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Cloning the broccoli HMG-I/Y gene as an endogenous reference for transgene copy number determinations using real-time PCR

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ABSTRACT. Transgenic technology has become an indispensable tool for crop improvement. However, transgene integration and copy number insertion affect transgenic expression and stability. TaqMan® quantitative real-time PCR (qRT-PCR) was developed as an alternative way to determine transgene copy numbers and has several advantages over other methods: smaller amounts of test materials required, higher throughput capacity, large quantification scope, no post-PCR gel analyses, etc. Nevertheless, the TaqMan qRT-PCR requires an endogenous DNA sequence with confirmed low copy numbers per genome for its internal control. The current reports that use the HMG-I/Y (high-mobile-group protein I/Y) gene as the internal standard for *Brassica oleracea* are discrepant. Using the *HMG-I/Y* gene found in rapeseed and *Arabidopsis* as a reference, we cloned and characterized a full cDNA sequence (796 bp) of the broccoli (*Brassica oleracea* var. *italica*) HMG-I/Y gene that shared 75% identity with the deduced amino acid sequences of *Brassica napus* in our laboratory. We also conducted phylogenetic analyses from the deduced amino acid sequences of the HMG-I/Y gene among known plant species. These corresponded with traditional taxonomy. We demonstrate here that the cloned *HMG-I/Y* gene, being a single copy in broccoli, can serve as an internal reference gene for estimating copy numbers of transgene among the tested populations of isopentenyl transferase (*ipt*) and *Arabidopsis* embryonic flower gene *EMF1* (*AtEMF-1*) transgenic broccoli.

Keywords: Quantitative real time polymerase chain reaction (qRT-PCR); *AtEMF1* gene; *ipt* gene.

INTRODUCTION

Rapid developments in genomic sequencing and functional genomics have paved a magnanimous avenue toward identifying genetic traits within gene sequences. Traditional plant breeders conducted genetic recombinations for desired traits without this sequence information. Trait-sequence link studies are now more feasible than in the past, coincidentally, transgenic technology has become an indispensible tool for plant improvement. The growing amount of acreage planted with GM (genetically modified) crops over the past decades (James, 2009) should be an indicator of its potential. The key factor in using transgenic technology, however, is how to avoid transgenic gene silencing. Although there are some controversies on the effects of transgene copy numbers toward transgene expressing or transgene silencing (Flavell, 1994; Vaucheret et al., 1998; Craig et al., 2005; Mirza, 2005; Tang et al., 2007), it is essential to estimate the inserted transgene copy numbers for stable integration and inheritance. An efficient method for transgene copy numbers determination is always the need for transgenic study. Copy number estimation methods have evolved from the traditional Southern blotting analysis to the handy Polymerase Chain Reaction (PCR)-based quantitative real time PCR (qRT-PCR). The qRT-PCR has recently been adapted for transgene expression, copy number determination, GM food contamination and mixed events (Ginzinger, 2002; Mason et al., 2002; Huang and Pan, 2004; Huang and Pan, 2005; Battistini and Noli, 2009; Beltrán et al., 2009). Several varieties of transgenic species have used qRT-PCR transgene copy number estimation including wheat, barley, (Ingham et al., 2001), rice (Yang et al., 2005), and maize (Song et al., 2002) for monocots; watermelon, tomato, sugar beet (Ingham et al., 2001), cotton (Yi et al., 2007), citrus (Omar et al., 2008), rapeseed (Weng et al., 2004) and soybean (Harikai et al., 2009) for dicots. The advantages of qRT-PCR in these studies noted the relatively minimal amount of test materials needed, the higher throughput capacity, the large scope of quantification, no post-PCR gel analyses, etc. (Mason et al., 2002; Weng et al., 2004). Transgene quantification employed two typical qRT-PCR methods, i.e. absolute quantification (or standard-curve quantitation) and relative quantification (Livak and Schmittgen, 2001; Ginzinger, 2002). The absolute quantification for determining the copy number is largely dependent on the standard curve obtained from serious di-

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lution of a known sample, while the relative quantification relies on comparing the amplified signal with the control gene. Estimating this requires exploring the endogenous genes and their reference gene potentials. Ding et al. (2004) propose that a reliable reference gene should meet three requirements: be species specific, have low copy number, and have low heterogeneity among the cultivars. They provided reference plant gene examples for maize, soybean, rapeseed and rice. The most intensive research on endogenous reference genes for qRT-PCR studies in *Brassica* have been with rapeseed (*Brassica napus*) (Masek et al., 2000; Weng et al., 2004; Weng et al., 2005; Wu et al., 2010). Wu et al. (2010) indicated that there were five endogenous reference genes in *B. napus*: acetyl-CoA carboxylase gene (BnACCg8), phosphoenolpyruvate carboxylase (PEP), oleoxl hydrolase gene (Fat A), high-mobility-group protein I/Y gene (*HMG-I/Y*) and cruciferin A gene (Cru A), The presence of these genes in other *Brassica* species is currently contested. For example, Weng et al., 2005, pointed out that *HMG-I/Y* is a single copy in rapeseed and that its amplification was not observed in other *Brassica* genus or species, such as broccoli, stem mustard, cauliflower, Chinese cabbage, cabbage, sprouts, etc. This gene was also absent in *B. oleracea* according to Southern blotting analysis of the *HMG-I/Y* gene. Wu et al. (2010), however, recently located *HMG-I/Y* in *B. napus, B. nigra* and *B. oleracea.* In this study, we demonstrate the presence of HMG-I/Y as single copy in broccoli (*B. oleracea* var. *italica*) and its utility for copy number determination in transgene broccoli transformation.

MATERIALS AND METHODS

Plant materials and transgene constructs

Broccoli (*B. oleracea* var. *italica*) cultivars Green King and Elegance seeds were obtained from the Know-You Seed Company (Kaoshiung, Taiwan). We used constructs of transgene *ipt* and *AtEMF1* as indicated in Figure 1A and 1B. We also used transgenic *ipt* T_5 lines derived from self-progeny of selected T_4 individuals from our previous study (Chen et al., 2001). Three T_4 lines were used first to check the stability and purity among selfed or sib-mated (due to the occasional lack of pollen) progeny. For each line, five sib or self-pollinated progeny were randomly selected for transgene zygosity examination via qRT-PCR. Among those, Lines 101 and 103 derived from the same T_0 transformant and Line 102 derived from another independent transformant. At least ten individuals of each T_5 from the self-pollinated progeny of each line were further confirmed for the copy number. The agronomic performance of the developed inbred lines (T_5) has been reported elsewhere (Chan et al., 2009). The later transgene construct for polycomb gene study (Figure 1C) was kindly supplied by Professor Zinmay Renee Sung of the Department of Plant and Microbial Biology, University of California, Berkeley, US). The *AtEMF1* transgenic lines were obtained following the method of Chen et al. (2001) using *Agrobacterium*-mediated transformation. We used twenty-four T_0 plants

