The Correlation between Expression and Localization of a Foreign Gene Product in Rice Endosperm

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Glucagon-like peptide 1 (GLP-1) is a 30 amino acid peptide hormone involved in insulin stimulation that is dependent upon blood glucose levels. We have previously reported that when this short peptide gene was directly expressed under the control of a glutelin promoter and its signal peptide, it was not accumulated in transgenic rice seed due to gene silencing. However, when the modified GLP-1 (mGLP-1) gene was enlarged to 5xGLP-1 (mGLP×5) by tandem repeat, no silencing was observed. The mGLP×5 peptide could be accumulated in rice seed and its localization was mainly limited to the endoplasmic reticulum (ER). We also investigated alternative cellular localization sites that would increase accumulation. The relationship between the expression level and localization was examined by attaching the chitinase signal peptide to mGLP×5 to direct it into the intercellular space (apoplast), or by expression as a fusion protein with glutelin by insertion into a variable region of the acidic subunit, thus directing the peptide to protein body II (PB II). Attachment of the KDEL ER retention signal to the 6xGLP-1 (mGLP×6) or its fusion to the C-terminus of the 13 kDa prolamin directed the peptide to the ER or PB I, respectively. Unexpectedly, these results indicated that mGLP×5 without any signal except for the glutelin signal peptide was accumulated to the greatest extent in rice endosperm. It can thus be concluded that the ER is a suitable intracellular organelle for accumulation of mGLP×5 peptide.

Keywords: Endosperm — GLP-1 — Rice — Subaleurone cell — Transgenic plant.

Abbreviations: BSA, bovine serum albumin; DAF, days after flowering; ER, endoplasmic reticulum; GFP, green fluorescent protein; GLP-1, glucagon-like protein 1; PB, protein body; PBS, phosphate-buffered saline.

Introduction

Many industrial enzymes and therapeutic proteins have been produced using plants as a bioreactor (Giddings et al. 2000). Plant-based production systems have many advantages over other production systems using bacteria, yeasts or animals, including (i) control of the production scale; (ii) production technology is limited to the agronomic level; (iii) there is a low risk of contamination by animal pathogens such as prions and viruses; and (iv) long-term stability at room temperature. These characteristics give rise to low cost production. It has been estimated that this system can provide recombinant products at a 10- to 50-fold lower cost than other systems (Kusnadi et al. 1997).

Recombinant proteins are not always expressed at the expected levels, and have in some cases yielded <0.01% of total soluble protein. The expression level is partly dependent upon the target crop tissues and the promoters chosen to direct the recombinant genes. When foreign genes were expressed in seed under the control of seed-specific promoters, accumulation increased to >1% of total seed protein (Katsube et al. 1999, Takagi et al. 2005). However, gene silencing has also been observed, which results in no accumulation, irrespective of the presence of a transgene. We have recently reported that the gene coding for a short peptide (30 amino acids) of modified glucagon-like peptide 1 (mGLP-1), a candidate as a therapeutic agent for diabetes, was frequently silenced in transgenic rice plants when directly expressed under the control of the endosperm-specific glutelin promoter and its signal peptide. However, silencing was not observed in rice transformants which expressed mGLP-1 as a fusion protein with green fluorescent protein (GFP) and 2A protein (Yasuda et al. 2005).

In this study, we generated transgenic rice plants that accumulate large amounts of mGLP-1 peptide in the endosperm. It has been reported that attachment of signal peptide sequences leading to the endoplasmic reticulum (ER) secretory pathway and the KDEL ER retention signal at the N- and C-terminal ends of a foreign gene are generally required for high levels of accumulation of its product (Herman et al. 1990, Gomord et al. 1997, Takagi et al. 2005). In fact, several reports indicate that the KDEL tetrapeptide increases accumulation of the fusion proteins (Schouten et al. 1996, Gomord et al. 1997), and transport of a protein into the extracellular space (bulk flow transport) or to the lumen of the ER has been achieved by binding signal peptides and/or KDEL to the target proteins (Firek et al. 1993, Conrad and Fiedler 1998, Stoger et al. 2000).

