running head: *Arabidopsis* chromosomal HMGB proteins

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Nucleocytoplasmic distribution of the *Arabidopsis* chromatin-associated HMGB2/3 and HMGB4 proteins

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\[W\] online version of this article contains Web-only data.
ABSTRACT

High mobility group (HMG) proteins of the HMGB family are chromatin-associated proteins that as architectural factors are involved in the regulation of transcription and other DNA-dependent processes. HMGB proteins are generally considered nuclear proteins, although mammalian HMGB1 can be also detected in the cytoplasm and outside of cells. Plant HMGB proteins studied so far were found exclusively in the cell nucleus. Using immunofluorescence and fluorescent microscopy of HMGB proteins fused to the green fluorescent protein (GFP), we have examined the subcellular localization of the *Arabidopsis thaliana* HMGB2/3 and HMGB4 proteins revealing that in addition to a prominent nuclear localization, they can be detected also in the cytoplasm. The nucleocytoplasmic distribution appears to depend on the cell type. By time-lapse fluorescence microscopy it was observed that the HMGB2 and HMGB4 proteins tagged with photo-activatable GFP (paGFP) can shuttle between nucleus and cytoplasm, while HMGB1 remains nuclear. The balance between the basic N-terminal and the acidic C-terminal domains flanking the central HMG-box DNA-binding domain critically influences the nucleocytoplasmic distribution of the HMGB proteins. Moreover, protein kinase CK2-mediated phosphorylation of the acidic tail modulates the intranuclear distribution of HMGB2. Collectively, our results show that in contrast to other *Arabidopsis* HMGB proteins such as HMGB1 and HMGB5, the HMGB2/3 and HMGB4 proteins occur preferentially in the cell nucleus, but to various extents also in the cytoplasm.
INTRODUCTION

Within the cell nucleus, the genomic DNA is organized with histones and other proteins into a nucleoprotein complex termed chromatin. This packaging of the DNA has crucial consequences for DNA-dependent processes including the transcription of genes. The chromatin structure is highly dynamic and is modulated by a variety of chromatin-associated proteins. Among these proteins are the high mobility group (HMG) proteins that represent a heterogeneous class of proteins that after the histones are the second most abundant family of chromosomal proteins (Bustin and Reeves 1996; Thomas and Travers 2001; Reeves 2010). It has been estimated that they bind to ≤10% of the nucleosomes (Johns 1982). Because of their abundance, HMG proteins are thought to serve a global structural function in the nucleus, and they act as architectural factors, facilitating various DNA-dependent processes including transcription, recombination and DNA repair (Bustin and Reeves 1996; Thomas and Travers 2001; Agresti and Bianchi 2003).

In mono- and dicotyledonous plants, members of the HMGA and HMGB families have been identified that are expressed throughout the plant at different levels (Grasser et al. 2007). Plant HMGB proteins (~13 – 27 kDa) have a distinctive three-domain structure with a central HMG-box DNA-binding domain that is flanked by a basic N-terminal and an acidic C-terminal domain. Plant HMGB proteins are structurally more diverse than their animal counterparts, in particular within the domains flanking the HMG-box domain (Pedersen and Grasser 2010). In addition, plants encode around six different HMGB proteins, whereas fewer HMGB variants are found in animals and yeast (Stros et al. 2007). Mediated by the HMG-box domain, the plant HMGB proteins interact non-sequence-specifically with linear DNA (Pedersen et al. 1991; Webster et al. 1997; Ritt et al. 1998; Wu et al. 2003), but they bind with high affinity certain DNA structures including four-way junctions and DNA minicircles (Ritt et al. 1998; Wu et al. 2003; Zhang et al. 2003), and they interact with nucleosomes (Arwood and Spiker 1990; Lichota and Grasser 2001). Moreover, by functional interaction with certain transcription factors, members of the HMGB family contribute to the regulation of gene transcription (Grasser et al. 2007).

