Endoxyloglucan Transferase is Localized both in the Cell Plate and in the Secretory Pathway Destined for the Apoplast in Tobacco Cells

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Intracellular trafficking of enzymes responsible for constructing and modifying the cell wall architecture in plants is mostly unknown. To examine their translocation pathways, we employed an endoxyloglucan transferase (EXGT), a key enzyme responsible for forming and rearranging the cellulose/xyloglucan network of the cell wall. We traced its intracellular localization in suspension-cultured cells of tobacco bright yellow-2 by means of green fluorescent protein-fusion gene procedures as well as by indirect immunofluorescence. During interphase the protein was extensively secreted into the apoplast via the endoplasmic reticulum–Golgi apparatus network, whereas during cytokinesis, the protein was exclusively located in the phragmoplast and eventually transported to the cell plate. These results clearly indicate commitment of EXGT protein to the construction of both the cell plate and the cell wall. This study also visualized the process of phragmoplast development at a level of vesicle translocation in the living cell.

Key words: Cell plate — Cell wall — Endoxyloglucan transferase — Green fluorescence protein — Secretion — Tobacco.

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Abbreviations: ER, endoplasmic reticulum; EXGT, endoxyloglucan transferase; GFP, green fluorescent protein; LS, Linsmaier and Skoog medium.

Introduction

The cell division in plants is accomplished by formation of a cell plate, which begins at the late anaphase when Golgi apparatus-derived vesicles associated with phragmoplast microtubules are delivered to the equatorial plane of the dividing cell. These vesicles fuse to each other to form a membrane network. The assembly of the membrane network gives rise to a disc-shaped immature cell plate, which enlarges centrifugally and eventually fuses with the mother cell wall, thereby separating daughter cells (Samuels et al. 1995).

On the other hand, the cell enlargement process, which occurs during the interphase of the cell cycle, is achieved through architectural changes of the existing cell wall, which is composed of crystalline cellulose microfibrils embedded in a matrix of amorphous polysaccharides (Carpita and Gibeaut 1993). Cellulose microfibrils are polymerized and crystallized by the cellulose-synthesizing complex located at the plasma membrane (Giddings et al. 1980, Arioli et al. 1998, Kimura et al. 1999), whereas matrix polysaccharides are synthesized in the Golgi apparatus and secreted into the apoplast or cell wall space via membrane trafficking machinery (Bolwell 1988, Moore et al. 1991, Zhang and Staehelin 1992). Newly secreted cellulose microfibrils and matrix polysaccharides are assembled and integrated into the framework of the existing cell wall. These processes are considered to be mediated by cooperative actions of various types of enzymes, such as endoxyloglucan transferases (Ito and Nishitani 1999), glucanases (Hoj and Fincher 1995) and expansins (Cosgrove 2000), all secreted via Golgi vesicles. Secretory pathways for the matrix polymers during cell division and cell enlargement have been explored by biochemical (Kakimoto and Shibaoka 1992) and immunofluorescence methods (Moore et al. 1991, Moore and Staehelin 1988, Northcote et al. 1989, Brummell 1990). On the other hand, despite their importance in plant cells, the secretion processes of the individual enzymes responsible for cell wall construction and modification are still poorly understood, and their regulatory systems remain elusive.

Endoxyloglucan transferase (EXGT) is responsible for rearrangement of the cellulose/xyloglucan framework in the cell wall of seed plants. The enzyme catalyzes transfer of a large segment of one xyloglucan molecule and thereby mediates molecular grafting between the polysaccharide molecules, a molecular process essential for rearrangement of the cell wall architecture (Nishitani and Tominaga 1991, Nishitani and Tominaga 1992, Okazawa et al. 1993). Many homologues of this enzyme have been found and characterized. Together, these homologues constitute a large multi-gene family designated as xyloglucan-related proteins (XRP) (Xu et al. 1996, Nishitani 1997, Yokoyama and Nishitani 2000).

In this study, we used a member of the XRP gene family as a representative of the cell wall construction enzyme, and traced its secretory pathway during a cell cycle. We found that this EXGT was secreted into the apoplast via the endoplasmic reticulum (ER)–Golgi apparatus pathway during interphase, and that the same protein was transported exclusively to the cell plate, i.e. not to the apoplast, during cytokinesis. This study is the first to visualize the trafficking process of a protein that is involved in both the cell plate formation and the cell wall construction in the apoplast.

