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ISOLATION AND CHARACTERIZATION OF THE LIPOXYGENASE-3 PROMOTER IN *NICOTIANA TABACUM*

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Abstract

The jasmonates (JA) play key roles in defending mechanical wounding, herbivore attack and various stresses in plants. Lipoxygenase (LOX) is one of the key enzymes in the biosynthesis of JA which also catalyzes the green leafy volatiles synthesis at the first step. Wounding and herbivore attack can induce the expression of *LOXs*. To investigate the molecular mechanism regulating the *LOX* genes in tobacco, we isolated and identified the *LOX3* gene promoter from *Nicotiana tabacum*. Analysis from transgenic plants and GUS activity fluorimetric assay revealed that 1847bp of *NtLOX3* promoter sequence was sufficient for wounding and methyl jasmonic acid (MeJA) induction. For wounding and MeJA induction, the deletion analysis identified the minimal effective regions which were located in -546bp to -199bp. And this region contained the *cis*-acting elements of CATG-box (CATG), W-box (C/TTGACY) and a T/G-box (AACGTG), which were thought to play a key role in wounding and MeJA elicitation. These results may provide information for further transcriptional regulation studies of *LOX* genes.

Key words: Lipoxygenase; Wounding; JA; Transgenic plants.

Introduction

Lipoxygenases (LOXs; EC 1.13.11.12) are lipid-oxidizing enzymes containing non-heme iron which is ubiquitous not only in plants, but also animals, bacteria and fungi (Viswanath *et al*., 2020). Lipoxygenases act on the substrate of polyunsaturated fatty acids including the (Z, Z)-1, 4-pentadiene composition to form hydroperoxy fatty acids by oxygenation reaction (Liavonchanka & Feussner, 2006). These products may serve as precursors of oxylipins which have important roles in defense response for pathogens, herbivores and other environmental stresses. Plant LOXs usually oxidize linoleate and α-linolenate. Oxygen can be added to the 9th or 13th carbon atom of polyunsaturated fatty acids, so that the plant LOX is classified as 9-LOX and 13-LOX (Andreou & Feussner, 2009).

It is well known that plant LOXs can catalyze oxygenation of polyunsaturated fatty acid to produce precursors of regulators, such as jasmonic acid (JA). JA and its derivatives, usually named as jasmonates (JAs), have many significance functions in plant growth and development, senescence and defense response (Ahmad *et al*., 2016). The biosynthesis of JA requires many enzymes and LOX catalyzes the initial reaction as a key enzyme in JA biosynthesis. In animals, LOXs may catalyze to produce the arachidonic acid derivatives and leukotriene, which can be further converted to leukotrienes (Ueda *et al*., 1986), anti-inflammatory lipoxins (Serhan, 2002) and 5-oxo-ETE (Powell & Rokach, 2005). These components may have functions in many diseases such as atherosclerosis, inflammation and cancer (Mashima & Okuyama, 2015).

With the advanced development in the sequencing technology and functional genomics study, the lipoxygenase genes are being identified in various plant species. LOX gene expression induced by wounding or MeJA treatment have been examined in Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), and soybean (*Glycine max*) and so on. Six LOX genes (LOX1~LOX6) were found in *A. thaliana* genome which may be involved in the defense pathway. LOX1 and LOX5 belong to the type of 9-LOXs and LOX2, LOX3, LOX4, LOX6 are the type of 13-LOXs (Bannenberg *et al*., 2009). Green leaf volatiles (GLVs) synthesis may begin with the oxidation of LOX, which involves biotic responses (Matsui & Engelberth, 2022). AtLOX2 is the isoform exclusively involved in GLV burst in Arabidopsis (Mochizuki *et al*., 2016). Studies have shown that both *AtLOX3* and *AtLOX4* were activated in response to nematode attack (Ozalvo *et al*., 2014). Three distinct types of LOX were reported in soybean, LOX1, LOX2, LOX3 (Axelrod *et al*., 1981). The soybean *LOX1* can be inhibited by some endocannabinoids containing polyunsaturated acyl moiety and phenolic group (Nguyen *et al*., 2013). The LOX2 isozyme in soybean is mainly responsible for the "beany" flavor (Shin *et al*., 2012). Six LOX genes were identified in tomato genome (LOXA~F). TomLOXC is involved in the synthesis of C5 and C6 products. While C5 and C6 volatiles can be stimulated by mechanical wounding in TomLOXC dependent response reaction, but not response to treatment with *Xanthomonas campestris PV. Vesicatoria* (Shen *et al*., 2014). TomLOXD is involved in the wounding-induced pathway of JA (Yan *et al*., 2013).