Studies addressing the biological function of HMGB proteins suggest that HMGB proteins have important cellular roles. In yeast, inactivation of one of the two NHP6A/B genes (encoding HMGB proteins) did not result in a phenotype distinct from the wild type, but the inactivation of both genes led to growth aberrations such as temperature-sensitive growth and morphological defects (Costigan et al. 1994). Knock-out of the HMGB1 gene caused pleiotropic defects in mice and they die soon after birth, but cell lines could grow normally without HMGB1 (Calogero et al.
Mice lacking HMGB2 (which is ~80% identical to HMGB1) were viable, but male mice had reduced fertility, as HMGB2 seems to play a role in germ cell differentiation (Ronfani et al. 2001). Mice deficient in HMGB3 were also viable, but had an altered rate of generation and differentiation of primitive hematopoietic progenitor cells and HMGB3 appears to be required for the proper balance between hematopoietic stem cell self-renewal and differentiation (Nemeth et al. 2005; Nemeth et al. 2006). Ectopic expression of maize HMGB1 in tobacco seedlings caused reduced length of the primary root, whereas HMGB4 did not affect root development (Lichota et al. 2004). In Arabidopsis, over-expression of HMGB2 reduced seed germination under salt and drought stress (Kwak et al. 2007). Altering the expression of HMGB1 influenced plant growth, stress response and transcriptome in Arabidopsis (Lildballe et al. 2008).

Chromosomal HMGB proteins are generally considered nuclear proteins (Grasser et al. 2007; Reeves 2010), although it is well documented that mammalian HMGB1 can be detected also outside the nucleus, acting as a kind of cytokine (Müller et al. 2004; Yang and Tracey 2010). The Arabidopsis genome encodes seven HMGB-type proteins that differ in structure, expression pattern and DNA-binding properties (Moehs et al. 1988; Stemmer et al. 1997; Grasser et al. 2004; Grasser et al. 2006; Kwak et al. 2007; Launholt et al. 2007), suggesting (partially) specific functions of the different family members. The Arabidopsis HMGB1, HMGB5 and HMGB6 proteins as well as the HMGB-type protein encoded by the locus At2g34450 were found to localize to the nucleus (Grasser et al. 2004; Grass et al. 2006; Launholt et al. 2006). Within the cell nucleus, Arabidopsis HMGB1 and HMGB5 are highly dynamic, binding DNA/chromatin only transiently before moving on to the next binding site (Launholt et al. 2006). Here, we have examined the subcellular localization of the closely related Arabidopsis HMGB2/3 proteins and of HMGB4. Our experiments revealed that these three HMGB proteins occur predominantly in the nucleus, but variable amounts of the proteins are also detected in the cytoplasm, while HMGB1 and HMGB5 are exclusively nuclear proteins.

RESULTS

Subcellular localization of Arabidopsis HMGB2/3 and HMGB4

Since the subcellular localization of plant HMGB proteins has not been examined systematically, we raised an antiserum against Arabidopsis HMGB2, whose distribution has not been studied in plant cells. The antibody was tested in immunoblots, revealing that it reacted specifically with a protein band of the expected size of ~15 kDa, when Arabidopsis Col-0 leaf protein extracts were
probed (Fig. 1A). When compared with an antiserum directed against Arabidopsis HMGB1 (Launholt et al. 2006), relative to the bands of the recombinant proteins the HMGB2 band appeared markedly more prominent, consistent with the higher expression level of HMGB2 (http://www.arabidopsis.org/portals/expression/index.jsp). In addition, the HMGB2 antiserum reacted equally well with recombinant HMGB2 and HMGB3 (data not shown), as both proteins share ~89% amino acid sequence identity and co-migrate in SDS-PAGE. The HMGB2 antiserum was used for indirect immunofluorescence microscopic analyses of Arabidopsis root tip cells (Fig. 1B), while the HMGB1 antiserum served as a reference. In line with previous experiments (Launholt et al. 2006; Lildballe et al. 2008), the HMGB1 antiserum reacted specifically with interphase nuclei that were stained in parallel with DAPI. Anti-HMGB1 signals were enhanced in heterochromatic interphase regions. Conversely, anti-HMGB2 labeled cells displayed uniformly stained interphase nuclei and additional weak immunofluorescence signals were found in the cytoplasm. Dividing chromosomes were hardly stained with either anti-HMGB1 or anti-HMGB2. This finding suggests that HMGB2 (and/or HMGB3) to some extent also occur outside the cell nucleus.