Rice endosperm cells have two kinds of protein storage vacuoles, designated type I protein body (PB I) and type II protein body (PB II). Prolamins are accumulated in PB I, whereas...
glutelins and globulins are deposited in PB II. Glutelins are the most abundant seed storage protein of rice, accounting for about 70% of total seed protein. Prolamins are accumulated in PB I, which reside in the ER, and comprise 20–25% of total seed protein.

In this report, we found that tandem repeat mGLP-1 peptides could be expressed in the endosperm of rice transgenic plants. To find out which inter- or intracellular spaces in the endosperm would be suitable for its accumulation, we expressed the mGLP-1 peptide under the control of the glutelin GluB-1 or chitinase signal peptide, and/or KDEL tetrapeptide. Furthermore, we produced chimeric transgenes, in which the C-terminal variable region of the GluA-2 acidic subunit was substituted for the mGLP-1 peptide and the 13 kDa prolammin was fused to the mGLP-1 peptide to transport the tandem repeat mGLP-1 peptide into PB I or PB II. We examined expression levels and localization of the peptides in those transformants when expressed under the control of the same glutelin GluB-1 promoter and terminator. To achieve the most efficient expression of foreign gene products in rice endosperm, we investigated the relationship between expression level and localization of mGLP-1 peptide.

Results

Expression of 5× tandem repeat mGLP-1 (mGLP×5) in rice endosperm cells

We previously reported that the expression of the mGLP-1 gene was silenced with high frequency by co-suppression when directed by the 2.3 kb glutelin GluB-1 promoter and its signal peptide. In these silenced transgenic rice seeds, expression of glutelin was highly depressed by co-suppression due to the presence of the 5′ and 3′ untranslated regions and the signal peptide of the glutelin GluB-1 gene used for expression of the mGLP-1 peptide gene (Yasuda et al. 2005). Therefore, in order to avoid co-suppression, a pentamer mGLP-1 (mGLP×5) was inserted between the GluB-1 promoter containing the signal peptide and its terminator [Fig. 1, pSP(GluB)-mGLP×5]. As shown in Fig. 2B, the mGLP×5 peptide was detected in mature seed, indicating that the tandem repeats solved the gene silencing problem. Several mGLP-1 constructs were expressed under the control of the same promoter and terminator (Fig. 1) to increase the accumulation level of the mGLP×5 peptide in rice endosperm, and their expression levels and localization were also examined. These chimeric transgenes were designed to transport the peptide into the intercellular space [pSP(chi)-mGLP×5], PB II [pGluA(mGLP×5)], ER [mGLP×6(KDEL)] or PB I [prolammin-mGLP×6(KDEL)].

Expression of mGLP×5 peptides in seed of SP(GluB)-mGLP×5, SP(chi)-mGLP×5 and GluA(mGLP×5) transformants

To estimate relative amounts of mGLP×5, total seed protein was extracted from one mature seed of transgenic rice plants (T1 generation) expressing pSP(GluB)-mGLP×5, pSP(chi)-mGLP×5 and pGluA(mGLP×5) (Fig. 1), and subjected to Western blot analysis using anti-GLP-1 antibody after SDS–PAGE.

For the SP(GluB)-mGLP×5 construct, mGLP×5 peptide could be detected in 11 of 30 regenerated transgenic rice lines. As shown in Fig. 2B, high levels of accumulation were observed in lines 5 and 6. It should be noted that higher levels of the glutelin precursor relative to the mature acidic and basic subunits are also observed in the high accumulation lines as compared with the low expression lines (Fig. 2A, lines 2, 14, 21 and 24). This suggests that high expression of mGLP×5 may have an effect on trafficking or processing of native storage proteins in maturing seed. For the SP(chi)-mGLP×5 and GluA(mGLP×5) constructs, the mGLP×5 peptide was detected in only four and seven of 30 regenerated transgenic lines,
respectively. As shown in Fig. 2B and C, their accumulation levels are remarkably low, although driven by the same promoter as the SP(GluB)-mGLP×5 construct. The apparent molecular weights of the mGLP×5 peptides from these three constructs were about 14, 13.5 and 47 kDa, based on electrophoretic mobility (Fig. 2B). The difference between the former two could be accounted for by the presence of an extra four amino acids at the N-terminus of the mature mGLP×5 peptide after processing of the signal peptide in the pSP(GluB)-mGLP×5 construct. It should be noted that the predicted molecular weight of the mGLP×5 peptide deduced from the amino acid sequence is 16.4 kDa, if the signal peptide is processed normally. The mobility of the peptide on SDS–PAGE was greater than predicted from its sequence, possibly due to its highly helical structure (Thornton and Gorenstein 1994, Neidigh et al. 2001).

