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Overexpression of an endo-1,4-β-glucanase V gene (*EGV*) from *Trichoderma reesei* leads to the accumulation of cellulase activity in transgenic rice

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ABSTRACT. The ectopic expression of cellulase in biomass can reduce the cost of biofuel conversion. This trait modification technique is highly beneficial for biofuel production. In this study, we isolated an endo-1,4-beta-glucanase gene (*EGV*) from *Trichoderma reesei* and inserted this gene downstream of a fragment encoding the signal peptide Apo-SP in a modified pCAMBIA1301 vector to obtain an Apo-SP and AsRed fusion protein. Transient expression of this fusion protein in onion epidermal cells showed that the Apo-SP signal was localized to the plastids. *EGV* transgenic rice plants that did not carry screening marker genes were obtained through overexpression of the pDTB double T-DNA vector. Western blotting showed that EGV was expressed in the dry straw of T_0 generation transgenic rice plants and in fresh leaves of the T_1 generation. More importantly, our results also showed that the peptide product of EGV in the transgenic plants folded correctly and was capable of digesting the cellulase substrate CMC. Additionally, cellulase activity remained stable in

the straw that had been dried at room temperature for three months. This study presents an important technical approach for the development of transgenic rice straw that has stable cellulase activity and can be used for biofuel conversion.

Keywords: *Trichoderma reesei*; *EGV*; Cellulose; Double T-DNA vector; Transgenic rice

INTRODUCTION

The use of bioethanol is the most promising approach to addressing the energy crisis. Initially, bioethanol was mainly generated from food starch. Currently, many countries are striving to develop second generation bioethanol based on lignocellulosic raw materials. Lignocellulose is the most abundant renewable resource on earth, with a global annual yield of approximately 10-50 billion tons (Sticklen, 2006; Wang et al., 2009). Some of the annual yield of lignocellulose is a byproduct of crop production. For example, rice is an important food crop globally; China alone produces nearly 2.5 GT (billion tons) of rice straw per year (Wang et al., 2009) that might contribute to the generation of bioethanol. The most efficient method for producing bioethanol from rice straw is to convert lignocellulose into fermentable monosaccharides through hydrolysis using cellulase. Thus, having an efficient source of cellulase is critically important for commercial bioethanol production from rice straw (Stocker, 2008; Sainz, 2009; Banerjee et al., 2010).

Cellulase can be obtained from a wide variety of sources, such as bacteria, fungi, and insects. The fungus *Trichoderma reesei* is considered to be the best species for the industrial production of cellulase (Aden and Foust, 2009) as it produces cellulase extracellularly, with high yields and activity. The cellulase generated by *T. reesei* is a multienzyme system consisting of three synergistic enzyme types, including endoglucanase (EG), cellobiohydrolase (CBH) and cellobiases (CB). During cellulose hydrolysis, EG randomly digests the β-1,4-glycosidic bonds in cellulose to produce new chain ends. CBH then acts on the exposed chain ends to produce cellobiose. Finally, CB breaks the cellobiose down into monosaccharides.

Studies worldwide have focused on both expressing cellulase in crops through genetic engineering and on identifying cellulases with high activity. Typically, heterologous proteins comprise part of the total soluble proteins in plants. These proteins are stable in fresh or dry transgenic crops biomass. Heterologous proteins are also extractable and play a role in the production of fermentable monosaccharides from pretreated biomass. More importantly, the total soluble proteins extracted from fresh or dried transgenic plants can be used to produce biofuels in ethanol fermentation facilities (Oraby et al., 2007; Ransom et al., 2007). The production of cellulase in transgenic plants might greatly reduce the cost of ethanol production, as the process would require the addition of fewer enzymes during the fermentative production of ethanol than is required for microorganism-based systems (Sticklen 2006; Wang et al., 2009). The expression of cellulase in specific regions of plant cells can significantly improve the cellulase content, leading to the production of correctly folded, glycosylated cellulase with higher activity levels than cellulase expressed in the cytoplasm. This reduces hydrolysis and avoids the adverse effects of exogenous cellulase expression on the cellulose of plant cell walls (Ziegler et al., 2000; Ziegelhoffer et al., 2001; Hood et al., 2007; Kim et al., 2010).