To further analyze the subcellular localization of HMGB2/3 and of HMGB4, we generated plasmids that could direct the expression of GFP fusion proteins in plant cells. Tobacco protoplasts were transformed with these plasmids and examined by confocal laser scanning microscopy (CLSM) (Fig. 2). The previously observed nuclear localization of HMGB1 and HMGB5 (Launholt et al. 2006; Lildballe et al. 2008) served as a reference. While GFP-HMGB1 and GFP-HMGB5 were found exclusively in the cell nucleus (Fig. 2A,B), the fluorescence signals of HMGB2/3 and HMGB4 were detected primarily in the nucleus, but to various extents also in the cytoplasm (Fig. 2C-H). Each fluorescent image (except G) is shown in normal exposure and overexposed to illustrate more clearly the cytoplasmic fluorescence seen with HMGB2/3 and HMGB4. The localization of HMGB2/3 and HMGB4 in both cytoplasm and nucleus was consistently observed for both the N- and C-terminal GFP fusion proteins, although quantitative differences were observed in the nucleocytoplasmic distribution with different protoplasts and with N- vs. C-terminal GFP fusions (HMGB2, HMGB4). These experiments showed that in contrast to the strictly nuclear proteins HMGB1 and HMGB5, the HMGB2/3 and HMGB4 proteins are also found in the cytoplasm.
To examine the subcellular distribution of HMGB2 and of HMGB4 \textit{in planta}, we generated transgenic \textit{Arabidopsis} plants expressing HMGB-GFP fusion proteins. Roots of the transgenic plants were analyzed by CLSM (Fig. 3), and compared with plants expressing HMGA and HMGB5 fused to GFP (which were constructed in the same way). The primary root tip was observed (Fig. 3A,D,G,J) and meristematic and cortical cells were inspected in more detail. Consistent with previous experiments (Launholt et al. 2006), HMGA and HMGB5 were detected exclusively in the nuclei both of the meristematic and cortical cells (Fig. 3B,C and E,F). For the HMGB2 and HMGB4 fusion proteins, in meristematic cells GFP fluorescence was detected preferentially in nuclei (Fig. 3H,K). In cortex cells, the GFP fusion proteins occurred predominantly in nuclei, but they were also clearly visible in the cytoplasm (Fig. 3I,L). The cytoplasmic GFP signal in cortex cells was observed both in the GFP fluorescence images (left panels) and in the overlays of GFP fluorescence and the corresponding bright field images (right panels). This experiment confirmed that HMGB2 and HMGB4 can occur in the cytoplasm and it suggested that the nucleocytoplasmic distribution of HMGB2 and HMGB4 varies between cells.

**Contribution of the basic N-terminal and acidic C-terminal domains on the subcellular localization of HMGB2 and HMGB4**