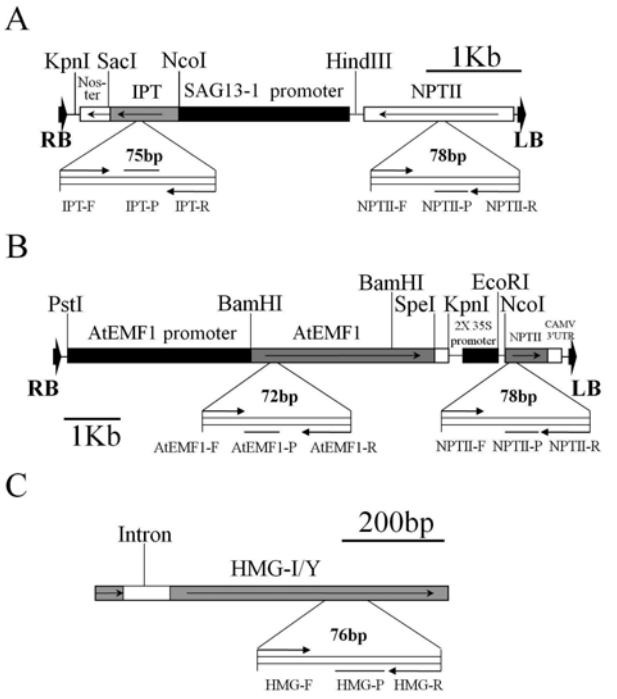

Figure 1. Maps of transgene constructs and sites of primer sets designed for qRT-PCR, (A) *ipt*&*NPTII*; (B) *AtEMF1*&*NPTII*; and (C) endogenous *Brassica oleracea* var. *italica HMG-I/Y.*

for copy number determination to validate the established methodology.

Plant DNA extraction, total RNA isolation and cDNA synthesis

The plant genomic DNA samples used for quantitative PCR detection were extracted and purified using a DNA extraction kit: Plant Genomic DNA Extraction Miniprep System (VIOGENE, Taipei, Taiwan, R.O.C.). The plant genomic DNA samples used for the Southern blot analysis and chromosome walking were extracted and purified from fresh leaves according to the CTAB method (Gawel et al., 1991). Genomic DNA was quantified spectrophotometrically using a DU® 640B DNA/Protein calculator (Beckman Company) and analyzed by 1% agarose gel electrophoresis in 1×TBE with ethidium bromide staining.

Total RNA was extracted from broccoli leaves using an RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Total RNA were reverse transcribed with Reflection™ Kit (ACTIVE MOTIF) and oligo (dT) following the enzyme manufacturer's instructions.

Cloning and identifying the broccoli *HMG-I/Y* gene

Primer pairs were designed to amplify the partial HMG-I/Y cDNA of broccoli through comparisons of NCBI blastn (http://www.ncbi.nlm.nih.gov/BLAST/) alignments on HMG-I/Y cDNAs of *Arabidopsis thaliana* (GenBank accession number CAA71797) and

rapeseed (*Brassica napus*; GenBank accession number AAF22135). We then noted several conserved DNA sequences (Figure 2). Oligonucleotide primers: HMG-F, 5′-TATCCTCAGATGATCATGGAAGCGAT-3′; HMG-R, 5′-TCTTCGGCGGTCGTCCACGTGGCCT-3′ were designed to clone the broccoli HMG-I/Y partial sequence. Primers from the partial HMG-I/Y cDNA were then designed to conduct chromosome walking and RACE (Rapid amplification of cDNA ends) for full sequence determination.

RACE, chromosome walking and HMG-I/Y full sequence cloning

To get the full-length cDNA of the putative *HMG-I/Y*, gene specific primers (GSPs) and nested gene specific primers (NGSPs) were designed with *HMG-I/Y* partial sequence for 3′,5′ RACE according to FirstChoice® RLM-RACE kit (Ambion) instructions. We used the GSPs and NGSPs as follows: 5′RACE: GSP1, 5′-GGGAGCAGGGATCGC AC-3′; NGSP1, 5′-CTTGGGCGGAGCATGTGGAT-3′; 3′RACE: GSP2, 5′-GGC ATCGCGAAGCACATC-3′; NGSP2, 5′-GCTGCTCAACTACCATCTCAACCA-3′.

The HMG-I/Y gene flanking region was determined using a GenomeWalker™ Universal Kit (BD Biosciences Clontech) according to the manufacturer's instructions. Restriction endonucleases, *Dra*I, *EcoR*V, *Pvu*II and *Stu*I were used to generate several DNA fragment libraries, which were separately ligated to corresponding adaptors. Adaptor-ligated genomic DNA fragment libraries were subjected to an initial round of polymerase chain reaction (PCR) amplification with the outer adaptor primer (AP1) (5′-GTAATACGACTCACTATAGGGC-3′) and an outer gene-specific primer (5′GSP1) (5′-CAAAGCTTATTCGGATTGCT-3′) and (3′GSP1) (5′-CCCACACAGCTTCTCCTCACG-3′), while an inner adaptor primer (AP2) (5′-ACTATAGGGCACGCGTGGT-3′) and inner gene-specific primer (5′GSP2) (5′-GGCTTACGCAACAGGCACCATCACT-3′) and (3′GSP2) (5′-CTCTCTGTGCGAATTCATGT-3′) were used for the second round of PCR amplification.

The amplified fragments were isolated, gel-purified using the Gel-M™ Gel Extraction System (VIOGENE, Taipei, Taiwan, R.O.C.), cloned into a yT&A vector (Yeastern Biotech, Taipei, Taiwan, R.O.C.), and the inserts were sequenced entirely on both strands with M13 pairs by Mission Biotech Co., Ltd. (Taipei, Taiwan, R.O.C.).

