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# Ectopic expression of the maize chromosomal HMGB1 protein causes defects in root development of tobacco seedlings☆

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**Abstract**

Chromatin-associated high mobility group (HMG) proteins of the HMGB family are versatile architectural factors assisting various DNA-dependent processes such as transcription and recombination. Here, transgenic tobacco lines were generated that ectopically express the maize HMGB1 protein, as detected by immunoblot analyses. The shoot morphology of HMGB1 expressing plants does not differ from that of control plants. By contrast, tobacco seedlings expressing HMGB1 are impaired in the growth of the primary root, relative to control plants. The reduced primary root length is correlated with the accumulation of small cells in the cell division zone (but not in the elongation zone) of the roots of transgenic plants. This "short-root" phenotype is specific for HMGB1, as is not observed with HMGB4 expressing plants, and it is transient in that it is restricted to young seedlings ($\leqslant$28 days), as the effect gradually disappears with older plants.


*Keywords:* Chromatin; Ectopic expression; HMGB protein; *Nicotiana tabacum*

The chromatin-associated high mobility group (HMG) proteins belonging to the HMGB family [1] act as architectural factors facilitating the formation of nucleoprotein structures, which regulate DNA-dependent processes such as transcription and recombination. HMGB proteins contain one or two copies of the characteristic HMG-box DNA-binding domain of ~75 amino acid residues. They bind DNA non-sequence-specifically, but they can recognise pre-bent and distorted DNA structures [2,3]. Upon binding HMGB proteins can bend the DNA by more than 90°, which increases the structural flexibility of DNA, assisting the efficient assembly of nucleoprotein complexes. In some cases, protein/protein interactions between the HMGB proteins and sequence-specific regulators are required [2–5].

The presence of chromosomal HMGB proteins in all tissues of eukaryotes supports the possibility that they are required for proper cellular function. Accordingly, knock-out experiments of *hmgb* genes in yeast and mammals revealed severe phenotypes. Inactivation of the *hmgb1* gene causes pleiotropic defects in mice and they die soon after birth [6]. Mice lacking HMGB2 (which is ~80% identical to HMGB1) are viable, but male mice have reduced fertility, since HMGB2 seems to play a role in germ cell differentiation [7]. In yeast, knock-out of the two *nhp6a/b* genes (encoding HMGB proteins) leads to growth aberrations such as temperature-sensitive growth and various morphological defects [8]. Analyses of gene expression in the strain lacking the NHP6 proteins revealed that the induction of transcription and the expression levels of a variety of genes are altered relative to the control strain [9,10].

In plants, HMGB proteins typically contain a central HMG-box domain that is flanked by a basic N-terminal and an acidic C-terminal domain. The *hmgb* genes appear to be expressed in all plant tissues, but at

☆ *Abbreviations:* HMG, high mobility group; DAPI, 4′,6-diamidino-2-phenylindole; TCA, trichloroacetic acid; CaMV, cauliflower mosaic virus.

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different levels [11–14]. Plant HMGB proteins bind linear DNA non-sequence-specifically, but interact with certain DNA structures with high affinity, and bend the DNA severely upon binding [15–18]. Both maize and *Arabidopsis* express at least five different HMGB proteins, termed HMGB1–HMGB5 [5]. The various maize HMGB proteins differ in their chromatin association [19], in some of their DNA interactions [15,20], in their post-translational modification by protein phosphorylation [21], and in their interaction with the transcription factor Dof2 [22]. Therefore, the different plant HMGB protein variants may be specialised to act in different DNA-dependent processes [5]. Plant HMGB proteins have been studied biochemically in quite some detail, but little is known about their in vivo function. Therefore, we have expressed here the best-characterised plant HMGB protein, maize HMGB1, in tobacco plants. We show that the ectopic expression of maize HMGB1 results in reduced root growth in young tobacco seedlings, correlating with a decreased size of cells at the root tips.

## Materials and methods

*Generation of tobacco plants expressing maize HMGB1.* The DNA sequence encoding full-length HMGB1 was obtained by cleaving the original HMGB1 cDNA clone with *Eco*RI/*Apa*I. After creating blunt ends using the Klenow DNA polymerase, the DNA fragment was cloned into the *Sma*I site of the plant expression vector pKRF9 [23], between the CaMV 35S promoter and *nos* terminator. The resulting plasmid pKRF9-HMGB1 carrying the HMGB1 coding sequence in sense orientation (as well as the insert-less pKRF9) was introduced into the genome of tobacco leaf cells by biolistic transformation essentially as described [24]. Transgenic calli were selected by their resistance against kanamycin and plants were regenerated [24]. *Nicotiana tabacum* cv. Petit Havana was used and plants were grown in a phytochamber (16 h light, 8 h darkness) at 24 °C.

