Title: Production of recombinant thanatin in watery rice seeds that lack an accumulation of storage starch and proteins

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Abbreviations: SPK, seed-development specific protein kinase

ABSTRACT

Molecular farming is a promising method for producing materials of commercial interest. Plants can be expected to be appropriate hosts for recombinant protein production. However, production in genetically modified plants has two major challenges that must be resolved before its practical use: insufficient accumulation of products and difficulty in establishing methods for their purification. We propose a simple procedure for the production of a desired protein using watery rice seeds lacking an accumulation of storage starch and proteins, a phenotype induced by the introduction of an antisense *SPK*. We produced a transgenic rice plant containing a gene for an antimicrobial peptide, thanatin, together with antisense *SPK*. Bioassay and proteome analysis indicated that recombinant thanatin accumulated in an active form in these watery rice seeds. These results suggest that our system worked effectively for the production of thanatin. This procedure enabled easy removal of impurities and simplified the purification process compared with production in leaves. Our system may therefore be a useful technique for the production of desired materials, including proteins.

Keywords: Molecular farming; Simple purification; *SPK*; Thanatin; Watery rice seeds

1. Introduction

Molecular farming methods using genetically modified plants have emerged as a promising approach to the production of materials of commercial interest. For the past two decades, transgenic plants have been successfully used in the production of pharmaceuticals (e.g. antibodies, vaccines, human growth hormone and interleukins) and industrial materials (e.g. xylanase, amylase, and cellulase) (Daniell et al., 2009; Hood and Requesens, 2012). Plant-based production systems have many advantages over other production systems, such as potentially low production costs, rapid scalability, the absence of human pathogens and endotoxins, and carbon neutrality (Obembe et al., 2011; Twyman et al., 2003). Moreover, plant systems can produce antibacterial products such as antimicrobial peptides (Breen et al., 2015; Imamura et al., 2010). However, there are two major challenges that prohibit the economical production of plant-made recombinant proteins, namely, insufficient accumulation of the products and difficulty in establishing an efficient method for their purification.

To obtain a sufficient level of product accumulation, several strategies could be used, including taking advantage of suitable promoters, codon optimization for expression of the target gene, adaptation of the untranslated sequences involved in mRNA stability, coordination of subcellular localization, and choosing a host plant suitable for production of the desired materials (Obembe et al., 2011; Twyman et al., 2003). Furthermore, systems that enable high levels of expression of the desired gene have been developed, such as chloroplast transformation and transient production using a plant virus vector system carrying an appropriate gene (New et al., 2012; Wang, 2012).

These expression systems often lead to high productivity compared with conventional methods (Chebolu and Daniell 2009; Pogue et al., 2010).

However, some difficulty remains in the isolation and purification of the desired substances from plant cells. Some materials present in plant cells require additional purification steps that cause a decrease in productivity and reduce the cost performance. The purification cost of a plant-based production can account for 80% of the total cost (Schillberg et al., 2005). To improve the cost performance, the establishment of an alternative purification system and the enrichment of product accumulation are required. A fusion protein with a specific tag may lead to a simple purification procedure for the desired protein. Recently, high-level accumulation of the desired protein was achieved using three fusion partners, the N-terminal domain of γ -zein (Zera), hydrophobin, and elastin-like polypeptide (ELP) (Joensuu et al., 2010; Scheller et al., 2006; Torrent et al., 2009). Protein fused to these partners can be purified easily using density centrifugation (Zera), aqueous two-phase separation (hydrophobin), and inverse transition cycling (ELP), which may contribute to establishing a simple procedure for production of the protein in plant cells (Joensuu et al., 2010; Scheller et al., 2006; Torrent et al., 2009).

A suitable host contributes to efficient protein production. Therefore, choosing a suitable host plant is important for establishing an efficient production system. Rice is an attractive host that has many advantages for recombinant protein production, such as high grain yield, capability of rapid scale-up, and self-pollination (Stoger et al., 2005). Rice seeds, which are a natural storage organ with a large amount of protein and storage starch, are an ideal production platform. Many substances are sustainably produced in recombinant rice seeds (Takaiwa et al., 2007; Wakasa and Takaiwa 2013). However, to obtain the purified materials, removal of a massive amount of storage protein and starch

is needed, and this process is problematic. We have reported a transgenic rice strain harboring the antisense gene for *SPK*, which results in the formation of watery seeds (Asano et al., 2002). *SPK* is a calcium-dependent protein kinase involved in the activity of sucrose synthase in developing seeds, and is specifically expressed in developing rice seeds. Watery seeds contain watery sap and lack the usual accumulation of storage proteins and starch (Shimada et al., 2004) (Fig. 1).

