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ORIGINAL PAPER

Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants

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Abstract To examine the relationship between gene expression and DNA methylation, transcriptionally activated genes were screened in hypomethylated transgenic tobacco plants expressing an anti-DNA methyltransferase sequence. Among 16 genes initially identified, one clone was found to encode a glycerophosphodiesterase-like protein (NtGPDL), earlier reported to be responsive to aluminium stress. When detached leaves from wild type tobacco plants were treated with aluminium, *NtGPDL* transcripts were induced within 6 h, and corresponding genomic loci were demethylated at CCGG sites within 1 h. Direct bisulfite methylation mapping revealed that CG sites in coding regions were selectively demethylated, and that promoter regions were totally unmethylated regardless of the stress. Salt and low temperature treatments also induced similar demethylation patterns. Such effects could be attributable to oxidative stress, since reactive oxygen species generated by paraquat efficiently induced the same pattern of demethylation at coding regions. Pathogen infection induced neither transcripts nor genomic demethylation. These results suggested a close correlation between methylation and expression of *NtGPDL* upon abiotic stresses with a cause–effect relationship. Since DNA methylation is linked to histone modification, it is conceivable that demethylation at coding regions might induce alteration of chromatin structure, thereby enhancing transcription. We propose that environmental responses of plants are partly mediated through active alteration of the DNA methylation status.

Keywords Aluminium · 5-Methylcytosine · DNA methylation · *Nicotiana tabacum* · Oxidative stress

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Introduction

The typical plant genome contains relatively high levels of 5-methylcytosine (m^5C), ranging from 6 to 25% of total cytosines, depending on the species (Steward et al. 2002). Thus DNA methylation is likely to be a regulatory mechanism. In the plant genome, m^5C is found in three nucleotide-sequence contexts: symmetrical CG, and two categories of non-CG sites, symmetrical CNG and asymmetric CNN sites (where N is A, T or C). Each sequence has different genetic requirements for de novo or maintenance methylation (Chan et al. 2005). The physiological role of DNA methylation is not completely understood, yet it is generally considered to be involved in regulation of gene expression, for example with reference to restriction of foreign DNA species that invade host organisms (Bender 2004), as well as epigenetic inheritance of traits (Zilberman and Henikoff 2005).

DNA methylation has in fact been shown to be involved in gene silencing at both transcriptional and posttranscriptional levels. Transcriptional gene silencing is associated with hypermethylation of promoter sequences, while post-transcriptional gene silencing is linked with hypermethylation of transcribed or coding

sequences (Paszkowski and Whitham 2001). Methylation of both DNA and histone tails is also critical for maintenance or formation of heterochromatin (Peters and Schübeler 2005), and its alteration results in a serious modification of gene expression, and transcriptional and transpositional activation of transposons (Bender 2004). DNA methylation has also been suggested to be involved in epigenetics, defined as mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in the DNA sequence (Bird 2002). This typically occurs during somatic cell differentiation in animal cells (Reik et al. 2001), and in plants and filamentous fungi as well (Kakutani et al. 1996; Martienssen and Colot 2001).

Methylation of plant DNA is catalyzed by three distinct enzymes: DNA methyltransferase 1 (MET1) is primarily responsible for maintenance of CG methylation (Finnegan and Kovac 2000); domains rearranged methyltransferase (DRM) contributes to de novo methylation in all sequence contexts (CG, CNG and CNN) (Wada et al. 2003; Wada 2005); and chromomethylase (CMT) is thought to maintain CNG methylation in heterochromatin and silencing of methylated loci (Bartee et al. 2001; Papa et al. 2001; Lindroth et al. 2001). Genetic analyses using mutants and transgenic plants have revealed close correlations with plant development and morphogenesis (Finnegan et al. 1998; Bender 2004).

In a previous study, we constructed transgenic tobacco plants expressing an antisense RNA for type 1 DNA methyltransferase (NtMET1), and found that growth and organ development were seriously affected (Nakano et al. 2000). In order to identify genes responsible for the observed abnormalities, differential display was performed and 16 genes were initially isolated (Wada et al. 2004). Sequence analyses indicated that more than half of them were related to stress responses, leading us to speculate that external stress might affect the methylation status of individual genes. Subsequent assays using one representative gene, *NtAlix1*, showed this to indeed be the case. When wild type tobacco was challenged with tobacco mosaic virus (TMV), transcripts of *NtAlix1* temporarily accumulated, and concomitantly its genomic locus showed a temporal change in the DNA methylation pattern (Wada et al. 2004). In the present study, we selected another so far uncharacterized gene from the above-mentioned 16, designated as hypomethylation associated (HMA) with serial numbers, and further analyzed the relationship between expression and methylation patterns. We report here that *HMA3* (*NtGPDL*) responds to metal and oxidative stresses, and that its genomic locus is in fact simultaneously demethylated.

Materials and methods

Plant materials and stress treatments

Wild-type tobacco plants (*Nicotiana tabaccum* cv Xanthi nc) were grown in a greenhouse at 23°C under a 14 h/10 h light/dark photocycle. About 2 month-old healthy mature leaves were detached and placed in water for 4 h to diminish cutting stress, and then used for subsequent experiments. Samples were then subjected to osmotic stress by transferring them into a solution containing 300 mM NaCl. Similarly, metal stress was applied by transferring leaves into a solution containing 100 μM $AlCl_3$. Low-temperature treatment was performed by exposing plants to 4 ± 1°C under continuous light conditions. Inoculation by pathogenic bacterium, *Pseudomonas syringae pv. glycinea*, was performed by infiltration method as described (Sugimoto et al. 2004). Leaves were harvested at appropriate time points, immediately frozen in liquid nitrogen and stored at −80°C until use.

