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RESEARCH ARTICLE

Developing transgenic maize (*Zea mays* L.) with insect resistance and glyphosate tolerance by fusion gene transformation

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Abstract

Using linker peptide LP4/2A for multiple gene transformation is considered to be an effective method to stack or pyramid several traits in plants. *Bacillus thuringiensis* (Bt) *cry* gene and *epsps* (5-enolpyruvylshikimate-3-phosphate synthase) gene are two important genes for culturing pest-resistant and glyphosate-tolerant crops. We used linker peptide LP4/2A to connect the Bt *cry1Ah* gene with the *2mG2-epsps* gene and combined the wide-used *manA* gene as a selective marker to construct one coordinated expression vector called p2EPUHLAGN. The expression vector was transferred into maize by *Agrobacterium tumefaciens*-mediated transformation, and 60 plants were obtained, 40% of which were positive transformants. Molecular detection demonstrated that the two genes in the fusion vector were expressed simultaneously and spliced correctly in translation processing; meanwhile bioassay detection proved the transgenic maize had preferable pest resistance and glyphosate tolerance. Therefore, linker peptide LP4/2A provided a simple and reliable strategy for producing gene stacking in maize and the result showed that the fusion gene transformation system of LP4/2A was feasible in monocot plants.

Keywords: LP4/2A, gene stacking, transgenic maize, insect resistance, glyphosate tolerance

1. Introduction

According to International Service for the Acquisition of Agri-Biotech Applications (ISAAA) statistics, global biotech crop coverage has reached 170.3 million ha, and 26% of these crops contain stacked traits with two or three genes resulting from gene stacking or pyramiding (James 2012). These data indicate that hybrid gene products will become increasingly significant for biotech crop development. Due to gathering multiple genes' excellent characteristics simultaneously in hybrid gene products, the advantages of 'stacking' or 'pyramiding' traits in crops are obvious. It is known that a few different multi-transgene-stacking methods are available for co-expressing multiple transgenes in plants (Halpin 2005). Compared with crossing and co-transformation, using linker peptides to transform multiple genes is considered as a better method for various reasons. The peptides included 2A (Ryan *et al.* 1991), LP4 (Tailor *et al.* 1997), LP4/2A, the internal ribosome entry sites (IRESs) (Chappell *et al.* 2000), NIa protease (Liang *et al.* 2005), and native protease (Zhang *et al.* 2011).

Linker 2A is a peptide from the foot-and-mouth disease virus (FMDV), and it has the property to cleave itself (Ryan *et al.* 1991; de Felipe *et al.* 2006). LP4 is a peptide from

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Raphanus sativus seeds. It has a recognition site and is cleaved by a protease (Tailor *et al.* 1997). LP4/2A is a hybrid peptide that contains the first 9 amino acids of LP4 and 20 amino acids from 2A. The cleavage site of the LP4/2A sequence includes both the enzyme-digested positions of the LP4 peptides and the self-cleaving position of 2A. The LP4/2A sequence possesses one more cleavage site than either the 2A sequence or the LP4 sequence alone, so the excess amino acid residues from the mature protein can be removed to avoid influencing the protein activity. In addition, removing the 2A sequence will reduce the risk of biosafety considerations in transgenic plant. In 2004, Francois *et al.* (2004) connected genes DmAMP1 and RsAFP2 with LP4/2A and achieved higher antibacterial activity in *Arabidopsis* plants in comparison to genes linked with the LP4 peptide.

Maize is an important staple crop, and pests and weeds are main factors that reduce maize production. Developing stacked traits for insect resistance and herbicide tolerance in maize is becoming increasingly significant to improve the corn yield and alleviate the loss.

The Bt *cry* gene and *epsps* gene are two important genes for culturing pest-resistant and glyphosate-tolerant crops (Schnepf *et al.* 1998). Some Lepidopteran insects are highly susceptible to the Cry1A class of insecticidal proteins (Cry proteins or δ-endotoxins) from Bt; however, these proteins are not toxic to non-target insects, birds, or mammals (including humans) because of their high specificity for certain species of insects (Bravo *et al.* 2007; Pigott and Ellar 2007; Kim *et al.* 2009). *Cry1Ah* is a novel insecticidal gene cloned from the BT8 isolate. The Cry1Ah protein is highly toxic to Lepidopteran insects; this protein is more toxic than Cry1Ac to *Helicoverpa armigera*, *Ostrinia furnacalis* and *Chilo suppressalis*, and it is more toxic than Cry1Ab to *O. furnacalis* (Xue *et al.* 2008).