We consider that this watery sap is a potential medium for the production of foreign proteins by recombinant gene technology, because purification methods can be simple and impurities in the sap are easily removed. In the present study, we examined a simple system for the production of recombinant thanatin in the watery seeds of transgenic rice plants. Thanatin is an antimicrobial peptide consisting of 21 amino acid residues, which originates in the hemipteran insect *Podisus maculiventris* (Fehlbaum et al., 1996). Thanatin is an antimicrobial substance with an expected wide spectrum of activity for Gram-negative and Gram-positive bacteria and some species of fungi (Fehlbaum et al., 1996). Here, we report the construction of a simple production system using *thanatin* and antisense *SPK*, which were predicted to be expressed together in the transgenic rice, and determined the production of thanatin in watery seeds.

2. Materials and methods

2.1. Plant materials and growth conditions

Oryza sativa L. cv. Nipponbare was used as the wild-type plant. Seeds were germinated at 30°C in a dark chamber. The seedlings were grown under continuous light (13 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for three days. The rice plants were cultivated in a growth

chamber with 80% humidity under a long day condition, with a cycle of 14 h light (30°C, 270 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 10 h dark at 25°C.

2.2. Plasmid construction

A plasmid containing the antisense *SPK* was constructed as described in a previous report (Asano et al., 2002). The region of antisense *SPK* used in the present study was taken from this plasmid by digestion with *Asp*718, then inserted into the *Asp*718 site of the plasmid p35S-TAN, which contained *thanatin* following a CaMV 35S promoter (Imamura et al., 2010). The resultant plasmid was named p35S-TAN-ASPK (Fig. 2A). The antisense *SPK* region was also inserted into the *Asp*718 site of pCAMBIA1300 (Cambia, Canberra, Australia), creating a vector named p35S-ASPK.

2.3. Plant transformation

Rice was transformed using an *Agrobacterium*-mediated method (Hiei et al., 1994). Transformants were grown on 1/2 Murashige–Skoog plates (Murashige and Skoog, 1962) supplemented with 50 mg L⁻¹ hygromycin B (Wako Pure Chemicals, Osaka, Japan) to screen for hygromycin-resistant lines before their cultivation in soil. The plants were grown in a growth cabinet or green chamber. The gene introduced into the transformants was confirmed by PCR using the primer set 5'-GAATCCATGGGCTCCAAGAA-3' and 5'-ATGTCATCCATGCCATGTGT-3' for *thanatin* and 5'-CGTGAAATCCAGATAATGCA-3' and 5'-CTTGAACACTGAGCTGTAC-3' for antisense *SPK*.

2.4. RNA isolation and reverse transcription (RT)-PCR

Total RNA was extracted from rice tissue as described previously (Imamura et al., 2007). The first-strand cDNA was synthesized from 1 µg of total RNA using a ReverTra Ace cDNA synthesis kit (Toyobo, Osaka, Japan) with an oligo-dT(20) primer. Semiquantitative RT-PCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The procedure for amplification of the *thanatin* transcript comprised initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The amplification protocol for *Actin I* transcript included an initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The *thanatin* mRNA was quantified relative to the amount of *Actin I* mRNA (acc. no. X16280). The primers used for semiquantitative RT-PCR were 5'-CCCTCCTGAAAGGAAGTACAGTGT-3' and 5'-GTCCGAAGAATTAGAAGCATTTC-3' for *Actin I*, 5'-GAATCCATGGGCTCCAAGAA-3' and 5'-ATGTCATCCATGCCATGTGT-3' for *thanatin*.