The apparent molecular weight of the mGLP×5 peptide from plants with the GluA(mGLP×5) construct was estimated to be about 47 kDa. This result indicated that the modified glutelin GluA-2 cDNA containing mGLP×5 as a fusion protein was normally processed into acidic and basic subunits in endosperm cells. As shown in Fig. 2B, the mGLP×5 peptide was detected as a part of the glutelin acidic subunit, since the highly variable C-terminus of the acidic subunit (between positions L276 and C304) was substituted for the mGLP×5 peptide.

mGLP×5 peptide was accumulated at the highest levels with the SP(GluB)-mGLP×5 construct in mature seed (Fig. 2C). It is notable that the expression level of mGLP×5 peptide in the SP(GluB)-mGLP×5 transformant (line 6) was >17-fold higher than the other transformants (Fig. 2C). Expression of mGLP×5 peptide from the SP(chi)-mGLP×5 transformant was nearly the same as in the GluA(mGLP×5) transformants (Fig. 2C).

Subcellular localization of the mGLP×5 peptides in subaleurone cells of SP(GluB)-mGLP×5, SP(chi)-mGLP×5 and GluA(mGLP×5) transformants

To investigate the subcellular localization of the mGLP×5 peptide in subaleurone cells, we observed immature seeds [15–20 days after flowering (DAF)] from the representative homozygous lines of the T1 generation for the three constructs. The mGLP×5 peptide expressed in the SP(GluB)-mGLP×5 construct was mainly localized in the ER (Fig. 3A, B), a conclusion based on the similarity to the SP(GluB)-mGLP×5(KDEL) construct, described later (Fig. 5A, B). It is interesting to note that localization of the mGLP×5 and mGLP×5(KDEL) peptides was much the same, irrespective of whether or not they had the KDEL ER retention signal. As shown in Fig. 3B, only a few PBII like green structures were also observed. It suggested that a part of the mGLP×5 peptide was secreted to the extracellular spaces (Fig. 3A).

When the SP(chi)-mGLP×5 construct was introduced, the fluorescence of the mGLP×5 peptide was mainly observed in the intercellular spaces (apoplast) of subaleurone cells, but was not seen in their intracellular space (Fig. 3C, D). This result suggested that the chitinase signal peptide is involved in transporting the mGLP×5 peptide into the intercellular space with high efficiency. Fluorescence of the mGLP×5 peptide in subaleurone cells of the GluA(mGLP×5) transformant was observed
as a distorted globular structure (Fig. 3E, F), a result suggesting that the globular structure was PB II, because the fluorescence did not overlap with that of rhodamine B, representing the localization of PB I. Furthermore, it should be noted that the size of the globular structure (approximately 3–4 µm) was greater than that of PB I (approximately 1–2 µm) (Fig. 3E).

The expression level of mGLP×6 peptides in seed of mGLP×6(KDEL) and prolamin–mGLP×6(KDEL) transformants

It has been observed that PB I is derived from the ER (Yamagata and Tanaka 1986). Given that the transgene product was synthesized in the ER and retained by the KDEL signal for retrieval to the ER lumen, the peptide would be efficiently transported to PB I compared with the peptide without the KDEL tetrapeptide. Therefore, the KDEL tetrapeptide was added to the C-terminus of the mGLP×6 fusion with prolamin. To estimate the relative amount of mGLP×6(KDEL) peptide, total protein was extracted from one mature seed of regenerated transformants (T1 generation) containing pmGLP×6(KDEL) or prolamin–mGLP×6(KDEL) (Fig. 1). Total seed protein was analyzed by SDS–PAGE and Western blot using anti-GLP-1 antibody.