Phylogenetic estimation on the known HMG-I/Y protein among plant species

We used the Neighbor-Joining method (Saitou and Nei, 1987) with MEGA4 (Molecular Evolution Analysis version 4.0) (Tamura et al., 2007) to estimate the phylogenetic trees of HMG-I/Y proteins based on the deduced amino acid sequences from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) for maize (*Zea mays*; CAB40848), sword-

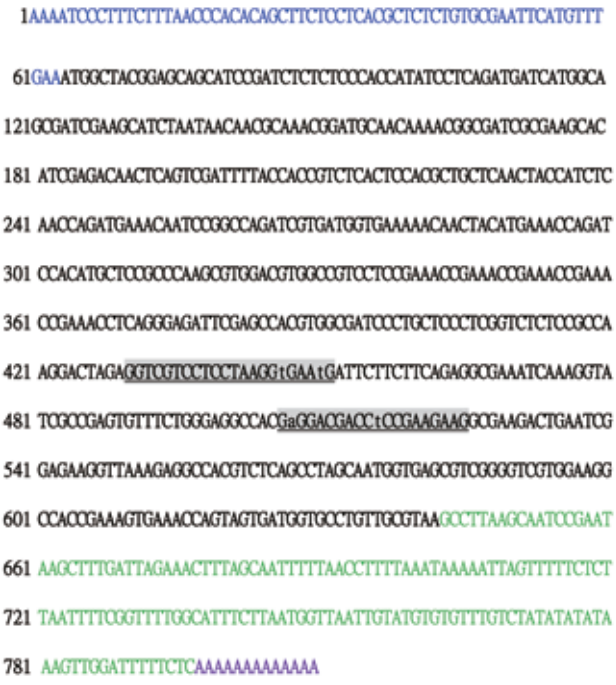

Figure 2. Broccoli HMG-I/Y full cDNA sequences (796 bp). The open reading frame is in black (576 bp), the 5′-untranslated region is in blue (63 bp), the 3′-untranslated region is in green (157 bp) and the poly(A) tail is in purple (13 bp). The grey underlined sites are primer sets in Weng et al. (2004) and the mismatched bases are in lower case letters.

bean (*Canavalia gladiata*; BAA11766), rapeseed (*B. napus*; AAF22135), *Arabidopsis* (*Arabidopsis thaliana*; CAA71797), rice (*Oryza sativa*; XP_482501), oats (*Avena sativa*; AAA32718), soybean (*Glycine max*; Q00423), wheat (*Triticum aestivum*; AAM22691), pea (*Pisum sativum*; CAA67752) along with broccoli (*B. oleracea* var. *italica*). The evolutionary distances were calculated by the Poisson correction method (Zuckerkandl and Pauling, 1965) and units are represented as the number of amino acid substitutions per site. The numbers in Figure 6 represent bootstrap percentages and the clustering significance was evaluated by bootstrap with 1,000 replications (Felsenstein, 1985).

Southern hybridization for genomic determination of *ipt*, *HMG-I/Y* and *AtEMF1* transgene in broccoli

A Southern assay on *ipt* transgenic inbred lines was previously demonstrated (Chan et al., 2009). In this study, genomic DNA was digested with *Hind*III (3-4U/μgDNA) and hybridized with respective probes DIG-labeled *ipt* and *NPTII* (Neomycine phosphotransferase II) based on similar procedures as in Chen et al. (2001) and Chan et al. (2009). To investigate the *HMG-I/Y* gene copy number in the broccoli genome, genomic DNA of cultivars Green King and Elegance were digested with 5U/μgDNA each of *Bam*HI, *Eco*RI, *Hind*III, *Sac*I and *Xho*I, respectively, for Southern

hybridization to confirm the copy number of *HMG-I/Y* gene. The digested genomic DNAs were fractionated on 1% agarose gels, transferred onto a nylon membrane and hybridized to DIG-labeled probes according to the Roche's instructions (Roche, Germany). The *HMG-I/Y* gene of genomic DNA was cloned into a yT&A vector (Yeastern Biotech, Taipei, Taiwan, R.O.C.) by PCR amplification using (3′GSP2) (5′-CTCTCTGTGCGAATTCATGT-3′) and (5′GSP1) (5′-CAAAGCTTATTCGGATTGCT-3′) primers, and the HMG-I/Y probe was labeled with non-radioactive digoxigenin (DIG)-11-dUTP (Roche, Germany). The hybridization signal was visualized on x-ray film using an alkaline phosphatase anti-DIG-Fab conjugate in the presence of a chemiluminescent substrate CDP-Star (Roche, Germany).

A similar method was used for transgene AtEMF-1 identification except that DIG labeling required a 700 bp PCR AtEMF1 DNA probe amplified by the primer set of forward AtEMF1-SF, 5′-CTTATTGAATGGGAAAAGGGTGGG-3′ and reverse AtEMF1-SR, 5′-CTGTATTGATTAGGAACACTCTGG CA-3′.

Oligonucleotide primers and probes

The oligonucleotide primers and TaqMan probes (Figure 1, Table 1) were designed and synthesized by Applied Biosystems (USA). The primer pairs for HMG1-F/HMG1-R, ipt-F/ipt-R, NPTII-F/NPTII-R and AtEMF1-F/AtEMF1-R are listed in Table 1. The TaqMan probes, HMG1-P for quantitative PCR, were designed to detect a 76 bp broccoli-specific fragment of *HMG-I/Y* (Figure 3) while in the ipt-P for a 75 bp fragment of transgene *ipt,* the NPTII-P for a 78 bp fragment of selection marker gene *NPTII* and another AtEMF1-P for a 72 bp fragment of transgene *AtEMF1*, as indicated in Figure 1. The probes were labeled with fluorescent reporter dyes 5′-FAM and the nonfluorescent quencher NFQ at the 3′-end.

The PCR reaction volume of 20 μL contained 5 μL of template DNA, 1 μL of 20x primer pair and probe mix (Applied Biosystems, USA), 4 μL ddH_2O and 10 μL of 2x TaqMan® universal PCR Master mix (includes ROX as a passive reference) (Applied Biosystems, USA). The PCR reactions were run on an ABI 7000 Sequence Detection System (Applied Biosystems, USA), with the following program: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The fluorescence signals obtained were measured once for each cycle at the extension step. The PCR results were then analyzed using ABI Prism 7000 SDS Software (Applied Biosystems, USA).

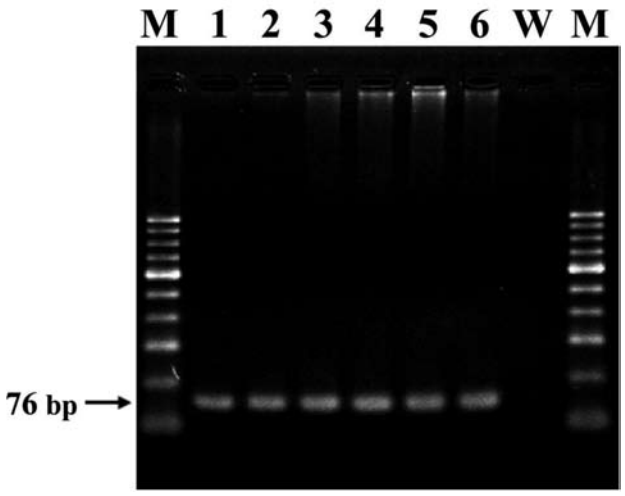

Figure 3. PCR identification of broccoli-specific 76 bp HMG-I/Y fragment by HMG1-F and HMG1-R primer set on tested lines. M: 50 bp DNA ladder; 1: Green King; 2: Elegance; 3: 93-101; 4: 93-102; 5: 93-103; 6: 93-104; W: water control.

