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

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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.

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

Abbreviation: SPK, seed-development specific protein kinase.

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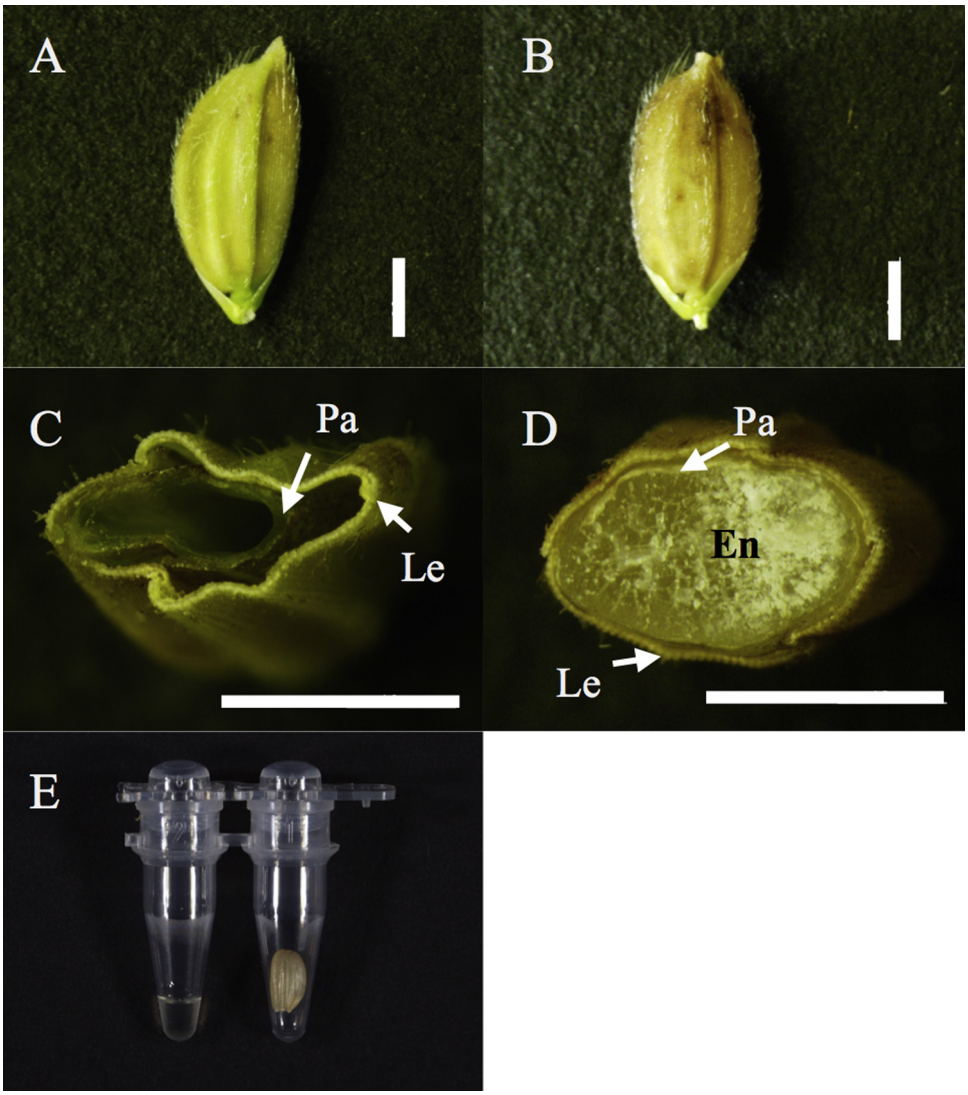

Fig. 1. The watery rice seed phenotype. Photographs of a watery seed (A) and wild-type seed (B). Cross section of a watery seed (C) and wild-type seed (D). (E) Watery sap in the watery seeds (left) and grain of the wild-type seed (right). Le, lemma; Pa, palea; En, endosperm. Bars indicate 2 mm.

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.