Since the \textit{Arabidopsis} HMGB2/3 and HMGB4 proteins were detected both in nuclei and cytoplasm, while HMGB1 and HMGB5 were exclusively found in nuclei, the amino acid sequences of the HMGB proteins were searched for motifs that possibly are responsible for this difference in subcellular distribution. However, we were unable to identify differences in potential nuclear localization signals (NLSs) or nuclear export signals (NESs) (Merkle 2003) that could explain the observed differential localization. Moreover, incubation of protoplasts that expressed GFP fusion proteins with the inhibitor of the nuclear export receptor XPO1, leptomycin B (LMB), had no effect on the subcellular distribution of HMGB2 or HMGB4 (data not shown). To examine experimentally the contribution of HMGB protein domains on the subcellular localization, we constructed a variety of plasmids that direct the expression of truncated and chimeric HMGB proteins fused to GFP. Tobacco protoplasts transformed with these plasmids were examined by CLSM (Fig. 4). For comparison, the localization of full-length HMGB1 and HMGB5 (localized in the nucleus) and of HMGB2 and HMGB4 (localized in nucleus and cytoplasm) are shown (Fig. 4A-D). Deletion of the basic N-terminal domain of HMGB2 and HMGB4 had no marked influence on the localization of the proteins, when compared to the full-length proteins (Fig. 4E,F). However, deletion of the acidic
C-terminal domain of HMGB2 and HMGB4 resulted in an increased accumulation of the proteins in the nucleus (Fig. 4G,H). Replacement of the basic N-terminal domains of HMGB2 and HMGB4 by the basic N-terminal domain of HMGB1 (that contains a strong NLS, (Launholt et al. 2006)), caused efficient nuclear accumulation of the chimeric proteins (Fig. 4I,J). When the acidic C-terminal domain of HMGB5 was replaced by the acidic C-terminal domains of HMGB2 or HMGB4, the chimeric proteins (unlike intact HMGB5) in addition to the nucleus were found in the cytoplasm (Fig. 4K,L). Replacement of the acidic tails of HMGB2 and HMGB4 by the short acidic C-terminal domain of HMGB5 resulted in chimeric proteins that accumulated in the nucleus (Fig. 4M,N). In summary, the comparative analyses of the subcellular localization of these full-length, truncated and chimeric HMGB proteins indicated that the basic N-terminal and the acidic C-terminal domains are involved in directing the nucleocytoplasmic distribution of the Arabidopsis HMGB proteins – possibly by intramolecular interaction.

Since no amino acid sequence motif(s) could be detected that may confer the different subcellular localization of HMGB1 and HMGB5 on one hand, and of HMGB2/3 and HMGB4 on the other hand, the contribution of post-translational modifications was considered. To date, the only post-translational modification that has been identified for plant HMGB proteins is the protein kinase CK2-mediated phosphorylation of Ser/Thr residues within the acidic C-terminal domains of maize HMGB proteins (Stemmer et al. 2002). When Arabidopsis HMGB proteins were tested for CK2-mediated phosphorylation, HMGB2/3 and HMGB4 were readily phosphorylated, while HMGB1 was phosphorylated only weakly (and not within the C-terminal region that does not contain Ser/Thr residues) and HMGB5 was not phosphorylated at all (Stemmer et al. 2003). Thus, the three HMGB proteins that in addition to the nucleus are found in the cytoplasm, are particularly good substrates for protein kinase CK2. In line with that, the C-terminal amino acid sequences of these proteins contain serine residues within CK2 consensus sequences (Pinna and Ruzzene 1996; Guerra et al. 1999) that resemble the sites known to be phosphorylated with maize HMGB proteins (Stemmer et al. 2002), while these sites do not occur in the C-terminal region of HMGB1 and HMGB5 (Fig. 5A). To test, whether the CK2-mediated phosphorylation of these C-terminal serine residues is involved in directing the subcellular localization of HMGB2, the three candidate serine residues (Ser120, Ser123, Ser137) were changed to alanines. Phosphorylation assays using purified recombinant maize CK2α and [γ-32P]ATP were performed with wild type HMGB2 and the S→A mutant version of HMGB2 lacking the three C-terminal serine residues. The substrate proteins were
reacted with CK2α for different times and as expected HMGB2 was vigorously phosphorylated by the enzyme (Fig. 5B). By contrast the S→A mutations abolished phosphorylation of HMGB2 by the protein kinase. The S→A mutant form of HMGB2 along with the corresponding phosphomimic mutant (S→D, the three Ser residues changed to Asp) fused to GFP were expressed in tobacco protoplasts and analyzed by CLSM in comparison to wild type GFP-HMGB2. The wild type and mutant HMGB2 forms showed a similar nucleocytoplasmic distribution, but in contrast to GFP-HMGB2 that often showed GFP fluorescence in the nucleolus (Fig. 5G,H), this effect was never seen with the mutant versions of GFP-HMGB2. In addition, the S→A and S→D mutant versions of HMGB2 displayed a more pronounced speckled pattern of fluorescence in the nucleus. Thus, CK2-mediated phosphorylation did not appear to influence the nucleocytoplasmic distribution of HMGB2, but it modulated the protein localization within the cell nucleus.