Gene isolation

Total RNA was isolated from tobacco samples by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987) with a slight modification, and used for cDNA library construction with the λZapII vector (Stratagene, La Jolla, CA, USA). In brief, cDNAs were ligated to the vector at the *Eco*RI and *Xho*I sites, and after transformation, the library was screened with ^{32}P-labeled probes. Positive clones were rescued in the pBluescript SK-phagemid vector by in vivo excision, and amplified in *Escherichia coli* DH5α. The nucleotide sequences were determined by the dideoxynucleotide chain termination method (PRISM BigDye Terminator, ABI, Sunnyvale, CA, USA), and analyzed with appropriate computer software (GeneWorks, National Center for Biotechnology Information, and PSORT server). Genomic sequence was obtained by PCR using genomic DNA as the template and appropriate primer sets designed after the cDNA sequence. Resulting fragment was 10,243 bp, including a 1,918 bp upstream region from the transcription initiation site (accession number AB267678).

DNA and RNA hybridization analysis

For DNA hybridization, genomic DNA was isolated from green leaves by the cetyl-trimethyl-ammonium bromide method (Murray and Thompson 1980). A 25-μg aliquot was digested with an appropriate restriction

enzyme at 37°C for 16 h, separated by electrophoresis on a 1% (w/v) agarose gel, and transferred onto a nylon membrane (Hybond N^+, Amersham, Buckinghamshire, UK). After cross linking using a UV cross linker (RPN 2501, Amersham), the membrane was subjected to hybridization with appropriate ^{32}P-labeled probes at 65°C for 16 h. After successive washing with 0.1× standard saline citrate (SSC) and 0.1% (w/v) SDS at 65°C, samples were used to expose either BAS (Fuji Photo Film, Tokyo, Japan) or X-ray film (Eastman-Kodak, Rochester, NY, USA). For RNA hybridization, total RNA was isolated by the aurintricarboxylic acid method (Gonzalez et al. 1980), and 20-μg aliquots per lane were fractionated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane (Hybond N^+, Amersham). Hybridization was performed in the same way as for the DNA hybridization described above, except that the hybridizing temperature was 42°C. The hybridization probe was a full length cDNA for *GPDL*, prepared from corresponding plasmids.

Epifluorescence analysis

The entire coding region of *NtGPDL* was amplified by PCR and inserted into the *Sal*I and *Nco*I sites of psGFP(S65T) vector to yield the fusion in frame with GFP. Particle bombardment was performed on a Bio-Rad Biolistic PDS 1000/He system to introduce the fusion construct into onion (*Allium cepa*) epidermal cells. The initial pressure of bombardment was 1,100 psi, and the traveling distance of the particles to the plant tissues was 6 cm. Bombarded tissues were placed on the water plates and incubated at 4°C for 24 h in the light, followed by monitoring the localization of GFP with a confocal microscope (AX70; Olympus, Tokyo, Japan).

Bisulfite methylation mapping

Five microgram of DNA was digested with *Eco*RI and subjected to the bisulfite modification as described (Xiao et al. 2003) with modification. Briefly, 120 μl of 5 M bisulfite was added to 20 μl of denatured DNA solution, incubated under five cycles of 55°C for 3 h and 95°C for 5 min. After samples were desalted with Wizard DNA Clean-up System (Promega, Madison, WI, USA), NaOH was added to a final concentration of 0.3 M and samples were incubated at 37°C for 5 min. DNA was then ethanol-precipitated, and 1–2 μl aliquot containing100 ng DNA was amplified with PCR under a 35-cycle of 95°C 1 min, 60°C 1 min, 72°C 2 min. Two regions were selected (see Fig. 3a) for amplification with sets of primers; Primers for region I are: RI-F, 5′-GGATTATGGTTGAATATYYAGGTAT-3′ and RI-R, 5′-CTCAATACCATCCTCRCCRARAA-3′, where Y stands for C and T, and R for A and G. Primers for region II are: RII-F1, 5′-TGGATGYTYYGATAGTTGAATAG-3′, RII-R1, 5′-TTACCRRCTCTTTCTRCCRRCTT-3′, RII-F2, 5′-GAAATTAAYYTGATATGYYAAYTAGTGA-3′, RII-R2, 5′-AARCRAACTTARTAATRRCCATTTCCTA-3′. PCR products were then purified using Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pGEMT-Easy vector system (Promega). Individual clone was sequenced using an ABI sequencer (PRISM BigDye Terminator, ABI, Sunnyvale, CA, USA). The conversion efficiency of cytosine into thymine was over 95% as judged from equal sequences in examined clones (see Fig. 5a).

Reverse transcription-PCR

Total RNA was prepared from aluminium-treated leaves by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987), and subjected to RT-PCR using an RNA PCR Kit (AMV) ver 2.1 (Takara, Japan) with gene specific primers. Primer #1: 5′-CCAAACACCATCTCTCTCTC-3′, Primer #2; 5′-TTACCTCAAGGCCCAAACAC-3′, Primer #3; 5′-AGCCTGAAATTTCCTACAGC-3′, Primer RV; 5′-CCGGTCAATGTAAGCCATTTAGAGGT-3′. PCR was carried out under the condition of a 30-cycle of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. After fractionation on agarose gel electrophoresis, products were identified by visualization with ethidium bromide staining.

High pressure liquid chromatography analysis

High pressure liquid chromatography (HPLC) was performed as described previously (Steward et al. 2002). A 100 μg aliquot of genomic DNA was incubated with 110 ng RNAaseA (Nacalai Tesque, Kyoto, Japan) at 37°C for 2 h to remove RNA contamination. After ethanol precipitation and denaturation at 100°C for 5 min, DNA was digested with two units of nuclease P1 (Sigma, Saint Louis, MO, USA) at 37°C for 20 h, followed by dephosphorylation with 20 units of calf intestine alkaline phosphatase (Takara, Otsu, Japan) at 37°C for 2 h. The sample was fractionated by Ultrafree-MC™ PL-10 microcentrifuge tubes (Millipore, Bedford, MA, USA) and the permeate was injected into a Supelcosil™ LC-18-S column (Supelco, Bellefonte, PA, USA). Separation was performed with

a 2.5–20% methanol gradient in the presence of 50 mM KH_2PO_4 (pH 4.3).