Many *LOX* genes have been identified in different plant species, while there is little information available on molecular mechanism of *LOXs* in tobacco (*Nicotiana tabacum*). Recently, JA has also been confirmed as an important signal in response to wounding, herbivore and pathogen attack in tobacco (Zhang *et al*., 2015). Antisense *LOX3* expression from *Nicotiana attenuata* plants has been shown to reduce wound-induced JA accumulation, and decrease both direct and indirect resistant defense

responses in *LOX3*-silenced plants (Halitschke & Baldwin, 2003). Thus, the change on *LOX* expression may influence the biosynthesis of JA. It may be providing a tool for studying the function of JA in plant defense responses and growth.

To investigate how the developmental and environmental signals that regulate the expression of the tobacco lipoxygenase, we have isolated the tobacco *LOX3* promoter and characterized the function of *LOX3* promoter conducted to the reporter gene of β-glucuronides (GUS) in transgenic plants. We found that 1847bp of *NtLOX3* promoter region was adequate for wounding and MeJA induction. The *cis*-acting elements of CATG-box (CATG), W-box (C/TTGACY) and T/G-box (AACGTG) may play a key role in wounding and MeJA elicitation of *NtLOX3* promoter. Hope these results can provide information for further research of *LOX*.

Material and Methods

Plant materials and treatment: Tobacco plants (*Nicotiana tabacum* L. cv. HONGDA) were transformed and used throughout this study. All seeds were sterilized using 75% ethanol for 45 seconds, 10% sodium hypochlorite twice for 5 minutes, and washed by sterile water three or more times, then sown on half MS medium at 26℃ in controlled chamber with 16/8h light/dark. Four-week-old seedlings were used for transformation. For transgenic experiments, the leaf pieces infected with the *Agrobacterium* were cultured on MS differentiation medium for screening. Regenerated shoots were selected and transferred to rooting medium containing hygromycin. Rooted plants were grown in glasshouse at 26℃～30℃.

Approximately two months old rosette stage tobacco leaves of the T2 generation were used as materials. For wounding treatment experiment, the leaves at nodal position +1 were wounded using the hemostat to cause about 6-8 bites per leaf. For MeJA treatment plants, the leaves at nodal position +1 were treated with 100 μM of MeJA as described previously (Kang & Baldwin, 2006). Then the samples were frozen in liquid nitrogen or reserved at -80℃ before detected.

Genomic clone of *NtLOX3* promoter: Extracted the genomic DNA using the TIANGEN plant genomic DNA extraction kit from the tobacco leaves. The 5'-upstream sequences of the *NtLOX3* gene about 1.9kb were cloned from genomic DNA by PCR. We used the different primers to amplify four progressive truncations of *NtLOX3* promoter regions (Table 1). And the PCR fragments were conducted into pEASY-T1 simple vector and sequenced.

Construction of *NtLOX3* promoter-gus reporter gene fusion: About 1.9kb of the *NtLOX3* promoter region was isolated by PCR and fused to *gus* in the plant binary vector pCAMBIA1301, using the P1 primers (Table 1), which contain *Kpn*Iand *Bgl*IIRestriction site, separately. The 1847bp fragment sequence was linkage to the pEASY-T1 simple vector. Then the 1847bp *NtLOX3* promoter fragment was cut from pEASY-T1 vector by *Kpn*Iand *Bgl*IIdigestion and cloned into pCAMBIA1301 vector replacing the 35S promoter. This vector was named pLOX1847. To search *cis*-regulatory elements in the *NtLOX3* 5'-upstream sequence, three different deletions were conducted. For pLOX1035 PCR products were cut with *Nco*Iand *Xba*Ifrom pEASY-T1 vector, and inserted into the pCAMBIA1301. For pLOX546 and pLOX199, the PCR products were cut with *Nco*Iand *EcoR*Ifrom pEASY-T1 vector, and inserted into the pCAMBIA1301.

Plant transformation: The plasmids of promoter deletion constructions were inserted into agrobacterium tumefaciens (strain LBA4404) (Kruegel *et al*., 2002). These agrobacteria were cultured in YEB liquid medium screening with kanamycin (50μg/ml) and rifampicin (50μg/ml) at 28℃ overnight. Harvest the agrobacterium cells by centrifugation at 4500rpm for 10min, and suspended them to a final OD600 of 0.8 in liquid MS medium supplemented with 20μg/L acetosyringone. About 1.0cm × 1.0cm leaf discs were inoculated into *Agrobacterium* inoculum for infection, and put them into the artificial climate chamber at 26℃, humidity 40% in darkness for 3 days. Then the leaves were transferred upwards to the S1 differentiation MS medium (0.5g/L MES, 1mg/L N6-benzylaminopurine, 0.1mg/L indole-3-acetic acid, 500mg/L cefradine, 5mg/L hygromycin). Subsequent transferred the long clustered shoots to S2 MS medium (0.5g/L MES, 0.5mg/L N6-benzylaminopurine, 0.1mg/L indole-3-acetic acid, 500mg/L cefradine, 8mg/L hygromycin) after 2 weeks. And transferred 2 week later to the same medium as above. Then the robust seedlings were inoculated into rooting medium R (0.5g/L MES, 0.1mg/L indole-3-acetic acid, 500mg/L cefradine, 20mg/L hygromycin). Rooted plants were grown in a greenhouse at 26℃～30℃.