**HMGB2 and HMGB4 shuttle between nucleus and cytoplasm**

The occurrence of HMGB2/3 and HMGB4 in both nucleus and cytoplasm raised the possibility that these proteins can shuttle between the two locations. To test this possibility, HMGB2 and HMGB4 fused to photoactivatable GFP (paGFP) were expressed in tobacco protoplasts and examined by multifocal two-photon laser scanning microscopy (Martini et al. 2007). The dynamics of the diffusion of HMGB2 and HMGB4 fused to paGFP was recorded before (0 s) and at different times during and after activation of the paGFP in the nucleus (Fig. 6A-E and F-K, respectively). For both paGFP fusion proteins, over time a decrease of nuclear fluorescence was observed. In parallel, an increase of fluorescence was seen in the cytoplasm. This demonstrated that significant amounts of both HMGB2 and HMGB4 fused to paGFP can migrate from the nucleus to the cytoplasm within the analyzed period. When HMGB1 fused to paGFP was examined in parallel, no cytoplasmic fluorescence was observed (Fig. 6L-O), indicating that in line with previous experiments (Launholt et al. 2006) HMGB1 remained in the nucleus and could not migrate to the cytoplasm. To study the migration from cytoplasm to the nucleus, paGFP fused to HMGB2 and HMGB4 were activated in a small area of the cytoplasm (indicated by a rectangle) and the fluorescence was recorded over time (Fig. 6P-Y). This experiment showed that HMGB2 and HMGB4 first spread from the activation site in the cytoplasm, and consistent with their preferential nuclear localization in the following they accumulated in the cell nucleus. Monitoring the distribution of paGFP fused to HMGB2 and HMGB4 demonstrated that both proteins can shuttle between nucleus and cytoplasm, whereas HMGB1 remained nuclear localized.
DISCUSSION

