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New gene construction strategy in T-DNA vector to enhance expression level of sweet potato *sporamin* and insect resistance in transgenic *Brassica oleracea*

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Abstract

Sporamin, an abundant storage protein in tuberous roots of sweet potato, possesses strong inhibitory activity against trypsin and pest-resistance. To promote consistent high-level expression of sporamin and insect resistance in transgenic *Brassica* plants, a wound-responsive *sporamin* promoter (P*spoa*) alone or combined with matrix-attached-region-like DNA segment (*spo*MAR) were constructed for driving *sporamin* cDNA. The results showed the transgenic plants containing P*spoa*-drived *sporamin* and *spo*MAR displayed the highest level and low inter-transformant variability of *sporamin* expression, and the ability of insect resistance of transformants positively correlated with sporamin activity. Furthermore, expressions of P*spoa*-drived *sporamin* especially combined with the *spoa*MAR retains high and steady levels in the T_1 and T_2 generations, in marked contrast to the variable expression patterns observed in *CaMV35S* promoter-driven transformants. This study evidently indicates that the P*spoa* and *spoa*MAR would be very efficient for high transgene expression in plants and obtaining inherently stable transformants in consecutive progenies.

Keywords: *Brassica*; Genetic variability; Matrix-attached region; Sporamin; Trypsin inhibitor

1. Introduction

Sporamin is a major storage protein accounted for over 80% of the total soluble protein in sweet potato tuberous roots first described by Maeshima et al. [1]. Spatial expression of *sporamin* has been shown to be mainly associated with tuberous roots and a very low amount in the stem, but not in leaves [2,3]. Systemic expressions, however, could be induced by wounding infliction and other stress-related chemicals [4,5]. Importantly, it has been demonstrated that sporamin is a strong trypsin inhibitor (TI) based on the in-gel activity analysis of recombinant proteins on SDS–PAGE [6].

During the past years, *sporamin* gene has been introduced to tobacco and cauliflower plants via *Agrobacterium tumefaciens*-mediated transformation. It showed that the ectopic over-expression of *sporamin* driven by cauliflower mosaic virus 35S (*CaMV35S*) promoter could confer an effective insect/nematode-resistance on transgenic plants [7–9]. The survey on transgenic plant lines indicated the magnitude of nematode resistance of transgenic plants was closely correlated with the expression level of *sporamin* transgene [9]. However, *sporamin* expression level in most transgenic tobacco and cauliflower lines gradually declined during serial propagation in tissue culture and in successive generations. It was suggested that this occurrence of gene silence was happening in *sporamin* transformation driven by *CaMV35S* promoter. For agricultural application of genetic modified organism technology, high-level and stable expression of transgene under field conditions is an essential requirement. In order to develop functional and efficient transgenic crops as an integral part of agricultural systems, we have considered several factors to improve *sporamin*-expression in transgenic population. The factors, such as promoter strength, enhancer DNA segment, and intron segment are thought to influence transgene expression level [10–12]. Promoters, for instance, not only affect transgene expression but affect the magnitude of expression variability

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among individual transformants also [13]. It has been reported that the widely used *CaMV35S* promoter yields a bimodal expression pattern with high expression levels in a limited number of transgenic plants and very low expression levels in the majority of the transformants [13,14].

In continuation with the previous works, we have taken up this study to find out a method to elevate the gene expression level and insect-resistant efficiency of sweet potato *sporamin* in transgenic plants. To achieve this, two major factors, i.e. wound-inducible *sporamin* promoter, P*spoa*, and matrix attachment region (MAR)-like sequence of *sporamin* gene (*spoa*MAR) were incorporated into the improvement strategy. It has been shown that P*spoa* exhibits high-level of regulatory activity, and efficiently and rapidly expressed in response to wounding in transgenic tobacco [5]. MAR is found in eukaryotic genome as non-transcriptional regions that attach to the proteinaceous matrix in the nucleus. It appears that they trigger the formation of chromatin loops and thereby shielding genes from chromosomal position effects [15]. MAR sequence located in the proximity of transgenes can affect expression level and variability [16,17]. In this report, the potential function of P*spoa and spoa*MAR on increasing the *sporamin* expression for improving the insect-resistance were demonstrated in transgenic *Brassica*.

2. Materials and methods

2.1. Plant material

The *Brassica oleracea cv. alboglabra Boa2301* seeds kindly provided by Ming-Fong Seed Company in Taiwan were used as the transgene receptor.

2.2. Vector constructs and bacterial strain

Binary vector, pBI121, was used as backbone to generate transgenic constructs. For p*35S-sporamin* construct, *sporamin* cDNA (0.9 kb) was subcloned at *Bam*HI and *Sma*I site under the driving of *CaMV35S* (P*35S*) promoter. For p*spoa-sporamin* construct, P*35S* promoter was replaced by 1.25 kb *sporamin* promoter (P*spoa*) [5] at *Pst* I and *Xba* I site. For p*spoa-sporamin-spoa*MAR, a 2.0 kb DNA fragment encompassing the full-length *sporamin* cDNA (0.9 kb) and the extension of 1.1 kb 3′ downstream DNA sequence was amplified from the genomic clone encoded a protein member of sporamin A family, g*SPO-A* [18]. This DNA fragment was subsequently subcloned to replace *sporamin* cDNA insert in p*spoa-sporamin* construct. In the latter two vector constructs, *sporamin* gene was drove by its self-promoter region. In the third vector construct, *sporamin* cDNA was extended with its own downstream sequence. These three complete constructs were transformed to *A. tumefaciens* LBA4404 following the method described by Hofgen and Willmitzer [19].