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Materials and Methods

GFP-fusion gene constructs

We amplified the coding region of EXGT-A1 (Okazawa et al. 1993) by PCR with two oligonucleotides, 5'-GTT TAC GAT GAC TGT TTC TTC ATC-3' and 5'-CAT GGA TCC TGC TCT GTC CCT-3'. The primers introduced XhoI and BamHI restriction sites at the 5' and 3' end of the coding region, respectively (underlined). The amplified DNA fragment was digested with XhoI and BamHI and inserted into the XbaI/BamHI sites of pBluescript II SK+ (Stratagene, La Jolla, CA, U.S.A.) to generate the pBluescript-EXGT-A1. A BamHI-EcoRI fragment containing the sequence for the plant adapted green fluorescence protein (sGFP) and the nopaline synthase (Tnos) (Chiu et al. 1996) was inserted into the BamHI/EcoRI site in the pBluescript-EXGT-A1 to generate an EXGT-GFP fusion gene construct. A DNA fragment containing the EXGT-GFP fusion gene and Tnos terminator was finally cloned into the XhoI/EcoRI restriction site of pBI122 (Jefferson et al. 1987) to generate pBI-35S-EX-GFP, which contained a cauliflower mosaic virus 35S promoter, a coding sequence for the EXGT-GFP and the Tnos terminator. To construct a coding sequence for a GFP fusion protein of the signal peptide of EXGT-A1 and GFP, we amplified a DNA fragment containing the cauliflower mosaic virus 35S promoter and the signal sequence using an oligonucleotide 5'-CAA TGG ATC GCC GTG GAA TAG CCA-3' as a primer and the pBI-35S-EX-GFP as the template. The amplified fragment was digested with HindIII and BamHI and cloned into the HindIII and BamHI restriction sites of pBI-35S-XE-GFP. The full length sequence encoding the EXGT-A1 protein in the plasmid was replaced with a short sequence encoding a signal peptide consisting of 24 amino acid residues and 4 amino acid residues derived from the two restriction enzyme sites, to generate pBI-35S-SP-GFP. The pBI 35S-GFP, which was used as the control vector, was prepared by replacing the BamHI-EcoRI fragment which was used as the control vector, was prepared by replacing the

Microscopic observation

The fluorescence images of tobacco cells expressing GFP fusion proteins were observed under an epifluorescence microscope equipped with a plant GFP filter set and differential interference contrast optics (DMRXP; Leica). The microscopic images were recorded using a 3CCD video camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan) using MacAspect software. Images of immunofluorescence were recorded using appropriate filters for FITC and DAPI fitted to the epifluorescence microscope (DMRXP; Leica). For optical sectioning, transgenic tobacco cells living in LS medium were mounted under a glass cover slip and observed under a laser scanning confocal microscope (Fluoview: Olympus, Tokyo, Japan) equipped with a krypton-argon laser and filter sets suitable for detection of fluorescein. Confocal images were acquired and composited using the Fluoview microscope and Adobe Photoshop software.

Immunofluorescence localization

Tobacco BY-2 cells were fixed in 3.7% formaldehyde in PMEG buffer solution consisting of 100 mM 1,4-piperazinediethanesulfonic acid (PIPES), 2 mM magnesium sulfate, 10 mM (ethylene glycol-O-bis(2-amino-ethyl)-N,N,N',N'-tetracetic acid (EGTA)) and 2% glyceral at pH 6.8 for 12 h at 4°C. After being rinsed with the PMEG buffer, fixed cells were transferred to a poly-l-lysine coated slide glass, followed by air-drying for 30 min. Cells on the slide glass were digested in a solution containing 1% Cellulase Y-C and 1% Pectolyase Y23 in the PMEG buffer for 5 min followed by treatment with a phosphate-buffered saline containing 1% Nonidet, 0.4 M mannitol, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 µg ml-1 leupeptin. Immunofluorescence microscopy was performed following a standard method using affinity-purified anti-Vigna EXGT IgG as the first antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit goat IgG as the second antibody. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 10 µg ml-1 for 5 min at room temperature. Photographs were taken using appropriate filters for FITC and DAPI fitted to an epifluorescence microscope (DMRXP; Leica, Heerbrugg, Switzerland).

