS, intercellular space; Pt, plastid and Mt, mitochondrion. Arrows show gold particles with anti-miraculin antibody. Scale bars = 2 μm (a), 1 μm (b), and 500 nm (c–f).

in the intercellular junctions (Fig. 2d, e) and the intercellular layer (Fig. 2f) of leaf tissues in transgenic tomato. In addition, immunogold particles were also observed in the intercellular layers of tomato fruits pericarp at both green (Fig. 3e, f) and red-ripening (Fig. 3g, h) fruit stages, but no immunogold particles were detected in non-transgenic tomato lines (Fig. 3a–d). These results demonstrate that recombinant miraculin is also specifically accumulated in intercellular layers and junctions of transgenic tomato as in miracle fruit.

Imaging localization of miraculin–GFP fusion protein in tomato

To obtain further evidence of the subcellular localization of miraculin protein, we transiently expressed miraculin fused to the amino-terminal of sGFP under the control of the cauliflower mosaic virus (CaMV) 35S promoter in epidermal cells of tomato hypocotyl. GFP fluorescence was detected only as an experimental control and was observed in the intercellular spaces (Fig. 4). This demonstrated that the miraculin–GFP fusion protein was secreted and accumulated in the intercellular spaces of tomato hypocotyl.

Detection of miraculin in culture filtrates from liquid cultures of transgenic tomato

To investigate the secretion of miraculin, transgenic tomato line 56B stably expressing the miraculin gene under the control of the 35S promoter and the accumulating miraculin protein (Sun et al., 2007) was cultured in liquid medium. Miraculin secretion into culture media was checked by protein gel blot analysis as well as miraculin accumulation in tomato roots of the transgenic line (Fig. 5). Miraculin was detected in culture media after 3, 5, and 7 days of culture in roots, but was not detected in culture medium from the non-transgenic tomato line. However, in culture medium of the transgenic tomato line, miraculin accumulation did not increase with culture times of 3–7 days. This indicates that miraculin was secreted from the roots of transgenic tomato expressing miraculin, and suggests that secreted miraculin was not stable in the culture medium.

Discussion

Protein secretion is important for protein production in biotechnology. The miraculin precursor has a cleavable N-terminal signal sequence rich in hydrophobic amino acids and the mature protein is an N-glycosylated glycoprotein (Takahashi et al., 1990; Masuda et al., 1995). Therefore, miraculin is expected to be secreted from cells. However, no data were available regarding the localization of miraculin. In this study, we confirmed the localization of miraculin in miracle fruit and in transgenic tomato plants. Miraculin is located in the intercellular layers of both plant tissues, indicating that it is a secreted protein. To elucidate the role of the signal peptide, we prepared transgenic tomato plants without the original signal peptide and analyzed them. Although a faint band was found in a protein gel blot analysis, no miraculin signals were detected under immunoelectron microscopy (data not shown), suggesting that the signal peptide of the miraculin precursor may play an important role in expression, post-translational modification, translocation, or secretion of the protein.

Transgenic tomato plants expressing the natural miraculin gene without modification unexpectedly accumulated a high level of miraculin protein, nearly 1% of the total soluble protein (Sun et al., 2007). In general, to achieve high-level heterologous protein production in plants, modifications of the transgene are required (Daniell et al., 2001; Streatfield, 2007), For example, high-level expression of the human alpha-1-antitrypsin protein in transgenic tomato was achieved by transgene modifications, including adoption of the codon usage pattern, elimination of mRNA destabilizing sequences, modification around the 5′ and 3′ flanking regions, and substitution of native signal peptide sequences with modified signal peptide sequences (Agrawal et al., 2008). Our results show that the miraculin secreted into intercellular spaces probably is a function of the signal peptide; therefore, the high level of miraculin