2.3. Plant regeneration system

For gene transformation process, the plant regeneration protocol was prior established as the following protocol [20]. Seeds of *B. oleracea* were sterilized with 70% (v/v) ethanol containing 1–2 drops of tween-20 and 0.1% (w/v) mercuric chloride ($HgCl_2$), and then rinsed several times with sterilized distilled water. Following, the seeds were germinated on 1/2 MS basal medium supplemented with 2% (v/v) sucrose for 5 days. Hypocotyl segments were excised from five-day-old seedlings and cultured on callus-induction MS medium containing 1 mg L^{-1} picloram, 2 mg L^{-1} BA, 2% (w/v) sucrose and 10 mg L^{-1} silver nitrate. Shoot buds were elongated on shoot-induction medium consisting of 0.3% (w/v) hyponex, 100 mg L^{-1} myo-inositol, 1 mg L^{-1} NAA, 0.5 mg L^{-1} BA and 3% (w/v) sucrose. After 8–12 weeks, shoots with 3–4 cm length were excised and cultured on root-induction medium (3 g L^{-1} hyponex, 100 mg L^{-1} myo-inositol, 1 mg L^{-1} NAA and 2% (w/v) sucrose). All the media were gelled with 0.8% agar and cultures were incubated under white florescent light (28 μmol m^{-2} s^{-1}) with 16 h-light/8 h-dark photoperiod at 25 °C.

2.4. Gene transformation and plant growth condition

The *A. tumefaciens* LBA4404 harboring the three respective *sporamin* gene constructs were used to transform hypocotyl explants. Single colony of *Agrobacterium* LBA4404 was transferred to liquid YEP medium with 50 mg L^{-1} kanamycin, 30 mg L^{-1} streptomycin, and incubated at 20 °C for 2 days. The bacteria was centrifuged and re-suspended in liquid MS medium supplemented with 50 μM acetosyringone and incubated for further 4–6 h. For gene transformation, hypocotyl segments were infected with this *Agrobacterium* (OD_{600} = 0.4–0.5) for 8 min, and then filter dried to remove the excesses of bacteria present on the surface of explants. The explants and bacteria were co-cultured in callus-induction medium for 2 days in dark. The co-cultivated explants were washed three to five times in steriled water for 10 min each, followed by washing in MS medium containing 500 mg L^{-1} carbenicillin for 45 min. Then, explants were inoculated in shoot-induction medium with 500 mg L^{-1} carbenicillin for 2 weeks. In the subsequent subcultures, 50 mg L^{-1} of kanamycin were added to shoot-induction medium for transgenic plants selection. The selected shoots of transgenic and control clones were transferred to other medium for rooting before the plants were transferred to the greenhouse.

2.5. Trypsin inhibitor activity assay

Trypsin inhibitor activity of sporamin was both qualitatively and quantitatively analyzed. In qualitative assay, proteins were extracted from the transgenic leaves after 30 min for wounding-treatment. The extraction method and buffer were as the description of Yeh et al. [6]. The extract was centrifuged twice at 10,000 × *g* for 15 min to remove pellet, and total protein was quantified following the method described by Bradford [21]. Crude protein extract (100 μg per each sample) was separated on 15% SDS–PAGE, Then, the gel was subsequently treated as described by Yeh et al. [6]. In brief, the gel was immersed in trypsin solution, and then gently shaken in dark at room

temperature with *N*-acetyl-D,L-phenylalanine-β-naphthyl ester (APNE) and *o*-dianisidine dye for 30 min. After the procedure, sufficient 7.5% (v/v) acetic acid was applied to stop the reaction.

In addition, the quantitative activity assay was performed following the method described by Yao et al. [22]. Protein extract of plant tissues was reacted with trypsin (1:1 molar ratio) at 37 °C for 10 min, and then *N*-benzoyl-D,L-arginine-β-nitroanilide (BAPA) was added to a final concentration of 500 mg L^{-1} for further reaction for 30 min. The optical density of the reaction mixture was then measured at 410 nm. The TI activity, i.e. percentage inhibition of trypsin by sporamin, was calculated using the below equation:

$$\text{TI activity} = \left[\frac{(T - T^*)}{T}\right] \times 100\%$$

where T denotes the OD_{410} in the absence of sporamin, and T^* is that in the presence of sporamin.

2.6. Reverse transcriptase (RT)-PCR analysis

For analysis of *sporamin* gene expression, total RNA of transgenic plants was extracted from the leaves harvested after wounding-treatment for 30 min. Total RNA was isolated using Trizol reagent (Invitrogen, CA, USA). RT-PCR analysis was performed by OneStep RT-PCR Kit included Omniscript/Sensiscript Reverse Transcriptases mix and HotStar Taq polymerase following the instruction (Qiagen, CA, USA).

2.7. Insect bioassays

Transgenic and control leaves of same size were randomly cut and placed in 9 cm petri dish which contained 2% (w/v) agar. A filter paper was covered on it to retain proper moisture. Twenty early second instar larvae of maize cutworm (*Helicoverpa armigera Hubner*) were put in the petri dish to feed the leaves. The fresh leaf disc was supplied every 24 h. The body weight and insect survival rate were measured after 4 day feeding test.