Table 1. Oligonucleotide primers used to amplify promoter fragment of *NtLox3*.

Name	Primers	Sequences (5'→ 3')	Tm(℃)
pLOX1847	P1-F	GGGGTACCGCCAGGTCTAAGGAAGG	49.3
	P1-R	GAAGATCTAATAATAATAGTTCTCTCTTCAATT	
pLOX1035	P2-F	GCTCTAGATATCTACTGAAGAAACTTGGGC	50
	P2-R	CATGCCATGGAATAATAATAGTTCTCTCTTCAATT	
pLOX546	P3-F	CGGAATTCAGTCCATTCGTTTTACATTG	49.5
	P3-R	CATGCCATGGAATAATAATAGTTCTCTCTTCAATT	
pLOX199	P4-F	CGGAATTCAAATGAAAACCCCACTTAC	49.1
	P4-R	CATGCCATGGAATAATAATAGTTCTCTCTTCAATT	

Histochemical analysis of GUS activity: The method we performed in transgenic tobacco for histochemical staining of GUS activity was in accordance with Jefferson *et al*., (Jefferson *et al*., 1987). The transgenic tobacco materials were treatment in GUS staining reaction solution at 37℃ (50mM sodium phosphate buffer [pH 7.0], 0.5mM $K_3Fe(CN)_6$, 0.5mM $K_4Fe(CN)_6 \cdot 3H_2O$, 0.1% Triton X-100, 20% methanol, 10mM EDTA and 0.8 mg/ml X-Gluc). After overnight incubation, the samples were washed away chlorophyll with 75% ethanol at 37℃. And then take pictures.

For GUS quantitative analysis, the transgenic leaves were ground with tissuelyser and extracted with GUS assay buffer (0.5M EDTA [pH 8.0], 0.1M sodium phosphate buffer [pH 7.0], 10mM β-mercaptoethanol, 0.1% Triton X-100, 10% SDS and 20% methanol), centrifuged and collected the supernatant. Protein concentration in supernatant was detected with Tecan Sunrise at 595nm using the Bradford assay method (Bradford, 1976). The total supernatant was mixed using GUS assay buffer including 2mM 4-methylumbelliferyl-β-D-glucuronide and kept at 37℃ for 30min. Then 50µl reaction solution was transferred into 950µl stop buffer (0.2M Na_2CO_3). GUS activity was measured according to the amount of 4-methylumbbelliferone (4-mu) produced by the catalysis of GUS enzyme per minute in 1mg protein (Jefferson *et al*., 1987; Xu *et al*., 2010). Three biological repeats were performed for each conducts.

Results

Sequence analysis of the *NtLOX3* promoter: In order to elucidate the mechanism about *NtLOX3* gene in response to wounding, a 1847bp fragment of *NtLOX3* 5′ upstream region was isolated from tobacco genomic DNA. To identify the *cis*-elements of *NtLOX3* promoter, we use the PlantCARE (Lescot *et al*., 2002) and PLACE (Higo *et al*., 1999) database to analyze the *NtLOX3* 5′ upstream sequence. Sequence analysis showed that at -166 to -159 present a typical TATA-box and at the position -193 to -190 was a possible CAAT-box element. And the transcription start sites were also predicted. Several types of possible *cis*-acting elements present in the *NtLOX3* 5′ upstream region (Table 2), such as W-box (C/TTGACY), CATG-box (CATG) and T/G-box (AACGTG), which were probably wounding and MeJA responsive elements (Fig. 1a) (Kang & Baldwin, 2006; Samach *et al*., 1995). Other types such as: *cis*-elements involved in ABA or/and drought response, for example ABRELATERD1, ABRERATCAL, ACGTATERD1, DRE2COREZMRAB17, MYB1AT, MYB2CONSENSUSAT and MYCCONSENSUSAT; enhancer elements such as EECCRCAH1; *cis*-elements related to light-regulated, including EBOXBNNAPA, GATABOX and GT1CONSENSUS; T/GBOXATPIN2 for responding to JA and WBOXATNPR1 for responding to SA, respectively; seven motifs of GT1GMSCAM4 induced by salt and pathogen were also present in *NtLOX3* promoter; water stress-responsive elements of MYBCORE were also found. The promoter also had some elements responsible for organ specific expression, such as CACTFTPPCA1 for mesophyll specific induction, POLLEN1LELAT52 for pollen specific expression, RAV1AAT required in rosette leaves and roots high expression and ROOTMOTIFAPOX1 for root specific induction. Other elements such as CPBCSPOR response to cytokinin and DOFCOREZM involved in carbon metabolism. G-box (CACGTG) or GCC-box (GCCGCC) known as typical JA responsive elements were not found in *NtLOX3* promoter sequence (Kim *et al*., 1992; Brown *et al*., 2003).

