

***In silico* analysis and transient expression of wound-inducible promoter *MPI* in tomato (*Lycopersicon esculentum* Mill. cv. CH)**

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Abstract

Various genetic promoters are applied to generate transgenic plants. Constitutive promoters such as CaMV35S are mostly being used; however, the constitutive expression of a gene could be harmful and lead to many changes in plant metabolism, growth and development. Subsequently, using inducible promoters in plant transformation has become more useful. In this project, efficiency of the wound-inducible promoter of *Maize Proteinase Inhibitor (MPI)* in tomato *Lycopersicon esculentum* Mill. cv. CH, as a new genetic background, was investigated. Therefore, the *MPI* was amplified following insertion into pTZ57R/T, sequencing and subsequently ligation into binary vector pBI121. Recombinant plasmids were transferred into *Agrobacterium tumefaciens* AGL1 strain. The results of *in silico* sequence analysis of *MPI* specified methyl jasmonate (MeJA) and abscisic acid (ABA) as responsive motifs, which might play a role in wound signaling pathway. Therefore, tomato leaves were transformed by PBI:*MPI*:*GUS* using agroinfiltration approach following *GUS* expression analysis by applying histochemical *GUS* and qRT-PCR assays. Our results demonstrated that induction of the *MPI* promoter only occurred in the wounded leaves and not detected in the control leaves. *GUS* transcripts in the wounded leaves containing *MPI* promoter were 4.1 folds higher than those containing CaMV35S, and *GUS* histochemical assays also revealed that the *MPI* give higher color intensity (2.64 folds) compared to the CaMV35S. Thus, the *MPI* could be considered as an acceptable working and inducible promoter to generate dicot transgenic plants harboring insecticidal genes such as different types of *cry* genes, which just expressed when plants are being attacked by pests.

Keyword: *GUS* reporter, *MPI*, Tomato, Wound inducible promoter.

Abbreviation: ABA_Abscisic acid; CaMV35S_Cauliflower mosaic virus 35S; JA_Jasmonate; MeJA_Methyl jasmonate; *MPI*_Maize Proteinase Inhibitor; PBS_Phosphate buffered saline, Ubi_Maize ubiquitin.

Introduction

Many foreign genes have been introduced into plant genomes. Constitutive promoters such as cauliflower mosaic virus 35S (CaMV35S) and maize ubiquitin (Ubi) have been widely used in various plant genetic engineering approaches. The constitutive expression of a gene is harmful to their host and lead to several changes in plant metabolism as well as plant growth and development. For instance in absence of biotic stresses, it can sometimes cause metabolic drain (Zhang et al., 2013). Constitutive expression of genes is normally unregulated; therefore, take a lot of energy for their transcription and translation. Therefore, it is much more efficient if expression of a foreign gene just occurs when the gene product is needed. The constitutive expression of promoters cause presence of transgenic protein in all tissues at all stage of growth and development. It is much more acceptable to limit transgene transcription to only target tissues (Breitler et al., 2001; Andow and Ives, 2002; Bates et al., 2005; et al., 2001; Kim et al., 2008). The CaMV35S promoter has been widely used in Bt crops and after two decades, cultivation of these crops has been questionable due to the increase of pest resistance (Gassmann et al., 2011; Tabashnik et al., 2013; Van den Berg et al., 2013). Using CaMV35S promoter in production of transgenic plants has also provided concerns regarding the food safety of genetically modified plants (Kuiper et al., 2001; Shelton et

al., 2002; Conner et al., 2003). Hence, it might be desirable to use inducible or tissue-specific promoters such as RSRE (general stress- inducible promoter) (Zhang et al., 2013). Therefore, use of wound-inducible promoters, which drive expression of genes encoding insecticidal proteins such as various types of CRY proteins, could be more practical to keep plants in normal growth, development as well as saving their energy in absence of biotic stress. In addition to the aforementioned advantages, the expression of transgenes should occur after pest attack in order to reduce likely unwanted effects on non-target organisms. Therefore, using wound-inducible promoter could be an important strategy and be helpful for insect resistance management strategies.

Plants have developed diverse physiological and biochemical mechanisms as defense systems against insects, pests and pathogens. These mechanisms are reinforcement of cell wall, limitation of cell energy consumption (translation arrest of some nuclear-encoded photosynthetic genes) and activation of defensive compounds (synthesis of chitinase, glucanase, phytoalexine, amylase inhibitor and serine proteinase inhibitor). Among a large number of proteins induced by wounding, plant serine proteinase inhibitors are particularly activated against insect pests and herbivores and can inhibit animal and microbial proteinase, (Heitz et al., 1999; Stotz et al., 1999; Hartl et al., 2011; Jamal et al., 2013).