3. Results

3.1. Generation of constructs for transgenic expression of sporamin in Brassica

Three binary vector constructs, pP*35S-sporamin*, pP*spoa-sporamin* and pP*spoa-sporamin-spoa*MAR, were generated based on the backbone of pBI121 binary vector (Fig. 1). pP*35S-sporamin* construct played a role as control. P*spoa* used in the second and third constructs was a 1.25 kb DNA fragment located at the upstream of *sporamin* cDNA, and employed as a promoter to drive *sporamin* cDNA (Fig. 1). The third construct

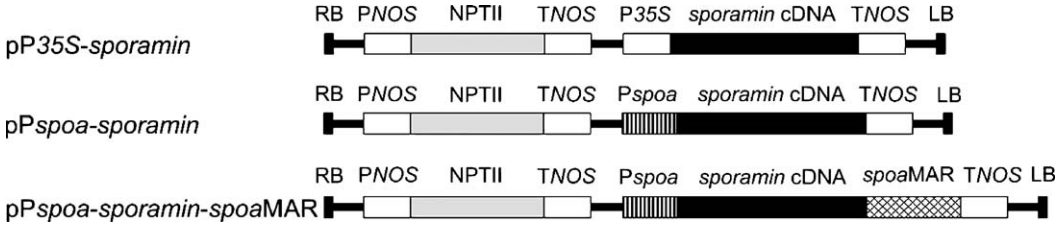

Fig. 1. Schematic representation of T-DNA vectors pP*35S-sporamin*, pP*spoa-sporamin* and pP*spoa-sporamin-spoa*MAR. pBI121 binary vector was employed as backbone to construct the three derivatives. P*35S*, CaMV35S promoter; NPTII, neomycin phosphotransferase II; PNOS, nopaline synthase promoter; P*spoa*, *sporamin* promoter; *spoa*MAR, sporamin matrix attached region-like segment; TNOS, nopaline synthase terminator; RB and LB, right and left T-DNA border, respectively.

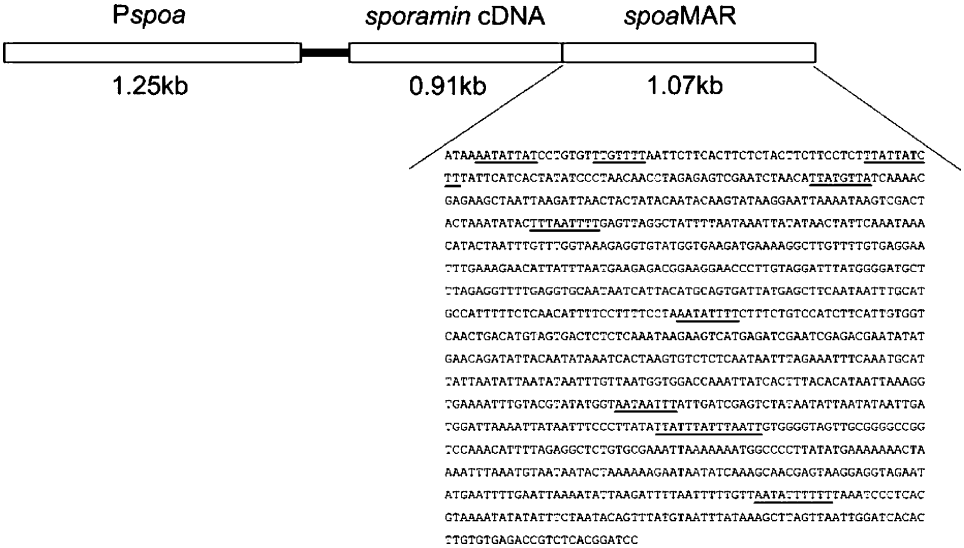

Fig. 2. Nucleotide sequence of matrix-attached region-like of *sporamin* gene. *spoa*MAR, matrix attached region-like sequence from 3′-flanking of *sporamin* gene. The nucleotide sequence of *spoa*MAR is characterized with some MAR motifs, such as TTTAATTT and AATATTTT etc.

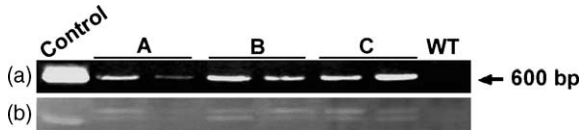

Fig. 3. RT-PCR analysis and gel staining of trypsin inhibitor (TI) activity to detect *sporamin* gene expression level in T_0 transgenic *Brassica* and wild type plant. A indicated the two transgenic *Brassica* lines of p*35S-sporamin*. B indicated the transgenic *Brassica* of p*spoa-sporamin*. C was the transgenic *Brassica* lines of p*spoa-sporamin-spoa*MAR. Wt, wild type *Brassica*. (a) *Sporamin* gene expressions detected by RT-PCR with *sporamin* gene specific-primers, and the PCR product size is 660 bp. Control for RT-PCR was using the plasmid containing the *sporamin* cDNA as the template of PCR. (b) TI activity analyzed by gel activity analysis. The sample for the control of TI activity assay was the protein of trypsin inhibitor extracted from soybean and the molecular weight was 20 kDa.

has an additional 1.1 kb flanking sequence attached to the downstream site of 3′-end of *sporamin* cDNA (Fig. 1). This flanking sequence was originally located at the 3′ downstream non-transcriptional region of *sporamin* genomic clone [18]. Nucleotide sequence analysis showed this DNA fragment was A-T rich (75% A/T bp) and contained several conspicuous characters of matrix attached region (MAR) such as MAR box (5′ AATATTTTT 3′) and T-box (5′ TTA/TTA/TTTA/TTT 3′) (Fig. 2).