Results

Identification of *NtGPDL*

The previously reported *HMA3* was only a fragment of an uncharacterized gene (Wada et al. 2004). In order to further examine its properties, we screened a tobacco cDNA library and isolated a full-length clone of 2,439 bp. Homology searches suggested *HMA3* to encode a polypeptide consisted of 752 amino acids with a molecular mass of 82 kDa, with high similarity to glycerophosphodiesterase-like protein (Fig. 1a), and consequently it was designated as NtGPDL (*Nicotiana tabacum* glycerophodiesterase-like) (accession numbers, AB120519/AB120520). Phylogenetic analysis indicated that NtGPDL belongs to the glycosylphosphatidylinositol-anchored protein (GAP) family (Fig. 1b), but its physiological function has not yet to be determined. Genomic complexity was analyzed by genomic DNA hybridization (Fig. 1c), which showed two or at most three signals on digestion with different restriction enzymes. Since *N. tabacum* is an amphidiploid, this rather simple pattern of hybridization suggests that the gene exist as a single copy per haploid genome. Cellular localization was examined by GFP-fusion proteins, which were expressed in onion epidermal cell layers by the bombarding method. Epifluorescence observation suggested that the protein was localized to the nucleus and may also be at the plasma membrane (Fig. 1d).

Stress-induced hypomethylation and expression

As *NtGPDL* (*HMA3*) was initially identified in hypomethylated transgenic tobacco expressing antisense-*NtMET1*, a DNA methyltransferase of maintenance-type 1 (Wada et al. 2004), it was speculated that hypomethylation is correlated with gene expression. Subsequently, the relationship between expression and methylation status was examined in wild type plants. If hypomethylation is linked to gene expression, the question arises as to what are the circumstances under which genes are demethylated. Database surveys indicated that transcripts of *NtGPDL* are induced by aluminium (Wada et al. 2004). This information lead us to speculate that, in wild type plants, such a stress might trigger off demethylation of relevant genes, resulting in transcriptional activation. In order to test this hypothesis, we first examined change in transcript levels for *NtGPDL* upon aluminium stress in wild type tobacco plants. Transcripts for *NtGPDL* were not detectable by RNA blot hybridization in leaves under non-stressed conditions, but began to accumulate 6 h after aluminium treatment, reaching maximal levels after 12 h with a gradual decline by 24 h (Fig. 2a). In addition, cold and salt stresses also induced transcripts after 3 and 6 h of treatment, respectively (Fig. 2a). Accumulation was temporary, showing gradual decline after 24 h. Hydrogen peroxide generated by paraquat treatment also induced expression within 1 h, indicating oxidative stress to be a powerful inducer. Effects of biotic stress were then examined. Healthy leaves were detached and inoculated with an incompatible pathogen, *Psuedomonas syringae* pv. *glycinea*, which caused severe necrotic lesions on leaves (data not shown). Subsequent RNA blot analysis showed that the level of *NtGPDL* transcripts scarcely changed during the infection up to 24 h (Fig. 2b), suggesting that *NtGPDL* transcription does not respond to biotic-stress. The molecular properties of this gene will be described elsewhere, as the aim of the current study was to determine its response to stressful environments at the methylation and transcription levels.

Methylation status of the genomic locus

The global methylation status of genomic DNA was first examined. Healthy leaves were treated or untreated with aluminium for 12 h, and isolated DNA was hydrolyzed and subjected to HPLC analysis. The amount of m^5C over total cytosine residues was $13.00 \pm 0.87\%$ in the control, while it was $10.95 \pm 0.67\%$ in aluminium-treated samples (triplicate measurements). This suggested that global levels of DNA methylation were slightly reduced upon aluminium treatment. The methylation status of the *NtGPDL* locus was then examined during aluminium stress by DNA blot hybridization using a pair of methylation-sensitive restriction enzymes, *Msp*I and *Hpa*II, both recognizing CCGG. *Msp*I is inactive with m^5CCGG and *Hpa*II with both m^5CCGG and Cm^5CGG. Under the non-stressed conditions, digestion of genomic DNA with *Msp*I yielded multiple fragments, ranging between 1.3 and 5.1 kb (Fig. 3a, see 0 h of aluminium treatment), indicating the majority of the first cytosine in each CCGG to be unmethylated. In contrast, digestion with *Hpa*II yielded two major fragments of 17 and 8.2 kb and one minor 5 kb fragment (Fig. 3b). These patterns clearly indicated that the frequency of Cm^5CGG was extremely high while that of m^5CCGG was low, suggesting that CG methylation is prevalent throughout the locus under non-stressed conditions.