A cDNA encoding a serine proteinase inhibitor of maize (MPI) has been isolated from maize in 1994 (Cordero et al., 1994). It has been reported that the *mpi* is locally and systemically inducible by mechanical and fungal wounding as well as treatment by methyl jasmonate (MJ) and abscisic acid (ABA). Moreover, it has been shown that 689 bp upstream to 197 bp downstream region of start site of *mpi* is responsive to wounding (Breitler et al., 2001), meaning that this region could be probably used as a wound-inducible promoter. Consequently, the efficiency and performance of MPI promoter has been investigated in tomato as a model plant for dicot species.

Transient expression of foreign genes in plant tissues is a valuable and functional method for investigating gene function in a short time period with low expenses (Yang et al., 2000). This method is simple, rapid and there's no need for any specialized equipment. In this regards, agroinfiltration is an *in planta* method to analyze gene function. In this approach, the *Agrobacterium* suspension harboring gene of interest is injected into plant leaves using a needle-free syringe. After 3-7 days (in order to integrate foreign gene in plant genome), expression of interesting genes can be investigated without costly equipments and complicated methods. In this system, bacteria suspension normally enters leaves through stomata following T-DNA transfer to nucleus. Agroinfiltration includes several advantages such as time and cost saving in comparison to other transient expression systems such as particle bombardment. This technique has been used in many experimental researches for example, studying foreign gene function (Chen et al., 2013), investigation of plant promoter expression (Liu et al., 2003), study of hypersensitivity responses (Palanichelvam et al., 2000) and gene silencing (Voynet et al., 2003).

In this study, in order to evaluate in order to evaluate inducible efficiency of the MPI in a new background (tomato as a dicot model), *in silico* sequence MPI promoter analysis was first performed. Then transient expression of *GUS* reporter gene was assessed using an agroinfiltration system and qRT-PCR approach. The obtained results showed that MPI could be considered as an acceptable inducible promoter to generate dicot transgenic plants.

Results and discussion

It would be very convenient to use inducible or tissue-specific promoters in generating transgenic plants for reduction of damages as well as decrease in the amount of plant metabolic changes. Regarding this purpose, working efficiency of the wound-inducible MPI promoter in dicot plants (tomato as a model) was investigated by conducting *in silico* sequence analysis, histochemical *GUS* and qRT-PCR assays. The advantages of employing wound-inducible promoters over constitutive promoters are: 1) wound-response is independent of plant age; and 2) the gene under control of wound-inducible promoter is induced in response to phytophagous insects attack and toxin (such as Cry), and does not produce and accumulate during plant development leading to energy saving (Breitler et al., 2004).

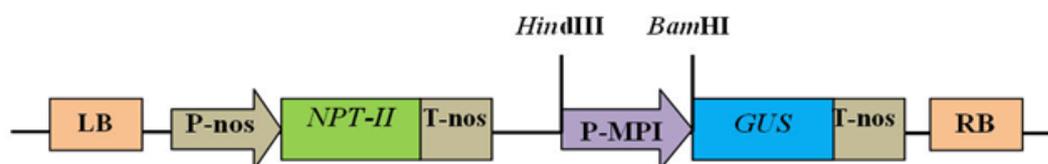
In Silico detection of the various MPI promoter elements

Computational prediction of promoter elements is an important approach to improve understanding of gene expression regulation. Before conducting an experiment, *in silico* sequence analysis actually help to design appropriate experiments. Based on our results, the length of sequenced