3.2. Comparison of sporamin expression levels in transgenic Brassica plants harboring with different T-DNA constructs

Three constructs were transformed to *B. oleracea* mediated *Agrobacterium* infection. An appropriate scale of T_0 transgenic lines was regenerated and selected through kanamycin contained-medium. Forty independent lines of T_0 transgenic plants of each T-DNA construct transformation were selected. RT-PCR analysis using *sporamin* gene-specific primers was employed to detect *sporamin* gene expression level in leaves of kanamycin-resistant transformants. As shown in Fig. 3a, most kanamycin-resistant T_0 transformants positively displayed a RT-PCR product of 660 bp DNA fragment. Furthermore, these selected transformants showed trypsin inhibitor activity of sporamin, and the activity level positively correlated to the RT-PCR results (Fig. 3b). Difference in the levels of expression of *sporamin* gene was among these three construct transformants. In general, the transformants of gene constructs, p*spoa-sporamin* and p*spoa-sporamin-spoa*MAR, showed higher expression level than those of p*35S-sporamin* constructs. Ten independent lines, randomly selected from 40 T_0 transgenic plants of each T-DNA construct transformation, were assayed for TI activity level. It revealed that the transformants of p35S-*sporamin* resulted in most of the individuals (7 out of 10 individuals) showing trypsin-inhibited efficiency lower than 50%, and few individuals showing the inhibited efficiency higher than 70%. The maximal activity was 95%, whereas minimum inhibited efficiency was only 2% (Fig. 4a). It showed a marked bimodal expression pattern in p*35S-sporamin* construct. On the other hand, 8 out of 10 transformants of both p*spoa-sporamin* and p*spoa-sporamin-spoa*MAR constructs showed the trypsin-inhibited efficiency higher than 50%, respectively (Fig. 4b and c). The average TI activity of T_0 transformants of the each three constructs was 42% in p*35S-sporamin*, 68% in p*spoa-sporamin* and 70% in p*spoa-sporamin-spoa*MAR, respectively.

To further investigate the variability of the TI activity within individual T_1 transformant at various organs, leaves, stems and flowers from three primary transformants of each T-DNA constructs were sampled at 45 day after germination (DAG). In all p*35S-sporamin* transformants, young leaves (L1), upper-section stem (US) and flower tissue expressed high level TI activity, whereas TI activity was dropped to a very low level in mid-aged leaves (L4), aged leaves (L7) as well as in lower-section stem (LS) (Fig. 5). On the other hand, the transformants harboring the constructs with P*spoa* retained almost consistent and high level of TI activity in whole plants (Fig. 5).

3.3. Stable trypsin inhibitor expression in second-generation of the sporamin-transformants

Based on T_1 segregation test (data not shown), three of the single-locus transformants of each T-DNA constructs were randomly selected to determined the stability of sporamin expression in second generation (T_1). At least, 10 progeny plants from each parental line were further grown to study TI activity. TI activity in T_1 population of all the three constructs-trangenic plants is shown in Fig. 6. T_1 population of p*35S-sporamin* transformation showed high variation of TI activity, and the TI activity level of T_1 generation deviated strongly from that of the parental plants (Fig. 6a). p*spoa-sporamin* showed a moderately reducing TI activity compared to that of the

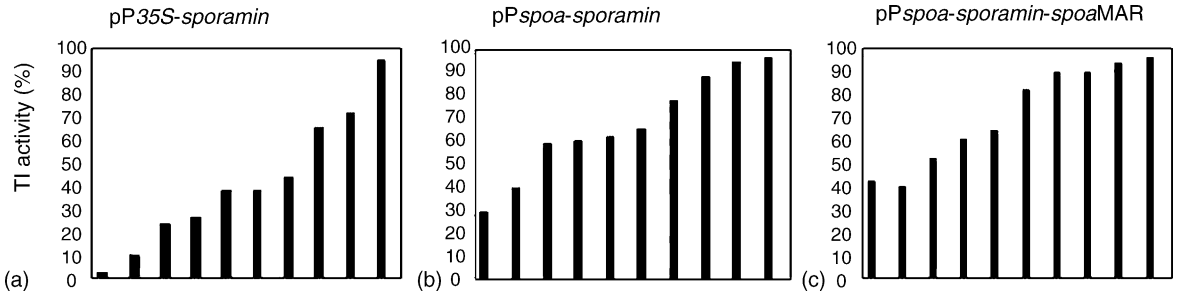

Fig. 4. Trypsin inhibitor (TI) activity of *sporamin* gene expressed in inhibitory percent (I %) in T_0 transgenic *Brassica*. The TI activity was presented as the percentage of trypsin activity was inhibited. Ten individual T_0 plants were randomly selected to assay from each construct transformation.