2.5. Bioassay of antimicrobial activity

Antimicrobial activity was determined as previously described, using *Micrococcus luteus* in 96-well microtiter plates, to which were added 10 µL of transgenic sap and 90 µL of assay medium (Imamura et al., 2008). They were diluted by the assay medium as necessary. Approximately 300 cells of *M. luteus* were added to every well for each assay, and the cells were incubated at 30°C for two days. The antimicrobial activity against *M. luteus* was estimated as the degree of turbidity of the medium (Imamura et al., 2010).

2.6. Purification of thanatin in watery saps of transgenic plants

Sap was collected from watery transgenic seeds, and filtered through a 0.45 μm filter (Millipore, Billerica, MA, USA) to remove insoluble materials. The sap of watery seeds was analyzed by HPLC on a reversed-phase column, with a combined solution of solvent A (0.1% trifluoroacetic acid [TFA] in H_2O) and solvent B (0.1% TFA in acetonitrile) as the mobile phase. The gradient system of these solvents was: 0% solvent B (3 min), 0% to 20% solvent B (12 min), then 20% to 30% solvent B (60 min), and 30% to 100% solvent B (5 min). The flow rate was 0.5 mL min^{-1} and the eluate was monitored by absorbance at 225 nm. Fractions of the HPLC separation were collected. These fractions were concentrated using a centrifugal concentrator (CC-105, Tomy Seiko Inc., Tokyo, Japan). They were dissolved in water, and assayed for antimicrobial activity. Authentic thanatin peptide (GSKKPVPIIYCNRRRTGKCQRM) was synthesized by Scrum Inc. (Tokyo, Japan)

2.7. Protein digestion and identification

Purified materials showing antimicrobial activity were digested with trypsin using an In-Gel Tryptic Digestion Kit (Thermo Scientific, Vantaa, Finland). Digested peptides were applied onto a Magic C18 AQ nano column (Michrom Bioresources, Auburn, CA, USA) in an Advance UHPLC system (Michrom Bioresources) equilibrated with 0.1% formic acid in acetonitrile, and eluted using a linear gradient from 5% to 45% acetonitrile at a flow rate of 500 nL min^{-1} . The mass spectrometer used was an LTQ Orbitrap XL (Thermo Scientific) operated with the Xcalibur software (version 2.0.7, Thermo Scientific). Peptides were identified using an in-house Mascot server (MS/MS ion search, Mascot version 2.5, Matrix Science Inc.) using the thanatin sequence.

3. Results

3.1. Production of transgenic rice carrying both *thanatin* and antisense *SPK*

We produced a plasmid containing two genes for expression, *thanatin* and antisense *SPK*, both of which were driven by the CaMV 35S promoter (p35S-TAN-ASPK). This plasmid was introduced into rice, and 30 recombinant plants, named 35S-TAN-ASPK lines 1 to 30, were obtained (Figs 2A and 2B). Expression of *thanatin* in each of the 35S-TAN-ASPK plants was determined by semiquantitative RT-PCR. A 780 bp fragment that was derived from the introduced *thanatin* was detected in the transformants (Fig. 2C). A 660 bp fragment was also detected (Fig. 2C). This fragment was generated by an alternative splicing of the transcript of the introduced gene and, based on a previous study (Imamura et al., 2010), was suggested to contribute to the production of active thanatin. This indicated that sufficient expression of *thanatin* occurred in these transformants.

The expression of antisense *SPK* in transgenic rice results in a reduction of starch storage and the production of watery seeds (Asano et al., 2002). We analyzed the morphology of immature seeds and determined the proportion of watery seeds produced by these transgenic lines. Formation of watery seeds was observed in all transgenic lines, and the proportion of watery seeds was between 15.0% and 32.8%, whereas wild-type plants produced normal seeds (Figs 1 and 2D). These findings suggested that the antisense *SPK* functioned effectively in these lines. In the transgenic rice lines, many seeds were empty (without starch or watery sap), and the proportion of normal seeds was very low. Because watery seeds were no longer viable, the levels of *SPK*

expression were not determined in the watery seeds of the transgenic lines (Fig. 2D). We chose six transgenic lines that produced a high proportion of watery seeds, and used these for further experiments. We also generated transgenic rice harboring antisense *SPK* without any *thanatin* using p35S-ASPK. The transgenic line that produced the highest proportion (41.3%) of watery seeds was selected as a control (Fig. 2D).

