Is DNA methylation modulated by wounding-induced oxidative burst in maize?

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Summary

Plants respond to environmental changes by modifying gene expression. One of the mechanisms regulating gene expression is methylation of cytosine to 5-methylcytosine (m^5C) which modulates gene expression by changing chromatin structure. Methylation/demethylation processes affect genes that are controlled upon environmental stresses. Here, on account of the regulatory role of m^5C, we evaluate the content of m^5C in DNA from normal and wound-damaged maize leaves. Wounding leads to a transient decrease of the global DNA methylation level ca 20-30% 1 hour after the treatment followed by a return to the initial level within the next hours. Similar results were obtained using of radio-labelled nucleotides separated by Thin Layer Chromatography (TLC) or using m^5C-specific Enzyme-Linked Immunosorbent Assay (ELISA). Wounding induced in maize leaves a two-step oxidative stress, an early one just after wounding and the second two hours later. It coincides with the transient changes of the cytosine methylation level. In the stress-inducible maize calcium-dependent protein kinase *ZmCPK11* gene wounding transiently reduced methylation of cytosines 100 and 126 in the first exon.

Keywords: abiotic stress; DNA methylation; maize; oxidative stress; ZmCPK11 maize calcium-dependent protein kinase;

Abbreviations:

BER, Base Excision Repair;

BSP, bisulfite sequencing PCR;

CMT, chromomethyltransferase;

DME, Demeter (5-methylcytosine DNA glycosylase);

DML2, Demeter-Like 2;

DML3, Demeter-Like 3;

Dnm2, *Mus musculus* dynamin 2;

DNMTases, DNA methyltransferases;

DRM, domains rearranged methyltransferase;

ELISA, Enzyme-Linked Immunosorbent Assay;

HPLC, high performance liquid chromatography;

m^5C, 5-methylcytosine;

MALDI-TOF-MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry;

MET1, DNA methyltransferase 1;

NBT, nitroblue tetrazolium;

ROS, reactive oxygen species;

ROS1, Repressor of Silencing 1 (5-methylcytosine DNA glycosylase);

TLC, Thin Layer Chromatography;

ZmCPK11, Calcium-Dependent Protein Kinase from maize;

1. Introduction

Plants are continuously exposed to numerous stresses, including wounding caused by wind, rain, hail or herbivore attack. Wounding not only physically damages tissues, affecting the growth and reproduction of plants, but also provides gates for pathogen invasion (Koo and Howe, 2009). The plant response to stresses involves numerous genes encoding proteins with various functions. The stress-induced genes are regulated by non-coding RNA, histone modifications, and DNA methylation (Chinnusamy et al., 2008; Chinnusamy and Zhu, 2009). Methylation of DNA cytosines at position 5 is one of the most important epigenetic modes of regulation. The pattern of methylation is non-random. Some regions of the genome are heavily

methylated while in others the level of methylation is low. Changes in the DNA methylation pattern in response to developmental or environmental cues may result in changes in gene expression (Furner and Matzke., 2010).

The average level of cytosine methylation in vertebrates is 3 - 8% of all cytosines, but the highest levels among all eukaryotes have been observed in plants, usually 6 - 30% (Chen and Li, 2004), up to 50% in some plants, e.g., maize (Suzuki and Bird, 2008). Such a high methylation in plants is due to the methylation of both CpG and CpHpG sequences, in which H is any nucleotide and p is phosphate group, while in animals the methylation usually concerns only cytosines located in CpG dinucleotides (Pradhan and Adams, 1995).

The first high-resolution genome-wide map of DNA methylation was obtained for *Arabidopsis thaliana* using the mCIP-chip method (based on methylcytosine immunoprecipitation combined with whole-genome microarrays) (Zhang et al., 2006). Since then, many other studies on the distribution of DNA methylation in plant genomes have been performed (Zilberman et al., 2007; Cocus et al., 2008; Li et al., 2008; Lister et al., 2008; Wang et al., 2009). The results of those studies, although obtained using different methods for DNA methylation profiling and various plant tissues and species, indicate a connection of DNA methylation with chromatin structure, which in turn is correlated with gene expression.