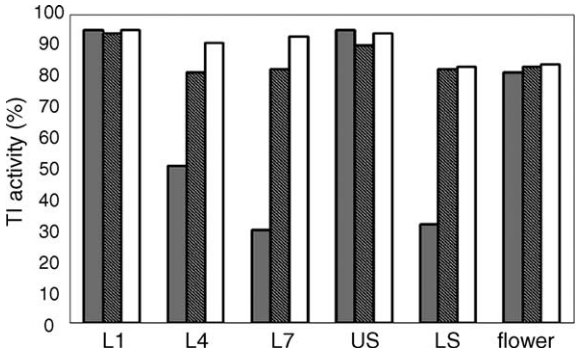

Fig. 5. Variation of trypsin inhibitor activity expressed in different tissues (leaf, stem, and flower) in T_1 *Brassica* plants transformed with pP*35S-sporamin*, pP*spoa-sporamin* and pP*spoa-sporamin-spoa*MAR. Three replicates of individual transgenic *Brassica* of each T-DNA construct were used to assay TI activity. L1, L4 and L7 represent corresponding leaves located from apical bud; US and LS represent the upper portion and lower portion of stem. ■ represents pP*35S-sporamin* transformants; ▩ represents pP*spoa-sporamin* transformants; □ represents pP*spoa-sporamin-spoa*MAR transformaants.

parental plants (Fig. 6b). Trypsin inhibitor activity of T_1 generation of pP*spoa-sporamin-spoa*MAR, however, showed highly stable expression levels (Fig. 6c).

Investigation on T_2 *Brassica* grown on field, individuals of pP*35S-sporamin* transformation almost lost the TI activity (Fig. 7). However, individuals of the T_2 transgenic population harboring the construct containing *sporamin* promoter retained TI activity expression and insect-resistance (Fig. 7).

3.4. Comparison of resistance to H. armigera among transgenic plants harboring different constructs

In order to understand the relationship between the TI activity expression level and the insect resistance in transgenic *Brassica*, the same size of transgenic leaves, detached from randomly selected T_2 populations of each three T-DNA constructs and wild type plants, were put in petri dish to feed second instar larvae of *H. armigera* individually. As expected, the weight of twenty insects fed with the *sporamin*-transgenic *Brassica* has the lighter weight than that fed with wild type plants (Table 1), and the size of *sporamin*-transgenic plant fed larvae were generally smaller than that of controls (Table 1 and Fig. 8). Moreover, the average survival rates of the larva fed with pP*35S-sporamin* was 40%; however, the survival rate of the larva fed with pP*spoa-sporamin* and pP*spoa-sporamin-spoa*MAR transgenic were only 16 and 4%, respectively (Table 1). In addition, transgenic population of *CaMV35S* promoter construct showed marked variability in insect resistance (data not shown).

4. Discussion

Previously it has been shown that P*spoa* promoter is highly activated in response to wounding [5]. The *cis*-acting elements on the P*spoa* promoter region were completely characterized, and several regulatory elements responded to wounding and pathogenic stresses have been observed (unpublished data). In this study, the expression of TI activity in individual T_0