As shown in Fig. 4B, the mGLP×6(KDEL) peptide was detected in seven of the 30 lines of regenerated mGLP×6(KDEL) transformants and in six of 30 lines of regenerated prolamin–mGLP×6(KDEL) transformants. Their molecular weights were estimated to be about 19 and 32 kDa for the mGLP×6(KDEL) and prolamin–mGLP×6(KDEL) transformants, respectively, based on their mobility on SDS–PAGE, or slightly smaller than what would be predicted from their amino acid sequences (20.5 and 36.7 kDa). The expression of the mGLP×6(KDEL) peptide was 2–3 times higher than that of the prolamin–mGLP×6(KDEL) peptide (Fig. 4C). Furthermore, it is notable that an additional faint band (approximately 20.5 kDa) was detected in mGLP×6(KDEL) transformants. This may be accounted for by a lack of signal peptide processed from the mGLP×6(KDEL) peptide (Fig. 4B).

Subcellular localization of mGLP×6(KDEL) peptides in subaleurone cells of mGLP×6(KDEL) and prolamin–mGLP×6(KDEL) transformants

The subcellular localization of the mGLP×6(KDEL) peptide was observed in immature T1 generation seeds (15–20 DAF) of both transformants. The fluorescence of mGLP×6(KDEL) peptide in subaleurone cells of the mGLP×6(KDEL) transformant was confined to the ER (Fig. 5A, B), because the peptide has an ER retention signal. The subcellular localization of the prolamin–mGLP×6(KDEL) fusion peptide in the prolamin–mGLP×6(KDEL) transformant was mainly restricted to PB I, because the fluorescence of mGLP×6(KDEL) peptide co-localized completely with the fluorescence of rhodamine B (Fig. 5C, D). PB I develops inside the cisternae of the ER (Yamagata and Tanaka 1986). It was expected that the transgene product expressed from the prolamin–mGLP×6(KDEL) construct would be efficiently transported to PB I, because the transgene product had an added KDEL tetrapeptide at the C-terminus.

The comparison of signal intensities of mGLP-1 peptides between lines; which transformant has the highest expression level

In order to determine which transformant has the highest expression level, we selected the line showing the highest expression of mGLP-1 peptide in each transformant (line 6 of SP(GluB)-mGLP×5, line 1 of SP(chi)-mGLP×5 and line 10 of the GluA(mGLP×5) transformant in Fig. 2B and line 6 of
Localization of a foreign gene product in rice seed

mGLP×6(KDEL) and line 11 of the prolamin-mGLP×6(KDEL) transformant in Fig. 4B] and compared the signal intensities obtained by Western blot analysis (Fig. 6). Although this direct comparison of the expression levels of mGLP-1 peptide for each transformant might be difficult due to the difference in mGLP-1 repeat number, the result indicated that the signal intensity in line 6 of the SP(GluB)-mGLP×5 transformant was the highest among the five transformants (T3 generation). Given that binding of the anti-mGLP-1 peptide antibody is dependent upon the number of mGLP-1 peptides, the relative amount of mGLP-1 peptide in line 6 of the mGLP×6(KDEL) transformant is about 2-fold higher than that of line 6 of the mGLP×5(KDEL) transformant. The result of relative quantitation to standard His-tagged mGLP×5 peptide prepared from Escherichia coli, showed that the highest expression level of mGLP-1 in the SP(GluB)-mGLP×5 transformant was approximately 150 µg per grain.