DNA methylation is catalyzed by DNA methyltransferases (DNMTases). Four groups of plant C-5 DNMTase families (MET1, CMT, DRM and Dnm2) have been identified *in silico* by searching databases for motifs highly conserved in DNMTases (Pavlopoulou and Kossida, 2007).

Hypermethylation of DNA usually decreases gene expression, while reduction of the methylation level (hypomethylation) leads to higher gene expression (Finnegan et al., 1998). The removal of methyl group from cytosine (demethylation) can proceed in a passive or active way. In *Arabidopsis* active demethylation is carried out by 5-methylcytosine DNA

glycosylases DME and ROS1 (Choi et al., 2002; Gong et al., 2002). Biochemical studies have shown that ROS1, DME, and two related proteins, DML2 and DML3, initiate the base excision repair (BER) for active DNA demethylation (Zhu, 2009).

DNA demethylation also can happen due to the activity of reactive oxygen species (ROS), mainly hydroxyl radical (·OH). This oxygen species has a half-life time of 10^{-9} s and reacts efficiently with all DNA components. The reaction of ·OH with m^5C leads to deamination and thymine formation, or to methyl group oxidation, which through formation of the 5-hydroxymethylcytosine intermediate causes demethylation and cytosine formation (Cerda and Weitzman, 1997). It is known that one of the consequences of cell injury is an oxidative burst and DNA damage (Apel and Hirt, 2004). The resulting miscoding (damaged) bases are thought to be a major reason of the induction of heritable changes in plants.

Maize, a plant of a rapid metabolic turnover, remarkable genome plasticity, exhibits high level of DNA methylation. Due to its large leaves is particularly exposed to mechanical wounding. Therefore, the aims of the present work was to elucidate the effect of wounding of maize leaves on the methylation of genomic DNA and ROS production. In addition, to studying the overall (global) of methylation level of genomic DNA, the methylation of *ZmCPK11* gene was analyzed. Previously, we have shown that wounding of maize leaves stimulates the expression and activity of *Calcium-Dependent Protein Kinase ZmCPK11* (Szczegielniak et al., 2005; 2012). Here, we looked for effect of the stress on the methylation of this wound-inducible gene.

2. Materials and Methods

2.1. Plant material and stress induction

For stress treatment maize plants (*Zea mays* inbred line Rd 17-25) were cultivated in soil in a greenhouse under controlled conditions: day/night 16h/8h, temperature 25/20 °C. Leaves of 2-week-old plants were wounded, at 9 am, by perforating the lamina with 50-grit sandpaper. At indicated times the wounded leaves were detached from plants and plunged into liquid nitrogen and stored at - 80 °C prior to analysis.

2.2. Genomic DNA isolation

Genomic DNA was extracted from wounded and non-wounded (control) maize leaves. Plant material (2.5 g FW) was ground in a mortar with liquid nitrogen, 15 mL of extraction buffer (100 mM Tris/HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM 2-mercaptoethanol) and 1 mL of 20% SDS was added. Samples were mixed vigorously and incubated at 65 °C for 10 min. Then, 5 mL of 5 M potassium acetate was added, samples were mixed and incubated at 0 °C for 20 min. Next, the mixture was centrifuged at 10 000 ×g for 20 min at 4 ºC. The supernatant was filtered through sterile Miracloth, and 10 mL of cooled (to -20 °C) isopropanol (≥ 99.7%) was added. The genomic DNA precipitated in the interphase was coiled on a glass Pasteur pipette, washed in 70% ethanol and resuspended in buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA, pH 8.0) overnight at 4 °C. Insoluble material was removed by centrifugation at 17 000 × g for 10 min. To the supernatant, RNaseA was added (final concentration 10 $\mu g.mL^{-1}$) and samples were incubated for 10 min at room temperature. An equal volume of Tris-saturated phenol was added, gently mixed and centrifuged at 17 000 × g for 10 min at room temperature. Aqueous phase was transferred to a new tube and equal volume of chloroform-isoamyl alcohol (19:1) was added and gently mixed, centrifuged and the upper phase was collected. The chloroform-isoamyl alcohol mixture extraction was repeated. DNA was precipitated from the aqueous phase by adding sodium acetate to a final concentration 0.3 M and equal volume of isopropanol. After centrifugation at 17 000 × g for 10

min, the pellet was washed with 80% ethanol, air dried and dissolved in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0). For evaluation of DNA quality, electrophoresis on 0.8% agarose gel was performed. Edithium bromide staining showed only one band of high molecular mass DNA. DNA was quantified spectrophotometricaly (Eppendorf BioPhotometer) at 260 nm.