Truncated *NtLOX3* promoters have activity in transgenic tobacco: About 1.9kb of 5'-upstream regions of *LOX3* was isolated from tobacco, and attached to GUS reporter gene in pCAMBIA1301 vector. In order to identify the specific region involved in *NtLOX3* gene expression, truncated *NtLOX3* promoters fused to GUS had been conducted (Fig. 1b; Fig. 2). GUS staining in seedlings was examined in all transgenic tobacco lines (Fig. 3). Strong GUS staining was found in the seedlings of the pLOX1847 promoter and pLOX1035 promoter transgenic lines. The pLOX546 promoter transgenic lines showed slightly weaker staining compared with the longer promoters (Fig. 3), consistent with the GUS activity analysis results (Fig. 4). The results reveal that the promoter length of -546bp to -199bp probably include the key strong enhancer *cis*-elements for wounding and MeJA induction in this study.

***NtLOX3* promoter activities in response to stress treatment:** To detect the *NtLOX3* promoter activity in response to wounding and MeJA, four GUS reporter gene fusions with truncated promoters were used for analysis. Leaves from the four transgenic lines were harvested 24h after being wounded or treated with MeJA, and then used the fluorescence assay to analyze the GUS enzymatic activity. Fig. 4 showed the results from fluorimetric analysis of GUS enzymatic activity. In general, GUS activity of the same lines in wounding and MeJA treatment plants showed similar induction patterns. The plants of treatment had more GUS expression compared to the control plants. And transgenic lines containing longer *NtLOX3* promoters showed higher GUS expression compared to those containing shorter ones. The longer deletion of pLOX1847 had more GUS activity, but pLOX546 construct led to reduction of GUS activity compared to the longer ones, suggesting that these promoter regions contain positive regulatory *cis*-acting elements.

Discussion

The pathway mediated by jasmonic acid (JA), which is biosynthesized via 13-lipoxygenases (LOX), plays important roles in both plant growth and defense (Ahmad *et al*., 2016; Nguyen *et al*., 2019; Griffiths, 2020). In this study, we cloned and characterized a promoter fragment 1847 bp upstream of the ATG start site of *NtLOX3*. Different truncated of *NtLOX3* 5′-upstream regulatory sequence were conducted to plant vector pCAMBIA1301 and transferred into tobacco. And the analysis of transgenic experiments indicated that 1847bp of *NtLOX3* promoter was sufficient for wounding and MeJA elicitation.

Table 2 Putative *cis*-acting elements in *NtLox3* promoter predicted by PlantCARE and PLACE.