Most commercially available genetically modified plants are screened using antibiotic

markers or anti-herbicide marker genes. Most of these marker genes are isolated from microorganisms and are heterologous to plants. Therefore, transgenic plants that are resistant to antibiotics or herbicides may possibly be hazardous to human health and/or the environment (Sugita et al., 1999; Daniell et al., 2001). Methods have recently been developed to eliminate resistance marker genes, such as co-transformation, site-specific recombination systems, and the use of transposons or homologous recombination. These techniques provide the technical foundation for the efficient expression of exogenous genes without producing potentially harmful transgenic material.

To date, few studies have been reported on the accumulation of cellulase in the plastids of transgenic rice plants. In the present study, we isolated an endo-1,4-β-glucanase V gene from *T. reesei* and inserted it into a double T-DNA expression vector, which we then transformed into rice. The accumulation and activity of cellulase were detected in fresh leaves and dry straw of transgenic plants without the use of selectable marker genes. The results presented in this study provide a basis for breeding transgenic rice expressing cellulase, which can be used in the production of biofuel from rice straw.

MATERIAL AND METHODS

Bacterial strains

The following bacterial strains were used in this study: 1) *Escherichia coli*: *DH5α* (from Key Laboratory of Crop Biology, Anhui) and Top10 (from the National Crop Molecular Design Center, Beijing); 2) *Agrobacterium tumefaciens* AGL0 (from the National Crop Molecular Design Center, Beijing); and 3) *T. reesei*: KT-TP007 (obtained from fermented solid waste at the Beijing Nangong Solid Waste Treatment Plant and provided by the Chinese Academy of Sciences, Beijing).

Gene cloning

Total RNA was extracted from *T. reesei* using Trizol (Invitrogen, USA). First-strand cDNAs were synthesized from 1 μg total RNA using a Promega kit. PCR amplification was performed using the primers EGV-F and EGV-R, and the products were subcloned into pMD18-T and sequenced. The sequencing results were compared with *EGV* sequences in GenBank.

Vector construction and generation of transgenic rice plants

Using the *EGV* gene template, HA-EGV-F and EGV-R primers were used to add the HA-tag sequence to the 5' end of *EGV*. The HA-EGV fragment, under the control of a maize ubiquitin promoter, was inserted into a pDTB vector that had been digested with PstI and AflII. The plastid signal peptide Apo-SP was synthesized using the Apo-Sp1, Apo-SP2, Apo-SP3, Apo-SP4, Apo-SP5, Apo-SP6, and Apo-Sp7 primers (Chen et al., 2004). The Apo-SP fragment was inserted upstream of *EGV* in the pDTB-HA-EGV vector after digestion with PstI and NcoI contained in the primers. Primer sequences are listed in **Table S1**.

The construct pDTB-Apo-SP-HA-EGV was introduced into *A. tumefaciens* strain Agl0 by the freeze-thaw method. Positive clones were confirmed by PCR and transformed into rice *Oryza japonica* cv. Zhonghua 11 as previously described (Hiei et al., 1994).

Subcellular localization

The transient expression vector pCAMBIA1301-Apo-SP-AsRed was constructed to confirm the effect of the signal peptide Apo-SP. The plasmid pCAMBIA1301-Apo-SP-AsRed or the control pCAMBIA1301-AsRed were introduced into onion epidermal cells by particle bombardment with a PDS-1000/He system (Bio-Rad, USA) using a helium pressure of 1100 psi (Dai et al., 2007). After bombardment, the transformed cells were incubated on 1/2 MS agar medium in the dark for 24-48 h at 24°C. The red fluorescent protein (AsRed) signal was detected under a fluorescence microscope (Olympus, Japan).

Selection of positive transgenic lines without selectable marker genes

Total DNA was extracted from the leaves of T_0 transgenic rice plants with the hexadecyl trimethylammonium bromide (CTAB) method. Using the DNA as a template, PCR was performed using two pairs of primers: EGV-F and EGV-R to amplify *EGV*, and Bar-test-F and Bar-test-R to amplify the *Bar* screening marker. Positive transgenic plants contained both *EGV* and *Bar* genes. PCR was performed using the following conditions: 94°C for 5 min, followed by 34 cycles of 94°C for 1 min, 60°C for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 10 min. The PCR products were analyzed on a 1% agarose gel.