G2-epsps is a bacterial *epsps* gene isolated from glyphosate-contaminated soil. It is the isoenzyme of plant endogenous EPSP synthase, but conformational change reduces its affinity for glyphosate. Therefore, even if plant endogenous *epsps* activity is inhibited by glyphosate treatment, the activity of exogenous *epsps* in transgenic plant harboring *G2-epsps* gene can be maintained at normal levels to meet the plant's metabolic needs. *G2-epsps* was codon-optimized with monocotyledonous preferences and was designated as 2*mG*$_2$*-epsps*.

Phosphomannose isomerase (PMI) gene is a superior selectable marker gene for improving selection efficiency relative to antibiotic or herbicide selectable marker genes in plant transformation. The PMI/mannose selection system employs the PMI-expressing gene *manA* as the selectable marker and mannose as the selective agent (Joersbo *et al.* 1999). The PMI (*manA*) gene is present in most organisms and does not pose a risk to human or animal health, so the PMI/mannose selection system has been confirmed as a superior alternative to antibiotic selection for plant genetic engineering without bio-safety concerns (Stoykova and Stoeva-Popova 2011). The *manA* gene from *Escherichia coli* has been used to produce transgenic maize (Negrotto *et al.* 2000; Wang *et al.* 2000), cassava (Zhang *et al.* 2000), and sugar beet (Joersbo *et al.* 1998; Joersbo *et al.* 1999). Furthermore, a database search revealed no significant homology between the *E. coli manA* gene product and any known toxin or allergen.

In our prior study, Bt *cry1Ah* gene and *mG2-epsps* gene were connected by 2A or LP4/2A linker to construct four fusion expression vectors, and the vectors were transferred into tobacco. The detection data demonstrated that transgenic tobacco possessed good pest resistance and glyphosate tolerance, and plants with genes linked by the LP4/2A peptide showed better pest resistance and glyphosate tolerance than plants with 2A gene links (Sun *et al.* 2012). However, the function of LP4/2A in monocotyledonous plants is unknown, especially in staple crops, and it would be showing a great application prospect for culturing the stacking transgenic crops.

In this study, we used linker peptide LP4/2A to connect Bt *cry1Ah* with *2mG2-epsps* and combined *manA* as a selective marker gene to construct one coordinated expression vector called p2EPUHLAGN. Thus, once the fusion gene is spliced effectively to produce sole *cry1Ah* gene and *2mG2-epsps* gene, we could acquire transgenic maize with both pest resistance and glyphosate tolerance; furthermore this feasible gene-stacking strategy has potential to apply to other staple crops.

2. Results

2.1. Construction of LP4/2A linker expression cassettes and *Agrobacterium*-mediated transformation

The plant transformation construct p2EPUHLAGN is displayed in Fig. 1-A. The ubiquitin promoter drove the expression of the polyprotein, which included Bt *cry1Ah* gene and 2mG2-epsps gene connecting with LP4/2A linker, and the positive PMI selectable marker gene was driven by the enhanced cauliflower mosaic virus (CaMV) 35S promoter. Finally, 60 transformants were obtained *via Agrobacterium*-mediated transformation (Fig. 1-B).

2.2. Identification of transgenic maize plants

The regenerated maize plants were detected by PCR amplification for the presence of *cry1Ah* and 2*mG2-epsps* genes. PCR amplification produced the expected 900 bp fragment

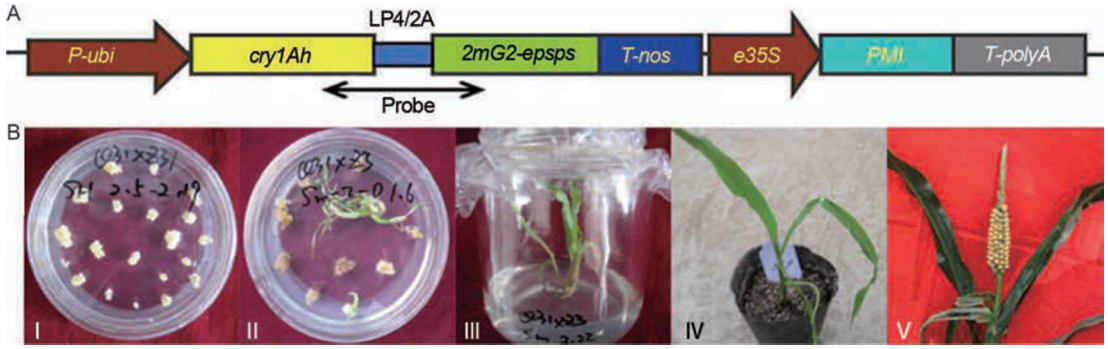

Fig. 1 A map of the expression vector and the regeneration of transgenic maize plants. A, a map of the expression vector. *P-ubi*, ubiquitin promoter; *cry1Ah*, Bt *cry1Ah* gene; *2mG2-epsps*, *2mG2-epsps* gene; *T-nos*, *nopaline synthase* gene terminator; *e35S*, enhanced cauliflower mosaic virus 35S promoter; *PMI*, *phosphomannose isomerase* gene; *T-polyA*, polyA terminator; LP4/2A, linker peptide LP4/2A; probe, a 900-bp fragment of the fusion gene was used as probe for Southern blotting. B, selection of resistant calli and regeneration of transgenic maize plants. I, selection of resistant calli; II, resistant shoot; III, resistant plant; IV, regenerated plant; V, T_0 transgenic maize seeds.