Synchronization of tobacco BY-2 cells

Suspension cultured tobacco BY-2 cells were maintained in modified LS-medium (Nagata et al. 1981) and synchronized essentially as described by Nagata et al. (1992). A 7-day-old cell culture was diluted 2:55 and incubated in the presence of 5 µg ml-1 aphidicolin for 24 h, followed by release from the inhibitor by washing with 1 liter of fresh medium by filtration. After the release from aphidicolin, cells synchronized at each stage of the cell cycle were subjected to analysis by immunofluorescence procedures and GFP-fusion procedures.

Fig. 1 Schematic presentation of three gene constructs. (a) GFP introduced via the pBI-35S-GFP vector. This construct was designed to express the plant adapted GFP (sGFP(S65T)) and was used as a control. (b) EXGT-GFP introduced via the pBI-35S-EX-GFP vector. The full length EXGT-A1 protein is fused to the plant adapted GFP. (c) EXGTsp-GFP introduced via pBI-35S-SP-GFP. A putative signal peptide of the EXGT-A1 (italicized) consisting of the N-terminal 24 amino acid residues and the 4 amino acid residues (roman) derived from restriction enzyme sites are fused to the plant-adapted GFP. These gene constructs are placed under the control of the cauliflower mosaic virus 35S promoter (CaMV35S) with a nopaline synthase gene poly(A) signal (Tnos).
Results

Expression of GFP fusion reporter in tobacco BY-2 cells

To investigate the secretory pathway of apoplastic enzymes involved in the cell wall construction in living plant cells, we prepared two types of GFP fusion reporter constructs, EXGT-GFP and EXGTsp-GFP, as shown in Fig. 1. In EXGT-GFP, the full-length EXGT-A1 polypeptide was fused to a plant-adapted GFP (sGFP (S65T)) (Niwa et al. 1999), while in EXGTsp-GFP, the first 24-amino-acid sequence of the EXGT-A1 was fused to the sGFP (S65T). To ensure high levels of gene expression, the two fusion genes were driven by the cauliflower mosaic virus 35S promoter (CaMV35S). These constructs were introduced into suspension-cultured cells of tobacco BY-2, and seven independent cell lines for each of the two GFP fusion constructs and the control construct (GFP) were selected and assayed for their green fluorescence intensities and growth rates. In cell lines expressing the EXGT-GFP fusion protein, the fluorescence intensity was found to be low and the growth rate was severely reduced (Fig. 2c, g). By contrast, the cell lines expressing EXGTsp-GFP exhibited high intensities of the green fluorescence and proliferated normally (Fig. 2d, h). Thus, we employed one of the EXGTsp-GFP expressing cell lines in further experiments.
Using a synchronized culture (Nagata et al. 1992) of the EXGTsp-GFP-expressing tobacco cells, we studied the intracellular localization of EXGT during mitosis. Figure 3 shows differential interference contrast images as well as epifluorescence images of a cell expressing EXGTsp-GFP at various stages of the cell cycle. At interphase (Fig. 3a, f), the fluorescent protein was observed at the surface of the nucleus. The protein was also found to exist as strings, radiating towards the periphery of the cell. At the beginning of mitosis the EXGT protein was concentrated into the central region of the cell and the fluorescence intensity of the radiating strings was diminished (Fig. 3b, g). During metaphase, the fluorescence was localized in the center of the cell (Fig. 3c, h). In the central region of the anaphase cell (Fig. 3d, i), fluorescent fibers were observed along with the spindle. At this stage, an intense fluorescent band appeared at the equatorial plate. As the cell plate formation progressed, the fluorescent fibers disappeared and a fluorescent band expanded centrifugally toward the parental cell wall (Fig. 3e, j).