MPI region was 886 bp. After confirmation of the MPI sequence by BLASTn, TSS site was determined by using SOFTBERRY database following determination of +1 position at the nucleotide sequence 701, and also TATA box sequence was found at -36bp upstream of TSS. Functions of different promoters is determined based on their cis elements (Hernandez-Garcia and Finer, 2014). Therefore, in the current study, PLANTCARE software was employed to determine wound inducible and other motifs of the MPI promoter (Fig. 2; Table 2). CAAT box, which is found to be at -57bp upstream of TSS, is a binding site signal for RNA transcription factors (Ke et al., 1997). Four methyl jasmonate (MeJA) inducible motifs were identified and subsequently classified into two groups; First group, CGTCA motif which is located at 215 upstream and 185 downstream of TSS site, and second group, TGACG motif was identified at -215 and +162 of TSS in complementary strand. G-box (CACGTG), which is involved in light response, UV and abscisic acid (ABA) stimuli was predicted at -640bp upstream of TSS (McKendree Jr and Ferl, 1992). MRE motif (5'-AACCTAA-3') is considered as a recognition element for MYB transcription factor and is necessary for light responsiveness (Feldbrügge et al., 1997). This motif was determined at -699 nucleotide position. Two other motifs, which are involved in response to light, were identified at -450 (5'-TACGTG-3') and -64 (5'-CACGTC-3') of TSS. The GT1 (5'-GTTGTGAA-3') and H (5'-ACCATTTTCACTC-3') motifs were recognized at -341 and -347bp of TSS, respectively. These motifs are involved in light-mediated regulation (Hagen et al., 1999). Two TC-rich motifs were predicted in the MPI promoter at -603 (5'-ATTTTCTCCA-3') and -368 (5'-ATTTTCTTCA-3') location involving in plant stress and defense responses. The LTR motif (5'-CCGAAA-3') at -283 is involved in low-temperature response. another report revealed that the LTR motifs regulate cold, drought as well as ABA related gene expression (Baker et al., 1994). Several ACGT elements were found in MPI promoter, as well. *In vivo* assessment of transgenic plants have illustrated that these elements are necessary for maximal transcriptional activity (Foster et al., 1994). Several W-boxes were determined in the MPI promoter (Table 2). Results of a microarray-based study revealed that w-box is a common promoter element in a group of defense-related genes (Maleck et al., 2000), therefore, W-box could be an important cis-acting element for transcriptional regulation after pathogen attack. WRKY transcription factors can bind to W-box and enhance transcription of the related gene in secondary metabolite biosynthesis process, as well (Yu et al., 2001; Kumar et al., 2009; Yang et al., 2013). plants normally use several molecules such as jasmonate (Farmer and Ryan, 1990), ethylene (Donnell et al., 1996) and ABA (Pena-Cortés et al., 1989) for activating of defense response through transcriptional activation of specific genes. It has been reported that MeJA and ABA can induce expression of genes through specific motifs, which are located in wound inducible promoters (Xu et al., 1993). Therefore, existence of jasmonate (JA), ethylene and ABA responsive elements suggests that probably the MPI has a good potential to be used as a wound-inducible promoter. JA (and its derivatives MeJA) and ABA act as wound signals in response to mechanical wounding, as well (Hernandez-Garcia and Finer, 2014). Herbivore pests activate proteinase inhibitor gene through JA synthetic pathway. Therefore, plants may use expression induction of insecticidal related genes as a defense system. By sequence Alignment of MPI promoter isolated from cultivar 704 with the *mpi* gene sequence in NCBI (B73, accession number: X78988.2), Kimura 2-parameter (K2P)

Table 1. The primer sequences designed for amplification of the *MPI* promoter designed by Oligo 7.

Primer name	Primer sequence	ΔG	bp	Annealing temperature
F-MPI	3'- AAGCTTTTATAGTTCTACACAAAACCCTC - 5'	-9.4	29	
R-MPI	5'- TCTAGACCGGACCAGTTGACGA -3'	-8.1	22	65

**Fig 1.** Schematic representation of the T-DNA cassette in the binary vector pBI:*MPI:GUS*. Abbreviation RB, right border; LB, left border; *nptII*, neomycin phosphotransferase II gene; P-nos, nopaline synthase promoter; T-nos, nopaline terminator; gus, beta-glucuronidase gene; P-MPI, Maize Proteinase Inhibitor promoter which replaced for CaMV35S promoter.**Table 2.** Cis-acting regulatory elements determined in the *MPI* promoter by PLANTCARE software.

Element	Sequence (5' → 3')	Position	Strand	Excepted function
ABRE	TACGTG	-450	+	ABA responsive element
AE-box	AGAAACAA	-296	+	Light responsive element
	AGAAACAA	-228	-	Light responsive element
CAAT-box	CAAAT	-603	-	Cis-acting element in promoter and enhancer regions
	CAAT	-388	-	Cis-acting element in promoter and enhancer regions
	CAATT	-406	-	Cis-acting element in promoter and enhancer regions
	CAAT	+43	+	Cis-acting element in promoter and enhancer regions
	CCAAT	-489	-	Cis-acting element in promoter and enhancer regions
	CAAT	-357	-	Cis-acting element in promoter and enhancer regions
	CAAT	-405	-	Cis-acting element in promoter and enhancer regions
	CAAT	+113	+	Cis-acting element in promoter and enhancer regions
CAG-motif	GAAAGGCAGAC	+37	-	Light responsive element
CAT-box	GCCACT	-107	-	Cis- acting regulatory element related to mersitem expression
CCGTCC-box	CCGTCC	-638	-	Cis- acting regulatory element related to mersitem expression
CGTCA-motif	CGTCA	-215	+	MeJA responsive element
	CGTCA	+162	+	MeJA responsive element
	CACGTA	-640	-	Light responsive element
G-box	TACGTG	-450	+	Light responsive element
	CACGTC	-640	+	Light responsive element
	GTGTTGAA	-341	-	Light responsive element
GT1-motif	GTGTTGAA	-341	-	Light responsive element
H-box	ACCATTTTCACTC	-347	+	Light responsive element
LTR	CCGAAA	-283	+	Cis-acting element involved in low-temperature responsiveness
MBS	CAAGTG	+165	+	MYB binding site involved in drought-inducibility
MRE	AACCTAA	-699	-	Light responsive element
Skn1-motif	GTCAT	-428	+	Cis-acting regulatory element required for endosperm expression
TATC-box	TATCCCA	-162	-	GA-responsive element
TC-rich repeat	ATTTTCTCCA	-603	+	Cis-acting element involved in defense and stress responsiveness
	ATTTTCTTCA	-368	+	Cis-acting element involved in defense and stress responsiveness
TCT-motif	TCTTAC	-440	-	Light responsive element
TGACC-motif	TCGAC	-215	-	MeJA responsive element
	TGACG	-148	-	MeJA responsive element
	TGAC	-567	+	WRKY DNA binding proteins
W-box	TTGAC	-590	+	WRKY DNA binding proteins
	TGACT	-403	+	WRKY DNA binding proteins
	TGAC	-183	+	WRKY DNA binding proteins
	ACCTAACTCC	-260	-	Part of a light responsive elements