for the fusion gene from the transgenic lines, and plasmid DNA was used as a positive control. Transgene DNA was not detected in wild type plants. Approximately 40% (24 individuals) of the transformants contained the chimeric gene and the PCR detection result of some transformants was showed in Fig. 2-A.

The fusion gene integration was further confirmed by Southern blot analysis of independent transgenic lines. The result (Fig. 2-B) indicated that the transferred foreign fusion gene cassette was intact in the maize genome and that the transferred gene had integrated into a single site in the genome of transgenic maize plants. No cross-hybridizing band was observed in the wild type. Single copy plants were further analyzed.

2.3. LP4/2A sequence function following transcription

To determine the splicing period of the linker, the total RNA and protein were isolated and subjected to RT-PCR and Western blotting. The locations of RT-PCR primers are shown in Fig. 3-A. These results showed that the foreign fusion genes were transcriptionally expressed as intact mRNA in the transgenic maize plants (Fig. 3-B). The actin cDNA fragment was amplified as the intrinsic control (Fig. 3-C).

The simultaneous expressions of Cry1Ah and 2mG2-EPSPS were detected as discrete proteins from a single open reading frame, as driven by the ubiquitin promoter in the transgenic maize plants. When probed with antiserum raised against the Cry1Ah protein, the extracts from the transgenic maize plants showed a major immunoreactive band of approximately 70 kD (Fig. 3-D). Similarly, when probed with antiserum raised against $2mG_2$-EPSPS, the

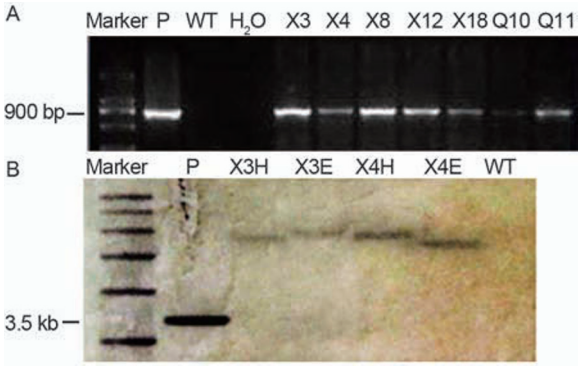

Fig. 2 PCR analysis of T_0 transgenic maize plants and Southern blot assay of independent events. A, PCR analysis of foreign fusion gene. Marker, 100 bp DNA ladder; P, p2EPUHLAGN positive control; WT, wild type plant; X3, X4, X8, X12, X18, Q10, and Q11 showed different events. B, Southern blot assay of independent events. Marker, 1 kb DNA ladder; P, fragment containing *cry1Ah* and *2mG$_2$-epsps* as positive control; X3H, event X3 digested by *Hin*dIII; X3E, event X3 digested by *Eco*RI; X4H, event X4 digested by *Hin*dIII; X4E, event X4 digested by *Eco*RI; WT, wild type maize plant.

transgenic maize plant extracts yielded a major band of 55 kD (Fig. 3-E). No band was detected in the wild type plant using Cry1Ah and 2mG2-EPSPS antibodies. An anti-β-actin antibody was used as a control (Fig. 3-F). These data indicate that the expected dissociation of LP4/2A-polyprotein into its component polypeptides occurred during translation. There was a small amount of uncleaved polyprotein present in some of the plants, as noted in the figures. About 10–50% of the target protein in transgenic maize plant was uncleaved polyprotein according to the band intensity measured by ImageJ software. The cleaved proteins from events X8 and Q11 are higher than those of the other events. The cleavage efficiency of events X8 and