To examine whether the intracellular accumulation pattern of the EXGTsp-GFP actually reflected the real localization pattern of the native EXGT protein in tobacco cells, we observed immunolocalization patterns of the native EXGT by using a polyclonal antibody raised against a recombinant EXGT protein (Fig. 4). The anti-EXGT antibody strongly labeled the cytoplasmic radiating strings and ER-like membrane system located around the nucleus at the interphase (Fig. 4f). As mitosis commenced, the labeling of the radiating strings by the antibody became less intense (Fig. 4g). During metaphase, the fluorescence labeling was found around the chromosome (Fig. 4h). At telophase, the phragmoplast and cell plate were clearly labeled with the antibody (Fig. 4j). Thus, the localization pattern of the immunofluorescence exactly coincided with that of EXGTsp-GFP, indicating that the EXGTsp-GFP reporter protein can properly represent the intracellular localization pattern of the native EXGT protein in tobacco cells.

On the basis of this result, we examined the intracellular localization of EXGTsp-GFP in living cells using a confocal laser scanning microscope. Optical sections of a single cell at interphase revealed that the fluorescence was located outside the nucleus throughout the cytoplasm. The inner surface of the plasma membrane or the cortical region of the cytoplasm was lined with the network-like tubular structure (Fig. 5e). This structure was found to exist throughout the cytoplasm (Fig. 5a–d). In control cells, in which the sGFP protein without the signal peptide was expressed, the green fluorescence was irregularly or indistinctly distributed inside the cell, and no network-like pattern was observed (Fig. 5f–i). This indicates that the network-like localization pattern of the EXGTsp-GFP reflected the real function of the signal peptide of EXGT-A1. A similar
network-like pattern has been observed in the cortical region of cells expressing a green fluorescent fusion protein with sequences ensuring retention in the lumen of ER (Boevink et al. 1998, Boevink et al. 1999, Ridge et al. 1999). The network-like pattern was also disclosed by staining tobacco cells with DiOC$_{6}$(3), a dye which specifically stains ER (Grabski et al. 1993). Taken together, these facts clearly indicate that EXGTsp-GFP is transported via the network-like ER tubules.

Effects of brefeldin A (BFA) on secretion of EXGTsp-GFP

To confirm the involvement of the ER–Golgi apparatus in the secretory pathway of EXGTsp-GFP, we incubated the cells in the presence of 10 µg ml$^{-1}$ BFA, a potent fungal toxin capable of specifically inhibiting the vesicle trafficking via the ER–Golgi apparatus (Driouich et al. 1993, Boevink et al. 1998, Boevink et al. 1999, Chardin and McCormick 1999). Within 1 h after application of BFA, the radiating strings diminished and small vesicles appeared at the periphery of the cytoplasm (Fig. 6A). An optical section through the cortical region of the BFA-treated cells revealed that the network-shaped tubular ER had begun to fuse, giving rise to a large irregularly perforated sheet (Fig. 6B). After prolonged treatment with BFA, the perforated membranous structure became disordered. It is likely that the disorganization of the ER was caused by an over-accumulation of the green fluorescent protein due to BFA-inhibited membrane trafficking between the ER and Golgi apparatus.

Cellular localization of EXGT during cell plate formation

Optical sectioning of the equatorial plate of the cell at telophase by a confocal laser scanning microscope revealed that the fluorescent reporter protein was accumulated in the expanding cell plate as well as in vesicles in the vicinity of the phragmoplast (Fig. 7). The median longitudinal optical section (Fig.
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7a) showed the short strings perpendicular to the cell plate near the parent cell wall. Bright spots were found to be present around the periphery of the growing cell plate. The short strings and spots were clearly observed in an optical section through a peripheral plane of the cell (Fig. 7d). Furthermore, the fluorescent spots to be translocated to the cell plate were observed (arrow, Fig. 7b).

Discussion

Secretory pathway for EXGT protein during the interphase

By using the EXGTsp-GFP reporter protein, we were able to visualize the pathway through which EXGT protein was

Fig. 6 Time course effects of BFA on intracellular localization of EXGTsp-GFP. (A) Fluorescence images of cells which had been incubated in 10 μg ml⁻¹ BFA for 0 to 24 h as indicated on individual plates. (B) Confocal laser scanning microscope images of the ER network located at the peripheral region of individual cells which had been treated with BFA as in (A).