Five biological replicates of leaves after wounding and four control ones were analyzed separately.

2.3. TLC-based determination of methylated cytosine in DNA

A TLC-based (Thin Layer Chromatography) method described by Barciszewska et al. (2007) was used for quantitative determination of m^5C levels in genomic DNA from wounded and non-wounded maize leaves. Briefly, genomic DNA (1 μg) was digested using 0.001 U of spleen phosphodiesterase II and 0.02 U of micrococcal nuclease and next labelled with 2 μCi [γ-^{32}P] ATP, 4.500 Ci.mmol^{-1} (ICN). Obtained 5' labelled nucleotides were separated by two dimensional chromatography on cellulose TLC plates (Merck). Spots containing labelled nucleotides were visualized on Typhoon Phosphoimager (Pharmacia) with Image Quant software. The analysis was repeated five times for each sample and evaluated with Statistica Annova test. For determination of the m^5C level in genomic DNA, R coefficient was calculated, $R=[m^5C/m^5C+C+T] \times 100$. The coefficient includes not only m^5C and C as the parent nucleoside, but also T which can be formed by deamination of m^5C.

2.4. ELISA-based method for determination of methylated cytosine in DNA

The ELISA (Enzyme-Linked Immunosorbent Assay) - based Imprint® Methylated DNA Quantification Kit (Sigma) is a tool to determine global DNA methylation levels and is

an alternative to the more traditional methods of analyzing global methylation, such as TLC, HPLC, or MALDI-TOF-MS.

Quantification of m^5C in genomic DNA isolated from maize leaves was performed according to manufacturer's recommendations. Genomic DNA (200 ng) in DNA Binding Solution was transferred to assay wells. Fully methylated DNA from the kit was used as a reference and the DNA Binding Solution as a blank. DNA binding to the assay wells was performed for 60 min at 37 °C and then Block Solution was added and incubated for 30 min at 37 °C. Next, each well was washed four times with Washing Solution and Capture Antibody was added. After 60 min of incubation at room temperature, the wells were washed five times and further incubated with Detection Antibody for 30 min at room temperature. After that washing was performed six times to remove the antibody and next Developing Solution was added. When the colour changed to blue (up to 10 min) the reaction was stopped by Stop Solution, which changed the colour to yellow. Next, absorbance at 450 nm was measured on a plate reader and the relative global methylation level [%] was calculated using the equation:

[Absorbance for sample DNA - Blank Absorbance/Absorbance for reference DNA - Blank Absorbance] × 100.

2.5. Identification of CpG islands in ZmCPK11 *gene and bisulfite sequencing PCR (BSP)*

For the detection of CpG islands the coding region of the *ZmCPK11* gene (accession number, AY301062) and 5' promoter region (1000 bp) were analyzed by the program Meth Primer Software (Li and Dahiya, 2002). The sequence of highest density of CpG pairs was located at the first exon of *ZmCPK11*. The sequence 200 bp length was selected for further analysis (Fig. 3A). To determine which cytosines are methylated in the CpG islands of the *ZmCPK11* gene bisulfite treatment of DNA followed by PCR and sequencing was used. Sodium bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosines unaffected.

Thus, bisulfite treatment introduces specific changes in the DNA sequence that depend on the methylation status of individual cytosine residues.