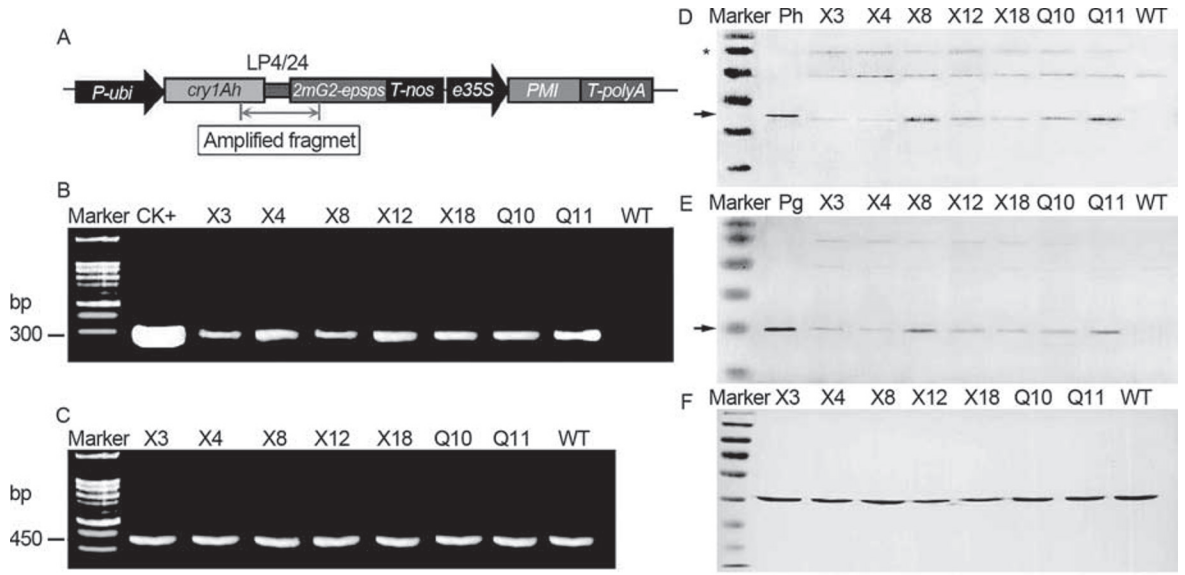

Fig. 3 RT-PCR and Western blot analysis of T_0 transgenic maize plants. A, the primers were designed according to the sequences of Bt *cry1Ah* and 2*mG2-epsps*, and the locations are shown. B, RT-PCR analysis for the mRNA expression of *cry1Ah* and 2*mG*$_2$-*epsps* in transgenic maize plants. Marker, 100 bp DNA ladder; WT, the cDNA of wild type maize plant as the template; X3, X4, X8, X12, X18, Q10, Q11, the template cDNA from different transgenic maize events; CK+, the p2EPUHLAGN plasmid as the template. C, the actin cDNA fragment was amplified with the primers ActinF and ActinR as the control. D, WB analysis of the cleavage efficiency of LP4/2A and the expression of Cry1Ah protein in transgenic plants using Cry1Ah antibody. E, WB analysis of the cleavage efficiency of LP4/2A and the expression of G2-EPSPS protein in transgenic maize plants using G2-EPSPS antibody. The asterisk denotes the full size of the polyprotein, and arrows indicate discrete proteins from polyproteins. F, an anti-β-actin antibody was used as a control. Marker, fermentas protein marker; Ph, Cry1Ah pure protein; Pg, G2-EPSPS pure protein; WT, protein from wild type maize plant.

Q11 is approximately 80–90%, and the cleavage efficiency of the other events is approximately 50–70% (Cleavage efficiency=Cleaved form/(Cleaved form+Uncleaved form)).

2.4. Analysis of foreign proteins levels

Parts of the PCR-positive plants were selected to analyze Bt Cry1Ah and 2mG2-EPSPS protein expression by enzyme-linked immunosorbent assay (ELISA) (Fig. 4). Each test consisted of three replicates. The results demonstrated that each transformed line showed a different level of protein expression, meanwhile the cleaved protein levels for events X8 and Q11 were higher than that of the other events, indicating that the amount of expressed protein was dependent upon the location of the gene insertion. The level of protein expression is in accordance with the protein cleavage efficiency, according to the results of the Western blot. These results demonstrated that Cry1Ah protein was expressed at the same level as that of 2mG2-EPSPS when placed upstream of the LP4/2A sequence, which means that the genes linked by LP4/2A were synergistically expressed.

2.5. Insect resistance to transgenic maize plants

To determine the function of Cry1Ah in maize resistance to Asian corn borers, wild type and transgenic plants were reared with Asia corn borer larvae. It was clear that the leaf, stem and tassel of wild type were eaten terribly in test, but the transgenic lines were intact and healthy with only a few tiny holes in leaf surface (Fig. 5). As shown in Table 1, the resistance grade was different among the events. Events X8 and Q11 were more resistant to Asian corn borers than other events. The reason of the high resistance of events X8 and Q11 to Asian corn borers is their higher protein expression levels, according to the Western blot and ELISA results.