Southern blotting analysis

For Southern blotting, the DNA of PCR-positive transgenic plants was digested overnight with *Eco*RI, separated on a 0.8% agarose gel, and transferred onto a Hybond-N+ nylon membrane (Amersham Pharmacia, UK). The membrane was probed with a digoxigenin (DIG)-labeled *Bar* selectable marker gene according to the manufacturer instructions (Roche, Germany).

Western blotting analysis

For western blotting, dry straw of transgenic rice was pulverized with a grinder and passed through a 60-mesh sieve. A total of 100 mg powder from each sample was evenly mixed in 400 µL citrate buffer, incubated for 10 min and centrifuged at 12,000 rpm for 10 min. The concentration of protein in the supernatant was measured using the Bradford method. One mg protein per sample was subjected to SDS-PAGE. The DNA was then transferred to a nitrocellulose membrane (Whatman) at 5V for 90 min. The membrane was sealed in TBST-prepared blocking buffer containing 0.5% BSA at 4°C overnight, and washed with TBST. It was then incubated at 4°C overnight in blocking buffer containing 0.01-0.05 µg/mL primary antibody (monoclonal anti-HA tag clone HA-7 isotype: mouse IgG1, Sigma), washed with TBST, and incubated in blocking buffer containing 0.01-0.05 µg/mL secondary antibody (alkaline phosphatase-rabbit anti-mouse IgG, Sigma). The membrane was washed with TBST and developed in NBT/BCIP (Furler et al., 2001).

Determination of Klason lignin and cellulose contents in rice straw

The lignin content was analyzed as acid-insoluble Klason lignin according to the Chinese National Standards GB/T 747-2003. Cellulose content was determined by the nitric acid-ethanol method (Shi and He, 2003).

Analysis of cellulase activity

To prepare cellulase plates, 1% CMC (carboxymethyl cellulose, cellulase substrates) and 0.8% agar were added to citrate buffer (pH = 4.4), sterilized at 121°C for 20 min, cooled and poured into petri dishes, holes were made with a puncher after polymerization, and the plates were stored at 4°C until use.

Proteins were extracted from dry straw and fresh leaves as described above, and 100 µL plant protein extract was added to each well of a cellulase plate. The plates were sealed and incubated at 37°C for 8 h, stained with Congo red staining solution for 10 min, rinsed and decolorized in 1 M NaCl solution for 10 min (Shimizu et al., 2002).

RESULTS

Cloning and construction of the *EGV* expression vector

Total RNA was extracted from *T. reesei* and the full-length *EGV* cDNA was synthesized by RT-PCR. The cDNA fragment containing the complete ORF was obtained with specific gene-primers designed using the sequence of *EGV*. Sequencing confirmed that this fragment contained a complete ORF of 729 bp encoding a 242-aa protein with a molecular weight of 24.4 kD. Sequence alignment indicated that this sequence shared 100% identity with the endo-1, 4-beta-glucanase gene (*EGV*), with a base mutation of T to A at base 137 (accession No. CAA83846.1) (Figure 1).

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1       ATGAAGGCAACTCTGGTTCTCGGCTCCCTCATTGTAGGCGCCGTTTCCGCGTACAAGGCC
1        M  K  A  T  L  V  L  G  S  L  I  V  G  A  V  S  A  Y  K  A

61      ACCACCACGCGCTACTACGATGGGCAGGAGGGTGCTTGCGGATGCGGCTCGAGCTCCGGC
21       T  T  T  R  Y  Y  D  G  Q  E  G  A  C  G  C  G  S  S  S  G

121     GCATTCCCGTGGCAGCACGGCATCGGCAACGGAGTCTACACGGCTGCCGGCTCCCAGGCT
41       A  F  P  W  Q  H  G  I  G  N  G  V  Y  T  A  A  G  S  Q  A