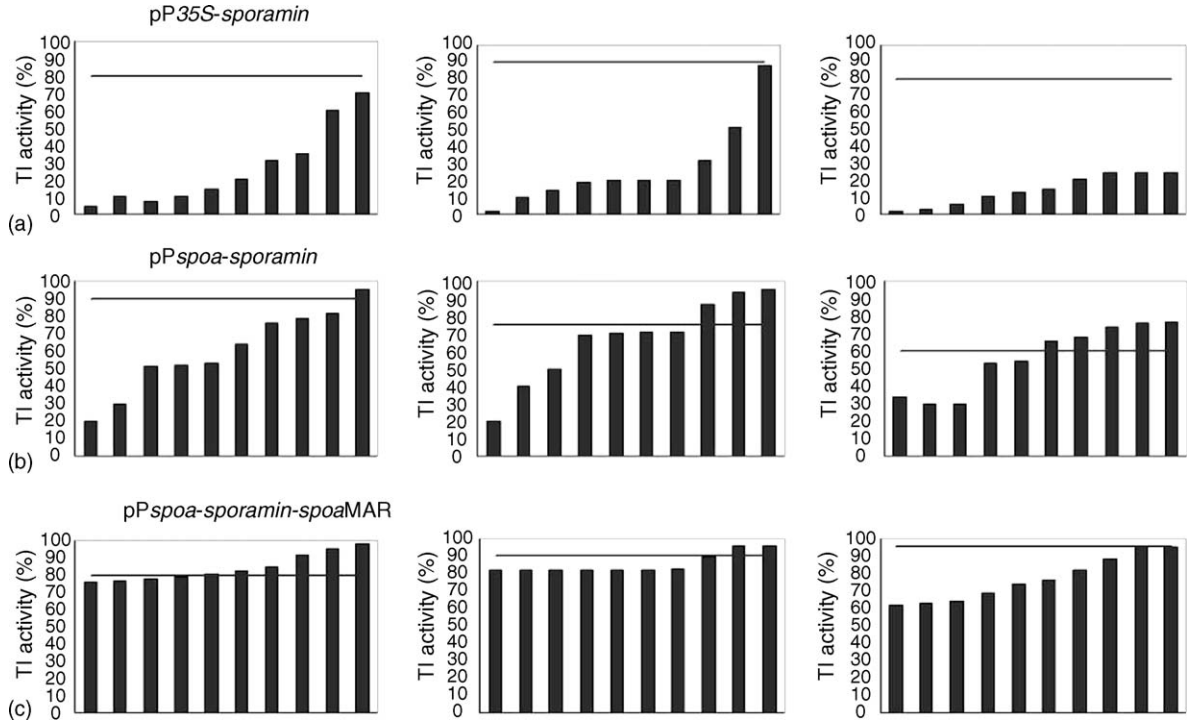

Fig. 6. Trypsin inhibitory activity of 10 transgenic progeny plants derived from each three single-locus T_0 *Brassica*. TI activity of the parental plant (in T_0 generation) is represented by a horizontal line in each figure. (a) TI activity of T_1 generation of pP*35S-sporamin* transformants. (b) TI activity of T_1 generation of pP*spoa-sporamin* transformants. (c) TI activity of T_1 generation of pP*spoa-sporamin-spoa*MAR transformants. The three groups of independent data in each (a)–(c) indicated the results got from the secondary generation of three different T_0 transgenic plants, respectively.

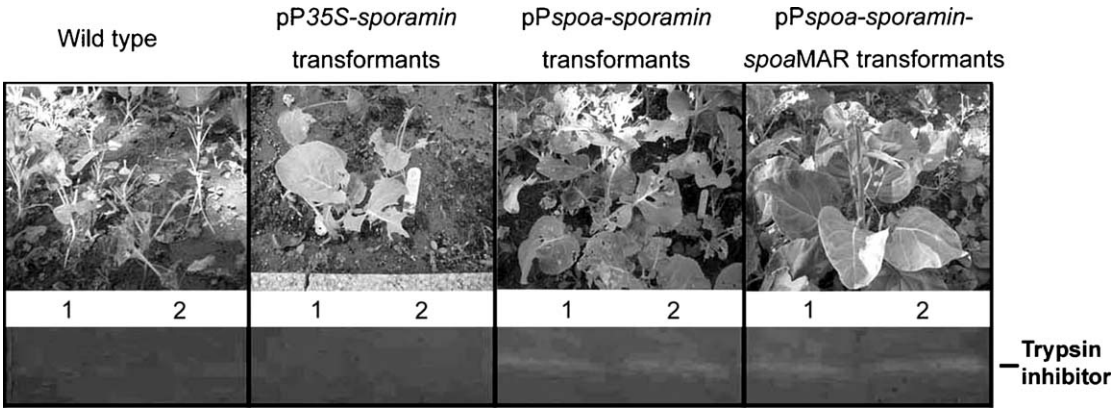

Fig. 7. Trypsin inhibitor activity of T_2 *Brassica* plants transformed with pP*35S-sporamin*, pP*spoa-sporamin* and pP*spoa-sporamin-spoa*MAR. Equal amount (100 μg) of crude protein extract from young leaves was assayed on SDS–PAGE. Panel (a) shows the T_2 and wild type *Brassica* plants growing in the trial field. Panel (b) shows the TI activity stain of crude leaf protein extracts on SDS–PAGE, which were represented by two selected independent lines.

Table 1
Results of maize cutworm (*Helicoverpa armigera*) biossays on T_2 transformed plants

Plants	[a]Number of sampled transformants	Average body weight of total incubated insects ± S.D. (mg)	Insect survival rate (%)
Wild type	4	31.6 ± 3.6	100
pP*35S-sporamin*	10	22.5 ± 2.1	40
pP*spoa-sporamin*	10	10.3 ± 1.5	16
pP*spoa-sporamin spoa*MAR	10	6.2 ± 1.81	4

[a] Number of sample plants used in the insect bioassay.

Brassica plant was not boosted by *sporamin* promoter. The average value of TI activity, however, is higher than those *CaMV35S* transformant population (Fig. 4). This is due to most of the transgenic *Brassica* harboring the P*spoa-sporamin* and P*spoa-sporamin-spoa*MAR expressed the high level sporamin and the prominent reduction in of inter-variability in these two transformant populations. The possible mechanism of the outcome is that *sporamin* promoter is an efficient inducible promoter [5], rather than a constitutive expression like *CaMV35S* promoter. Another advantage of P*spoa* is that it can be induced to express systemically by wounding in short term. This property of P*spoa* promoter can be of significant importance for application in transgene-overexpressing plants. Based on the spatial analysis of TI activity in transgenic plants, high level of TI activity is systemically consistent in transformants containing the construct with *sporamin* promoter (Fig. 5). These results indicated that P*spoa* promoter conferred the consistent systemic expression of *sporamin* throughout the whole plant, and P*spoa* was more stable compared to *CaMV35S* promoter in transgene expression.