Transgenes and endogenous gene copy number calculation

To calculate the number of transgene copies and copy number, we used the equation designed by Weng et al. (2004) below:

$$[1]\ X_0/R_0 = 10^{[(C_{T,X} - I_X)/S_X] - [(C_{T,R} - I_R)/S_R]}$$

where I_X and I_R are intercepts of the relative standard curves of target and reference genes, respectively, S_X and S_R are the slopes of the standard curves of target and reference genes, respectively, and $C_{T,X}$ and $C_{T,R}$ are the detected threshold cycles of the amplification of the target and reference genes to a tested sample. If the copy number of the reference gene (R_0) is well confirmed, the copy number of the target gene (X_0) can easily be deduced from the I_X, I_R, S_X, S_R, $C_{T,X}$, and $C_{T,R}$ in the tested sample.

The accurate number of target gene copies in the tested

Table 1. The primers and probes designed by ABI.

Gene	Primer1 (Sense)	Primer2 (Antisense)	TaqMan Probe	Length
HMG-I/Y	HMG1-F CGTCCTCCTAAGGTGAATGATTCTT	HMG1-R CTCGTGGCCTCCCAGAAA	HMG1-P FAM CACTCGGCGATACCTT NFQ*	76 bp
Ipt	ipt-F GAGTGCAGATTTTCGTTGGCATATT	ipt-R GCCTTGGCCGCTTTCATG	ipt-P FAM CCGACCAAGAGACCTT NFQ*	75 bp
NPTII	NPTII-F GGATTGCACGCAGGTTCTC	NPTII-R GCAGCCGATTGTCTGTTGTG	NPTII-P FAM CCCAGTCATAGCCGAATAG NFQ*	78 bp
AtEMF1	AtEMF1-F GGAGAAAGTGGGTGTAAACTGTGA	AtEMF1-R CGACCACGTGCTTTCTTGAGAA	AtEMF1-P FAM TAGCTGTCTGATCATCATTC NFQ*	72 bp

*NFQ: Non-fluorescent quencher.

samples, I_X, I_R, S_X, and S_R in equation [1] can be deduced from relative standard curves of transgenic plants containing the target and endogenous genes without knowing the exact number of target gene copies. To prepare the relative standard curves of three PCR reaction systems (endogenous gene *HMG-I/Y* and transgenes *ipt*, *NPTII* and *AtEMF1*) by 10-fold, were serially diluted its genomic DNA over four grades (12ng, 1.2ng, 0.12ng and 0.012ng for *ipt/NPTII* and 50ng, 5.0ng, 0.5ng and 0.05ng for *AtEMF1*) with each in duplicate. We were thus able to determine the standard curve correlation coefficients.

RESULTS AND DISCUSSION

Cloning and identification of broccoli *HMG-I/Y* gene

Based on the comparison between the *HMG-I/Y* cDNA sequences of *Arabidopsis* and rapeseed, primer pairs were designed to amplify the partial *HMG-I/Y* cDNA from broccoli (Table 1). The partial *HMG-I/Y* cDNA sequence was aligned on NCBI Blastn, where we noted their high similarity to *Arabidopsis* and rapeseed *HMG-I/Y* genes (Data not show). We then designed some primers from partial *HMG-I/Y* cDNA to do chromosome walking and RACE (Rapid amplification of cDNA ends). Through chromosome walking and RACE we determined that 796 bp cDNA contains an open reading frame of 576 bp along with 63 bp of the 5′-untranslated region and 157 bp of the 3′-untranslated region exclusive of a 13 bp poly(A) tail (Figure 2). We deduced that the broccoli *HMG-I/Y* gene encodes a protein of 192 amino acid residues containing four copies of the AT-hook motif with a calculated molecular mass of 20.78 kDa (Figure 4). The translational start site of the HMG-I/Y coding region (TTGAA**ATG**GC) is very similar to that of the dicots consensus sequence (aaAa(A/C)**ATG**GC) (Cavener et al., 1991). We also compared the *HMG-I/Y* genomic DNA and cDNA that found a single intron (data not shown). This intron has a 89 bp in the broccoli *HMG-I/Y* gene, starts 45 bp downstream of the translation start site, follows the consensus (AG/GT) at the 5′ exon/intron boundary, but diverges (AG/AT) at the 3′ end of the intron. The intron is located in exactly the same place as the single introns in the pea (Gupta et al., 1997), rapeseed (Masek et al., 2000) and *Arabidopsis HMG-I/Y* genes (Gupta et al., 1998). The protein is very similar to HMG-I/Y proteins from other plant species, with the highest similarity to *B. napus* (75% identity at the protein level).

The endogenous copy number of the broccoli *HMG-I/Y* gene was confirmed with Southern analyses, probing with DIG-labeled broccoli *HMG-I/Y* genomic DNA. As indicated in Figure 5, genomic DNA of two cultivars digested by *Bam*H1, *Eco*RI, *Hind*III, *Sac*I and *Xho*l all exhibited a single band pattern, and the single major band of hybridization in each digest suggested it was a single copy of the *HMG-I/Y* gene in the broccoli genome.

Phylogenetic studies among plant species

In addition to the model plant *Arabidopsis thaliana*, we studied two *Brassica species* (*B. oleracea* var. *italica* and *B. napus*), three legume (*Canavalia gladiate*, *Glycine max* and *Pisum sativum*) and four Graminae (*Oryza sativa*, *Avena sativa*, *Triticum aestivum* and *Zea mays*). The phylogenetic relationship of the deduced amino acid se-

Figure 4. Sequence alignment of *B. oleracea* HMG-I/Y protein (deduced from cDNA sequence) using the CLUSTALX program to *A. thaliana*, *B. napus* HMG-I/Y proteins. Gaps introduced to improve the alignment are shown as dashes (-). Color identical amino acids are black, and similar amino acids are light gray. The 'AT-hook' sequences are underlined.