181     CTCTTCGACACGGCCGGAGCTTCATGGTGCGGCGCCGGCTGCGGTAAATGCTACCAGCTC
61       L  F  D  T  A  G  A  S  W  C  G  A  G  C  G  K  C  Y  Q  L

241     ACCTCGACGGGCCAGGCGCCCTGCTCCAGCTGCGGCACGGGCGGTGCTGCTGGCCAGAGC
81       T  S  T  G  Q  A  P  C  S  S  C  G  T  G  G  A  A  G  Q  S

301     ATCATCGTCATGGTGACCAACCTGTGCCCGAACAATGGGAACGCGCAGTGGTGCCCGGTG
101      I  I  V  M  V  T  N  L  C  P  N  N  G  N  A  Q  W  C  P  V

361     GTCGGCGGCACCAACCAATACGGCTACAGCTACCATTTCGACATCATGGCGCAGAACGAG
121      V  G  G  T  N  Q  Y  G  Y  S  Y  H  F  D  I  M  A  Q  N  E

421     ATCTTTGGAGACAATGTCGTCGTCGACTTTGAGCCCATTGCTTGCCCCGGGCAGGCTGCC
141      I  F  G  D  N  V  V  V  D  F  E  P  I  A  C  P  G  Q  A  A

481     TCTGACTGGGGGACGTGCCTCTGCGTGGGACAGCAAGAGACGGATCCCACGCCCGTCCTC
161      S  D  W  G  T  C  L  C  V  G  Q  Q  E  T  D  P  T  P  V  L

541     GGCAACGACACGGGCTCAACTCCTCCCGGGAGCTCGCCGCCAGCGACATCGTCGAGTCCG
181      G  N  D  T  G  S  T  P  P  G  S  S  P  P  A  T  S  S  S  P

601     CCGTCTGGCGGCGGCCAGCAGACGCTCTATGGCCAGTGTGGAGGTGCCGGCTGGACGGGA
201      P  S  G  G  G  Q  Q  T  L  Y  G  Q  C  G  G  A  G  W  T  G

661     CCTACGACGTGCCAGGCCCCAGGGACCTGCAAGGTTCAGAACCAGTGGTACTCCCAGTGT
221      P  T  T  C  Q  A  P  G  T  C  K  V  Q  N  Q  W  Y  S  Q  C

721     CTTCCTTGA
241      L  P  *
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Figure 1. *EGV* nucleotide sequence and the deduced amino acid sequence.

Subsequently, *EGV* was subcloned into the pDTB double T-DNA vector to construct an overexpression vector controlled by a signal peptide Apo-SP fragment.

Transient expression of signal peptide Apo-SP in onion epidermal cells

The fragment encoding the signal peptide Apo-SP was inserted upstream of AsRed to obtain an Apo-SP and AsRed fusion protein under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 2F). AsRed controlled by the 35S promoter was used as a control (Figure 2E). Constructs were transformed into onion epidermal cells and observed under a fluorescence microscope. Cells transiently transformed with the control vector that lacked the signal peptide exhibited a diffuse fluorescent signal; there was more intense red fluorescent signal in the nucleus than in the cytoplasm, and the signal in the cytoplasm was diffuse (Figure 2A, B). However, in cells transiently expressing the expression vector containing the signal peptide, the signal was clearly concentrated in the plastids, with less red fluorescent signal in the cytoplasm outside the plastids or in the nucleus (Figure 2C, D). These results indicate that the signal peptide Apo-SP could transfer peptides to plastids in onion epidermal cells.