2.6. Glyphosate tolerance in T_1 generation transgenic maize plants

To measure glyphosate tolerance, transgenic maize seeds were grown in greenhouse and sprayed with Roundup. From the results of the pre-experiment, 0.1% Roundup was selected to test the glyphosate tolerance of the plants (data not shown). The wild type plants experienced severe vegetative damage and died in 2 wk of being sprayed at the lowest tested dose (0.05%). The transgenic events showed different herbicide tolerances and events X8 and Q11 were better than other transgenic events, which could be tolerant up to 0.1% glyphosate (Fig. 6).

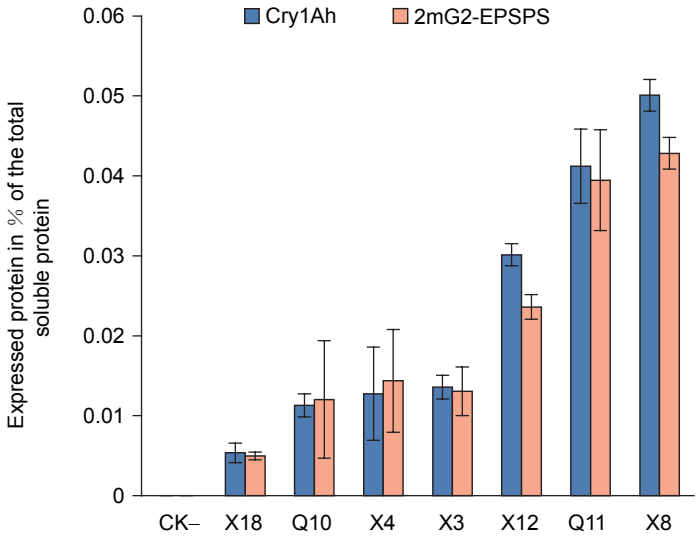

Fig. 4 Enzyme-linked immunosorbent assay of Cry1Ah and 2mG2-EPSPS expressions in different transgenic maize plants. CK, the wild type maize plant. X18, Q10, X4, X3, X12, Q11 and X8 represent different transgenic maize plants. The error bars represent the standard deviations of biological replicates from each transgenic event.

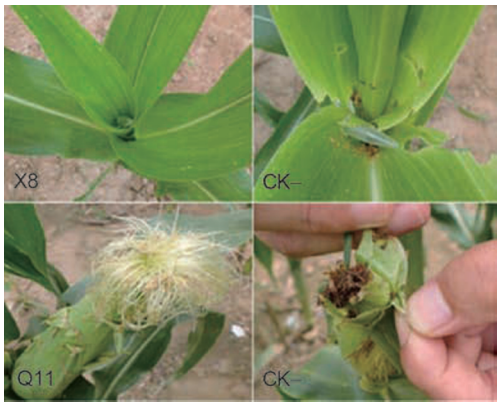

Fig. 5 Bioassay of T_1 transgenic maize plants after 2 wk of infestation. X8, the leaf appearance of transgenic event X8 after infestation; Q11, the silk appearance of transgenic event Q11 after infestation; CK–, the leaf and silk appearance of wild type plants after infestation.

3. Discussion

Because of the obvious advantages of gene-stacking, multiple gene transformation has become an area of intense study in biotechnology research, and multiple gene transformation is becoming more popular in commercial GM (genetic modified) crops. Multiple gene transformation is advantageous for combining several traits which are controlled by more than one gene. ''Golden rice'' is a well-known example of a genetically engineered plant in which three different genes in the carotenoid synthesis pathway were combined to obtain a new vitamin A-rice variety (Ye *et al.* 2000).

Table 1 Statistics of T_1 generationplant resistance to Asian corn borers

Name of events	Average resistance grade
X3	7.5
X4	8.3
X8	3.4
X12	5.7
X18	7.3
Q10	6.8
Q11	2.4
CK–	9.0

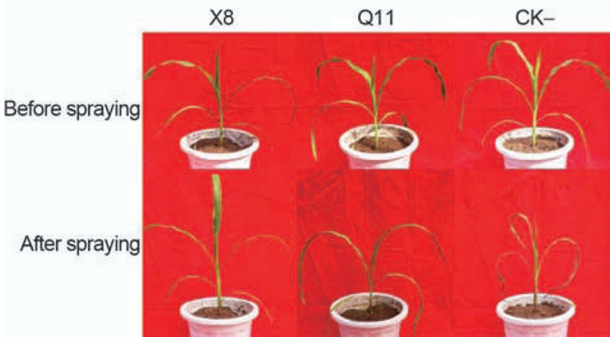

Fig. 6 The glyphosate tolerance of T_1 transgenic maize plants. X8, maize plant of transgenic event X8; Q11, maize plant of transgenic event Q11; CK–, wild type maize plant.