3.2. Evaluation of antimicrobial activity of the sap of the transformants

A transgenic rice plant harboring *thanatin* showed antimicrobial activity and the acquisition of tolerance to rice blast fungi (Imamura et al., 2010). We evaluated the antimicrobial activity of the sap in the watery seeds of seven 35S-TAN-ASPK lines. The watery seeds of six lines (#1, #2, #5, #6, #9, and #13) showed antimicrobial activity, whereas no significant activity was detected in those of line #3 or in those of controls (Fig. 3A). We analyzed the production of thanatin in the representative strain. HPLC analysis of the watery seeds of 35S-TAN-ASPK line #5 plants showed a specific peak corresponding to thanatin (Fig. 3B).

3.3. Identification of thanatin from transgenic sap

We attempted to purify and identify the thanatin in the sap of watery seeds. Each watery seed contained approximately 10 μ L of sap. We collected approximately 2 mL of sap from watery seeds of the 35S-TAN-ASPK line #5 plant. Preparative HPLC was performed to collect the thanatin fraction, which corresponded to the specific peak detected in the sap of 35S-TAN-ASPK plants, with a peak retention time coinciding with that of authentic thanatin. Thanatin activity in this fraction showed an antimicrobial activity, with a specific activity approximately equal to that of authentic

thanatin. This fraction was concentrated and was subjected to mass spectroscopic analysis. As shown in Fig. 3C, the signal for this substance corresponds to a part of thanatin (KPVPIIYCNR) produced from the introduced gene. Therefore, the recombinant thanatin produced by these transgenic lines may have the same structure as the native form. The yield of thanatin from the sap of the transgenic line was estimated by HPLC to be approximately 0.11 µg per watery rice seed.

4. Discussion

Molecular farming facilitates both the production of good-quality products and high productivity, and is desirable for extensive commercialization of materials production. Improvement of the purification process is highly desirable because this accounts for a large part of the cost of protein production. Production systems using a fusion protein, in which the desired protein is easily isolated using an affinity or nonaffinity tag, are widely applied to purification processes (Joensuu et al., 2010; Lichty et al., 2005; Scheller et al., 2006; Terpe, 2003; Torrent et al., 2009). These tags are commonly used in laboratory-scale production. However, for commercial use the tag portion of the fused protein must be removed, and production of the native-form protein is required.

Here, we focused on the importance of the host plant used to establish a suitable purification method, which provided advantages over conventional techniques. Plant seeds are a good medium in which to produce recombinant protein, because they have a high ability to accumulate a massive amount of storage substances. However, these storage substances, such as starch and protein, often act as impurities that interfere with the isolation of the desired product. We have reported that transgenic rice harboring antisense *SPK* produced watery seeds as its phenotype (Asano et al., 2002). The watery

seeds lacked an accumulation of many of the usual storage substances, and contained a watery sap that was derived from phloem (Shimada et al., 2004). Mutants defective in storage protein accumulation are available as a platform that may reduce the requirement for removal of impurities and enrich the product accumulation in seeds (Lin et al., 2013; Schmidt and Herman, 2008; Shigemitsu et al., 2012). Here, we created transgenic rice containing both thanatin and antisense *SPK*, and developed a simplified procedure for protein purification using watery seeds. The resultant plants produced a large proportion of watery seeds with the aid of the antisense *SPK*, and produced active thanatin in their watery sap that showed antimicrobial activity similar to that of authentic thanatin (Figs 1-3). This indicates that our system worked effectively as a method for protein production. Although the productivity of our system is still low compared with that of other production systems such as chemical synthesis, this could be improved by applying an appropriate promoter. Because the watery seeds are sterile, the development of an efficient method for obtaining fertile seeds for the establishment of a sustainable production system is desirable. An inducible promoter could be applied to regulate the expression of antisense *SPK*.

Sieve tube elements contain various materials (Aki et al., 2008; Gaupels et al 2008; Giavalisco et al., 2006; Walz et al., 2004) that are transported over long distances in plants (Aoki et al., 2005). In rice, phloem sap contains various proteins ranging in size from 10 to 250 kDa (Aki et al., 2008). Materials in the watery sap of the transformant carrying the antisense *SPK* were transferred from phloem sap (Shimada et al., 2004). These findings suggest that the watery seed production system induced by the antisense *SPK* may be applied to the production of various recombinant proteins in sieve elements.