AAGCTTTT TAGGTTCTACACAAAACCCTCTATGGCATCGTGGATGGTCCACCACCTACAAGTGGAC**CACG**
TGWUNGGTCCACGCGCCTGCAGAGAAGAGCATAGTTATTTGCTCCATGGTGACCTACACGCGAACGGTCCA
 GTGACCTATCACGAACCGTTCAGCACCTTGCAGAGAGATCTCCAAGGTCTCGAC**TACTC**^{JRE}ATGAGCGGA
 ATCTAGTGATTATTGGTGTTAGCGCAAAAACGGCGCCAACAGTAGGGTTT**ACGTGC**^{ABRE}GAAAGTAAGAT
 GGAGGGTCATAGAGTTAAGATATAGAAAATTGACAGATTCCCTCCTATTGTTGGATAACTCGGCGGATTTT
 GTTCAGATTGATTTAGACCATTTTCACACACTATCAGATGCCGATCCTAGCTAATGGCCAGAATCTGAGA
 AACAAACACCCGGAACGTACAGCGGTGGGCGAGG**TGGTTAG**^{MeJRE}GTCACTGTTCCACCGAGTTTGGATA
 AGTTTCTGTCCG**CGTCA**^{MeJRE}GCGCCAGCTACGCGCGCTGGAAAAATGCTGACTATTTTGGCTGCAAGCAT
 GGGATAACCCAGGATTTTTTTTCTCGAGTGTGCTATCTGTTCTTTGTTTCGTAGTGGCTGTTTATCCT
 CGTGTGCCTCGCGTGCGTATATACACACACGTCCTGCCTGCACGCAACGCAGCTC**TATAAATA**^{TATAbox}CC
 TCATCACCGGTGCACTCGCTTAATATATATCCAACAACCAGCAGTGCAACAAGCAAGATTTACCTGCCCG
 TGAGTGTGCCTTTCAATAATATAATGTCTTATTTTATATATGTTTTTGCAGTGATAGTAGTCTGTAAT
 AAATCTCTCCTTCAATATATATAATTACATGCCAGATCGATCATATCGTCAACTGGTCCGGTCTAGA

Fig 2. The nucleotides sequence of cloned *MPI* promoter from *Zea mays* L. cv. 704. This sequence has been deposited to GeneBank (Accession number: KP793074). The TATA box and wound inducible motifs are shown. The bold letters indicate some boxes which are induced by abscisic acid (ABRE), methyl jasmonate (MeJA), Wound (WUN) and jasmonate (JRE). The PLANTCARE software has been use to identified the *MPI* promoter cis elements.

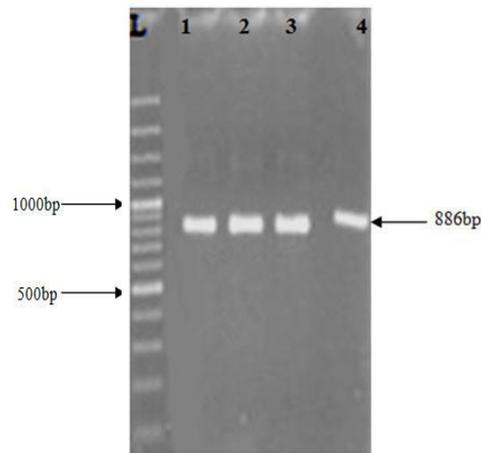


Fig 3. Confirmation of the presence of *MPI* promoter in several *Agrobacterium* strain AGL1 colonies by PCR. An 886 bp fragment of the *MPI* promoter (from *Zea mays* L. cv. 704) was amplified by PCR from the recombinant colonies. L) 1 Kb ladder. Lane 1-4 presents the transgenic lines.