According to our previous experiment, comparing with linkers 2A and LP4, LP4/2A is a superior linker for acquiring gene stacking in tobacco plants (Sun *et al.* 2012). This study is a continuation of preceding results, and our aim was to confirm the function of LP4/2A in monocot plant and

to generate transgenic maize with insect resistance and glyphosate tolerance. In this study, the p2EPUHLAGN fusion gene plant transformation vectors containing the *2mG2-epsps* gene and the *cry1Ah* gene, linked by LP4/2A, were introduced into Q31×Z3 maize *via Agrobacterium*-mediated transformation, with mannose as a selection agent. The construct was successfully transformed into maize plants and showed good insect resistance and glyphosate tolerance.

The RT-PCR analysis showed that the mRNAs were intact during transcription. This finding indicates that cleavage occurred at the translation level, which is consistent with RT-PCR results in tobacco (Sun *et al.* 2012) and the cleavage mechanism of the linker (Francois *et al.* 2004).

Western blot and ELISA results further confirmed that the fusion genes were simultaneously expressed in these maize plants. The results of the Western blot showed that the linker peptide system played a very significant role, but the cleavage was not complete. In 2004, Francois *et al.* (2004) used LP4/2A to fuse the anti-bacterial gene sequences RsAFP2 and DmAMP1 and integrated them into the *Arabidopsis thaliana* genome; the cleavage efficiency of LP4/2A was improved significantly relative to LP4 alone. Our Western blot results showed that the LP4/2A linker peptide system played a substantial role and the cleavage efficiency was high (50–90%). The cleaved proteins from events X8 and Q11 were higher than those of other events. The cleavage efficiency of events X8 and Q11 was approximately 80–90%, and the cleavage efficiency of other events was approximately 50–70%. The ELISA results demonstrated that the two genes in the fusion vector were coordinately expressed which is consistent with the ELISA results in tobacco (Sun *et al.* 2012). The Cry1Ah protein placed upstream of the LP4/2A sequence was expressed at the same level as that of 2mG2-EPSPS, which meant that the genes linked by LP4/2A were synergistically expressed, thereby providing an advantage for multiple gene transformation. Each transformed event showed a different level of protein expression. The highest level of Cry1Ah expression was 0.50 μg mg^{-1} of total soluble protein, and the lowest level of Cry1Ah expression was 0.05 μg mg^{-1} of total soluble protein. The expression level of Cry1Ah protein in fusion gene transformation system was similar with those in single gene expression (Yue *et al.* 2010; Li *et al.* 2014). The cleaved protein levels of events X8 and Q11 were higher than those of other events, indicating that the amount of expressed protein was dependent on the location of the gene insertion. The protein expression level is in accordance with the protein cleavage efficiency, according to the Western blot results.

The bioassay results showed that transgenic maize had insect resistance and glyphosate tolerance. Events X8 and Q11 were highly resistant to Asian corn borers, but other events ranked a higher resistance grade, which meant their resistance to Asian corn borers was not sufficient. According to the Western blots and ELISA, the cause of the high resistance of events X8 and Q11 to Asian corn borers is their higher protein expression levels. Additionally, transgenic maize could tolerate up to 0.1% Roundup; however, the wild type plants died after being sprayed with 0.05% Roundup.

Our results also clearly showed that the PMI (*manA*) gene could be used as an efficient selectable marker gene, replacing of the antibiotic resistant markers in maize transformation, to acquire high transformation efficiency. In our study, 24 transgenic plants were obtained with positive frequencies of up to 40% when a combination of 10 g L^{-1} mannose and 20 g L^{-1} sucrose was used in the selection medium.

4. Conclusion

We verified the function of the LP4/2A linker in monocot plant. The linker peptide LP4/2A provided a more versatile and simple strategy for producing gene stacking in monocot plant and it allows for coordinated expression from a single promoter. We developed transgenic maize plants that not only had pest resistance but also were tolerant to glyphosate, which was achieved by using fusion gene transformation and a PMI/mannose selection system. However, there was one little imperfection: We did not create a highly glyphosate-tolerant maize plant. Although the transgenic maize plant exhibited tolerance to glyphosate in comparison with wild type plants, the transgenic maize did not reach the field standard (tolerant to 0.2% Roundup). This deficiency may have occurred because our number of transformation individuals was small, so it was difficult to select an event with both high protein expression levels and high glyphosate tolerance.

Many aspects contributed to influence the foreign gene expression in transgenic plants, such as the gene's codon preference (Perlak *et al.* 1991), regulating element, gene subcellular location, transgenic plants population size, etc. Modifying *cry1Ah* gene codon preference and chloroplast-targeted gene expression have significantly increased protein expression levels (Li *et al.* 2013). In the future, we will combine the linker and subcellular-targeted strategy to generate more gene-stacking crops with high protein levels to meet commercial demands.