Fig. 7 Confocal laser scanning microscopic images of a transgenic tobacco cell expressing EXGTsp-GFP at the late stage of cell plate formation during cytokinesis. Consecutive optical sections perpendicular to the cell division plane from the center of the cell (a) toward the cell surface (d). The arrow in (b) indicates vesicles to be translocated to the cell plate.
secreted into the apoplast, confirming our previous prediction based on both the primary structure of EXGT protein (Okazawa et al. 1993), and its presence in the apoplastic fluid (Nishitani and Tominaga 1992). This finding demonstrates that the EXGTsp-GFP that contained only a 24-amino acid sequence in common with the EXGT-A1 protein was correctly recognized as the signal sequence destined for the initial transfer to the ER, and ultimately destined for the apoplast. Since the transcription of this reporter gene was driven by the powerful CaMV35S promoter in our experiment, we must be extremely careful in considering the biological implications of the tentative retention of the EXGTsp-GFP in the lumen of the ER. However, our finding that the intracellular localization pattern of the native EXGT protein (Fig. 4) resembled that of the EXGTsp-GFP reporter fusion protein definitely excludes the possibility that the visible retention of the EXGTsp in the ER lumen was an artifact caused by its overexpression. On the basis of these findings, we conclude that a certain amount of EXGT is present in both the ER and Golgi apparatus.

Boevink et al. (1998) visualized a network of ER tubules by infecting leaf cells of Nicotiana clevelandii with a viral vector expressing an ERD2-GFP fusion protein. More recently, using a similar viral expression system, Boevink et al. (1999) investigated the secretory pathway of a GFP fusion protein with a signal peptide of sporamin. In their system, the signal peptide-GFP fusion protein was not clearly observed in the lumen of the ER, whereas a signal peptide-GFP-KDEL fusion protein was clearly shown to be located in the ER. By contrast, in tobacco cell lines expressing the EXGTsp-GFP fusion protein, a substantial amount of the EXGTsp-GFP was found in both the cortical network and the radiating strands of the tubular ER throughout the cytoplasm. The different localization patterns of the two signal peptide-GFP fusion proteins might indicate their different rates of processing and/or traffic through the lumen of the ER in the two different expression systems. A high rate of secretion would result in a low level of GFP fusion protein in the ER lumen. Furthermore, since complete maturation of GFP is considered to require a few hours (Cubitt et al. 1995), rapid trafficking of the ER lumen would cause retention and secretion of an immature GFP fusion protein, which would not fluoresce. Thus, the visible retention of the secretory protein in the ER lumen strongly implies the existence of a mechanism that regulates the flow rate of ER-mediated protein traffic or the amount of protein tentatively retained in the ER lumen. Such a regulatory system would be mediated either by post-translational modification of individual secretory proteins or by any mechanism responsible for controlling vesicle trafficking. At present, we have no clue either to the possible post-translational modification or the molecular device involved in the rate-regulation of vesicle trafficking. Clearly, such a molecular mechanism would be essential in the secretory system and would form the basis for spatially and temporally regulated intracellular trafficking of secretory vesicles.

EXGT is specifically targeted to the cell plate during cytokinesis

At the beginning of mitosis, the radiating network of ER tubules distributed throughout the cytoplasm was disconnected. As a result, the cortical domain of the ER network was separated from the central ER domain located around the nucleus (Fig. 3, 5). The segregation of the two ER-network domains seemed to result in the blockade of the secretory pathway for both the matrix polysaccharide and enzymes, both of which are required for construction of the cell wall outside the plasma membrane. Although matrix polysaccharides (Kakimoto and Shibaoka 1992) and certain proteins directly related to cell division have been shown to be transported to the cell plate at the end of mitosis, localization of the so-called cell wall enzymes in the cell plate has not been investigated. To our knowledge, the present study is the first to demonstrate the localization of a cell wall enzyme in either the phragmoplast or cell plate at an early stage of the cytokinesis. Taken together, these facts imply the presence of a switching system that can alternate the direction of the secretory pathway for cell wall proteins. Such a switching system is important particularly in plants, because the plant cell needs to construct a new cell wall promptly at the end of mitosis in order to separate daughter cells. Clearly, cell-cycle-dependent alteration of the membrane trafficking system carries an advantage over a constitutive secretory system, in which the apoplast-directed membrane trafficking continues even during cell plate formation.