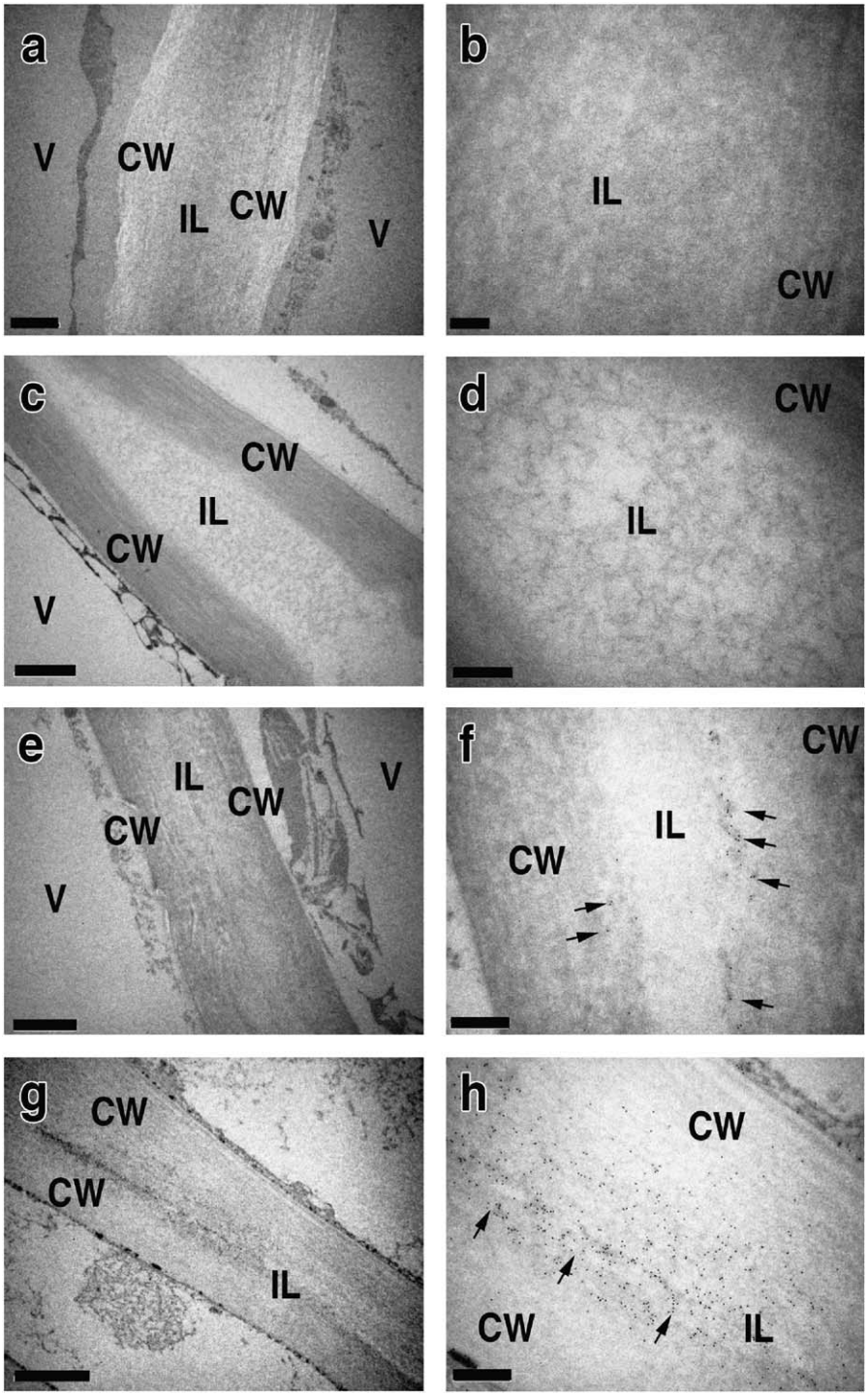

Fig. 3. Immunoelectron microscopy of miraculin protein in fruits of transgenic tomato. (a, b) Fruits pericarp of non-transgenic tomato at the green fruit stage. (c, d) Fruits pericarp of non-transgenic tomato at the red-ripening fruit stage. (e, f) Fruits pericarp of transgenic tomato at the green fruit stage. (g, h) Fruits pericarp of transgenic tomato at the red fruit stage. (b), (d), (f), and (h) are higher magnification images of (a), (c), (e), and (g), respectively. V, vacuole; CW, cell wall; IL, intercellular layer; IS, intercellular space; arrows show gold particles with anti-miraculin antibody. Scale bars = 1 μm (a), 200 nm (b), 2 μm (c, e, g), and 500 nm (d, f, h).

accumulation in transgenic tomato plants appears to be caused by secretion into intercellular layers.

This study provides new insight into miraculin production in transgenic plants. Genetic engineering of the signal peptide, such as replacement with a peptide derived from other secretory proteins, will provide a strategy to enhance miraculin accumulation in intercellular spaces, thus increasing the final miraculin content in transgenic plants.