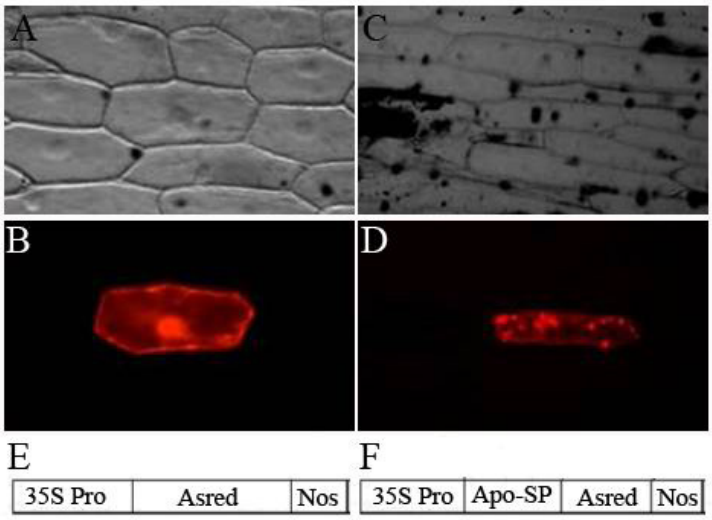

Figure 2. Transient expression in onion epidermal cells of red fluorescent protein (AsRed) under the controlof the Apo-SP signal peptide. The transient expression vector with the signal peptide produced signals throughout the entire cell; the signals wereparticularly concentrated inside the plastids (**C**, **D**, and **F**). The control vector without the signal peptide showed diffuse distribution of signals throughout the cell, with a slightly more intense signal in the nucleus (**A**, **B** and **E**).

Generation of transgenic rice without a selectable marker gene

The construct pDTB-Apo-SP-HA-EGV (Figure 3A) was transformed into Zhong hua 11 rice by *Agrobacterium*-mediated transformation. We obtained 97 phosphinothricin (PPT)-resistant rice seedlings of T_0 generation plants. After PCR confirmation, 78 positive transgenic plants were obtained that contained both the *EGV* and *bar* genes (Figure 3B). PCR-positive transgenic plants were further confirmed by Southern blotting using digoxigenin (DIG)-labeled *Bar* as a probe. The *bar*-positive plants contained 1-4 copies of T-DNA (Figure 3C). To screen *EGV* transgenic plants

without the selectable marker gene, nine transgenic T_0 rice plants containing the *Bar* selectable marker and *EGV* were subjected to further analysis. A total of 30 T1 generation plants from each transgenic line was screened by PCR using primer pairs for EGV-F/R and for Bar-test-F/R (Figure 4). From this screen, we identified 27 *EGV* overexpression transgenic plants lacking the selectable marker gene.

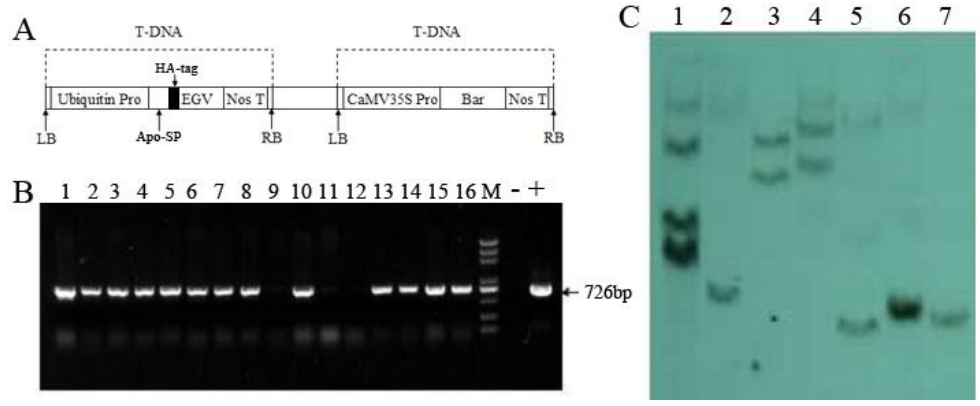

Figure 3. Molecular characterization of *EGV* transgenic plants. **A.** Double T-DNA construct of *EGV* for rice transformation. **B.** PCR confirmation of positive transgenic plants containingboth the *EGV* and *bar* genes. *Lane M* = Trans2K Plus DNA marker, (+) positive control; (-) negative control. **C.** Southern blot analysis of transgenic plants.