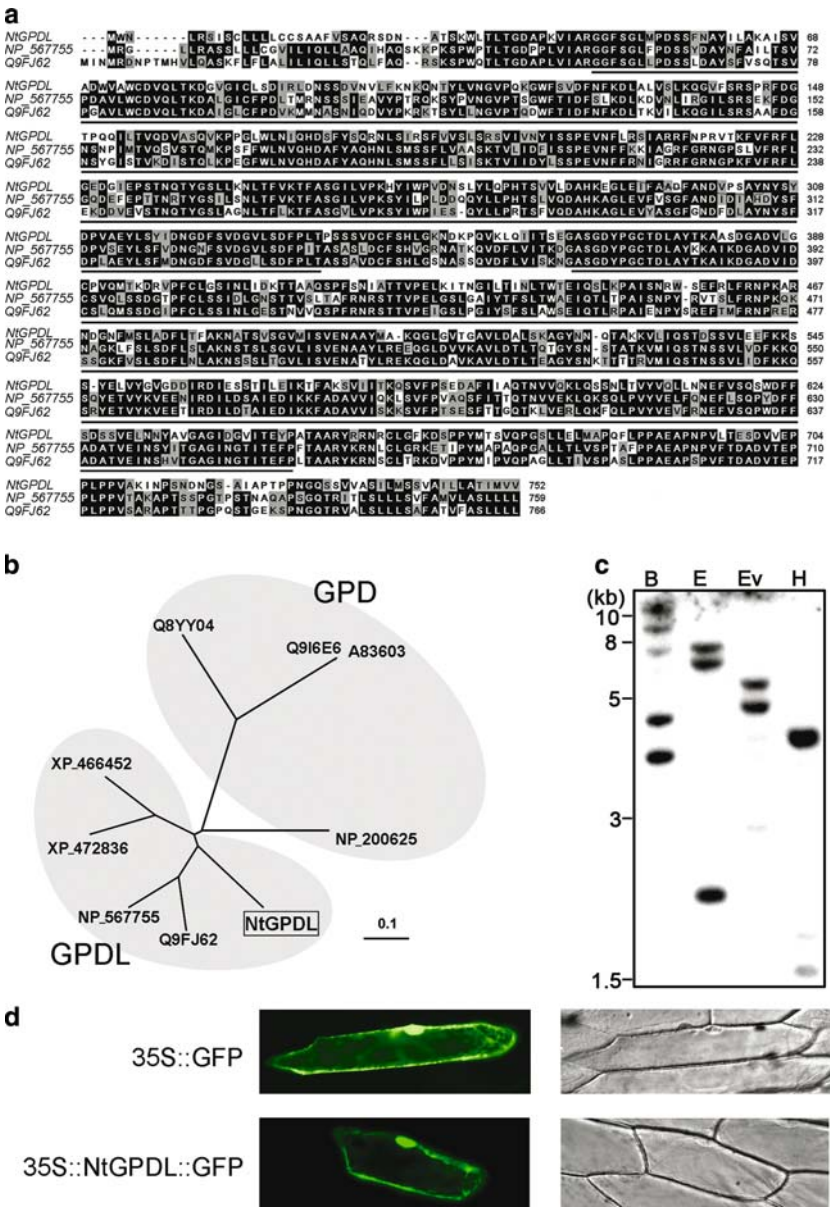

Fig. 1 Properties of *NtGPDL*. **a** Amino acid sequence and alignment with related proteins (*Arabidopsis thaliana* NP_567755 and Q9FJ62). *Black* and *grey boxes* indicate identical and similar amino acids, respectively. Two tandem located conserved GPD motifs are *underlined*. **b** Unrooted phylogenetic tree of *NtGPDL* with related GPDL proteins found in *A. thaliana* (Q9FJ62, NP_567755) and *Oryza sativa* (XP_472836, XP_466452) and GPD proteins from *E. coli* (Q8YY04, Q916E6, A83603) and *A. thaliana* (NP_200625). Each family is indicated by *shading*. **c** Southern hybridization analysis of *NtGPDL*. A 10-µg aliquot of genomic DNA from tobacco (*N. tobacum*, cv Xanthi) was digested with *Bam*HI (*B*), *Eco*RI (*E*), *Eco*RV (*Ev*) or *Hin*dIII (*H*), fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with radioactively labeled *NtGPDL* as the probe. **d** Intracellular localization. Onion epidermal cell layers were bombarded with gold particles coated with 35S*NtGPDL-GFP* or 35S*GFP* alone, and observed under interference contrast (*right panel*) and by epifluorescence for GFP (*left panel*)

Based on this observation, methylation patterns upon aluminium stress were examined at CCGG sites. When leaves were treated with aluminium and periodically isolated DNAs were digested with *Hpa*II, a new fragment of 13 kb was generated within 1 h, in addition to 17 and 8.2 kb fragments which were originally found in non-stressed leaves (Fig. 3b). The level of the 13 kb fragment appeared to increase thereafter, with a concomitant decrease of the 17 kb fragment (Fig. 3b, c), suggesting one Cm^5CGG site within the latter to be gradually demethylated, resulting in susceptibility to *Hpa*II. Digestion with *Msp*I equally generated multiple fragments throughout examined period, indicating m^5CCGG, if any, not to be affected (Fig. 3a). Effects of biotic stress were then examined by inoculating detached leaves with *P. syringae* pv. *glycinea*. Subsequent DNA blot analysis as described above revealed no change of methylation patterns, showing *Hpa*II-fragments with 17 and 8.2 kb throughout the infection period (Fig. 3d). These results indicated that abiotic stress induced transcripts and demethylation of *NtGPDL*, while biotic stress induced none of them, and therefore suggested a positive correlation between transcript induction and genomic loci demethylation.

Genomic organization

DNA blot analyses suggested methylation level at some CG sites to be decreased upon aluminium stress. However, due to multiple site numbers, identification

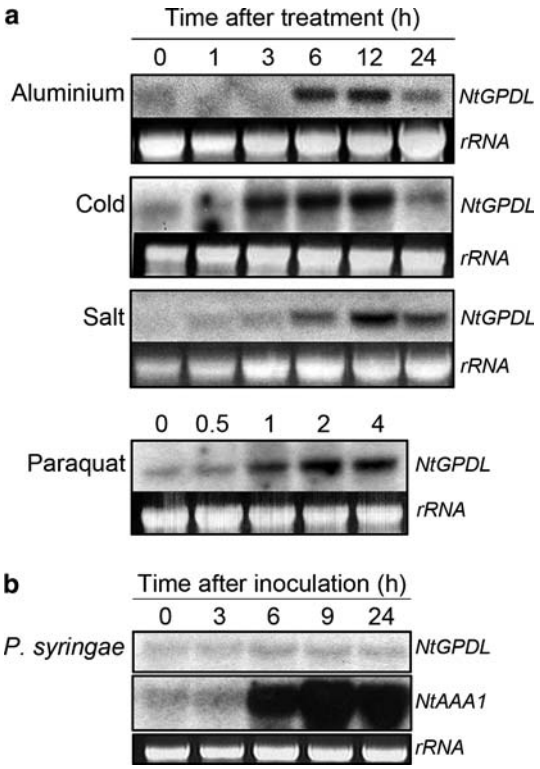

Fig. 2 Expression of *NtGPDL* upon stress treatment. **a** Abiotic stress response. Healthy leaves were detached from wild type mature tobacco plants, and subjected to stress with aluminium chloride (100 μM), cold (4°C), sodium chloride (300 mM) or paraquat (100 μM). Total RNA was isolated at the indicated time points, and subjected to hybridization analyses. A 20-μg aliquot of RNA from leaves after the indicated stress was fractionated on an agarose gel, and subjected to RNA blot hybridization with radioactively labeled full-length *NtGPDL* cDNA as the probe. Equal loading of RNA samples was confirmed by rRNA staining with ethidium bromide. **b** Biotic stress response. Detached healthy leaves were treated with *P. syringae* pv. *glycinea* (2×10^8 cfu/ml) using a syringe without a needle and incubated at 25°C. Total RNA was isolated at the indicated time points after infiltration and subjected to RNA blot hybridization using *NtGPDL* probe (*upper panel*). The successful infection was confirmed by expression of a hypersensitive responsive gene *NtAAA1* (*middle panel*) (Sugimoto et al. 2004). As the loading control, rRNA was used (*lower panel*)