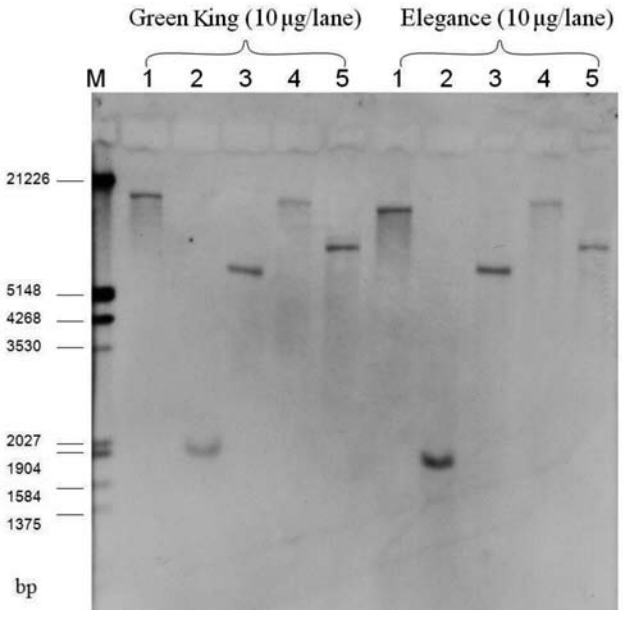

Figure 5. Southern blot confirmed that *HMG-I/Y* gene is a single copy gene on two broccoli cultivars, Green King and Elegance. M: DNA DIG-labeled Marker III (Roche); 1: *Bam*HI digestion; 2: *Eco*RI digestion; 3: *Hin*dIII digestion; 4: *Sac*I digestion; 5: *Xho*I digestion.

quences of the HMG-/Y gene were quite clear, with three distinct clusters as listed in Figure 6. *Arabidopsis* was clustered with the *Brassica* species and the three legume species clustered. *Brassica* species and the legumes are dicots, while the four Gramineae species, i.e. rice, maize, oat and wheat, are all monocots and form another group. The obtained results fit very well with the traditional taxonomic analyses.

Purity and copy number determination on *ipt* transgenic inbred lines by qRT-PCR and Southern analysis.

Standard curves of reference gene *HMG-I/Y* and two linked transgene, *ipt* and selection marker gene *NPTII,* were established through plotting the threshold cycle (Ct) values *vs* a logarithm of template concentrations from four 10-fold serial dilutions. The slopes were -3.139, -3.414 and -3.283, respectively, for *HMG-I/Y*, *ipt* and *NPTII,* and interceptions were 28.369, 28.425 and 29.184. The X_0/R_0 were then calculated from the equation [1] (Weng et al., 2004) as described in the materials and methods. Both T_4 and T_5 *ipt* transgenic inbred lines were estimated for the *ipt* and *NPTII* copy numbers. Random samples from five progeny of each transgenic T_4 inbred (sib-mated) indicated some heterozogosity. As indicated in Table 2, of the T_4 progeny of line 93-101, 3/5 of the *ipt-NPTII* transgene were hemizogotes, line 93-102 had all 5 individual progeny as single copy/haploid genome for both genes and line 93-103 had one progeny, A-17, that resulted in 2 copies of *ipt* and 1 copy of the *NPTII* gene (showing rearrangement had taken place). The last four were single copy/ haploid

Table 2. Estimated numbers of *ipt* and *NPTII* copies for T_4 transgenic ipt inbred line progeny from the qRT-PCR.

Sample	*ipt*		*NPTII*	
	(X_0/R_0)	Estimated number of copies	(X_0/R_0)	Estimated number of copies
93-101-A-4	0.39	0.5	0.58	0.5
93-101-A-9	0.76	1	0.97	1
93-101-A-15	0.43	0.5	0.48	0.5
93-101-A-23	0.96	1	1.21	1
93-101-A-35	0.45	0.5	0.49	0.5
93-102-3-20	0.82	1	1.26	1
93-102-4-12	0.78	1	1.04	1
93-102-1A-12	1.02	1	0.92	1
93-102-1A-26	0.97	1	1.09	1
93-102-2A-7	0.77	1	0.94	1
93-103-A-5	1.06	1	1.24	1
93-103-A-8	0.83	1	0.96	1
*93-103-A-17	1.73	**2**	0.95	1
93-103-A-26	1.14	1	1.23	1
93-103-A-27	0.98	1	1.18	1

Values of X_0/R_0 were calculated according to equation [1]. From standard curves of *HMG-I/Y*, *ipt*, and *NPTII* systems (*Rearrangement).

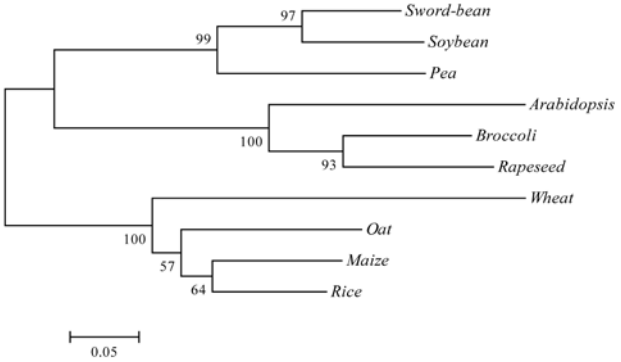

Figure 6. Evolutionary relationships of 10 known plant taxa including sword-bean (*Canavalia gladiata*, soybean (*Glycine max*), pea (*Pisum sativum*), *Arabidopsis* (*Arabidopsis thaliana*), broccoli (*Brassica oleracea* var. *italica*), rapeseed (*Brassica napus*), wheat (*Triticum aestivum*), oat (*Avena sativa*), maize (*Zea mays*) and rice (*Oryza sativa*) of HMG-I/Y proteins based on the deduced amino acid sequences searched from the GenBank as described in the materials and methods. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The scale on the bottom indicates the number of substitutions per amino acid site.

genome for both *ipt* and *NPTII* transgenes. Transgene identification through traditional PCR was not feasible for distinguishing these hemizygous individuals among the selfed or sib-mating populations. In this study, we used the developed inbred lines on the T_4 lines to check transgene zygosity. Hemizygous individuals and rearrangement occurred in lines 101 and 103, respectively. An explanation for the hemizygotes might be their accidental pollination by the non-transgenic progeny, as mentioned in the materials and methods. We noted the lack of pollen in some *ipt* transgenic inbred progeny, thus conducting sib-mating to obtain the seeds for further study. However, through selection of T_4 individuals with homozygous transgene we further self-pollinated for advance generation to derive the T_5 inbred lines. The estimated result from the T_5 progeny for the above three transgenic lines all exhibited an unique purity. We checked 20 progeny with regular PCR and randomly picked 10 individuals from each line for qRT-PCR and observed single copy/haploid genome for both *ipt* and *NPTII* transgenes (Table 3). The Southern confirmation had been proved elsewhere (Chan et al., 2009) from five random individuals selected from each T_5 line. In this

Table 3. Estimated numbers of *ipt* and *NPTII* copy of *ipt* and *NPTII* for T_5 transgenic ipt inbred line progeny from the qRT-PCR.