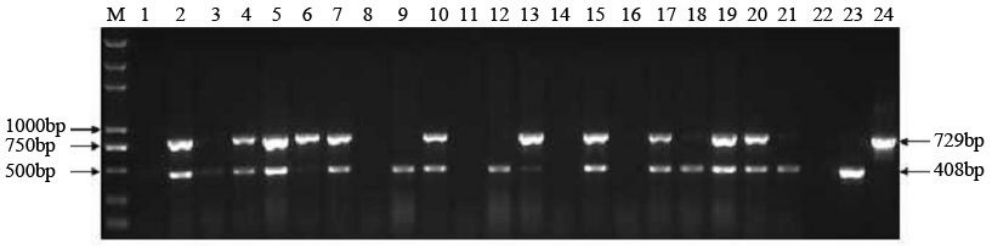

Figure 4. Screening of *EGV* T_1 transgenic plants without the*Bar* selectable marker. *Lane M* = Trans2K Plus DNA Marker. *Lanes 1-21* = transgenic plants. *Lane 22* = wild-type plants. *Lane 23* = positive control for the *bar* gene (plasmid DNA). *Lane 23* = positive control for the *EGV* gene (plasmid DNA).

Effects of *EGV* overexpression on lignin and cellulose contents

To examine the effects of *EGV* overexpression in rice, the Klason lignin content was measured in straw of wild type (WT) and transgenic plants. Compared to WT plants, the cellulose contents of transgenic plants showed a non-significant reduction. The average Klason lignin content of the transgenic plants was 10.35%, while the WT plants contained 13.34% Klason lignin. The largest reduction in Klason lignin content was detected in the L1-1 line (9.94%).

Cellulose contents were also compared between WT and transgenic plants. In contrast to the Klason lignin content, the cellulose content of transgenic plants was significantly higher than in WT plants. The cellulose content of transgenic plants ranged from 45.32 to 49.73%, while

the WT plants contained only 38.4%. Interestingly, transgenic line L1-1 had the highest cellulose content among the transgenic plants, suggesting that cellulose biosynthesis is regulated by the downregulation of ZmCoA in transgenic plants. Indeed, the downregulation of CCoAOMT in maize led to a 22.4% decrease in Klason lignin content and a 23.3% increase in cellulose content compared to WT plants. Based on these results, we conclude that lignin and cellulose, important cell wall components in plants, may be regulated in a compensatory fashion. This conclusion is consistent with previous reports (Hu et al., 1999).

Analysis of cellulase activity in transgenic plants

Crude proteins were extracted from dry straw of T_0 generation plants and fresh leaves of T_1 generation plants. Western blotting was performed using proteins extracted from WT plants as the negative control. The analysis showed that EGV was expressed in the tissues of T_1 generation transgenic rice (Figure 5, bands 5-7). More importantly, EGV showed high accumulation in the dry straw of T_0 generation transgenic rice that had been dried at room temperature for over three months (Figure 5, bands 2-4).

The crude protein extracts from dried straw and fresh leaves of transgenic rice plants were also subjected to enzyme activity plate analysis (Figure 6A, B). The results showed that the EGV protein produced in the T_1 generation transgenic plants folded correctly, and was capable of digesting the cellulase substrate CMC under the appropriate conditions, while the negative control did not digest the substrate. More importantly, cellulase activity remained stable in dry straw of T_0 generation plants that had been stored at room temperature for more than three months.

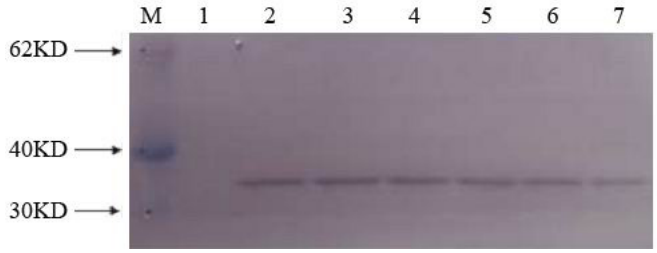

Figure 5. Western blotting of EGV transgenic plants. *Lane M* = Blue Plus Pre-stained Protein Marker. *Lane 1* = Fresh leaves of wild-type plants. *Lanes 2-4* = Dry straw of T_0 generation *EGV* transgenic rice. *Lanes 5-7* = Fresh leaves of T_1 generation *EGV* transgenic rice.