of particular CCGG sites which were demethylated was difficult. To address this question, direct methylation mapping with the bisulfite method was planned, and the genomic locus covering 10 kb was cloned and the sequence determined. The coding region was found to span over 8 kb with 9 exons and 8 introns (Fig. 4a). The transcription initiation site was determined by PCR with several overlapping forward primers covering positions between −210 and −157, and a fixed reverse primer at +110 from the first ATG codon (Fig. 4b). Results showed that an approximately 280-base fragment was efficiently amplified with the primers #1 and #2, but not at all with the primer #3, indicating transcription to initiate around the position between #2 and #3 (Fig. 4c). Subsequent computational analysis (http://www.fruitfly.org/seq_tools/promoter.pl) suggested that the adenine at position −192 from the ATG codon could be the putative transcription initiation site (Fig. 4b). This position was tentatively assigned as the position +1, from which genome map was numbered (Fig. 4a). The promoter region was scanned for transcriptional *cis*-elements, but no clear motifs were identified except for one light-responsive GATA box (position −205). However, subsequent RT-PCR assays showed *NtGPDL* not to be responsive to light conditions (data not shown). Based on above-described information, two regions were selected for direct methylation mapping; Region I was at the coding region spanning 389 bases including intron 3 and exon 4 (positions +2263 to +2651), and Region II was selected from the promoter region including exon 1 and upstream (positions −627 to +363) (Fig. 4a).

Direct methylation mapping

Healthy leaves of wild type plants were treated or untreated with aluminium for 12 h, and DNA isolated from each sample was treated with bisulfite, amplified with region-specific primers by PCR, and cloned. For Region I mapping, 25 and 21 clones from untreated and aluminium-treated DNA samples, respectively, were subjected to sequence determination (Fig. 5a). When a 70-base sequence was selected, and aligned, it was evident that the majority of cytosines in CG remained intact in the untreated control, indicating them to be originally m^5C (Fig. 5a, upper panel). In contrast, one third of cytosines in CG were converted into thymine in aluminium-treated samples, indicating them to be originally cytosines (Fig. 5a, lower panel). Cytosines in CNG and CNN were mostly converted into thymine, indicating them to be originally unmethylated. The distribution and frequency of m^5C were then estimated from the ratio between cytosines and thymines (Fig. 5b). When plotted to the nucleotide position of the region, m^5C was found to be preferentially distributed in CG sites (Fig. 5b). A notable feature is that demethylation appeared to have preferentially occurred at a CG cluster between 309 and 378 within the exon. In this region, there are seven CGs, among which six were found to be demethylated and one appeared to be overmethylated. This site was CCGG, consistent with DNA blot hybridization patterns which showed high methylation levels at many CCGGs (Fig. 3c). To determine whether or not similar demethylation occurs with oxidative stress, leaves were treated with paraquat and DNA was subjected to