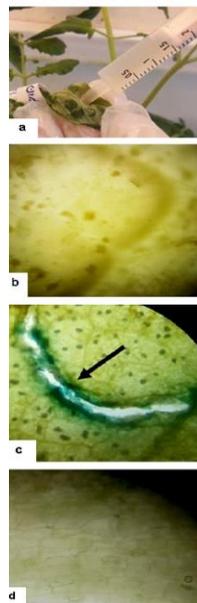


Fig 4. Agroinfiltration and Histochemical GUS assay. a) Injection of *Agrobacterium* suspension harboring the pBI:*MPI*:*GUS* plasmid into tomato leaf. b) Unwounded and c) Wounded leaves injected by the construct suspension *MPI* promoter (pBI:*MPI*:*GUS*); and d) negative control (injected by PBS buffer). The black arrow shows the wounded site. *GUS* has been expressed only at the wounded site (c). There was no *GUS* expression at a detectable level in unwounded leaves.

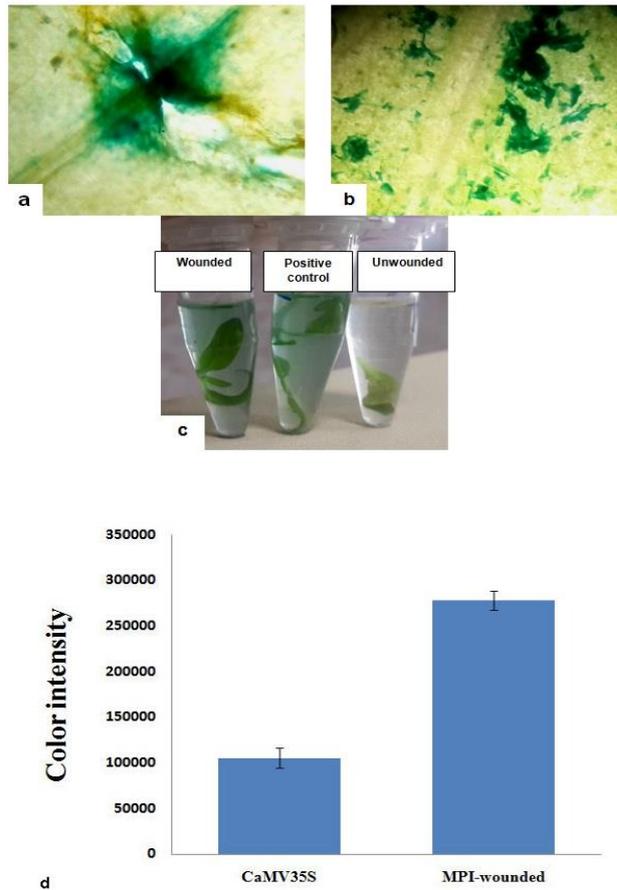


Fig 5. Histochemical GUS assay and comparison of color intensity using imageJ. a) Wounded sample containing the *MPI* promoter (pBI:*MPI:GUS*); b) Positive control using a constitutive promoter (pBI:35S:*GUS*) and c) Macroscopic comparisons of samples. As shown in the above figure, the color intensity of the *MPI* promoter (b) is approximately equal to that of constitutive CaMV35S promoter (a). The macroscopic assessment results consistent with the above findings. d) A comparison of the blue color intensity data obtained from GUS histochemical assay using imageJ revealed that the color intensity of *MPI* promoter is more than CaMV35S. Therefore, the *MPI* promoter has sufficient strength to express its downstream genes.

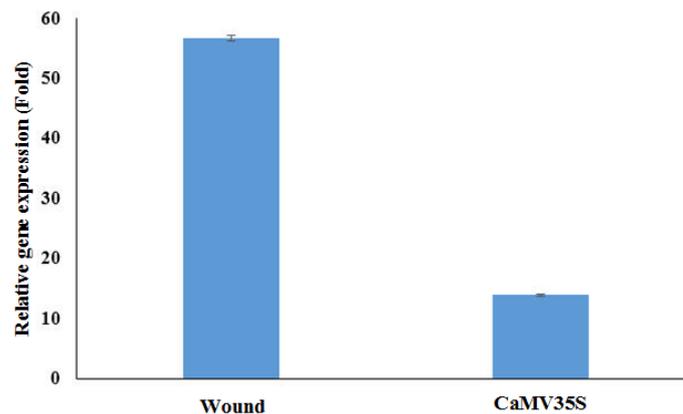


Fig 6. Quantitative measurement of relative expression of *GUS* gene in young and healthy leaves from one-month-old plants after 72 hours of agroinfiltration. The universal *Actin* gene was used as an internal reference gene. Each column calculated using $2^{-\Delta\Delta Ct}$ formula, as mentioned in material method, with standard errors from three replicates. As shown in the figure, the expression level of *GUS* in leaves with the *MPI* promoter is more than CaMV35S.