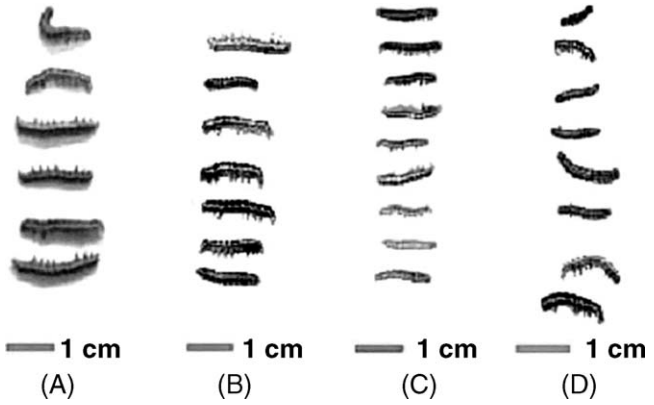

Fig. 8. Effect of sporamin on the maize cutworm fed with the *sporamin* transgenic *Brassica*. Maize cutworms (*Helicoverpa armigera*) were fed with leaves of wild type *Brassica* (A), pP*35S-sporamin* (B), pP*spoa-sporamin* (C) and pP*spoa-sporamin-spoa*MAR (D) transgenic *Brassica* for 4 days, respectively.

It would be advantageous that a transgenic plant keeps the characters including high-level transgene expression, low inter-transformant variability and consistent inherent stability in subsequent generations. However, it is not easy to combine all these traits in a transgenic plant. For example, strong promoters such as *CaMV35S* and cassava vein mosaic virus promoter are widely used and can lead to high transgene expression, but the expression is also more prone to gene silencing than the expression obtained by the weaker promoters [23,24]. Moreover, the strong promoters are always with the drawback of high inter-transformants variability. Our observation on the previous transgenic *Tobacco* and cauliflower overexpressed the sweet potato *sporamin* gene drove by *CaMV35S* promoter clearly displayed the situation, and it was not sufficient to guarantee stable expression in successive generation [7,8]. As shown in Fig. 6c and 7, *sporamin* promoter is generally better than *CaMV35S* promoter to maintain highly stable transgene expression in the subsequent generation of single-locus parental plants.

It has been shown that the 3′-flanking regions of some genes play a regulatory role in gene expression such as the 3′ region of

Pvlea-18 gene encoding a member of late embryogenesis-abundant (LEA) proteins in *Phaseolus vulgaris* has been proven to increase the gene expression level during growth and development, and response to dehydration [25]. The 3′-flanking sequence cloned from *sporamin* gene [26] showed the character of MAR sequence (motifs) (Fig. 2). To date, MAR effect on gene expression has been reported only in a few cases [13,17,26,27], however, it is recognized generally as an efficient suppressor of inter-individual variation of transgene expression. The *spoa*MAR sequence appears to enhance the function of P*spoa* promoter and stabilize the wound-inducible regulation of P*spoa* promoter expression in transgenic plants (Fig. 4). Moreover, it is effective in retaining the stability of TI expression level in successive generation (Figs. 6c and 7). It is quite possible that this DNA region might have the potentiality to play a function like MAR in stabilizing transgene expression and reducing position-induced quantitative difference among independent transformants [28–30]. These results suggested that *spoa*MAR got potential for transgenic crop production.

In conclusion, since the TI activity and insect resistance was not stable enough in pP*35S-sporamin* transgenic plants, the combination of P*spoa* and *spoa*MAR successfully improvement the efficiency and stability of both TI activity and insect-resistance in transgenic *Brassica*. The P*spoa* and *spoa*MAR on transgenic constructs reduced the variability of TI activity in inter-transgenic plants and inter-generation. These results suggested that the inducible *sporamin* promoter combined with MAR is an excellent strategy to express the insect-resistant protein, sporamin, in *Brassica*. Even the MAR function on transgene expression in crops has been reported, based on our knowledge, this is the first paper directly proved the MAR could apply to biotechnology for improving the physiological trait of crop such as insect-resistance.