Overall, the findings of the present study confirmed that LP4/2A could work well as a linker in monocot plant. We developed transgenic maize with both pest resistance and glyphosate tolerance, and this promising gene-stacking strategy could be applied to other staple crops.

5. Materials and methods

5.1. Plasmid construction

The coding sequence of *cry1Ah* (containing part of LP4/2A) was amplified using the primers 1AhF (5′-CATGCCATGGGAAAGAACAGCATCAAACT-3′) and 1AhR (5′-GGTAGCCACCTCGTCCGCCGCGTTGGAATCAATGTGGTAGTCAGTGACATTG-3′) from plasmid pSAh (Sun *et al.* 2012). The coding sequence of *2mG2-epsps* (containing part of 2A) was amplified using the primers LP42A2mG2F (5′-CCGCTCGAGTCCAACGCGGCGGACGAG-3′) and LP42A2mG2R (5′-AACTGCAGCCATAGGCCCAGGGTTGGACTCGA-3′) from plasmid pSmG2 (Sun *et al.* 2012). The *cry1Ah* fragments were cloned into a T vector (TaKaRa, Japan) to form pTcry1Ah and the *2mG2-epsps* fragments were cloned into the pTcry1Ah vector to form pHLAG. The pHLAG sequence was digested with *Nco*I and *Kpn*I and cloned into the corresponding restriction sites of plasmid pUAh (Yue *et al.* 2010) to form pUHLAGN. The coding sequence of *pmi* was amplified using the primers PMIF (5′-CATGCCATGGTTCCACATTAAAACAGGGATTGAT-3′) and PMIR (5′-CCGCTCGAGCTTAGCAAGAGATGTTAATTTTTTCA-3′) from *E. coli* genomic DNA. The *pmi* fragments were cloned into a T vector (TaKaRa, Japan) to form pTpmi. The authenticity of the sequence was confirmed by sequencing. The *Eco*RI-*Nco*I fragment of the pE35S2mG2 plasmid, which was deposited in the laboratory, was cloned into the corresponding restriction sites of the pTpmi plasmid to yield pEP. The *Xba*I-*Eco*RI fragment of plasmid pEP was cloned into the corresponding restriction sites of plasmid pCAMBIA2300 to form p2EP. The pUHLAGN sequence was digested with *Eco*RI and *Hin*dIII and cloned into the corresponding restriction sites of digested p2EP plasmid to yield p2EPUHLAGN.

5.2. Plant transformation and regeneration

The maize ear of 10 DAP (days after pollination) of hybrid line Q31×Z3 was harvested and the immature embryo was excised and placed on N6 induce medium (Chu 1975). The *Agrobacterium tumefaciens* strain LBA4404 was transformed with p2EPUHLAGN and used to infect maize calli. The inoculated calli were placed on N6 plates and cultured at 20°C for 3 d, then transferred to N6 recovery medium and cultured at 26–28°C in the dark for 7 d. Then, the calli were transferred to N6 selection medium containing 10 g L^{-1} mannose and 20 g L^{-1} sucrose. Resistant colonies were isolated 5 to 6 wk later. Transformant plants were regenerated by transferring embryogenic callus tissue to N6 medium containing 2 mg L^{-1} 6-benzylaminopurine. The shoots that developed after 2 to 4 wk on this medium were transferred to 1/2 MS (Murashige and Skoog 1962) medium with 30 g L^{-1} sucrose and solidified with 3 g L^{-1} gel after rooting occurred. Regeneration was performed at 25°C under light (2 500 lux). After approximately 2 wk, the developing plantlets were transferred to soil and grown to maturity in the greenhouse.

5.3. DNA isolation and PCR

Genomic DNA was isolated from the leaves of maize plants using DNA Extraction Kit (Tiangen Biotech,China). PCR was performed by amplifying the transgene coding regions by using oligonucleotide primers HGF (5′-TCAACAACATCATTGCATCGG-3′) and HGR (5′-TGGTGTAACGGTCACGGTCT-3′), which lied in *cry1Ah* gene and *2mG2-epsps* gene coding region respectively. The standard routine for the PCR reactions was as follows: cycling began with an initial denaturation at 94°C for 5 min, then 32 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 50 s, followed by a final extension at 72°C for 10 min. The PCR products were detected by electrophoresis in 1% (w/v) agarose gels.