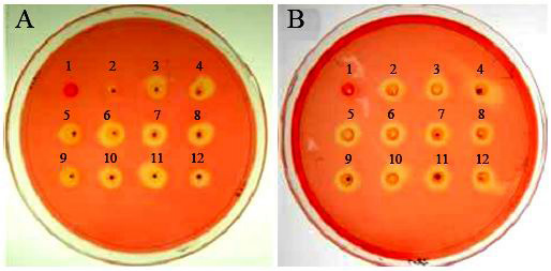

Figure 6. Cellulase activity of *EGV* transgenic rice. **A.** Cellulase activity of dry straw of T_0 generation of *EGV* transgenic rice after drying at room temperature for three months. **B.** Cellulase activity of fresh leaves of T_1 generation of *EGV* transgenic rice. *Lane 1* = negative control. *Lanes 2-12* = independent transgenic lines.

DISCUSSION

There is increasing interest in the use of lignocellulose in bioethanol production in place of grain starch. The critical factors restricting the commercialization of lignocellulose are the need for cellulase and the high cost of this product. Attempts have recently been made to produce cellulase using transgenic plants as bioreactors instead of adding microbial enzymes to plants. There are three problems with producing and using the cellulase in crops to produce biofuels: 1) cellulase may not accumulate in high enough quantities, and its activity may not be maintained after harvesting and storage; 2) the overexpression of cellulase in plants may harm plant growth and development; and 3) the production of transgenic plants is potentially harmful to the environment.

Previous studies have shown that the appropriate cellular location is required for the folding and accumulation of exogenous proteins. The best way to increase the level of exogenous proteins is to directly express them in specific cellular regions rather than in the cytosol (Ziegelhoffer et al., 2001). In this study, we directly expressed cellulase in plastids using plastid signal peptides to localize the protein. The *EGV* transgenic plants had high levels of EGV accumulation and activity after the straw was stored for three months at room temperature. Expression of protein in the plastids rather than in the cytoplasm may reduce the exposure of the protein to degradation by cellular enzymes and improve its accumulation levels. In addition, when an exogenous protein is expressed in a specific cellular region, protein activity increases due to correct folding, as more molecular chaperones are contacted by the protein (Horn et al., 2004; Sticklen, 2006). We determined that it was feasible to express cellulase in rice plastids using transgenic technology, resulting in the accumulation of active cellulase.

No significant difference was observed in growth and development between the *EGV* transgenic rice and WT plants (data not shown). In addition, there were no significant differences in cellulose and lignin contents between rice straw of *EGV* transgenic rice and WT plants, suggesting that the growth of *EGV* transgenic plants was not significantly affected by the overexpression of this gene. We conclude that directly expressing *EGV* in a specific cellular region may prevent this exogenous hydrolase from affecting cytoplasmic metabolism; by keeping the cellulose degrading enzyme away from the cell wall, potential damage to the plant is avoided. Plant cellulose normally exists in crystalline form. Cellulose closely binds with lignin and is therefore not prone to hydrolysis by cellulase. Additionally, the activity of EGV from *T. reesei* may be inhibited during plant growth, since the optimal temperature for the activity of this enzyme is high (Ziegler et al., 2000).

We also used a double T-DNA expression vector to eliminate the selectable marker gene in *EGV* transgenic plants. With this vector, double T-DNA fragments were independently inserted into different rice chromosome sites and separated during meiosis. The frequency of selecting transgenic plants containing only the target gene without the selectable marker in the T_1 generation was 9.28%. Such plants did not have selectable markers that could be transmitted to microbes or wild allied species, thus reducing environmental risks and improving the ecological effects of transgenic plants in the environment.

In this study, we examined the effect of expressing the *T. reesei EGV* gene in transgenic rice plastids using genetic engineering technology and provided a basis for the molecular breeding of rice straw for cellulose biofuel conversion. Further studies should be conducted on the effects of transforming multiple cellulose degrading genes in crops. In particular, the effects of these genes on crop growth and development should be examined, as well as the efficiency and cost of fermentative degradation of cellulose in transgenic rice straw.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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