In summary, we constructed a novel production system that allowed the efficient accumulation in watery seeds of native-form thanatin that retained its antimicrobial activity (Figs 1 and 3). This watery-seed system enables the use of a simple procedure for purification because the watery seeds contain only small amounts of impurities. Our system involves a purification process comprising the extraction of the crude extract by squeezing the watery seeds, filtration, and preparative HPLC. This purification process is greatly simplified compared with that for purifying thanatin produced in leaves, which involves extensive processing, including extraction by grinding leaves, sonication, ion-exchange chromatography, gel filtration, and preparative HPLC (Imamura et al., 2010) (Fig. 4). Our present results show that our system may work as a novel method to accumulate efficiently the desired recombinant proteins in the sap of watery rice seeds and to simplify the removal of impurities. We believe that our proposed new strategy for molecular biology-based farming will be widely utilized in many scenarios.

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Figure legends

Fig. 1. The watery rice seed phenotype. Photographs of a watery seed (A) and wild-type seed (B). Cross section of a watery seed (C) and wild-type seed (D). (E) Watery sap in the watery seeds (left) and grain of the wild-type seed (right). Le, lemma; Pa, palea; En, endosperm. Bars indicate 2 mm.

Fig. 2. Generation of transgenic rice harboring *thanatin* and antisense *SPK*. (A) Schematic representation of the *thanatin* gene. A stop codon was introduced downstream from the open reading frame of the *thanatin* gene. *Thanatin* is followed by 727 bp of noncoding sequence before a Nos terminator. RB: right border; LB: left border; ASPK: Antisense *SPK*; *HPT*: hygromycin phosphotransferase gene; 35S: CaMV 35S promoter; *Thanatin*: the coding region of the *thanatin* gene with its stop codon; *NosT*: terminator of the nopaline synthase gene; STOP: stop codon (TAG); P1 and P2: position of the RT-PCR forward primer and reverse primer, respectively. (B) Detection of *thanatin* and *SPK*. a: The fragment of the *thanatin* gene. b: 1337 bp corresponding the endogenous *SPK* gene (containing an intron). c: 873 bp corresponding the antisense *SPK*. Fragments b and c were amplified using the same PCR primers. (C) Detection of the transcript of the introduced *thanatin* gene. a: The 780 bp fragment corresponding to full-length *thanatin*. d: The 660 bp fragment indicating the *thanatin* transcript with unexpected splicing. Rice *Actin I* used as a control. (D) Fertility of transformants harboring the *thanatin* gene. Fertility is indicated as the ratio of the mature seeds to the total number of flowers. Solid, open, and shaded boxes indicate ratios of fertility of normal, empty, and watery seeds, respectively. Numbers

indicate transgenic lines. VC and WT show vector control and wild-type plants, respectively.

Fig. 3. Determination of antimicrobial substances in the transformants. (A) Antimicrobial activity in representative transformants carrying the thanatin gene. Activity is represented by a clear well. Numbers shown above correspond to the transformant line numbers. VC indicates the assay media including *M. luteus* with the watery sap of the vector control. Positive Control and Negative Control indicate the assay media including *M. luteus* with or without an antimicrobial substance, respectively. (B) Chromatogram from the HPLC. This chromatography was conducted using the antimicrobial substances in the watery seeds of transformant line #5. The arrow indicates the specific peak in the sap of the transformant. The eluted substances were monitored by their absorbance at 225 nm. The upper panel shows the chromatogram of the watery sap of 35S-TAN-ASPK line #5. The middle and lower panels show the chromatograms of the watery sap of the vector control and of authentic thanatin peptide, respectively. Arrowheads indicate peaks for thanatin. (C) MS spectra of the additional substance derived from the transformant. The horizontal axis indicates mass-to-charge ratio (m/z). The vertical axis indicates the relative abundance.

Fig. 4. Schematic representation of the process to produce recombinant thanatin in transgenic plants. The left column shows the procedure to purify recombinant thanatin from watery seeds (present study). The right column shows the procedure to purify recombinant thanatin from leaves (Imamura et al., 2010).

Figure 1

Figure 2

Figure 3

Figure 4