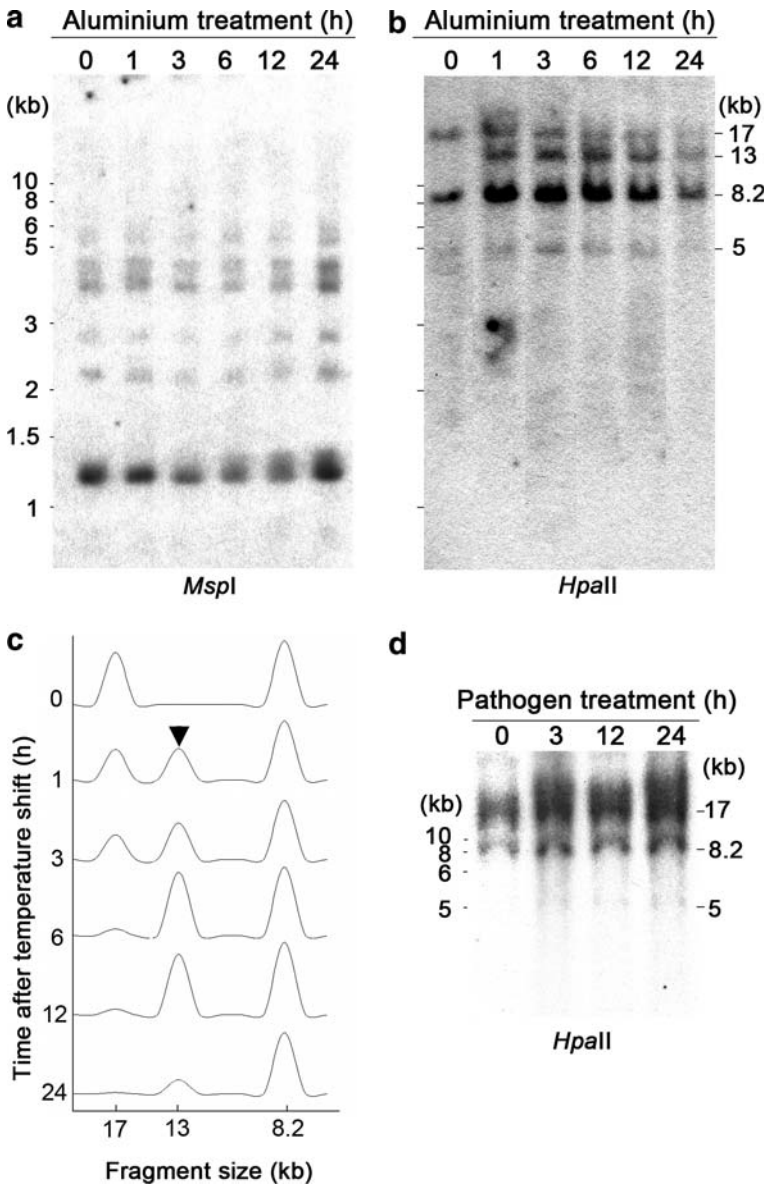

Fig. 3 Time course changes in methylation status. Total DNA was isolated from aluminium-treated leaves at the indicated time points, digested with *Msp*I (**a**) or *Hpa*II (**b**) for 16 h, and subjected to hybridization with the full length *NtGPDL* probe. Duplicate experiments were performed, and one is shown here. **c** Densitometric quantification of signal intensities observed in (**b**). The molecular size is shown on the *horizontal axis*, and the stress treatment time on the *vertical axis*. Note the progressive increase of the 13 kb fraction as indicated by the *arrowhead*. **d** Methylation status during pathogen infection. Total DNA was isolated from *P. syringae*-treated leaves at the indicated time points, digested with *Hpa*II for 16 h, and subjected to hybridization with the full length *NtGPDL* probe. The position of molecular size marker is indicated at *left*, and that of calculated molecular size of each fragment is indicated at *right* in kb

direct mapping as with aluminium-treated samples. The results were essentially consistent, showing a demethylation pattern at the CG cluster (Fig. 5b). Methylation at the promoter region (Region II) was then examined. Mapping primers were designed to amplify a 990-base fragment which included 627-base promoter region and exon 1 (627 bases upstream and 363 bases down-stream from the transcription initiation site) (Fig. 4a). The numbers of CG, CNG and CNN in this region were 22, 27 and 174, respectively, and CCGG sequences were six (Fig. 4a). Results of direct mapping of 20 clones from untreated and 25 clones from aluminium-treated DNA samples revealed that all cytosine residues were completely free of methylation in both untreated control and aluminium-treated samples (Table 1).

Differential methylation among sequence contexts

The average frequency of m^5C in Region I was plotted to CG, CNG and CNN contexts (Table 1). The numbers of methylatable CG, CNG and CNN in Region I were 11, 12 and 47, respectively. In untreated samples, the methylatable CG dinucleotides were methylated at over 93% efficiency, while cytosines in CNG and CNN were methylated at 0.7 and 2.8%, respectively. In the same region from aluminium-treated samples, CG methylation was evident for 84%, a reduction of 10%. Methylation frequency at CNG was 0% at CNG and 3.0% at CNN, resembling the control. In paraquat-treated samples, average methylation levels were 82.9% for CG, 0.5% for CNG and 2.9% for CNN, showing again a reduction for CG methylation.

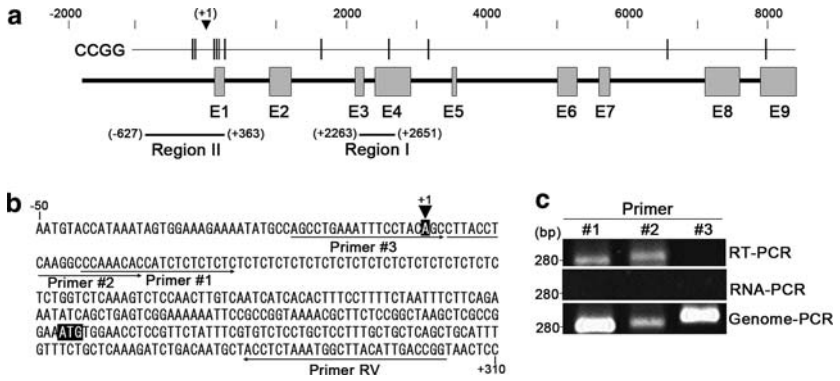

Fig. 4 Properties of *NtGPDL* locus. **a** Genomic organization. A 10 kb genomic locus is illustrated with indication of exons (*box*) and introns (*line*). Exons (*E*) are numbered in Arabic letter starting from the transcription initiation site (+1). CCGG sites are indicated by *vertical bars upper* the sequence and mapping regions are indicated by *horizontal bars*. The regions for direct methylation mapping are located at nucleotide positions 2263–2651 (Region I), and −627 to +363 (Region II). **b** Sequence of the promoter region spanning 360 bases including the ATG site. *Underlines* indicate positions of PCR primers used for determination of transcription initiation site. Three forward (Primer #1 through #3) and one reverse (RV) primers were prepared as shown in (**c**). Nucleotide positions relative to the transcription initiation site are indicated (−50 at the 5′ end and +310 at the 3′ end). The ATG start codon is shaded, and the putative transcription initiation site is indicated by an *arrowhead*. **c** Total RNA was isolated from aluminium-treated leaves of wild-type plants, subjected to RT-PCR using indicated forward (Primer #1 through #3) and reverse primers (*top panel*, RT-PCR). The possibility of genomic DNA contamination in RNA samples was excluded by no amplification under the same reaction condition without reverse transcription (*middle panel*, RNA-PCR). As the control, genomic DNA was similarly amplified by the same primers (*lower panel*, Genome-PCR). Products were fractionated on gel electrophoresis and visualized with ethidium bromide. Both RT and genomic PCR yielded ca 280 bp fragments (size indicated at the *left side*)

Tukey's studentized range test indicated that demethylation frequency is statistically significant at CG site, but not at CNG and CNN sites. Overall, direct mapping experiments indicated that methylation preferentially occurred at CG sites in untreated samples, and that demethylation selectively took place at CG clusters upon aluminium treatment.