**Discussion**

We recently reported that transgenic rice plants do not express mGLP-1 peptide in endosperm cells due to RNA silencing when the mGLP-1 gene is directly under the control of the glutelin 2.3 kb GluB-1 promoter (Yasuda et al. 2005). However, it has been demonstrated here that this silencing by co-suppression could be avoided by increasing the size of the insert, since tandem mGLP-1 repeats resulted in measurable expression. In a preliminary experiment, we found that a trimer (mGLP-1×3) was the minimum multimer required for expression of the mGLP-1 peptide, although its expression level was lower than that of the pentamer (data not shown).

We performed an immunohistochemical analysis using anti-mGLP-1 antibody to observe the subcellular localization of the mGLP×5 peptide derived from the SP(GluB)-mGLP×5 transformant. In general, a protein containing a signal peptide is transported to the intercellular space via default or the bulk-flow pathway, if the protein does not have any specific targeting signals for subcellular compartments (Denecke et al. 1990, Hunt and Chrispeels 1991, Boevink et al. 1996, Batoko et al. 2000). However, the results of the immunohistochemical analysis indicate that the mGLP×5 peptide is located in the ER of subaleurone cells (Fig. 3A, B). On the other hand, expression...
of the mGLP-5 peptide in transgenic rice seed containing the SP(chi)-mGLP×5 construct, which directed the peptide to the intercellular space by the chitinase signal (Fig. 3C, D), was very low when compared with that from the SP(GluB)-mGLP×5 transformant (Fig. 2C). These results suggest that the signal peptide derived from glutelin GluB-1 has retention activity in the ER, and/or that the secretion activity of the subaleurone cell is very weak. In fact, GFP attached to the soybean 11S glycycin signal peptide (Kawagoe et al. 2005) or the glutelin GluB-1 signal peptide (data not shown) was also never secreted from subaleurone cells. In rice endosperm, targeting of foreign gene products to the intercellular space is not adequate for high-level expression.

It is noteworthy that the mGLP×5 peptide inserted downstream of the rice chitinase signal peptide was secreted into the intercellular space (apoplast) (Fig. 3C, D). It had previously been demonstrated that a peptide following the chitinase signal peptide is secreted into the intercellular space of the cell (Batoko et al. 2000, Sharma et al. 2000). It was suggested that the signal peptide of chitinase could bind to a factor of a hypothetical 'secretory apparatus', but the signal peptide of glutelin GluB-1 could not. It is conceivable that signal peptides play a crucial role in localization of the downstream peptide in rice endosperm cells.

In the case of oral administration of transgenic rice grains, the expression level of the peptide is critical for delivery of a therapeutic dose of the mGLP-1 peptide. Therefore, a major goal is to develop transgenic rice that accumulate the peptide more abundantly. If the transgene product (mGLP×5) could be efficiently transported to one or both of the two PBs, it is expected that the peptide would be highly accumulated in the endosperm. Yang et al. (2003) indicated that human lysozyme with a globulin signal peptide expressed in transgenic rice plants was co-localized with endogenous globulin and glutelins in the endosperm. They pointed out that human lysozyme contains a targeting signal specific to the protein storage vacuole. Torres et al. (2001) indicated that transgenic rice plants expressing ScFvT84.66-KDEL with an N-terminal signal peptide derived from the murine immunoglobulin heavy chain cDNA transported the transgene product to both PB I and PB II. Furthermore, Takagi et al. (2005) have recently shown that the artificial 7Crp peptide with a glutelin GluB-1 signal peptide and C-terminal KDEL tetrapeptide was also transported into both PBs in transgenic rice seeds. These results indicate that foreign gene products with a signal peptide and the KDEL tetrapeptide are directed to protein bodies. However, in this work, we found that the mGLP×6(KDEL) peptide was not transported to these compartments. It is thus likely that both signals are necessary but not sufficient for targeting to protein bodies. Choi et al. (2000) and Hamada et al. (2003) suggested that the localization of mRNA is important for the transport of the gene product to each protein body. Kawagoe et al. (2005) suggested that the formation of a disulfide bond is responsible for sorting to PB I or PB II. In either case, localization of the foreign gene product is important for high-level expression.