*Verification of transgenic plants.* Genomic DNA extracted from tobacco leaves was analysed by PCR using primers A (5′-TCTCCACTGACGTAAGGGATGA, specific for the CaMV 35S promoter) and B (5′-CAGTGCTCTCGCCCTTGTT, specific for the maize HMGB1 coding sequence). Only plants carrying the *hmgb1* transgene resulted in the PCR fragment of the expected size of 460 bp. Total RNA extracted from the transgenic plants was examined by Northern blot analysis using the *hmgb1* coding sequence as hybridisation probe. HMG proteins were extracted from nuclei of 3- to 4-week-old seedlings or leaves of adult plants taking advantage of their solubility in 2% TCA [12]. HMGB1 was detected by immunoblot analysis using an antiserum directed against maize HMGB1 [15]. An endogenous tobacco HMGB protein was detected using an antiserum against the recombinant tobacco HMGB protein encoded by the cDNA with Accession No. T02252 (the recombinant tobacco HMGB protein used for immunisation was expressed and purified in this work as described previously for the maize HMGB proteins [15]), using the ECL detection system (Amersham Biosciences).

*Analysis of tobacco roots.* Tobacco seedlings (T3 and T4 generation) expressing the maize HMGB1 protein and control lines transformed with the insert-less expression vector were grown on solid MS medium [25] supplemented with kanamycin (50 μg/ml). The length of the primary roots of $\geqslant$30 seedlings of each analysed line was measured by stretching out the seedling roots on graph paper 9 or 28 days after germination.

*Microscopy.* Roots of 9-day-old tobacco seedlings were fixed for 15 min in ethanol:acetic acid (3:1) and DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) as described [26]. The cell nuclei in roots of transgenic tobacco plants were visualised using a fluorescence microscope (Leica DMR). For confocal laser scanning microscopy, roots of 9-day-old tobacco seedlings were stained with propidium iodide and examined using a microscope (Leica DM RBE TCS4D) and Leica Scanware as described [27]. Images were saved at the same scale and the size of the cells in the root tips was measured, before the average cell size for each line (HMGB1 expressing and control) was calculated.

## Results

### *Generation of tobacco plants expressing the maize HMGB1 protein*

The DNA sequence encoding maize HMGB1 was placed in the expression vector pKRF9 under control of the CaMV 35S promoter (which confers constitutive expression), and stably integrated into the tobacco genome by biolistic transformation of tobacco leaves. Tobacco plants were regenerated from transformed kanamycin-resistant calli and self-pollinated. DNA isolated from leaves of the progeny plants was examined by PCR for the presence of the *hmgb1*-transgene, proving that the expression cassette is present in the genome of these plants. Total RNA extracted from leaves of plants carrying the transgene was probed by Northern blot analysis using the sequence encoding maize HMGB1 as hybridisation probe, revealing that the *hmgb1* mRNA was detectable in the majority of transformants (data not shown). More importantly, leaf protein extracts were prepared from plants that gave a positive result in the PCR and Northern analyses, to test whether the transgenic tobacco plants express the maize HMGB1 protein. The proteins soluble in 2% TCA were examined by immunoblot analyses using an antiserum raised against recombinant maize HMGB1 [15]. Four independent transgenic lines were found to express the maize HMGB1 protein, as the antiserum reacted with a protein band of the expected size ($\sim$17 kDa), which is not present in two control lines transformed with the insert-less expression vector (Fig. 1A). In addition, the antiserum recognised an endogenous tobacco HMGB protein that is also detected in the control lines. Using an antiserum raised against a tobacco HMGB protein (which shares $\sim$62% amino acid sequence identity with maize HMGB1), it was examined whether expression of maize HMGB1 resulted in altered levels of endogenous tobacco HMGB protein. According to immunoblot analyses, the HMGB1 expressing lines and control lines contained similar amounts of the endogenous HMGB protein (Fig. 1B), suggesting that expression of maize HMGB1 did not interfere with endogenous HMGB levels.