cis-element	Consensus	Position	Function
ABRELATERD1	ACGTG	-1243, -212	ABRE-like sequence
ABRERATCAL	MACGYGB	-213, -367	ABRE related sequence
ACGTATERD1	ACGT	-212, -1243	Dehydration and dark induced senescence
ARR1AT	NGATT	-93, -145, -220, -498, -512, -682, -833, -1008, -1075, -1130, -1205, -1698, -1743, -1791, -1825	Transcriptional activator ARR1 binding site
CACTFTPPCA1	YACT	-112, -199, -238, -287, -372, -390, -420, -493, -586, -721, -855, -903, -969, -979, -1031, -1294, -1317, -1354, -1385, -1414, -1499, -1537, -1575, -1600, -1721, -1755, -1815, -1832	Mesophyll specific expression
CAATBOX1	CAAT	-24, -36, -193, -460, -462, -468, -487, -530, -574, -595, -880, -935, -959, -960, -1203, -1404, -1699,-1802	Common *cis*-acting element
CATGBOX	CATG	-319, -1041, -1101, -1127, -1181, -1307, -1376, -1579, -1808	MeJA responsive element
CPBCSPOR	TATTAG	-99, -667	Response to cytokinin
DOFCOREZM	AAAG	-49, -86, -1171, -255, -312, -388, -433, -439, -634, -687, -802, -1012, -1061, -1089, -1249, -1521, -1567, -1606, -1658, -1663, -1753	Carbon metabolism of maize
DRE2COREZMRAB17	ACCGAC	-870	ABA, Drought responsive
EBOXBNNAPA	CANNTG	-231, -319, -462, 1301, -1309, -1385, -1714	Light-responsive, tissue specific expression
EECCRCAH1	GANTTNC	-223, -497, -754, -768, -1373, -1705, -1762	Enhancer elements
GATABOX	GATA	-193, -796, -821, -1035, -1349, -1379, -1485, -1529, -1610, -1635, -1749	Light regulated, tissue specific expression
GTGANTG10	GTGA	-196, -200, -251, -316, -346, -722, -902, -965, -1116, -1226, -1257, -1293, -1469, -1533, -1631, -1831	*Cis*-regulatory element
GT1CONSENSUS	GRWAAW	-54, -55, -82, -83, -223, -360, -400, -430, -452, -478, -571, -614, -641, -642, -796, -1135, -1136, -1186, -1675	Light-inducible, cell specific expression
GT1GMSCAM4	GAAAAA	-54, -83, -400, -430, -571, -642, -1135	Pathogen and salt inducible
MYB1AT	WAACCA	-127, -1067, -1169, -1625	ABA and drought responsive
MYB2CONSENSUSAT	YAACKG	-482, -706, -888, -908, -1123, -1301, -1714	ABA and dehydration responsive
MYBCORE	CNGTTR	-244, -482, -706, -868, -888, -908, -1123, -1301, -1714, -1727, -1767	Water stress responsive
MYCCONSENSUSAT	CANNTG	-231, -319, -462, -1301, -1309, -1385, -1714	ABA, drought and cold responsive
POLLEN1LELAT52	AGAAA	-106, -170, -387, -398, -408, -431, -778, -804, -1025, -1097, -1333, -1364, -1739	Pollen specific expression
RAV1AAT	CAACA	-32, -598, -1044, -1240, -1727, -1766	High expression in rosette leaves and roots
ROOTMOTIFTAPOX1	ATATT	-525, -526, -562, -563, -652, -665, -840, -937, -938	Root specific expression
T/GBOXATPIN2	AACGTG	-213	*Cis*-element involved in MeJA induction
WBBOXPCWRKY1	TTTGACY	-335	WRKY proteins bind specifically to the W box
WBOXATNPR1	TTGAC	-299, -334, -486, -1586, -1701	Regulates NPR1, SA induced
WBOXHVISO1	TGACT	-234, -333, -1039, -1450, -1456, -1587,-1702, -1733	Binding site of WRKY
WBOXNTCHN48	CTGACY	-1616, -1734	Elicitor responsive, binding site of WRKY
WBOXNTERF3	TGACY	-202, -234, -333, -1039, -1292, -1450, -1456, -1587, -1615, -1702, -1733	Wound-responsive element
WRKY71OS	TGAC	-201,-233,-333,-485,-1039,-1225,-1292,-1401,-1450,-1456,-1561,-1586,-1615,-1701,-1717,-1733	Core of W-box, binding site of WRKY

Symbols used in the sequences are: B: T, C or G; M: A or C; N: A, C, G, or T; R: A or G; W: A or T; Y: T or C