Sample	*ipt*			*NPTII*		
	(X_0/R_0)	Estimated number of copies	Southern blotting (*Hind*III)	(X_0/R_0)	Estimated number of copies	Southern blotting (*Hind*III)
95-101-58	0.93	1	1	0.91	1	1
95-101-59	1.02	1	1	0.89	1	1
95-101-60	1.07	1	1	1.05	1	1
95-101-71	0.99	1	1	0.95	1	1
95-101-72	0.99	1	1	1.01	1	1
95-101-73	0.93	1	1	0.95	1	1
95-101-263	1.08	1	1	0.98	1	1
95-101-265	1.06	1	1	0.88	1	1
95-101-266	1.03	1	1	0.97	1	1
95-101-300	1.05	1	1	1.01	1	1
95-102-1	0.96	1	1	0.99	1	1
95-102-2	1.04	1	1	1.08	1	1
95-102-3	0.96	1	1	0.99	1	1
95-102-15	0.96	1	1	1.12	1	1
95-102-16	0.99	1	1	1.19	1	1
95-102-17	1.03	1	1	1.04	1	1
95-102-27	0.88	1	1	0.95	1	1
95-102-37	1.14	1	1	1.08	1	1
95-102-201	1.00	1	1	1.05	1	1
95-102-204	0.99	1	1	1.10	1	1
95-103-1	1.08	1	1	1.02	1	1
95-103-2	0.97	1	1	1.05	1	1
95-103-3	1.10	1	1	1.01	1	1
95-103-11	1.01	1	1	1.04	1	1
95-103-13	0.98	1	1	0.88	1	1
95-103-23	0.96	1	1	0.91	1	1
95-103-24	0.90	1	1	0.94	1	1
95-103-31	0.99	1	1	1.04	1	1
95-103-32	0.93	1	1	0.98	1	1
95-103-33	1.08	1	1	1.14	1	1

[a] X_0/R_0 are calculated from equation[1], with slopes -3.239, -3.315, -3.319 and intercept 23.019, 23.216, 23.505 from the standard curve of *HMG-I/Y, ipt* and *NPTII* respectively.

study, the *Hind*III digested genomic DNAs of lines 101, 102 and 103 from each of five randomly selected individuals within line also proved to be single insertion (Figure 7).

Copy number screening on the *AtEMF-1* transgene by qRT-PCR and Southern confirmation

We also randomly selected 24 T_0 transformants for AtEMF-endogenous 1 transgene determination. The relative standard curves of 3 PCR reaction systems (gene *HMG-I/Y* and transgene *AtEMF1*) were established by 10-fold, serial dilution of its genomic DNA over four grades with each in duplicate and three repeated measurements. The correlation coefficients of the standard curves were good (0.999 for *HMG-I/Y* and: 0.997 for *AtEMF1*) and the PCR efficiencies were all above 0.95. Copy number estimation by qRT-PCR was then calculated according to equation [1], relative standard curves of three reaction systems determined slopes (S_X and S_R) and intercepts (I_X and I_R) for the target genes *AtEMF1*, and reference gene *HMG-I/Y.* The slopes for *HMG-I/Y* and *AtEMF1* were -3.360 and -3.389, and the intercepts were 29.110 and 31.513, respectively (Figure 8). Relative standard curves allowed $C_{T,X}$ (*AtEMF1*) and $C_{T,R}$ (*HMG-I/Y*) calculations for each transgenic plant tested. Each reaction had three replications. Because the transgenic lines in the T_0 generation are hemizygotes for transgenes and homozygotes for endogenous genes, the copy number of the target gene in T_0 transformants should be calculated after the value of X_0/R_0 in equation [1] is doubled. The copy numbers of the *AtEMF1* in primary broccoli transformants (T_0) was calculated by comparing quantitative PCR result of the *AtEMF1*

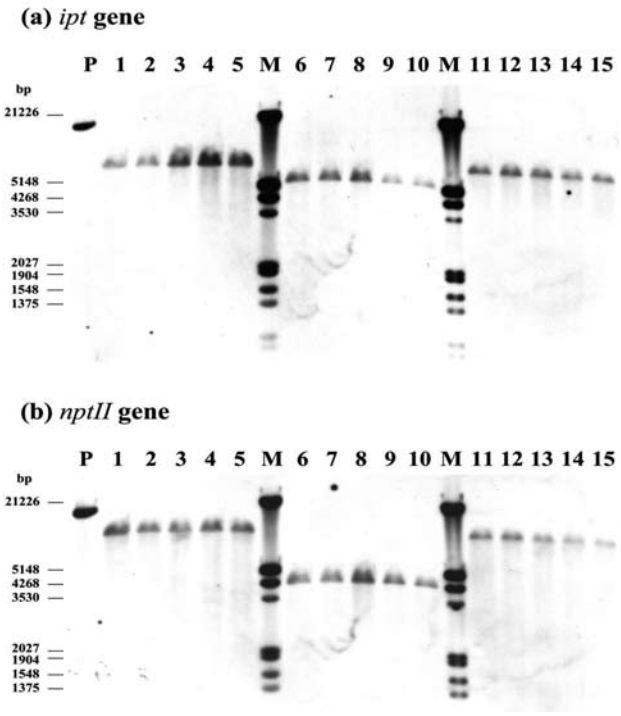

Figure 7. Transgene confirmation by Southern hybridization on transgene *ipt* and selection marker gene *NPT*II of transgenic T_5 inbred progeny based on *Hind*III digestion and exhibiting the single insertion in the three tested lines each with five randomly selected individuals. M : DNA DIG-labeled marker III; P: pSG766 plasmid DNA; 1-5: individuals from 95-101 inbred line; 6-10: individuals from 95-102 inbred line; 11-15: individuals from 95-103 inbred line.