was calculated. K2P and gene diversity were 0.003 and 0.34%, respectively. Three transition substitutions were found in the isolated *MPI* promoter at 26, 164 and 544 positions. These substitutions were not detected in wound inducible motifs. Based on the aforementioned *in silico* sequence analysis, the isolated *MPI* promoter probably can meet our goal to function as a wound-inducible promoter in plants. In order to verify the bioinformatic predictions, *pBI:MPI:GUS* was transferred into tomato leaves using agroinfiltration approach

Confirmation of recombinant clones following agroinfiltration of tomato leaves

Recombinant colonies containing the suitable segment (886bp) were identified (Fig. 3) based on colony PCR approach following transferring a single colony to LB medium containing kanamycin (100 µg/ml) to grow for 48 hours. The bacterial suspensions were injected into plant leaves by applying 2 ml needleless syringe. After 72 hours, the injected leaves were collected for further analysis. Agroinfiltration is a rapid technique in order to introduce gene of interest into plant cells for a broad range of applications such as promoter investigation (Liu et al., 2003) and production of pharmaceutical proteins. The simplicity of syringe agroinfiltration allows further quick assessments. Different parameters such as OD and temperature can affect the efficiency of agroinfiltration. According to previous evaluations, the best bacterial suspension OD for injection of tomato leaves is 0.5, and at 22-23°C, high level of *GUS* expression has been reported (Dillen et al., 1997; Yasmin and Debener, 2010). In this study, acetosyringone (200 µM) was used to induce the *vir* genes. Phenolic compounds such as acetosyringone are required to increase the *vir* genes induction and improve *Agrobacterium* transformation efficiency (Hiei et al., 1994)

Detection of GUS activity in wounded leaves

Histochemically *GUS* activity assay was carried out on the injected leaf tissues, which have been agroinfiltrated by *Agrobacterium tumefaciens* AGL1 strains harboring either *pBI:MPI:GUS* or *pBI:35S:GUS*. The related results demonstrated that induction of *MPI* promoter occurs only in wounded leaves, and no *GUS* activity (blue color) was detected in unwounded leaves harboring *pBI:MPI:GUS* (Fig. 4). No *GUS* staining was detected in any of the negative controls suggesting that our findings were not affected by environmental factors (Fig. 4). Moreover, *GUS* expression only occurs at the wounded leaf site (not the total leaf surface area). Therefore, genes under control of the *MPI* promoter are more probably expressed at the time and site of pest attack. These results are in agreement with the reports provided by Cordero et al. (1994) and Tamayo et al. (2000). Cordero et al. (1994) showed that the *mpi* mRNA accumulation was induced in response to fungal infection in maize embryos, while no *mpi* transcript was detected in maize unwounded tissues. In this regard, Tamayo et al. (2000) also revealed that *MPI* protein accumulation occurs in tissues adjacent to the wound created by *Spodoptera littoralis* in maize leaves (Tamayo et al., 2000). Consistent with our findings, Breitler et al. (2001) demonstrated that -689/+197 region of *mpi* gene is sufficient to express an insecticidal gene in transgenic rice to protect it against stem borer attack. Thus, the *MPI* promoter could be used to generate transgenic plants containing pest-resistant genes under control of this wound-inducible promoter. Interestingly, using these kinds of

transgenic plants is more sensible due to energy saving and expressing the insecticidal proteins at the site of pest attack. Moreover, expression of insecticidal genes in healthy parts of those plants is lacking. Although insect resistance plants harboring *cry* genes under *CaMV35S* promoter are grown in several countries (Kumar et al., 2009). However, the most desired strategy is to express the insecticidal genes under a promoter that would express only at the site of feeding by larvae (Kumar et al., 2009). Thus driving *cry* genes under control *MPI* promoter could be a helpful way to enhance expression of these genes at the site of wounding when insects start feeding.

In this study, recombinant vector *pBI: 35S: GUS* has been used as a positive control for *pBI: MPI: GUS*. As shown in figure 5, visual survey reveals that the strength of *MPI* promoter is approximately equal to the constitutive *CaMV35S* promoter. For further analysis, image J program was applied to evaluate the *GUS* color intensity in wounded leaves. The color intensity was calculated based on the formula: Integrated Density - (Area of selected cell X Mean color of background readings). The background reading should be an area close to the site of interest. The results of color intensity assessment confirmed that the *MPI* promoter give higher color intensity (2.64 fold) compared to the *CaMV35S* (Figure 5). To date, just few promoters, which are the same or stronger than *CaMV35S* have been identified (Zhang et al., 2013). However, according to the results presented in this study, the activity and expression of *MPI* promoter is more than *CaMV35S*. The *MPI* promoter could be also a very useful promoter to generate such constructs containing insecticidal genes (different types of *cry*) just expressed once plants are being attacked by insect pests.