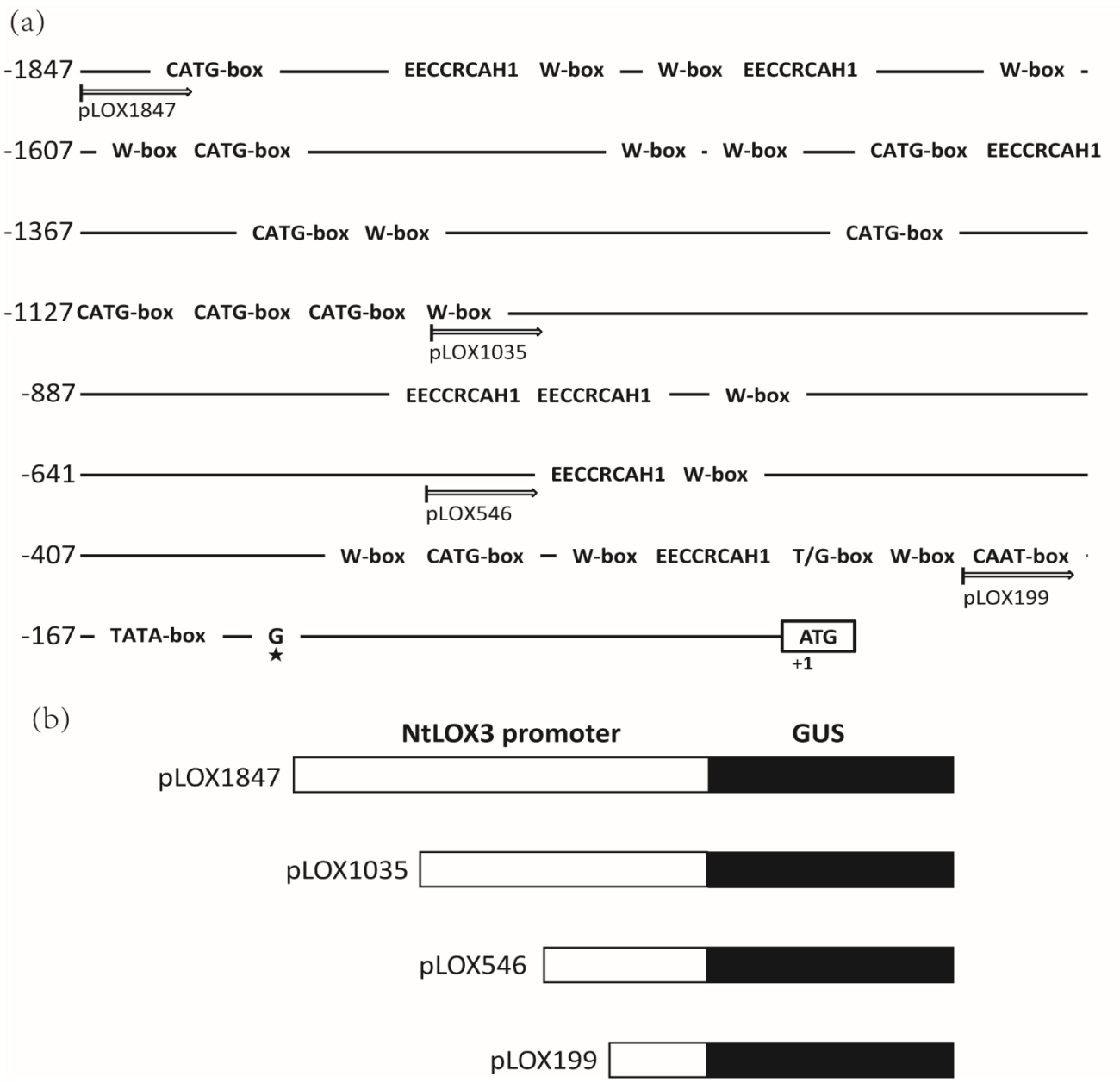

Fig. 1. (a) The putative wounding and MeJA responsive elements in the *NtLOX3* promoter. Asterisk (★) means the putative transcription start site of *NtLOX3*. The putative start codon (ATG) and transcription start site is indicated with +1. The putative TATA-box and CAAT-box are presented. The putative wounding and MeJA responsive elements are marked in different positions. Promoter position of truncated transformation constructs is also indicated with the arrow. (b) Schematic representation of the constructs used for transformation.

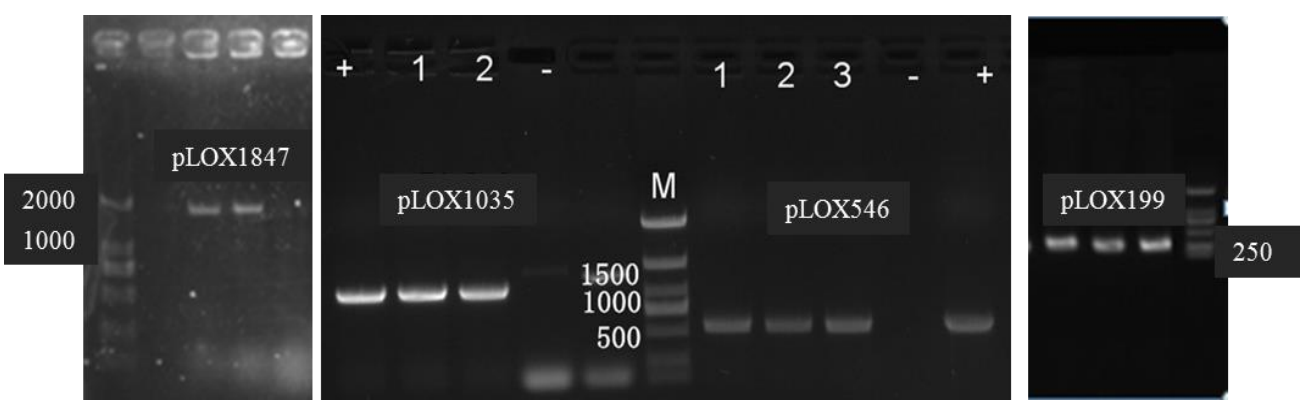

Fig. 2. Confirmation of promoter clones in pCAMBIA1301 vector. M: DNA ladder.