The Imprint® DNA Modification Kit (Sigma) was used for bisulfite conversion of cytosines in genomic DNA isolated from wounded and non-wounded maize leaves. The One-Step Modification Procedure was used according to the manufacturer's instructions. DNA (1μg) was denaturated by heating and incubated with Modification Solution at 99 ºC for 6 min and then at 65 ºC for 90 min. These conditions ensure C→U conversion exceeding 99%. The modified DNA was then purified using Kit columns and used as a template for PCR. Primers for amplification of a fragment of the *ZmCPK11* first exon containing the CpG island identified as above were designed using MethPrimer software (Li and Dahiya, 2002). The primers F1 5'-TTTTGATTAAGGATAGGTTTTTTTT-3 and R1 5'-ACTACCCCTACCCCAATTTCTTAC - 3' flank the region from -92 (relative to the ATG translation start codon, the "Adenine" defined as position +1) to +163 (see Fig. 3A). Two μl of the modified DNA solution was used for PCR in 50 μl of PCR mixture containing 0.5 U of Taq DNA polymerase (EURx), 200 μM each dNTP, 1 x PCR buffer B (EURx), 2% (v/v) DMSO and 0.4 μM of each primer. The PCR conditions were: 94 ^{o}C, 3 min (first cycle); 94 $^{\circ}$C, 45 s; 55 $^{\circ}$C, 45 s; 72 $^{\circ}$C, 45 s (30 cycles), and72 $^{\circ}$C, 7 min (final cycle). PCR was performed on a Primus 96 Advanced thermocycler (Peqlab Biotechnologie). The PCR product of 255 bp was cloned into pCR®II-TOPO® plasmid (Invitrogen) and subjected to sequencing. The sequencing shows 5-methylcytosines unconverted in bisulfite treatment as cytosines (m^5C→C), and unmethylated cytosines converted to uracil as thymidines (C→T).

2.6. Assay for reactive oxygen species (ROS) production

ROS production was evaluated by determining the reduction of NBT (nitroblue tetrazolium, (2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-[3,3'-dimethoxy-4,4'diphenylene]-ditetrazolium chloride, Sigma) in the medium by $O_2^{\cdot -}$ released from leaf tissues. The wounded and control (non-wounded) maize leaves were incubated for 2 h in a mixture containing NBT. The mixture was then heated at 85 ºC for 15 min, cooled and absorbance at 580 nm was measured according to a described procedure (Polkowska-Kowalczyk et al., 2004).

3. **Results**

3.1. Transient changes in DNA methylation level

The effect of wounding of maize leaves on 5-methylocytosine level in their DNA was determined by TLC and ELISA methods. In the TLC-based method the m^5C levels in total DNA were determined separately in samples between 0 and 3 h after wounding. The amount of m^5C decreased significantly ($p<0.05$) 1 h after wounding and returned nearly to the initial level thereafter (Fig. 1). Similar results were obtained using the ELISA-based Imprint® Methylated DNA Quantification Kit (Fig. 2).

The presented results obtained by TLC and ELISA methods show substantial scatter, especially 2 h after wounding when the level of m^5C returns to the initial status. Global DNA methylation in non-wounded plants shows only minor changes, possibly reflecting the circadian rhythm (Fig. 1).

Despite the scattering, the level of m^5C in genomic DNA from wounded maize leaves, as determined by both the TLC and ELISA-based methods, shows a similar statistically significant tendency. The content of m^5C decreases by 25- 30% 1 h after wounding, and then increases to the initial value after 2 - 3 h. Similar tendency was observed in 72-h-old etiolated

maize seedlings where the level of m^5C dropped also by about 30% 1 h after wounding (data not shown).

3.2. Methylation of ZmCPK11 *CpG island*

An analysis of the *ZmCPK11* nucleotide sequence reveals a high density of CpG dinucleotides around its 5' terminus, which could be associated with transcriptional regulation by cytosine methylation. The *ZmCPK11* CpG island starts 105 bp before the start codon and ends 581 bp after it and overlaps the first exon of the gene. The local percentage of CpG ranges from 50 up to 70% within the island. At the 3' end of *ZmCPK11* a second, smaller CpG island is present (Fig. 3A).

We determined the methylation status of the 5' CpG island of *ZmCPK11* by bisulfite sequencing PCR (BSP). Almost all cytosines present in the fragment analyzed were efficiently converted to thymines in DNA obtained from non-wounded as well as wounded leaves, indicating a lack of their methylation in either case. However, two cytosines in positions 100 and 126 behaved differently: in DNA from non-wounded leaves they remained unconverted by bisulfite treatment in 4 out of 6 PCR products sequenced, indicating their methylation. Notably, in a given PCR product the status of the two cytosines was identical, that is they were both unconverted (methylated) in four cases or both were converted to thymines in the two remaining cases. In contrast, in DNA from wounded leaves these cytosines (100 and 126) were converted to thymines upon bisulfite treatment in 3 out of 4 PCR products sequenced. These results indicate that wounding stimulates demethylation of both cytosines. Again, in a given PCR product these cytosines were either both converted or both unconverted (Fig. 3 B,C). Such a concerted behavior suggests an internal link between these cytosines in the structure of the gene.