In order to enhance the expression level of the mGLP×5 peptide, we also generated transgenic rice containing the GluA(mGLP×5) construct to transport mGLP×5 peptide to PB II. We expected that the high-level expression of the transgene product could be obtained from the GluA(mGLP×5) transformant, because high levels of expression of mGLP-1×1 as a fusion protein with the 26 kDa globulin protein were observed in transgenic rice plants harboring pGlbGLP130Hm, in which mGLP-1 was inserted into a variable region of the rice globulin gene (Sugita et al. 2005). It was expected that the modified globulin containing mGLP-1×1 might be targeted to PB II in the same way as the native globulin. The highest expression level of mGLP-1 in transgenic rice was 50 µg per grain. A similar level of expression was also detected in the construct in which mGLP-1×1 was inserted into the C-terminal highly variable region of the glutelin acidic subunit (data not shown). Unexpectedly, expression of the chimeric mGLP×5 peptide in GluA(mGLP×5) transformants was remarkably low, as shown in Fig. 2C, even though the peptide as a part of glutelin acidic subunit was processed normally (Fig. 2B) and was transported to PB II (Fig. 3E, F). These results suggest that the size of the mGLP×5 peptide (150 amino acids) inserted into the glutelin acidic subunit might be too large to accumulate stably as a chimeric glutelin in PB II. Given that accumulation levels are very different between mGLP-1×1 and mGLP-1×5 as inserts when
expressed as a part of the glutelin protein, it is important that an appropriately sized insert is introduced into the highly variable regions of seed storage proteins. Therefore, it will be necessary to determine what the most appropriate size would be on an individual basis for high accumulation when expressed as a fusion with storage proteins.

In the Western blots, a faint band that migrates slightly higher than the mGLP×6(KDEL) peptide was detected in mGLP×6(KDEL) transformants (Fig. 4B, 6A). This faint band was also detected in the T3 generation of SP(GluB)-mGLP×5 transformants (Fig. 6A). These bands suggested that the signal peptide might not be processed normally from both the peptides. The maize floury-2 mutant, in which the processing site sequence in the signal peptide of the 22 kDa α-zein gene is mutated, indicates that the signal peptide cannot be cleaved from the following peptide (Coleman et al. 1997; Coleman et al. 1997). The unprocessed zein remains anchored to the ER membrane (Gillikin et al. 1997), and this mutation produces pleiotropic effects such as reduction in the amount of seed storage proteins, and changes in the arrangement of proteins within PBs (Lending et al. 1988). In line 6 of transgenic rice containing the mGLP×6(KDEL) construct, the SDS–PAGE profile of seed proteins showed a reduction in the mature form of glutelins and an increase in glutelin precursor correlated with the levels of mGLP×6(KDEL) peptide (Fig. 4A). Furthermore, the amount of fluorescence of PB I was slightly less than in the wild type or the other transformants as shown in Fig. 5A (red signals). A similar observation was made for the SP(GluB)-mGLP×5 transformant (Fig. 3B, red signals). These results strongly suggest that both of the unprocessed mGLP-1 peptides might be anchored to the ER membrane, and exhibited a phenotype analogous to the floury-2 mutant. However, when expression was low [as in the other transformants containing SP(GluB)-mGLP×5, mGLP×6(KDEL) and SP(chi)-mGLP×5], or the peptide was transported to PB I [prolamin–mGLP×6(KDEL)] and PB II [GluA(mGLP×5)], this phenotype was not observed.

In these experiments, we succeeded in the production of transgenic rice in which transgene products (mGLP-1 peptides) were transported to the specified intracellular domains (i.e. ER, PB I and PB II) and to the intercellular space (apoplast) of rice endosperm. These inter- and intracellular localizations were confirmed by indirect immunohistochemical analysis using confocal laser scanning microscopy, although direct confirmation should be possible by immunogold analysis using electron microscopy. These results indicate that high level expression of transgene products can be obtained by transporting them to the most suitable sites for accumulation. In the case of tandem repeated mGLP-1, expression levels of the mGLP-1 peptide from SP(GluB)-mGLP×5 and mGLP×6(KDEL) transformants were higher than those of the other transformants. These results suggest that the ER of rice endosperm cells is a prime candidate as a storage compartment for foreign gene products.