Discussion

This paper documents a close correlation between demethylation and expression of a tobacco gene encoding a glycerophosphodiesterase-like protein upon exposure to abiotic stresses. A recent survey revealed that one of the molecular bases of regulation of gene expression is DNA methylation, the pattern of which spontaneously and reversibly changes during the life time of eukaryotes (Bird 2002) and with disease processes, a notable example being neoplasia (Ehrlich 2000). We also found that pathogen attack simultaneously induced expression and demethylation of one gene, *NtAlix* (Wada et al. 2004). To further examine the relationship between gene expression and demethylation by external stimuli, we here analyzed another gene, *NtGPDL*, for which transcripts were previously found to accumulate upon aluminium treatment (Wada et al. 2004).

GPDL possesses two glycerophosphoesterase-like domains, suggesting them to be involved in phospholipid metabolism, although no functional analyses have so far been carried out. As a protein group, GPDL appears to belong to the glycosylphosphatidylinositol-anchored protein (GAP) family, which are localized at plant cell surfaces and are thought to be involved in extracellular matrix remodeling and cell signaling (Borner et al. 2002, 2003). However, current evidence suggests that they are likely to have a wider range of functions (Borner et al. 2003). This speculation is in accordance with the present finding, showing that NtGPDL is distantly related to other members, and localized not only at cell surface (plasma membrane) but also in nucleus, suggesting a novel, so far unknown biological functions.

In healthy leaves grown under non-stressed conditions, *NtGPDL* was not expressed, and its coding region was found to be heavily methylated. Upon exposure to abiotic stress, its transcripts were induced and genomic locus was partly demethylated, while upon biotic stress, neither transcripts nor demethylation were induced. This can be a good example indicating the close correlation between gene expression and methylation. Direct methylation mapping showed that demethylation predominantly took place at CG doublets, which were clustered in exon but rare in intron. Assuming that examined clones were randomly derived from genomic DNA population, we speculated

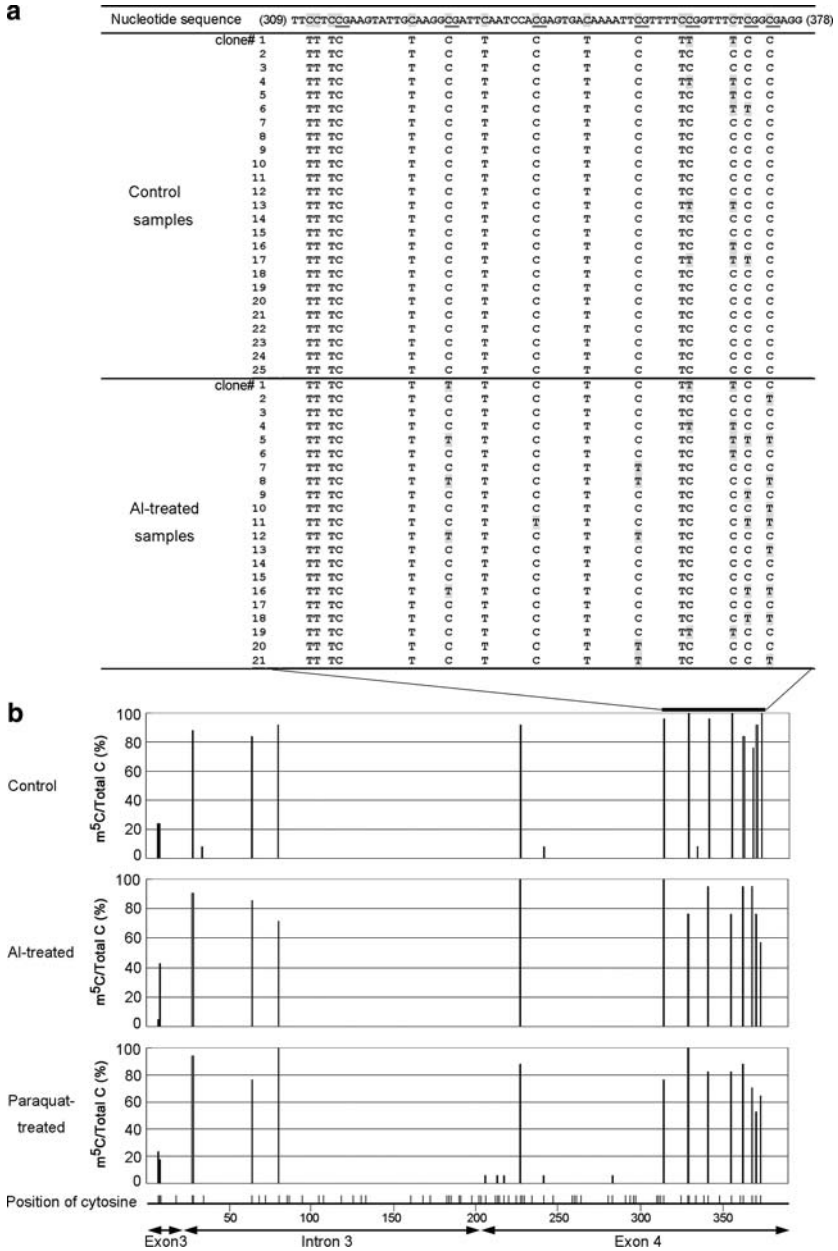

Fig. 5 Direct methylation mapping. **a** Identification of m^5C. Healthy leaves were untreated or treated with aluminium for 12 h, and total DNA was extracted and subjected to methylation mapping. After bisulfite treatment, the region I with 389-bp was amplified with PCR, cloned and sequence determined. The nucleotide sequence of 70 bases (positions between 309 and 378) at the 3′ end is shown. Cytosines (*C*) and m^5C in the original sequence were converted into thymine (*T*) and C, respectively. The original nucleotide sequence of wild-type without bisulfite treatment is shown at the top line with shading of the methylatable cytosines. CG is *underlined*. Thymines in treated samples which corresponded to cytosines in the control are *shaded*. Twenty-five and 21 clones were analyzed for untreated and aluminium-treated samples, respectively. **b** Distribution and frequency of m^5Cs. Histograms represent the percentages of m^5Cs over the total cytosines in the top strand (*vertical axis*) at the indicated nucleotide positions (*horizontal axis*) in the untreated (*top panel*) and aluminium-treated samples (*middle panel*). The distribution and frequency of m^5Cs in paraquat-treated (4 h) samples are also shown (*bottom panel*). The frame lines on the top indicate the sequence shown in **a**. All methylatable Cs in are marked by *vertical bars* over the *horizontal axis*

that demethylation have occurred in approximately 10% of total DNA population, or cell population, which might be appropriate for stress response in leaf tissues. Our observation is consistent with current concepts of reverse relationship between expression and genomic methylation (Bird 2002), and involvement of CG methylation in chromatin structure (Fuchs et al. 2006). In addition to methylation of coding regions, gene expression is often correlated with promoter methylation, which sometimes occurs at only one or