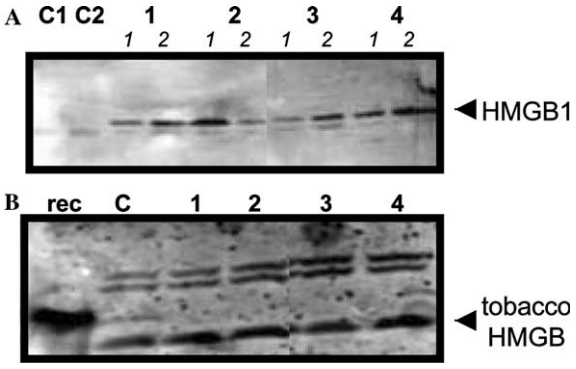


Fig. 1. Examination of transgenic tobacco plants expressing maize HMGB1 by immunoblot analyses. (A) Expression of maize HMGB1 in different tobacco lines. Two plants each of four independent tobacco lines (1–4) transformed with pKRF9-HMGB1 were analysed by immunoblotting using an antiserum raised against maize HMGB1. As control, two lines transformed with the insert-less pKRF9 vector (C1,C2) were examined in parallel. The protein band corresponding to maize HMGB1 is indicated, but an additional cross-reacting protein band (most likely of an endogenous tobacco HMGB protein) is also detected. (B) Expression level of an endogenous tobacco HMGB protein. The tobacco HMGB protein was detected both in HMGB1 expressing lines (1–4) and control plants (C) by immunoblotting using an antiserum directed against the recombinant protein encoded by the cDNA with Accession No. T02252. The detected native HMGB protein band is indicated. It displays a slightly higher electrophoretic mobility than the recombinant HMGB protein used for immunization (rec), due to the presence of a 6× His-tag in the recombinant protein. The antiserum reacts also with two other protein bands, most likely other tobacco HMGB proteins.

### *Expression of HMGB1 causes reduced root development in tobacco seedlings*

HMGB1 expressing plant lines were examined for possible phenotypic differences relative to the control lines. Adult plants of the tobacco lines looked indistinguishable and they did not display detectable differences in their germination times and rates, flowering time, and seed production. The early seedling development was inspected in more detail, revealing that shoot development of the HMGB1 expressing seedlings did not differ from that of control seedlings grown under identical conditions. In contrast, early in development the HMGB1 expressing seedlings displayed clearly shorter primary roots than the control seedlings. Reduced root growth of the seedlings was detected with 9- and 28-day-old seedlings (Fig. 2), while this effect gradually disappeared when the seedlings grew older (data not shown). The length of the primary root of at least 30 seedlings each from the individual HMGB1 expressing and control tobacco lines was measured (Fig. 3). This experiment showed that all HMGB1 expressing lines display shorter roots than control seedlings. With 9-day-old HMGB1 expressing seedlings, the length of the primary root is on average reduced by ~40%, while with 28-day-old seedlings the length of the root is reduced by ~25%, relative to control seedlings.

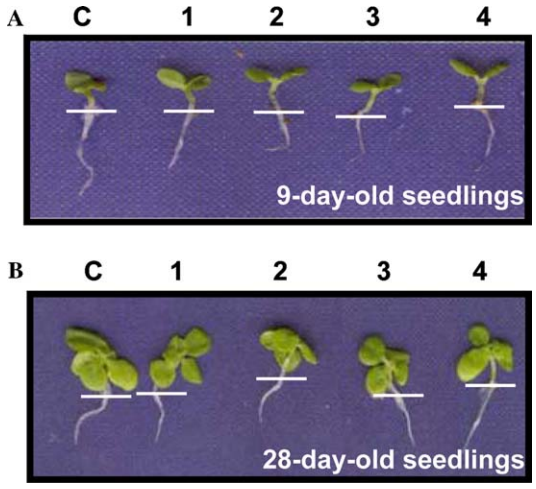


Fig. 2. Tobacco seedlings expressing HMGB1 display reduced growth of the primary root. (A) Nine-day-old seedlings of independent lines (1–4) expressing maize HMGB1 were compared to control seedlings transformed with the insert-less vector (C). (B) Same as in (A) but 28-day-old tobacco seedlings were compared. White bars indicate the root-hypocotyl transition.