Quantitative measurement of relative expression of GUS gene

Relative gene expression analysis of *GUS* using qRT-PCR revealed that the transcript levels of *GUS* gene in the wounded leaves containing *MPI* promoter (*pBI: MPI: GUS*) is 4.1 fold higher than the positive control wounded leaves containing 35S promoter (*pBI: 35S: GUS*) (Fig. 6). These findings are in agreement with the results of histochemical *GUS* assay. These results are also consistent with Breitler et al. (2001) in rice revealing that efficiency of *MPI* promoter in response to mechanical wounding induced 3-5 fold higher *GUS* activity in comparison to the constitutive promoter. Although, in contrast to 35S (constitutive promoter), induction of *MPI* is only localized to the site of leaf wounding, the transcripts level of *GUS* under control of the *MPI* is higher than that of 35S. Therefore, it could be concluded that in the case of localized expression, *MPI* promoter is much stronger than *CaMV35S*. The *MPI* reaction to wounding is likely related to the wound responsive elements including MeJA and ABA motifs, and therefore inducible promoters provide precise regulation of transgene expression through external control (Hernandez-Garcia and Finer, 2014). Defense responsive promoters are necessary in plant biotechnology applications to develop resistant transgenic crops. Otherwise, the biosynthesis of abundant recombinant proteins under control of constitutive promoters can impact plant energy and subsequently reduction in yield and biomass (Hernandez-Garcia and Finer, 2014). Thus, there are some important reasons to develop inducible expression systems in crop plant biotechnology.

The main aim of this study was to assess the possible function of *MPI* promoter in a new plant background (tomato). The presented results showed that *MPI* promoter,

which has been isolated from maize and subsequently transferred to tomato, could almost function in the same way in dicot plants as found in monocots (Breitler et al., 2001). Regardless of current study results, several plant promoters such as *Wip1* showed a different function in monocot and dicot plants. *Wip1* promoter originating from maize was not induced by wounding in transgenic tobacco plants (Zhang et al., 2013). Interestingly, our findings are in agreement with the results reported by Xu et al. (1993), who demonstrated that the *pin2* wound-inducible promoter from potato conferred the same expression of *GUS* gene in transgenic rice plants.

Materials and methods

Plant materials and MPI promoter isolation

Seeds of *Zea mays* L. cv. 704 and *Lycopersicon esculentum* Mill. cv. CH were provided by Pakan Bazr Seed Company (Isfahan, Iran). The maize seeds were grown for 15 days in the lab growth chamber with a photoperiod 16/8 hours light/dark at 23°C and 70% relative humidity following genomic DNA extraction using the modified CTAB method (Murray and Thompson, 1980). The quality and quantity of DNA has been determined by 1% agarose gel electrophoresis and nanodrop spectrophotometer (Thermo Scientific, USA). In order to design specific primers to amplify *MPI* promoter, first sequence of *Zea mays* L. (inbred line B73) *mpi* gene was obtained from GeneBank (Accession number: X78988.2), and then software Oligo 5.0 (Molecular Biology insights, Inc., cascade, CO, USA) (Table 1) were used. PCR reaction containing the final volume 15 µl was prepared as follows: 1.5 µl of 10X PCR buffer, 2 mM MgCl₂, 1 U of LONG PCR Enzyme Mix (Thermo Fisher Scientific), 0.2 mM dNTPs, 0.3 µM of each primer and approximately 20 ng of genomic DNA. The LONG PCR Enzyme Mix has a proofreading activity and mostly generates 3'-dA tailed PCR products. The thermocycler program (BioRad, USA) was conducted at " 5 min at 95°C followed by 30 cycles of 1 min 94°C, 1 min 65°C and 1 min at 72°C, with a final extension of 10 min at 72°C ". The right size PCR products were extracted from the agarose gel using gel extraction kit (Thermo Fisher Scientific) and then cloned into pTZ57R/T vector (Thermo Fisher Scientific), following transformation of *Escherichia coli* MC1061 using cloning kit based on manufacturer instructions. Plasmids containing the *mpi* fragment were purified by plasmid extraction kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Colony PCR following digestion by *Bam*HI and *Hind*III (Thermo Fisher Scientific) were performed for screening of colonies containing the desired insertions. Confirmed colony was subsequently sequenced (Bioneer Inc., South Korea) and then deposited in GeneBank (Accession number: KP793074). Therefore, nucleotide sequences of the *MPI* promoter of *Zea mays* L. cv. 704 were identified and confirmed afterward by using BLASTn (Altschul et al., 1990) tool in NCBI.

In silico sequence analysis of the MPI promoter

In order to calculate pairwise distance of sequenced *MPI* promoter to compare with the *mpi* gene sequence in NCBI (B73, accession number: X78988.2), the K2P model (Kimura 2-parameter), which calculates distance based on transitional and transversional substitution rates by using MEGA ver. 5, was employed (Tamura et al., 2011). Transition, transversion, substitution and InDel were calculated by ARLEQUIN version 3.1 (Excoffier and Lischer, 2010). The percentage of

genetic diversity was obtained by using the number of different nucleotides to the total number of nucleotides for each region. In order to determinate the transcriptional start site (TSS) of the *MPI*, SOFTBERRY database (<http://www.softberry.com>) was used. Searching for cis-acting elements was carried out using PLANTCARE (Lescot et al., 2002).