Acknowledgements

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References

[1] M. Maeshima, T. Sasaki, T. Asahi, Characterization of major proteins in sweet potato tuberous roots, Phytochemistry 24 (1985) 1899–1902.
[2] T. Hattori, N. Yoshida, K. Nakamura, Structural relationship among the members of multigene family coding for the sweet potato tuberous roots storage proteins, Plant Mol. Biol. 13 (1989) 563–572.
[3] T. Hattori, S. Nakagawa, K. Nakamura, High-level expression of tuberous root storage protein genes of sweet potato in stem of plantlets grown in vitro on sucrose medium, Plant Mol. Biol. 14 (1990) 595–604.
[4] M. Ohto, K. Nakamura-Kito, K. Nakamura, Induction of expression of genes coding for sporamin and β-amylase by polygalacturonic acid in leaf-petiole cuttings of sweet potato, Plant Physiol. 99 (1992) 422–427.
[5] S.J. Wang, Y.C. Lan, S.F. Chen, Y.M. Chen, K.W. Yeh, Wound-response regulation of the sweet potato *sporamin* gene promoter region, Plant Mol. Biol. 48 (2002) 223–231.
[6] K.W. Yeh, J.C. Chen, M.I. Lin, Y.M. Chen, C.Y. Lin, Functional activity of sporamin from sweet potato (*Ipomoea batatas*)—a tuberous storage protein with trypsin inhibitor activity, Plant Mol. Biol. 33 (1997) 565–570.
[7] K.W. Yeh, M.I. Lin, S.J. Tuan, Y.M. Chen, C.Y. Lin, S.S. Kao, Sweet potato (*Ipomoea batatas*) trypsin inhibitors expressed in transgenic tobacco plants confer resistance against *Spodoptera litura*, Plant Cell Rep. 16 (1997) 696–699.
[8] L.C. Ding, C.Y. Hu, K.W. Yeh, P.Y. Wang, Development of insect-resistant transgenic cauliflower plants expressing the trypsin inhibitor gene isolated from local sweet potato, Plant Cell Rep. 17 (1998) 854–860.
[9] D.G. Cai, T. Thurau, Y. Tian, T. Lange, K.W. Yeh, C. Jung, Sporamin-mediated resistance to beet cyst nematode (*Heterodera schachtii* Schm.) is dependent on trypsin inhibitory activity in sugar beet (*Beta vulgari*s L.) hairy root, Plant Mol. Biol. 51 (2003) 839–849.
[10] P. Meyer, Variation of transgene expression in plants, Euphytica 85 (1995) 359–366.
[11] P. Meyer, Transcriptional transgene silencing and chromatin components, Plant Mol. Biol. 43 (2000) 221–234.
[12] R.E. Down, L. Ford, S.J. Bedfod, L.N. Gatehouse, C. New, J.A. Gatehouse, A.M.R. Gatehouse, Influence of plant development and environment on transgene expression in potato and consequences for insect resistance, Transgenic Res. 10 (2002) 223–236.
[13] M.F.C. De Bolle, K.M.J. BUtaye, W.J.W. Coucke, I.J.W.M. Goderis, P.F.J. Wouters, N. Van Boxel, W.F. Broekaert, B.P.A. Cammue, Analysis of the influence of promoter elements and a matrix attachment region on the inter-individual variation of transgene expression in populations of *Arabidopsis thaliana*, Plant Sci. 165 (2003) 169–179.
[14] T. Elamayan, H. Vaucheret, Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionly, Plant J. 9 (1996) 787–797.
[15] J. Bode, C. Benham, A. Knopp, C. Mielke, Transcriptional augmentation: modulation of gene expression by scaffold/matrix-attached regions (S/MAR elements), Crit. Rev. Eukaryot. Gene Expr. 10 (2000) 73–90.
[16] R. Holmes-Davis, L. Comai, Nuclear matrix attachment regions and plant gene expression, Trends Plant Sci. 3 (1998) 157–162.
[17] G.C. Allen, S. Spiker, W.F. Thompson, Use of matrix attachment regions to minimize transgene silencing, Plant Mol. Biol. 43 (2000) 361–376.
[18] S.J. Wang, C.T. Lin, K.C. Ho, Y.M. Chen, K.W. Yeh, Nucleotide sequence of *sporamin* gene in sweet potato, Plant Physiol. 108 (1995) 829–830.
[19] R. Hofgen, L. Willmitzer, Storage of competent cells for Agrobacterium transformation, Nucleic Acids Res. 16 (1988) 9877.
[20] G.B. Poulsen, Genetic transformation of *Brassica*, Plant Breeding 115 (1996) 225–251.
[21] M.M. Bradford, A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[22] P.E. Yao, M.J. Hwang, Y.M. Chen, K.W. Yeh, Site-directed mutagenesis evidence for a negatively charged trypsin inhibitory loop in sweet potato *sporamin*, FEBS Lett. 24841 (2001) 1–5.
[23] Q. Que, H.Y. Wang, J.J. English, R.A. Jorgensen, The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence, Plant Cell 9 (1997) 1357–1368.
[24] K.M.J. Butaye, I.J.W.M. Goderis, P.F.J. Wouters, J.M.T.G. Pues, S.L. Delaure, W.F. Broekaert, A. Depicker, B.P.A. Cammue, M.F.C. Bolle, Stable high-level transgene expression in *Arabidopsis thaliana* using gene silencing mutants and matrix attachment regions, Plant J. 39 (2004) 440–449.
[25] L.P. Moreno-Fonseca, A.A. Covarrubias, Downstream DNA sequences are required to modulate *Pvlea*-18 gene expression in response to dehydration, Plant Mol. Biol. 45 (2001) 501–515.
[26] S.J. Oh, J.S. Jeong, E.H. Kim, N.R. Yi, S.I. Yi, I.C. Jang, Y.S. Kim, S.C. Suh, B.H. Nahm, J.K. Kim, Matrix attachment region from the chicken lysozyme locus reduces variability in transgene expression and confers copy number-dependence in transgenic rice plants, Plant Cell Rep. 24 (2005) 145–154.

[27] H. Xue, Y.T. Yang, C.A. Wu, G.D. Yang, M.M. Zhang, C.C. Zheng, TM2, a novel strong matrix attachment region isolated from tobacco, increases transgene expression in transgenic rice calli and plants, Theor. Appl. Genet. 110 (2005) 620–627.

[28] A. Stief, W.H. Winter, W.H. Straetling, A.E. Sippel, A nuclear DNA attachment element mediates elevated and position-independent gene activity, Nature 341 (1989) 343–345.

[29] P. Breyne, M. Van Montague, A. Depicker, G. Gheysen, Characterization of a plant scaffold attachment region in a DNA fragment that normalizes transgene expression in tobacco, Plant Cell 4 (1992) 463–471.

[30] W. Van Leeuwen, L. Mlynarova, J.P. Nap, L.H.W. van de Plas, A.R. van der Krol, The effect of MAR elements on variation in spatial and temporal regulation of transgene expression, Plant Mol. Biol. 47 (2001) 543–554.