Interestingly, while cytosine 100 sits in a CpG dinucleotide susceptible to methylation, cytosine 126 is part of a CAC sequence, rarely methylated. Possibly the methylation of neighboring cytosine 100 facilitates the modification of cytosine 126.

3.3 ROS production after wounding

ROS production in wounded leaves was compared with that in control, non-wounded leaves. Wounding clearly induced accumulation of ROS with a biphasic kinetics. After a rapid initial increase, ROS accumulation decreased transiently, then rose again and afterwards returned to the control level. After a rapid increase at 1 min after wounding (about 570% of control level, $p<0.05$), ROS accumulation decreased significantly to about the initial value at 0.5 – 1 h, increased again at 2 h up to 550% of control to return gradually to the control level at 4 h (Fig. 4).

ROS production *in situ* was visualized by reduction of NBT by superoxide radicals. After reduction, dark blue colour was observed in the wounded leaves (Fig. 5).

In accord with the quantitative data above, the ROS production was more pronounced in wounded maize leaves than in non-wounded maize leaves.

4. **Discussion**

It has been proposed that DNA methylation controls plant genomes by repressing their transcription (Pavlopoulou and Kossida, 2007). The stress-induced DNA methylation alteration shows substantial developmental and tissue specificity (Wang et al., 2011). The changes in cytosine methylation seem to be stress and tissue specific. Dynamic changes in the methylation pattern of genomic DNA upon biotic or abiotic stresses have been reported for several plant species (Chinnusamy and Zhu, 2009). For example, global hypermethylation is induced by

drought in pea (Labra et al., 2002). However, stress-induced hypomethylation is observed more frequently. It was induced by touching in white bryony (Galaud et al., 1993) and by heavy metals in clover (Aina et al., 2004). When maize seedlings were exposed to cold stress, a genome-wide demethylation occurred in root tissues, but not in leaf blades or stem (Steward et al., 2002). Hypomethylation was also induced upon pathogen infection in tobacco (Wada et al., 2004) and *Arabidopsis* (Pavet et al., 2006). Some hypomethylated genes involved in stress responses were found to be transcriptionally activated (Wada et al., 2004; Choi and Sano, 2007). A physiological effect of DNA methylation was shown by treatment of germinated rice seeds with 5-azadeoxycitidine, a powerful demethylation agent, which induced dwarfism at maturity (Sano et al., 1990). Finnegan et al. (1998) have demonstrated that the promotion of flowering by prolonged exposure to low temperatures (vernalization) is mediated by DNA demethylation which was illustrated by transient demethylation of DNA in *FLC* (Flowering Locus C) and its two flanking genes in *Arabidopsis*.

Abiotic stresses, like cold or drought, generate a noticeable decrease of global m^5C content within a few days (Steward et al., 2002; Wang et al., 2011). The drought-induced changes of DNA methylation are reversible and methylation level returns close to their original status after recovery. Our results are consistent with the pattern of m^5C changes (control>stress<recovery) caused by drought in rice (Wang et al., 2011). Our results show that the damage of maize leaves induces a reversible decrease, of the global m^5C content by about 20-30% within 1 h after wounding, which then returns to the initial level within 2 h after the stress. The scattering of the results, especially 2 h after wounding, may reflect a diversified sensitivity of individual plants/leaves to the stress and different capacity to recover. This suggests that wounding provokes the plants to a rather quick response which finally leads to regeneration and/or adaptation processes. **Some of the known Calcium Dependent Protein Kinases are involved in wound signal transduction in plants. These signaling pathways**

mostly include elevation of intracellular level of calcium, reversible protein phosphorylation and transcriptional activation of the specific genes (Leon at al., 2001). Most of the induced responses occur within a few minutes to several hours after wounding. Previously, we have shown that wounding of maize leaves stimulates both enzymatic activity and expression of *ZmCPK11*. This protein kinase participates in early events of local and systemic response to wounding and seems to be important transmitters of the stress signaling in maize (Szczegielniak et al., 2005; 2012).