**Materials and Methods**

**Construction of chimeric genes and plant transformation**

mGLP-1 cDNA (Yasuda et al. 2005) was amplified by KOD-Plus-TOYOBO (Osaka, Japan), and self-ligated by T4 DNA ligase (TOYOBO). The ligated cDNAs were cloned into p17Blue vector (Novagen, Madison, WI, USA), and a 5× tandem repeat mGLP-1 cDNA (mGLP×5) was selected. The mGLP×5 cDNA was linked to the glutelin 2.3 kb GluB-1 promoter (pGluB-1; Qu and Takaiwa 2004) containing the glutelin GluB-1 signal peptide [pSP(GluB)-mGLP×3].

The signal peptide derived from rice chitinase (chi-1, accession number D16221, Nishizawa et al. 1999) was amplified from a cDNA clone as a template, and was used to replace the signal peptide from GluB-1 in pSP(GluB)-mGLP×5 to form pSP(chi)-mGLP×5.

The modified GluA-2 cDNA clone (pREE99, accession number X05664, Takaiwa et al. 1987), which has an inserted Smal site and a deletion from L276 to C304, was fused to the glutelin 2.3 kb GluB-1 promoter, and the mGLP×5 cDNA was cloned into the Smal site to form pGluA(mGLP×5).

The mGLP×5 cDNA was inserted between the GluB-1 signal peptide and mGLP-1 in pmGLPHIs (Yasuda et al. 2005) and the His tag sequence was deleted to make pmGLP×6(KDEL). Furthermore, a 13 kDa prolamalin cDNA (without signal peptide, accession number XM477075) was inserted between the signal peptide and mGLP×6(KDEL) in pmGLP×6(KDEL) to form prolamalin–mGLP×6(KDEL).

All constructs were transferred into the modified binary vector pGPTV-35S-HPI, containing the glutelin GluB-1 terminator (Fig. 1, Yasuda et al. 2005). The binary vectors were individually transferred into Rhizobium radiobacter strain EHA105 by electroporation. Rhizobium-mediated rice (Oryza sativa L. cv. Kita-ake) transformation was performed as described previously (Goto et al. 1999).

**Quantification of mGLP×5 peptide**

Total protein extractions and Western blots were performed as previously reported (Yasuda et al. 2005). Expression levels of mGLP×5 peptides were calculated from the intensity of bands on X-ray films after Western blot analysis using anti-mGLP-1 antibody, measured with NIH image software.

**Indirect immunohistochemical analysis of mGLP-1 peptide**

Immature seeds (15–20 DAF, T3 generation) of rice transformants were cross-sectioned with a Microslicer DTK-1000 (DOSAKA EM Co., LTD., Kyoto, Japan) to approximately 200 μm in thickness. The sections were fixed in 3.7% formaldehyde solution in phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) for 1 h followed by washing three times in PBS. The specimens were incubated with an enzyme solution (1% cellulase, 0.1% pectolyase Y-23 in PBS) for 5 min at 37°C, and then were permeabilized in 0.3% Triton X-100 in PBS for 15 min. To prevent non-specific binding of antibody, the specimens were blocked with PBS/bovine serum albumin (BSA) (1% BSA, 0.03% Triton X-100 in PBS) for at least 1 h at room temperature and then treated with anti-mGLP-1 antibody diluted 1:200 in PBS/BSA overnight at room temperature. After washing with PBS, specimens were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR, USA) diluted 1:300 in PBS for 2 h at room temperature. To stain PB I, the specimens were incubated with 1 μM rhodamine B in PBS for 30 min (Muench et al. 2000). After washing with PBS, the specimens were mounted on slide glass in component A from a SlowFade Antifade kit (Molecular Probes) and observed with a confocal laser scanning microscope (Radiance 2000, Bio-Rad, Hercules, CA, USA) using GFP and rhodamine filter sets. Image processing was performed using Adobe Photoshop 5.0.
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References


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