In this study, we demonstrated that miraculin protein was secreted into the culture medium from the roots of transgenic tomato plants expressing miraculin, although the functions of secreted miraculin into the culture media remain to be elucidated. This suggests the possibility of producing miraculin using a root culture system. Hairy root culture has been used to produce plant secondary metabolites (Georgiev et al., 2007) and recombinant proteins

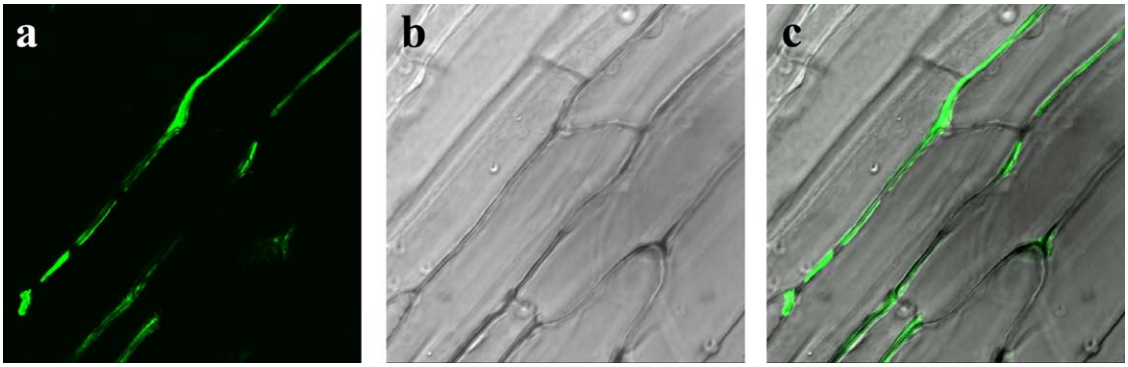

Fig. 4. Subcellular localization of miraculin–GFP fusion protein in epidermal cells of tomato hypocotyls. The GFP signal was detected in intercellular spaces. (a) GFP fluorescence; (b) bright field image, and (c) merged image of (a) and (b).

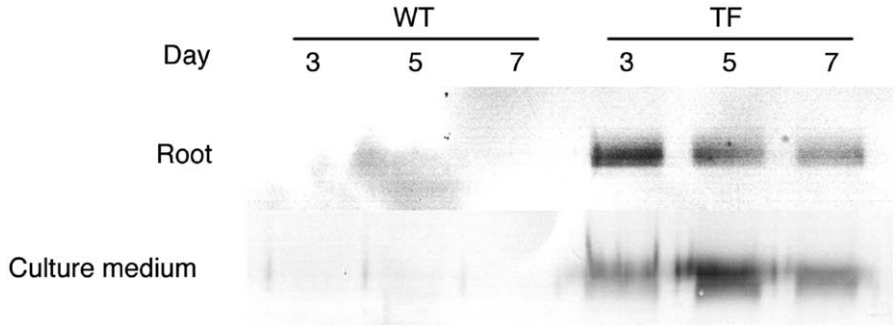

Fig. 5. Detection of miraculin protein in culture medium by protein gel blot analysis. Seedlings of transgenic tomato expressing miraculin protein were grown in MS liquid medium. The culture medium was subjected to protein gel blot analysis after culturing. WT, non-transgenic tomato line and TF, transgenic tomato line 56B.

(Skarjinskaia et al., 2008), and as such cultures have been reported in tomato (Shahin et al., 1986; Peres et al., 2001), miraculin protein production in tomato hairy root cultures may be possible.

The biological function of miraculin is unknown, although a tomato gene *LeMir* induced early after infection with root-knot nematodes has a high similarity to miraculin (Brenner et al., 1998). *LeMir* mRNA is found in roots, hypocotyls, and flower tissues, with the highest expression in roots. Rapid induction upon nematode infection occurs in root tips, and LeMir is secreted from roots into the surrounding environment. These results suggest that LeMir may interact with soil-borne microorganisms and may also be involved in plant defense. Our experiments showed that miraculin is secreted into the intercellular spaces, and when expressed in tomato, into the surrounding environment. Taken together, we speculate that miraculin is involved in plant defense. Transgenic tomato constitutively expressing miraculin (Sun et al., 2007) will provide a powerful tool for elucidating the biological roles of miraculin in plants.

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