One hour after wounding of maize leaves two methylcytosines in positions 100 and 126 located in a CpG island overlapping the first exon of the *ZmCPK11* gene are demethylated, and 3-6 h after wounding the transcript level of the kinase is markedly increased (Szczegielniak et al., 2005). This suggests that reversible methylation of cytosines 100 and 126 is involved in the regulation of *ZmCPK11* transcription under stress, in accord with the general opinion that cytosine demethylation results in activation of transcription (Zhang et al., 2006; Zilberman et al., 2007).

Cytosine in DNA can be methylated in promoters as well as in gene bodies (Maunakea et al., 2013). Feng et al. (2010) have shown that in plants m^5C in gene bodies occurs almost exclusively in CpG dinucleotides, and its distribution is conserved among diverse plant species. Methylation in CHG or CHH contexts is rare, especially in gene bodies. Methylation of cytosine 126 in *ZmCPK11* occurs in CHH, which may indicate that **this, not typical methylation, is promoted by methylation at cytosine 100.**

The rapidness and reversibility of DNA methylation changes upon stresses suggest an involvement of non-enzymatic reactions driven by massive production of reactive oxygen species (ROS) called oxidative burst. ROS signaling pathways appear to play a central role in the coordination of plant responses to a wide variety of stresses and can lead to alterations in DNA methylation (Wrzaczek et al., 2013). These changes in DNA methylation patterns can

strongly affect the regulation of expression of many genes. The present results showed that in maize leaves wounding induced a two-step oxidative stress, an early one just after the wounding and the second one two hours later. This is in concert with observations made by other researchers on various plant species. The kinetics of ROS production in response to wounding appears to differ substantially between species, varying from biphasic, such as in ryegrass and *Medicago truncatula* (Le Deunff et al., 2004; Soares et al., 2009) to long-term and multi-phasic one such as in potato (Razem et al., 2003). A biphasic ROS production has also been observed in plants in response to biotic stresses (Polkowska-Kowalczyk and Maciejewska, 2001; Bolwell et al., 2002). The initial burst appears to be non-specific, being produced in response to numerous stress factors. The second phase is thought to correlate with resistance of the plant to the stresses.

Oxygen radical injury in DNA results in the formation of 8-hydroxyguanosine as a prevalent product. The replacement of guanine with the oxygen radical adduct 8-hydroxyguanine profoundly alters methylation of an adjacent cytosine, suggesting a role for oxidative injury in the formation of aberrant DNA methylation patterns (Cerda and Weitzman, 1997). Choi and Sano (2007) showed decreased DNA methylation in tobacco plants exposed to paraquat, an effective reactive oxygen species generator. Moreover, those authors observed induction of glycerophosphodiesterase-like protein (*NtGPDL*) transcript within 6 h after the stress, preceded by a demethylation of corresponding the gene within 1 h after the stress.

Our results indicate that wounding of maize leaves transiently changes the DNA methylation status, most probably through an initial effect of reactive oxygen species and subsequent action of methyltransferases. Earlier work has shown that the transcript level of a DNA methyltransferase gene (*ZmMET1*) is decreased in maize after wounding (Steward et al., 2002). Moreover, the demethylation of two cytosines in *ZmCPK11* upon wounding is correlated with an increase in the transcript level of this gene reported earlier (Szczegielniak et

al., 2005). We have also found that the oxidative stress caused by wounding is correlated temporally with the transient DNA hypomethylation.

Contribution

Study conception and design: Muszyńska, Barciszewski, Lewandowska-Gnatowska; Acquisition of data: Lewandowska-Gnatowska, Polkowska-Kowalczyk, Barciszewska; Analysis and interpretation of data: Lewandowska-Gnatowska, Polkowska-Kowalczyk, Muszyńska, Szczegielniak; Drafting of manuscript: Muszyńska, Lewandowska-Gnatowska, Polkowska-Kowalczyk, Szczegielniak; Critical revision: Polkowska-Kowalczyk, Lewandowska-Gnatowska, Szczegielniak, Muszyńska;

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Legend to figures:

Fig. 1. Content of m^5C in genomic DNA in wounded and non-wounded maize leaves determined by TLC-based method. Results are shown as percentage of the initial content of m^5C (0 hours). The mean value of R coefficient in control leaves was 9, expressed as 100%. At least three in duplicate independent series of experiments were performed. Data are mean values ± SD (n=6). The significance of difference between mean values was determined by Student's *t*-test. The same letters (aa, bb) indicate significant differences ($p<0.05$) between values for non-wounded and wounded leaves.