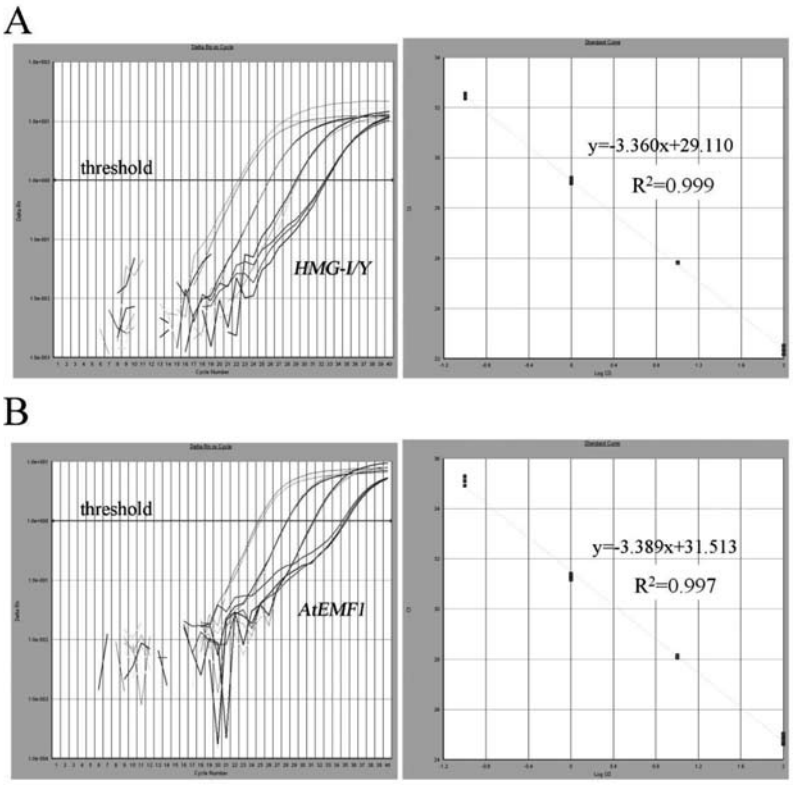

Figure 8. Real-time PCR amplification of the logarithmic plots resulting from the amplification of four 10-fold serial dilutions (Genomic DNA:50ng, 5ng, 0.5ng, 0.05ng) of DNA from lines AtEMF1-03 and its relative standard curve, obtained with the threshold cycle (C_T) value versus the log of each initial concentration on (A) *HMG-I/Y* gene; and (B) *AtEMF1* gene.

gene with the *HMG-I/Y* internal standard. We obtained the number of transgene *AtEMF1* copies from 24 tested T_0 transformants (Table 4).

As suggested by Ingham et al. (2001), samples with lower copies allowed better accuracy in the TagMan copy number assay. Lower copies of transgene insertion also simplified the explanation for transgene activity in transgenic studies. Our goal for establishing the qRT-PCR was also aimed at screening lower copy transformants. At the sampling stage, we thus used the seven transgenic broccoli lines (from 24 transformants) with the lowest estimated copy numbers (using real-time PCR) and with enough leaf tissue for DNA extraction for Southern analyses. As shown in Figure 9 and Table 4, the results from the Southern blotting and real-time PCR analyses were consistent in six samples (86%) and differed in one (14%). However, we cannot rule out that the inconsistent one was incompletely digested.

In this study, we successfully developed a rapid and reliable TaqMan real-time PCR method for estimating the number of integrated copies of a transgene in transgenic broccoli. This method can be used as an alternative to Southern blotting. Southern blotting showed that the *HMG-I/Y* gene was present as a single copy in the broccoli genome. We used TaqMan qRT-PCR systems and compared it to a novel broccoli endogenous reference gene coding for the *HMG-I/Y* gene to determine the copy numbers of the *Arabidopsis* embryonic flower 1 (*AtEMF1*) gene in transformed broccoli. We also found that this method can directly identify hemizygotes or homozygotes of the transgene in T_1 plants. Homozygous transgenic plants can thus be obtained in the T_1 generation, which can greatly reduce the time required to select the ideal plants for further breeding.

Table 4. Estimated numbers of *AtEMF1* copies for each line from the qRT-PCR and Southern blot results.

Sample	(X_0/R_0)	Estimated number of copies	Southern blotting (*EcoRI*)
AtEMF1-03	1.82	2	2
AtEMF1-05	1.88	2	2
AtEMF1-10	4.55	4~5	3~4
AtEMF1-13	1.30	1	1
AtEMF1-14	1.33	1	1
AtEMF1-19	9.89	10	ND[a]
AtEMF1-23	1.31	1	1
AtEMF1-24	1.34	1	1
AtEMF1-30	3.32	3	ND
AtEMF1-31	5.08	5	ND
AtEMF1-33	1.01	1	ND
AtEMF1-36	34.67	35	ND
AtEMF1-37	1.81	2	ND
AtEMF1-38	1.80	2	ND
AtEMF1-39	1.88	2	ND
AtEMF1-41	7.02	7	ND
AtEMF1-45	2.06	2	ND
AtEMF1-51	3.24	3	ND
AtEMF1-52	3.16	3	ND
AtEMF1-58	4.05	4	ND
AtEMF1-61	3.03	3	ND
AtEMF1-62	1.21	1	ND
AtEMF1-79	15.13	15	ND
AtEMF1-74	13.98	14	ND

[a]ND: not determined.

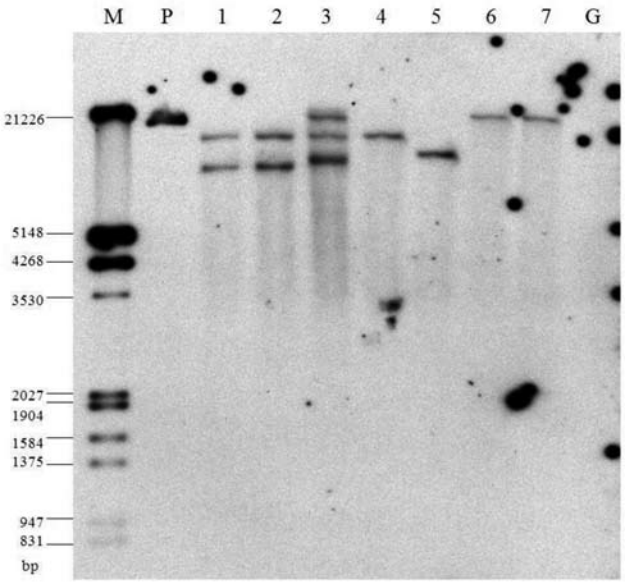

Figure 9. Southern blot confirmation of *AtEMF1* Transgene based on the genomic DNA digestion of *Eco* RI. M: DNA Molecular Weight Marker III (Roche); P: *AtEMF1* plasmid DNA; 1: AtEMF1-03; 2: AtEMF1-05; 3: AtEMF1-10; 4: AtEMF1-13; 5: AtEMF1-14; 6: AtEMF1-23; 7: AtEMF1-24; G: Green king.