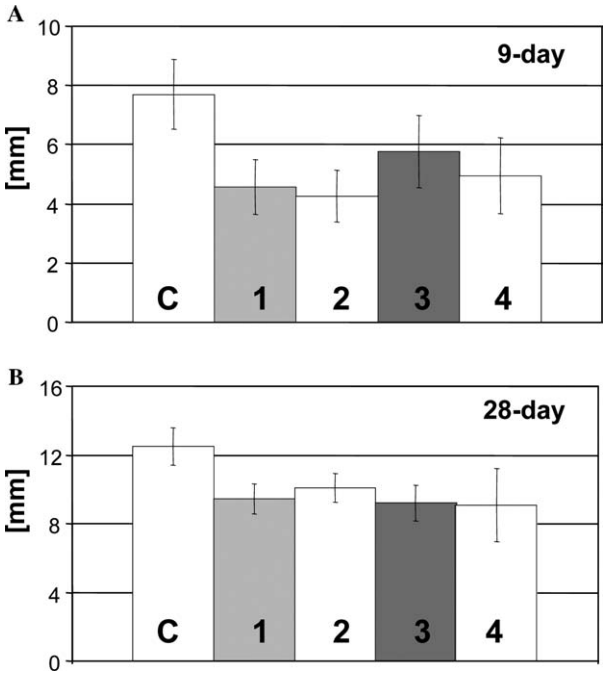


Fig. 3. Measurement of the root length of HMGB1 expressing seedlings. The length of the primary root of a representative number of seedlings (⩾30) of each independent HMGB1 expressing line (1–4) and of a control line (C) was measured for 9-day-old (A) and 28-day-old (B) seedlings. The indicated standard deviation was calculated for each sample.

### *HMGB1 expressing seedlings have a reduced cell size in the cell division zone of the root*

To examine the possible cause for the reduced root length in the tobacco plants expressing maize HMGB1,

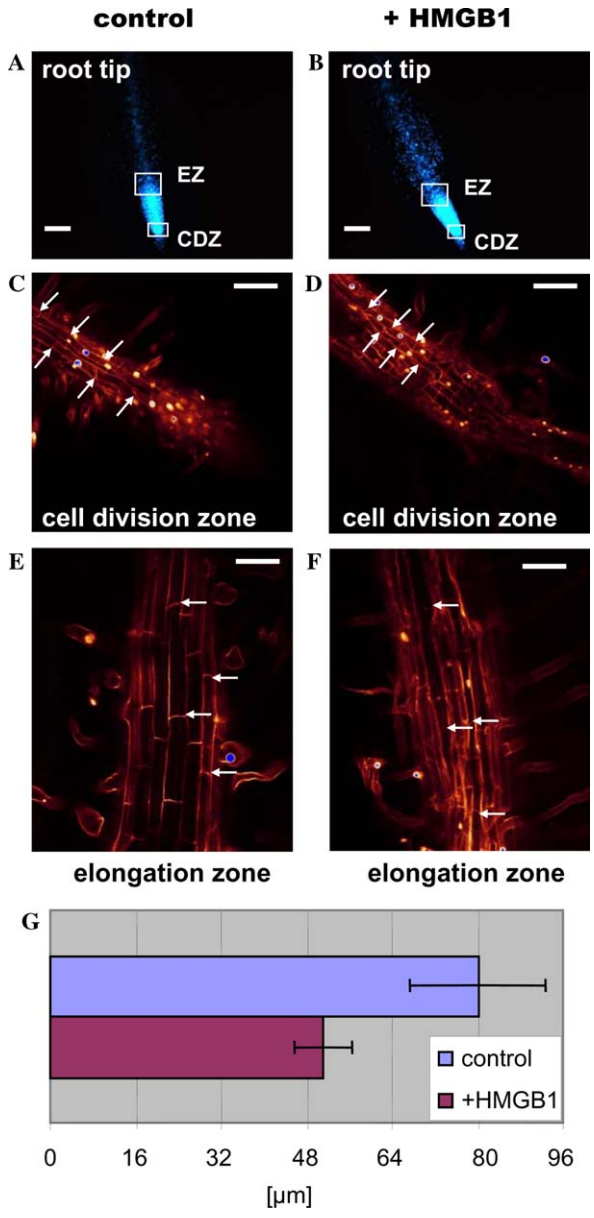


Fig. 4. Root morphology of 9-day-old HMGB1 expressing (+HMGB1) and control tobacco seedlings. (A,B) Root tips were stained with DAPI and examined by fluorescence microscopy. DAPI staining of the nuclei of the densely packed cells in the meristematic zone of the root results in a prominent staining at the root apex. There is a continuous transition of cell division zone (CDZ) and elongation zone (EZ) [33], and the boxes indicate the areas of the root, which have been further analysed in (C)–(F). Bars indicate 150 µm. (C–F) Root tips were stained with propidium iodide and analysed by confocal laser scanning microscopy. The cell division zone and the elongation zone are shown in (C,D) and (E,F), respectively. Arrows point at the proximal and distal cell walls of selected cells, indicating the cell length. Bars indicate 100 µm. (G) Representative number of cells in cell division zone of seedlings expressing HMGB1 and of control seedlings was measured (~100 cells each), and the average cell length is given in the graph. The indicated standard deviation was calculated for each sample.