Fig. 2. Content of m^5C in genomic DNA in wounded maize leaves determined by ELISA-based method. The results are in %, relative to the content of m^5C at initial time (0 hours). The mean value of m^5C in control leaves was 13,7% of fully methylated DNA, expressed as 100%. At least five series of experiments were done. Data are the mean values ± SD (n=5 at least). The significance of difference between mean values was determined by Student's *t*-test. The same letter (aa) indicate significant differences ($p<0.05$) between values.

Fig. 3. Methylation status of *ZmCPK11* gene. *In silico* determination of CpG islands (**A**), fragment of *ZmCPK11* nucleotide sequence (**B. I**), fragment of 200 bp nucleotide sequence of *ZmCPK11* from bisulfite-treated DNA isolated from non-wounded (**B. II**, 6 repeats) and wounded (**B. III**, 4 repeats) maize leaves, methylation (in %) of cytosines 100 and 126 in *ZmCPK11* from previously non-wounded and wounded maize leaves (**C**).

Fig. 4. Time course of ROS production in wounded maize leaves. At least three independent series of experiments were performed each determined in duplicate. Data are mean values ± SD ($n\geq6$). The significance of difference ($p<0.05$) between values was determined by Student's

t-test. The same letters (aa, bb, cc, dd, ee) indicate significant differences ($p<0.05$) between samples.

Fig. 5. Visualisation of ROS production in non-wounded and wounded maize leaves. Reduction of NBT by superoxide radicals results in production of dark blue formazan.

Fig. 1

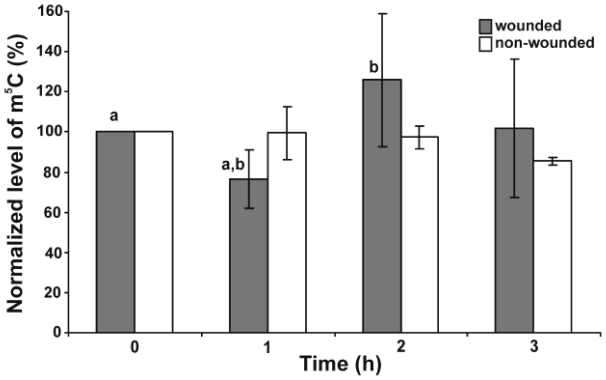
Normalized level of m5C (%)
160
140
120
100
80
60
40
20
0
a
a,b
b
0
1
2
3
Time (h)
wounded
non-wounded

Fig.2

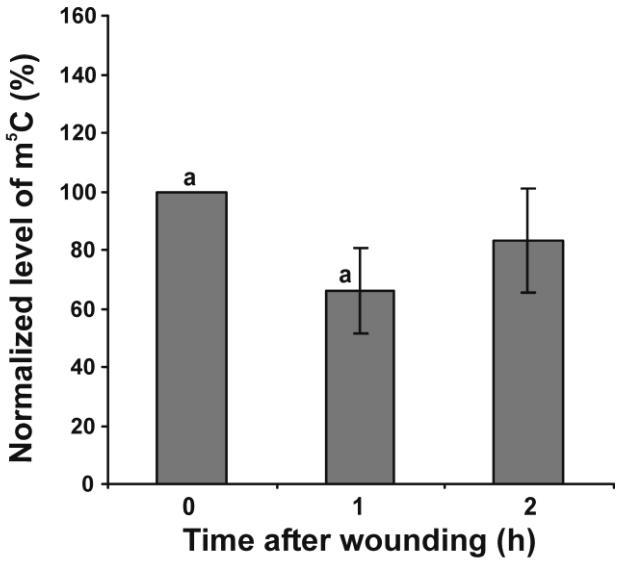
Normalized level of m5C (%)
160
140
120
100
80
60
40
20
0
a
a
0
1
2
Time after wounding (h)