High-mobility group (HMG) proteins are known as low molecular weight non-histone proteins that commonly exist in higher eukaryotic organisms (Bustin et al., 1990). The HMG-I/Y protein genes isolated from various plants such as rice, oats, Japanese jack bean, pea and *Arabidopsis* have been noted (Gupta et al., 1997) and adopted as endogenous reference genes for transgene copy number determination in *B. napus* for qRT-PCR assays (Weng et al., 2005; Weng et al., 2004; Wu et al., 2010). Documented occurances of *HMG-I/Y* in *B. napus*, however, have been somewhat controversial. *HMG-I/Y* cloned from a microspore-specific library of *B. napus* have expressed in all tissues, especially in pollen-derived embryos (Masek et al., 2000). We noted that four copies of the "AT-hook" motif had 62% homology with the *Arabidopsis thaliana* protein sequence. Our deduced protein sequence (Figure 4) showed similar conformation, having 75% homology with *B. napus*. However, the cloned *B. napus* HMG-I/Y protein genes were detected in closely related *B. carinata*,

B. campestris var. *chinesis* and *B. rapa* species, but not in *B. oleracea* using Southern analysis with a *HMG-I/Y* cDNA probe (Masek et al., 2000; Weng et al., 2004). No amplification was observed when template DNAs from other species of *Brassica* or other species such as broccoli, stem mustard, cauliflower, Chinese cabbage, cabbage, sprouts, *Arabidopsis*, carrot, tobacco, soybean, mung bean, tomato, pepper, eggplant, plum, wheat, maize, barley, rice, lupine and sunflower (Weng et al., 2005) were used. As indicated by our comparison in Figure 2, the primer set designed by Weng et al. (2004), sense primer 5′ GGTCGTCCTCCTAAGGC/tGAAA/tG 3′ was located at positions 430-451 with mismatches at positions 446 and 450 (Figure 2 underlined). This appeared as "t" (shown in lower case for the ease of differentiation) in both positions for the broccoli *HMG-I/Y* full cDNA sequence, while the reverse primer 5′ CTTCGC/aGGTCGTCCA/tC 3′ located at positions 506-525. Here we found a mismatch on the *HMG-I/Y* sequence at position 507,"a" for broccoli and "T" for rapeseed, and at position 516, "t" for broccoli and "G" for rapeseed. The inability to detect the *HMG-I/Y* on broccoli in the study by Weng et al. (2004) might be due to the mismatched bases in the designed sense primer having two mismatched codes at the 3′ end. However, Masek et al. (2000) used the cloned putative HMG-I/Y protein gene as a probe for Southern analyses, did not detect *HMG-I/Y* in *B. oleracea* either. *B. oleracea* had multiple origins and parents including *B. oleracea* var. *gemmifera*, *B. oleracea* var. *italica*, *B. oleracea* var. *botrytis*, *B. oleracea* var. *alboglabra*, *B. oleracea* var. *acephala*, *B. oleracea* var. *gongylodes*, *B. oleracea* var. *capitata*, etc. The controversial results might also be due to the taxonomic differences among the research groups. However, it is clear from our study that broccoli carries the HMG-I/Y protein gene. Through comparison of five endogenous reference genes for specific PCR detection and quantification of *B. napus,* two sets of PCR primers for *HMG-I/Y* could be positively amplified in tested *Brassica* species, even from some distant cruciferae species, including cultivars of *B. oleracea* such as cabbage (*B. oleracea* cv. Niuxin), Chinese kale (*B. oleracea,* ssp. *albaglabra* cv. Hongkong) and white Chinese kale (*B. oleracea,* ssp. cv. Tianjin) (Wu et al., 2010). Although, no direct tested sample of broccoli was involved, we cloned the *HMG-I/Y* from broccoli and proved it was a single copy in the broccoli genome (Figure 5). We also demonstrated the usefulness of designed primer sets in detecting the transgene copy number in transgenic populations.

In this study, we first cloned the *HMG-I/Y* through chromosome walking and then designed proper primer sets for qRT-PCR study. The established system was first tested for detecting heterogenous transgenic lines and further examined the purity of advanced self-pollinated generations screening on the T_4 and T_5 *ipt* transgenic inbred lines. We also demonstrated in our newly transgenic *AtEMF1* study how to screen for low copy number transgenic transformants. The merit of the established system is that it rapidly identifies low transgene copy numbers in broccoli and can be extended to the related *B. oleracea* species. The theoretical limitation of the TaqMan copy number assay suggests fewer than four copies (Ingham et al., 2001). As noted in Table 4, the estimated copy numbers of the T_0 transformant varied from single to multiple copies. This fit well with the collected low copy transgene samples that we selected from the Southern analysis.

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青花菜高移動性蛋白群 HMG-I/Y 基因之選殖及提供為定量 Real-time PCR內生性標準估算轉基因套數之分析評估

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植物基因轉殖技術已是作物改良不可或缺之工具，然一般亦知轉基因之嵌入基因組位置與轉基因之套數關係著轉基因之表現與穩定性。TagMan 定量式即時聚合酶連鎖反應 (Quantitative real-time Polymerase Chain Reaction, qRT-PCR) 係被發展出具若干優點如測試所需樣品量小、高流通量、高定量範圍及無後 PCR 之膠體電泳分析等之快速檢定轉基因套數方法。惟此方法之分析有賴建立一在基因組內低套數且穩定之內生性參考基因為基準。在蕓苔屬 (*Brassica*) 作物中，高移動性蛋白群 HMG-I/Y 基因曾被報導利用為 qRT-PCR 分析之參考基因，但該基因是否可在青花菜偵測得則有所爭議。本報告因此乃參考已報導之油菜 (*Brassica napus*) 及阿拉伯芥 *HMG-I/Y* 基因序列選殖得一全長 796 bp 青花菜 HMG-I/Y cDNA 序列並加以定性其衍生之胺基酸序列與油菜之相似度達 75%。利用已知作物 HMG-I/Y 基因衍生胺基酸序列在演化樹分析上，其結果與傳統分類分群極為吻合。本研究亦証實 *HMG-I/Y* 基因在青花菜基因組每單套基因組為單套存在，利用研究室之異戊丙烯轉移酶 (*ipt*) 基因及阿拉伯芥胚性開花基因 (*AtEMF1*) 青花菜轉殖材料進行檢測與篩選低套數轉基因，亦獲致該基因利用為內生性標準參考基因以估算轉基因套數之可行性。

關鍵詞：定量式聚合酶連鎖反應；轉殖青花菜；阿拉伯芥胚性開花基因；異戊丙烯轉移酶基因。