5.4. Reverse transcription-PCR

Total RNA was isolated from the leaves of transgenic maize plants and a non-transformed plant using Trizol solution. The quantity of extracted total RNA was determined spectrophotometrically, and 1 μg total RNA was used as a template in the reverse transcript reaction primed with oligo (dT)15 (Promega, USA). Reverse transcription PCR (RT-PCR) assays were performed according to the directions and materials supplied in the RT-PCR Kit (Invitrogen, USA). The primers used for amplifying cDNA were HGF2 (5′-GCCGTGAATGCCCTGTTTACCT-3′) and HGR2 (5′-CTCGTGGATGCTGCTGCGTCTT3′), which yielded a 300-bp product. The actin cDNA fragment was amplified with primers ActinF (5′-CACCTTCTACAACGAGCTCCG-3′) and ActinR (5′-TAATCAAGGGCAACGTAGGCA-3′) as the control.

5.5. Southern blot

To detect the integration of the fusion gene in the maize genome, a 900-bp fragment of the fusion gene was PCR amplified from p2EPUHLAGN using the primers HGF and HGR and used as probe in Southern blot analysis. 30 μg of each genomic DNA sample were digested with restriction enzyme *Eco*RI and *Hin*dIII and separated on a 0.8% (w/v) agarose gel. The gel was blotted to a Hybond$^+$ nylon membrane (GE Healthcare, USA) and hybridized with the probe. A Southern blot was carried out using a DIG-High

Prime DNA Labeling and Detection Starter Kit I (Roche, Germany) as directed by the manufacturer.

5.6. Protein detection in transgenic plants

A Western blot analysis and an ELISA (enzyme-linked immunosorbent assay) of the transgenic plant materials were performed. Leaves from 1-mon-old plants grown in the greenhouse were used for protein extraction. Plant material was ground in liquid nitrogen and dissolved in buffer (200 mmol L^{-1} Tris-HCl pH 8.0, 100 mmol L^{-1} NaCl, 400 mmol L^{-1} sucrose and 1 mmol L^{-1} PMSF; 14 mmol L^{-1} β-mercaptoethanol and 0.05% Tween-20 were added just before use), vortexed and centrifuged for 15 min at 12 000 r min^{-1}. The total protein levels were detected by Bradford assay (Bradford 1976). Protein samples (20 μg of soluble protein) were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore, USA). The primary antibodies (anti-Cry1Ah and anti-G_2-EPSPS) were used at 1/5 000 dilutions, and IgG-AP (Sigma, USA) was used at a 1/20 000 dilution. Detection was performed using the NPT/BCIP Kit (CWBIO Co., China) as described by the manufacturer. A Bt cry1Ab/1Ac ELISA Detect Kit (Envirologix, USA) was used to detect Cry1Ah protein. The 2mG2-EPSPS protein was detected using the following method. 100 μL of maize extract (about 100 μg solution protein) was added to each well of ELISA plate, and a coating buffer was added. The plate was then incubated at 4°C overnight. Each well was washed 3 times with 0.05% phosphate-buffered saline-Tween-20 (PBST) and incubated at 37°C for 2 h with antibody (anti-G2-EPSPS) at a 1/1 000 dilution in PBST+1% BSA. The wells were then washed three times with PBST, and a 1/10 000 dilution of IgG-HRP in PBST+1% BSA was added to each well and incubated for 1 h at 37°C. Enzyme activity was detected using the TMB Kit (CWBIO Co., China) as described by the manufacturer.

5.7. Insect bioassays

Asian corn borers were provided by the Institute of Plant Protection of the Chinese Academy of Agricultural Sciences. When the T_1 plants had 6–8 leaves, field insect resistance assays were performed. Newly hatched larvae were placed into the whorl of each plant. Each plant was infested with 40–60 corn borer neonatal larvae, and a wild type plant was used as the negative control. After two weeks of infestation, plant damage rank was determined according to the nine grading standards developed by the International Corn Borer Collaboration Team. The resistance level classifications were as follows:

Level 1, few pin holes (highly resistant); level 2, few shot holes on a few leaves (resistant); level 3, several shot holes on leaves (<50%)(resistant); level 4, several shot holes on leaves (>50%) or small lesions (<2 cm long) (moderately resistant); level 5, elongated lesions (>2 cm long) on a few leaves (moderately resistant); level 6, elongated lesions on several leaves (susceptible); level 7, several leaves with long lesions with leaf tattering (susceptible); level 8, several leaves with long lesions with severe leaf tattering (highly susceptible); level 9, plant dying due to death of growing points ('dead-hearts') (extensively sensitive to damage).

5.8. Glyphosate tolerance spray tests

Glyphosate tolerance spray tests were carried out when the T_1 plants had 5–6 leaves. The transgenic maize plants were sprayed with Roundup herbicide (Monsanto, 41.0% isopropylamine salt), and wild type plants were used as negative controls. The concentrations of glyphosate tested were 0.05, 0.1, 0.2, and 0.4%, and the solutions were prepared with distilled and deionized water (Millipore). Vegetative injury and fertility data were obtained by visual observation 2 wk after the plants were sprayed.

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