Fig. 3

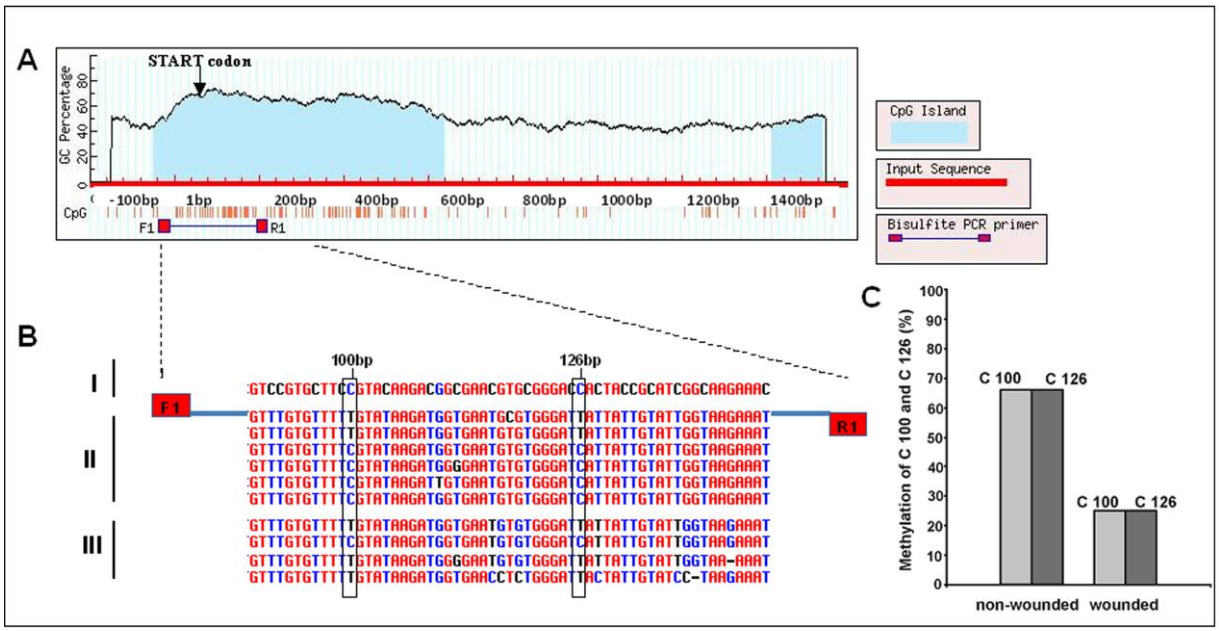
A
START codon
GC Percentage
0
20
40
60
80
-100bp
1bp
200bp
400bp
600bp
800bp
1000bp
1200bp
1400bp
CpG
F1
R1
CpG Island
Input Sequence
Bisulfite PCR primer
B
100bp
126bp
I
II
III
F1
R1
C
Methylation of C 100 and C 126 (%)
C 100
C 126
C 100
C 126
non-wounded
wounded

Fig. 4

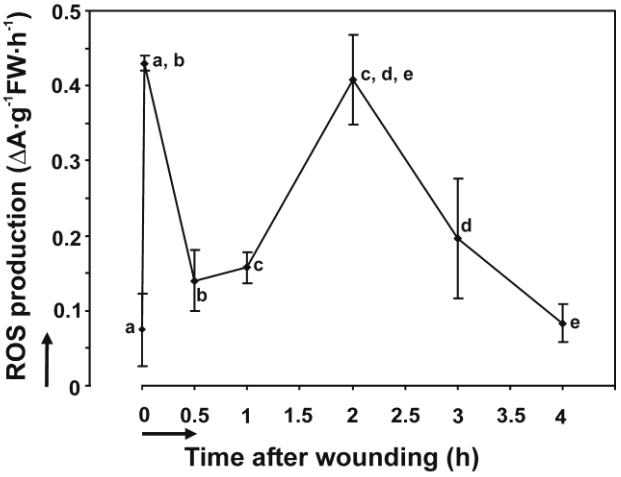
ROS production (ΔA·g-1FW·h-1)
0.5
0.4
0.3
0.2
0.1
0
a, b
c, d, e
d
c
b
a
e
0
0.5
1
1.5
2
2.5
3
3.5
4
Time after wounding (h)

Fig. 5

non-wounded wounded