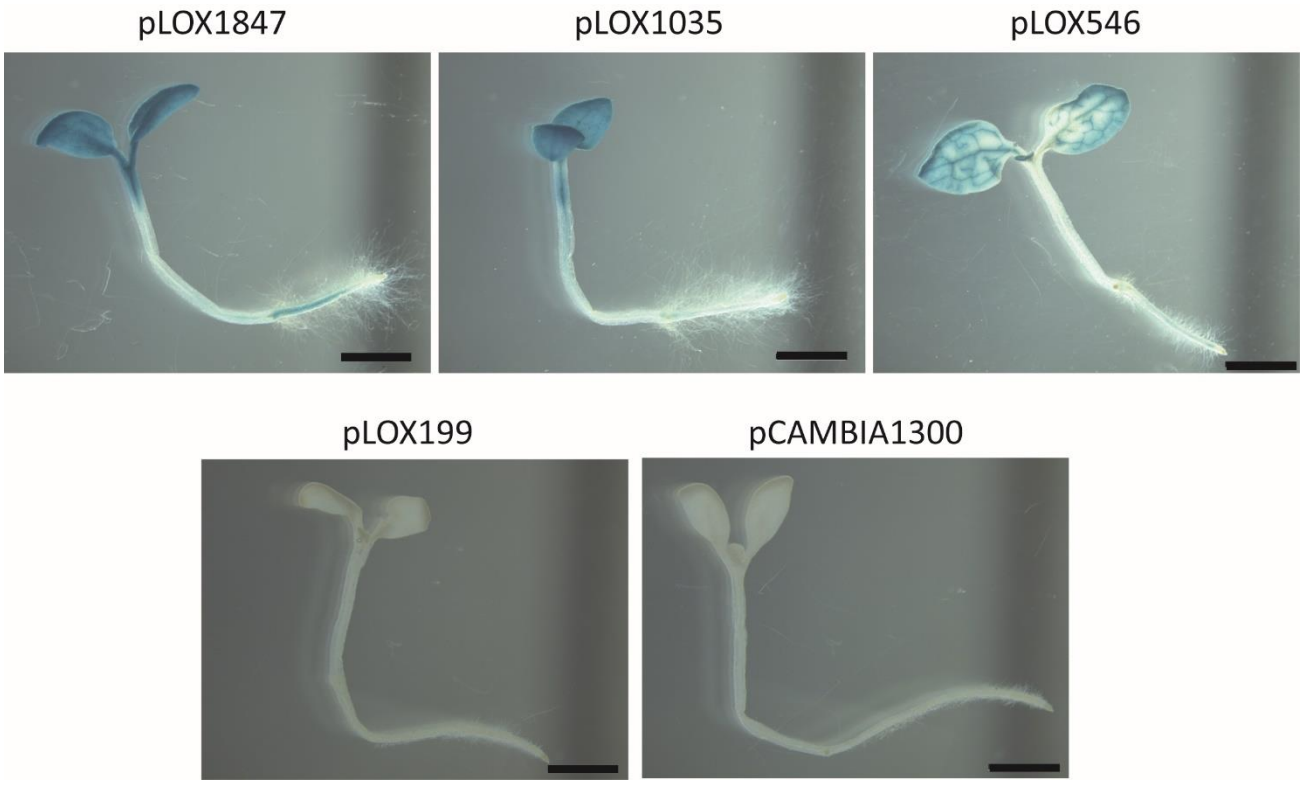

Fig. 3. Histochemical GUS staining of transgenic tobacco seedlings. Bar=2 mm.

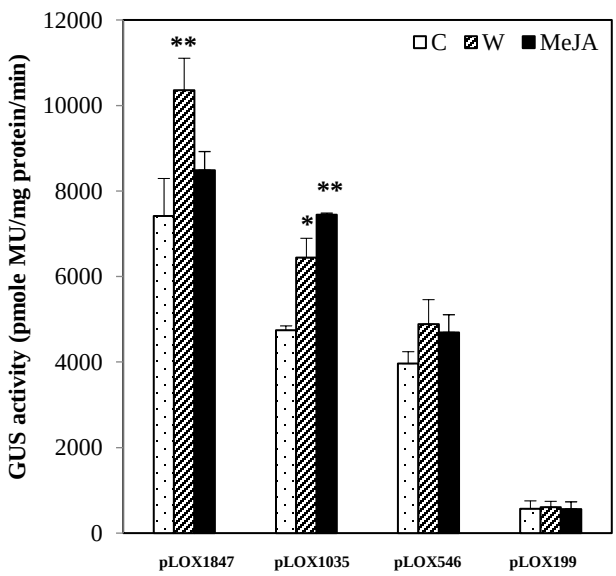

Fig. 4. GUS activity driven by truncated *NtLOX3* promoters after wounding and MeJA treatment. Transgenic leaves were harvested 24 h after wounding or MeJA treatment. "C" means the control samples that were untreated. "W" mean wounded samples with hemostatic forceps treated. "MeJA" means samples that treated with 100μM of methyl jasmonic acid. Error bars show the standard error of the replicates. Asterisks represent significant differences according to *t*-test (*p<0.05; **p<0.01).