Novel role of EXGT in cell division and expansion

Many studies on the function of EXGT have been focused on the modification of the pre-existing cell wall based on the view that EXGT can mediate molecular grafting between xyloglucan molecules, which function as load-bearing bridges among cellulose microfibrils (Fry et al. 1992, Nishitani 1997). Extensive secretion of EXGT into the apoplast during interphase supports this widely advocated role of EXGT in the reconstruction of the pre-existing cell wall.

On the other hand, our finding that the EXGT is localized in the cell plate clearly indicates its essential role in the formation of the cell plate in addition to its well-known role in the apoplast. Xyloglucan molecules have been shown to polymerize in the Golgi apparatus and to exist in the cell plate (Moore and Staehelin 1988). By contrast, cellulose microfibrils are not found in the cell plate (Kakimoto and Shibaoka 1992, Moore and Staehelin 1988). This means that the EXGT protein present in the cell plate will not mediate either splitting or formation of xyloglucan bridges among cellulose microfibrils. In other words, the EXGT in the cell plate may play a role distinct from that of EXGT in the apoplast.

What, then, is the function of EXGT in the cell plate? There are two possibilities. One is that EXGT might be responsible for the processing of xyloglucans secreted by the Golgi apparatus-derived vesicles. Certain molecular grafting reactions between xyloglucan molecules can mediate elongation of xyloglucan chains (Nishitani 1997). Repetition of such reac-
EXGTsp-GFP as a new tool for studying regulation of the secretory pathway

In this study, we chose EXGT as a molecular marker to explore the secretory pathway of apoplastic enzymes because this enzyme is considered to play various physiological roles in a wide range of cell wall dynamics. Comparison of intracellular localization images of the immunofluorescence (Fig. 4) and the GFP (Fig. 3) clearly demonstrated that the EXGTsp-GFP accurately represents the real distribution pattern of the endogenous EXGT protein in tobacco BY-2 cells, indicating that the EXGTsp-GFP fusion protein is of considerable utility as a precise molecular marker for the secretory pathway. In the present study, cell lines expressing EXGT-GFP (Fig. 2c, g) were not used for further analysis because they proliferated at a much lower rate than those expressing EXGTsp-GFP. The reduced proliferation rate of the EXGT-GFP lines might be due to a negative–dominant type of effect caused by the EXGT-GFP fusion protein.

The EXGTsp-GFP was used successfully to visualize the tubular network-structure of the ER due to its temporal retention in the ER. Use of this fusion protein system made it possible to visualize the vesicle trafficking during cell plate formation in a living cell. Until very recently, our understanding of the formation of the cell plate has largely been dependent on electron microscopic and immunocytochemical methods (Hepler 1982, Samuels et al. 1995). The most recent model of cell plate formation was reported by Samuels et al. (1995), who successfully observed the many new transient membrane structures by use of improved cell-preservation techniques adapted for electron microscopy. Although their excellent methods for visualizing the cell plate formation in fixed cells revealed a wealth of information, the formation process of the cell plate in living cells remained largely unclarified. Our confocal laser scanning microscopic analysis of cells expressing EXGTsp-GFP proteins has clearly disclosed the dynamic aspects of cell plate formation in living cells and provided explicit evidence in support of the model advanced by Samuels et al. (1995). That is, the GFP fluorescence was being targeted to the margins of the expanding cell plate, suggesting that vesicles filled with wall polysaccharides and enzymes for the cell plate formation were transported and fused to the margins of the cell plate. The GFP fluorescence also revealed the position to which GFP in the ER moved to participate in the formation of vesicles, i.e., the point at which vesicles were formed. This is the first observation of membrane trafficking occurring during the cell plate formation in living cells. Studies of living cells using EXGTsp-GFP should provide much additional novel information toward an enhanced understanding of the cell plate formation in terms of vesicle trafficking.

Acknowledgements

We are grateful to Drs. Toshiyuki Nagata and Seiichiro Hasezawa for their gift of the tobacco BY-2 cell line and for their kind advice on its synchronization. We also thank Dr. Yasuo Niwa for the gift of the sGFP vector. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (No. 10182102), a Grant-in-Aid for Scientific Research (No. 09440269), a grant from the Research for the Future Program (JSPS-RFTF96L00403) and by a grant of Rice Genome Project PR-1108, MAFF, JAPAN.

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(Received September 25, 2000; Accepted December 14, 2000)