the root morphology has been analysed in more detail. Roots of 9-day-old seedlings were stained with DAPI and examined using fluorescence microscopy. The characteristic pattern of the nuclei in the densely packed cells of the root tip of HMGB1 expressing plants was indistinguishable from that of control plants (Figs. 4A and B). This result suggests that the overall size of the entire meristematic zone of the root tips is not affected by the presence of maize HMGB1. Using confocal laser scanning microscopy, the cell division zone of roots stained with propidium iodide was analysed. Here, the size of the cells of HMGB1 expressing plants is clearly reduced, relative to cells of the same zone of control plants (Figs. 4C and D). The sizes of cells from different control and transgenic lines were measured, confirming that the cells in the HMGB1 expressing plants are significantly smaller (~40%) than those of control plants (Fig. 4G). In contrast to this finding, no difference in cell size was detected between the different tobacco lines, when cells of the elongation zone were compared (Figs. 4E and F).

## Discussion

The maize HMGB1 protein is the biochemically best-characterised plant HMGB protein. It binds specifically to distorted DNA structures, and it can facilitate the formation of specific nucleoprotein structures, which is regulated by protein kinase CK2-mediated phosphorylation [15,20–22]. Here, we have ectopically expressed maize HMGB1 in tobacco plants to learn more about its in vivo role. HMGB1 expressing seedlings were found to exhibit reduced length of the primary root, whereas the shoot of the seedlings had wild type appearance. This "short-root" phenotype of the transgenic lines correlated with a decreased size of the cells in the cell division zone of the root, resulting in densely packed, relatively small cells at the root tips of plants expressing HMGB1. However, according to the analysis of the root tips by DAPI staining, the size of the entire root apex including the meristematic and elongation zone is unchanged, when compared to the roots of control plants. Therefore, the cell division rate rather than the cell elongation may be affected in the HMGB1 expressing plants. This suggests that the cells remain longer in the process of cell division before they enter cell elongation. Accordingly, small retarded cells accumulate at the root tip. In older seedlings, the growth of the roots of HMGB1 expressing plants becomes gradually normal, reaching the growth rate of control plants, which indicates that HMGB1 is involved transiently in the regulation of root development only in early seedling development ($\leqslant$28 days). The shoot apical meristem is not affected in the HMGB1 expressing seedlings, suggesting that signalling and/or signal sensitivity partially differ between the root and shoot apical meristem. The observed effect is specific for HMGB1, since tobacco seedlings ectopically expressing the maize HMGB4 protein (at levels similar to those

observed here for HMGB1) do not display this "short-root" phenotype and have clearly the appearance of control plants (data not shown). Considering the fact that the abundant HMGB proteins occur rather ubiquitously in the plant and that several HMGB family members are expressed simultaneously [5], the HMGB1 expressing plants display a rather specific phenotype. This situation is similar for the linker histones, which are even more abundant proteins interacting (like the HMGB proteins) with the internucleosomal linker DNA in eukaryotic chromatin. The ectopic expression of an *Arabidopsis* histone H1 in tobacco specifically affected certain developmental programmes, but had only limited effect on basal cellular functions [28]. Moreover, the expression of *Arabidopsis* histone H1 was shown to affect the cell size in tobacco BY-2 cells [29].

Mammalian HMGB1 has been reported to contribute to the correct expression of certain steroid hormone-responsive genes [6,30]. Therefore, it is tempting to speculate that maize HMGB1 is also involved in the expression of specific genes regulated by hormones such as auxin that plays an important role in controlling root development [31], which may result in the "short-root" phenotype of young tobacco seedlings. Similarly, HMGB1 may be involved in cytokinin-dependent gene expression, since cytokinins can negatively regulate root growth in *Arabidopsis* and tobacco, which has been attributed to cytokinin-controlled exit of cells from the root meristem [26,32]. HMGB1 could (as mentioned above) contribute to the proper transition of cells from the phase of cell division to cell elongation. Specific HMGB1-mediated effects may be brought about by well-established functional interactions of HMGB proteins and certain transcription factors [3–5]. It will be interesting to examine in the future potential links of HMGB protein action and gene expression levels, for instance, by profiling expression levels of genes in plants containing different amounts of HMGB1.

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