Four progressive truncations of the promoter ranging from -1847bp to -199bp in length were constructed to identify the regulatory elements in the gene promoter. To investigate putative *cis*-acting elements, we use PlantCARE (Lescot *et al*., 2002) and PLACE (Higo *et al*., 1999) to analyze the *NtLOX3* promoter sequence. We are concerned on *cis*-elements usually involved in defense reactions, and found many W-boxes (C/TTGACY) recognized by the WRKY transcription factors and CATG-boxes (CATG) which all response to wounding and MeJA. The constructs design was also according to the position of putative enhancer elements, such as W-box, CATG-box and EECCRCAH1 (GANTTNC) (Fig. 1). But G-box (CACGTG) and GCC-box (GCCGCC) known as typical JA responsive elements were not found in *NtLOX3* promoter sequence (Kim *et al*., 1992; Brown *et al*., 2003).

We examined the GUS staining of seedlings in all truncated promoter transgenic tobacco lines. Almost full color staining was observed in the length from -1847bp to -1035bp , the promoter regions of -546bp showed slightly weaker staining compare with the longer ones (Fig. 3). The results reveal that the promoter length of -546bp to -199bp probably include the key strong enhancer *cis*-elements for wounding and MeJA induction. Analysis from GUS activity fluorimetric assay showed that the length of 1847bp *NtLOX3* promoter was sufficient in wounding and MeJA response. For wounding and MeJA induction, the deletion analysis identified the minimal effective regions which located in -546bp to -199bp (Fig. 3).

This region (from -546bp to -199bp) contains CATG-box (CATG), W-box (C/TTGACY) and one T/G-box (AACGTG), which are thought to play a key role in wounding and MeJA elicitation. The CATG-box is putative wounding and MeJA responsive element in the threonine deaminase promoter of tomato (Samach *et al*., 1995) and tobacco (Kang & Baldwin, 2006). Leucine aminopeptidase (LAP) and proteinase inhibitor II (pin2) genes from tomato were involved in JA response pathway. And the T/G-box was found in the promoters of them (Boter *et al*., 2004). The WRKY transcription factors can recognize the W-box

elements which seem to be rich in wounding induced genes, such as ERF3 gene (Nishiuchi *et al*., 2004). W-box (C/TTGACY) was also found in the promoter of parsley PR1 gene responding to elicitor (Rushton *et al*., 1996). The WRKYs regulate plant responses to pathogen attack and various abiotic stresses through JA, SA or other signals pathway (Jiang *et al*., 2017). Research has shown that WRKY3 and WRKY6 from *N. attenuate* response to herbivore-attacked by regulating JA signal pathway (Skibbe *et al*., 2008). Some WRKY transcription factors from *Arabidopsis* were induced by wounding, including AtWRKY11, AtWRKY15, AtWRKY22, AtWRKK33, AtWRKY40, AtWRKY53, AtWRKY60 and AtWRKY6 (Cheong *et al*., 2002; Robatzek *et al*., 2001). Genetic analysis revealed that *AtWRKY62* negatively regulated the expression of *LOX2* and *VSP2* which involved in JA signaling response process (Mao *et al*., 2007). The overexpression of *WRKY40* from cotton (*Gossypium hirsutum*) enhanced the plant tolerance to injury and increased the sensitivity to *R. solanacearum* (Wang *et al*., 2014). These findings show the importance of WRKYs in regulation of wounding-induced response. Thus the transcriptional regulations of *NtLOX3* gene for JA response probably involve the family of WRKY transcription factors.

In conclusion, the *NtLOX3* promoter sequence contains many different regulatory elements, such as drought, light and wounding responsive elements, and also MeJA, ABA and SA responsive elements. Our results revealed that the W-box, CATG-box or T/G-box (AACGTG) probably were necessary and sufficient elements for wounding and MeJA induction in *NtLOX3* expression. However, further study should be done to discover whether W-box, CATG-box, or other enhancer elements such as EECCRCAH1 are responsible for the *NtLOX3* induced expression. Future research will focus on the transcription factor proteins that bind to the *cis*-elements in *NtLOX3* promoter. This research may provide valuable information for further study on *NtLOX3* function and transcriptional regulation.

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