PBI:MPI:GUS construction

Binary vector pBI121 (pBI: 35S: *GUS*) has been employed for the agroinfiltration performance. Both plasmids pBI121 and pTZ57R/T (containing the confirmed *MPI* fragment) were extracted using plasmid extraction kit (Thermo Fisher Scientific) following digestion by *Bam*HI and *Hind*III (Thermo Fisher Scientific). Then the *MPI* promoter were extracted from agarose gel using gel extraction kit (Thermo Fisher Scientific) and subsequently cloned into pBI121 (Fig. 1). Colony PCR and restriction enzyme digestion approaches were subsequently used for screening colonies containing PBI:MPI:GUS vector.

Agro-infiltration

The PBI:MPI:GUS and pBI: 35S: *GUS* recombinant plasmids were transferred into competent cells of *Agrobacterium tumefaciens* strain AGL1 by micropulser electroporator (Biorad, USA), following bacterial growth at 28°C for 72 hours. Colony PCR approach was used to confirm colonies with interested recombinant vector. A confirmed single recombinant colony was transferred to LB medium containing kanamycin (100 µg/ml), ampicilin (100 µg/ml) and rifampicin (50 µg/ml) and subsequently incubated at 28°C and 250 rpm for 48 hours in darkness. The *Agrobacterium* cells were harvested at OD₆₀₀ 0.5 by centrifugation at 5000 rpm for 10 min and resuspended in PBS buffer (pH 7.4) plus 200 µM acetosyringone and subsequently injected into plant leaves by use of 2 ml needleless syringes. PBS buffer was also injected into plant leaves as negative control. Therefore, the following samples including injected leaves by either pBI: 35S: *GUS* (positive control) or pBI:MPI:GUS, unwounded leaves, and injected negative control leaves by PBS buffer was evaluated. Each assessment was carried out on three young and healthy separated leaves from one-month-old single plants. After injection, plants were incubated at 23°C in darkness for 72 hours. These plants were used for histochemical *GUS* assay.

Histochemical GUS activity assay

In order to assay promoter function and efficiency in transgenic leaves, 24 hours after the agroinfiltration, the injected leaves were wounded by forceps, and other leaves and control plants remained intact. After 72 hours, wounded, unwounded, negative and positive controls were collected following histochemical *GUS* activity assay according to the Jefferson et al. (1987). Then, plant leaf disks were decolorized by 70% ethanol at 50°C and were investigated by means of an optical microscope. The color intensity was calculated by image J software (Rasband, 1997).

RNA isolation and quantitative real-time PCR (qRT-PCR)

The injected leaves have been collected 72 hours after agroinfiltration, and total RNA was isolated from injected leaves (wounded and positive control leaves) using RNX-Plus (Sinaclon, Iran) following the manufacturer's

instruction. Complementary DNA (cDNA) was synthesized using cDNA synthesis kit (Pishgam, Iran) according to the manufacturer instruction. The qRT-PCR was performed using universal *GUS* specific forward 5'-TCACAGCCAAAAGCCAGA -3' and reverse 5'-AAGCGTAAGGGTAATGCGAG -3' primers. Universal *Actin* (as an endogenous control gene) forward 5'-GCTCCTCAGTTGAGAAGAGC-3' and reverse 5'-CCTTCCTGATATCCACGTCAC-3' primers were used. The qRT-PCR reactions were performed on a LightCycler-96 real-time PCR. Each reaction was performed in triplicate using the following program: 5 min at 95°C followed by 35 cycles of 95 °C for 20 s, 60 °C for 25 s, 72 °C for 25 s with a single fluorescent measurement and melting curve program (60-95 with a heating rate of 0.1 per second and continuous fluorescent measurement). Cycle threshold (C_t) values were determined for each sample and comparative CT method ($\Delta\Delta C_t$) was used to determine relative quantification of *GUS* transcript (Livak and Schmittgen, 2001).

Conclusion

In order to bring the paper in a final touchdown, *in silico* sequence analysis of the *MPI* promoter resulted in identification of cis-acting elements related to the wound-responsive motifs. The *GUS* histochemical activity assay and qRT-PCR findings indicated that the *MPI* promoter can work as a wound-inducible promoter the induction of which occurs only in the wounded site of tomato leaves. Therefore, the *MPI* promoter could also be expressed in dicot plants as occurred in monocot plants. Based on the obtained results, the *MPI* promoter can be very useful in plant engineering to develop insect-resistant transgenic plants containing strong wound-inducible promoter.

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