Table 1 Methylation frequency in sequence contexts

Region	Sample	No. of clones	m^5CG/total CG[a]	m^5CNG/total CNG[a]	m^5CNN/totalCNN[a]
I	Control	25	256/275 (93.1)[b,d]	2/300 (0.7)[f]	33/1175 (2.8)[h]
	Al-treated[c]	21	194/231 (84.0)[e]	0/252 (0)[f]	30/987 (3.0)[h]
	PQ-treated[c]	17	155/187 (82.9)[e]	1/204 (0.5)[f]	23/799 (2.9)[h]
II	Control	20	0/440(0)	0/540 (0)	0/3480(0)
	Al-treated[c]	25	0/550(0)	0/675(0)	0/4350(0)

[a] Numbers of CG, CNG and CNN in a single clone were 11, 12 and 47, respectively in Region I, and 22, 27 and 174, respectively in Region II

[b] The sum of m^5 C in the total methylatable Cs in the indicated sequences in a total clones examined are presented as numbers observed and as percentages (%) in parentheses. For example, the 275 CG sites were calculated by multiplying 25 clones by 11 sites of a 389 bases of the Region I

[c] Al, aluminium; PQ, paraquat

[d–i] Values with different letters are significantly different at $P<0.05$ by the test of significance

two cytosine residues (Ko et al. 2005). Methylation mapping at the promoter region of *NtGPDL* surprisingly showed that it was completely free of m^5C regardless of stress treatment. This finding was rather contradictory to general concepts described above, but compatible with recent studies on genome-wide methylation mapping of Arabidopsis (Zhang et al. 2006). Heavy methylation was found in heterochromatin, repetitive sequences and small RNA coding regions, consistent with previous studies (reviewed by Wada 2005). However, there were several unexpected findings. For example, over one third of expressed genes were also methylated in coding regions (body-methylated), and 95% genes were free of methylation in their promoter regions (Zhang et al. 2006). This indicates that promoter methylation is not necessarily prerequisite for controlling expression. *NtGPDL* is a heavy body-methylated, but promoter methylation-free gene, and is highly regulated to specifically respond to external stimuli. Although the genome-wide methylation mapping suggested that constitutively active genes were heavily body-methylated (Zhang et al. 2006), cases such as *NtGPDL* were not exemplified. Since cytosine methylation was shown to be closely linked with methylation of histones (Tariq and Paszkowski 2004; Fuchs et al. 2006), demethylation within *NtGPDL* locus might affect chromatin architecture. We speculate that stress-induced demethylation of coding regions may relax chromatin structure, thereby allowing enhanced transcription (Shilatifard 2006).

The question arises as to how demethylation is specifically activated upon stress. Active oxygen radicals, or reactive oxygen species have been proposed as possible triggers (Cerda and Weitzman 1997), formed through the action of environmental factors that cause oxidative stress, such as ozone, intense light, heat and cold, drought, heavy metals and pathogens. They are highly destructive to cellular components, including lipids, nucleic acids and proteins (Bray et al. 2000) and in the case of DNA, ROS attack is known to result in formation of 8-hydroxyguanosine as a major adduct, which is usually repaired by specific mechanisms that correct this oxidative lesion. However, when stress is too heavy, or when repair is inefficient, methylation of adjacent cytosines is affected, thereby inducing aberrant epigenetic effects, which may lead to carcinogenesis (Cerda and Weitzman 1997). It is tempting to speculate that, in plants, similar events take place, the DNA repair system being activated immediately after injury with consequent alteration of the levels and distribution of m^5C. Indeed, we found similar reduction in methylation and transcriptional activation of *NtGPDL* upon exposure to paraquat, an effective reactive oxygen species generator. Direct methylation mapping showed almost identical demethylation patterns as with aluminium treatment, featuring selective demethylation at CGs in exon regions. Since aluminium is known to generate reactive oxygen species (Boscolo et al. 2003; Apel and Hirt 2004), the present data suggest that the observed demethylation might have been mediated through oxygen radicals. If this were the case, induction of *NtGPDL* transcripts upon drought and cold stresses could also be triggered by similar mechanisms, as both stresses are known to generate reactive oxygen species (Apel and Hirt 2004).

Demethylation of genomic DNA with environmental stress has occasionally been reported in several plant species (Galaud et al. 1993; Steward et al. 2002; Lizal and Relichova 2001; Labra et al. 2002; Alina et al. 2004). Although the underlying process have yet to be clarified in detail, two alternative mechanisms are conceivable: active and passive. The active mechanism involves enzymes that excise m^5Cs from DNA to replace them with cytosines. DNA glycosylases are known to have such activity (Gong et al. 2002), the function being essentially to maintain DNA repair and

to serve as an epigenetic regulator (Gehring et al. 2006). The passive mechanism is considered to be the result of chance 'not-methylation' after DNA replication (Finnegan et al. 1998). In this case, demethylation becomes evident after several cycles of DNA replication, or cell division. Demethylation upon environmental stress must involve the former, since, in stressed tissues, DNA is scarcely replicated, while demethylation takes place rather rapidly (Steward et al. 2000). In this context, the recently identified enzyme, ROS1, is notable, since it shows clear demethylation activity towards m^5C in DNA and ubiquitously expressed in plant tissues (Aguis et al. 2006). Its involvement in stress response has yet to be confirmed, but the present findings indicate that this possibility warrants attention.

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