Chromatin-associated HMGB proteins occur in a wide variety of eukaryotes and are generally considered nuclear proteins (Thomas and Travers 2001; Grasser et al. 2007; Reeves 2010), but already early studies have revealed that HMGB1 can be also detected in the cytoplasm of mammalian cells (Bustin and Neihart 1979; Rechsteiner and Kuehl 1979). However, only in recent years the extra-cellular presence of mammalian HMGB1 was linked with its specific (additional) function as a cytokine during injury or inflammation (Müller et al. 2004; Yang and Tracey 2010). Plant HMG proteins, which structurally differ from their mammalian counterparts (Stros et al. 2007), traditionally were purified from chromatin or isolated nuclei (Spiker 1984; Grasser et al. 1991). All HMGB proteins, whose subcellular localization was studied by immunofluorescence and/or by fluorescent microscopic analyses of GFP-tagged proteins in plant cells, were found in the cell nucleus. Among these nuclear HMGB proteins were Arabidopsis HMGB1, HMGB5 and HMGB6 (Grasser et al. 2004; Launholt et al. 2006). While the (long) N-terminal basic domain was sufficient for nuclear accumulation of HMGB1, in the case of HMGB5 both the N-terminal and the HMG-box domains were required for efficient nuclear targeting (Launholt et al. 2006). Here, we report that the Arabidopsis HMGB2 and HMGB4 proteins in addition to the nucleus can occur in the cytoplasm. Although the subcellular distribution of HMGB3 was assayed only in the protoplast system, due to its high degree of similarity to HMGB2 (89% amino acid sequence identity) it is likely that it behaves as HMGB2 that was studied in detail. Analysis of the amino acid sequences of the proteins did not reveal an explanation to the question of why some Arabidopsis HMGB proteins (HMGB1, HMGB5, HMGB6) are exclusively nuclear, while others (HMGB2/3, HMGB4) are found both in nucleus and cytoplasm. However, the examination of the subcellular localization of a variety of truncated and chimeric HMGB proteins (in comparison to the wild type proteins) provided some insight. HMGB2 and HMGB4 lacking the basic N-terminal domain were also found in the nucleus and in the cytoplasm, but showed a more prominent cytoplasmic localization, when compared to full-length HMGB2 and HMGB4. In line with this finding, deletion of the acidic tails of HMGB2 and HMGB4 resulted in proteins that were exclusively nuclear, and these proteins were also found in the nucleoli. This demonstrated that the acidic C-terminal domain is critical for the occurrence of HMGB2 and HMGB4 in the cytoplasm. The nuclear accumulation of the chimeric proteins, in which the natural N-terminal domain was replaced by the N-terminal domain of
HMGB1, showed that the strong NLS in the N-terminal domain of HMGB1 (Launholt et al. 2006) can “overrule” the effect of the acidic tails that in the natural context direct some of the protein to the cytoplasm. Replacement of the acidic tail of HMGB5 with those of HMGB2 or HMGB3 resulted in some cytoplasmic localization of HMGB5 (that normally is a nuclear protein), confirming the role the HMGB2/HMGB4 tails play in cytoplasmic localization. In addition, replacement of the acidic tail of HMGB2 and HMGB4 by the acidic C-terminal domain of HMGB5 abolished the cytoplasmic localization of HMGB2 and HMGB4. Thus, both the basic N-terminal and the acidic C-terminal domains of HMGB2 and HMGB4 are critical for the subcellular localization of the proteins. DNA-binding experiments suggested that the basic N-terminal and the acidic C-terminal domains of maize and rice HMGB1 interact to regulate the affinity for DNA (Ritt et al. 1998; Wu et al. 2003). An interaction of the two terminal domains was also observed in spectrometric and cross-linking experiments, which in addition revealed that the intramolecular interaction is modulated by CK2-mediated phosphorylation of the acidic tail in maize HMGB1 (Stemmer et al. 2002; Thomsen et al. 2004). Similarly, this intramolecular interaction of the terminal domains was observed for Arabidopsis HMGB1 and HMGB4 and appears to be a general feature of plant HMGB proteins (Thomsen et al. 2004). Therefore, the intramolecular interaction between the two terminal domains offers the most likely explanation for the observed subcellular distribution of the truncated and chimeric HMGB proteins in comparison to the wild type proteins. The C-terminal domains of HMGB2 and HMGB4 play a critical role for the proteins to localize to both nucleus and cytoplasm, since deleting the acidic tail or replacing it by the acidic tail of HMGB5, essentially abolished the cytoplasmic localization, rendering the proteins nuclear. However, in cases where the C-terminal domains of HMGB2 and HMGB4 occurred in conjunction with the basic N-terminal domain of HMGB1 (containing an efficient NLS) the chimeric proteins accumulated in the nucleus. In these proteins the acidic tail of HMGB2 and HMGB4 could not confer cytoplasmic localization. In conclusion, we propose that the basic N-terminal and acidic C-terminal domains of HMGB2 and HMGB4 need to be in a delicate balance of interaction. This interaction allows preferential nuclear accumulation of the proteins that most likely is mediated by nuclear targeting information both within the N-terminal and the HMG-box domains. This is similar to the situation with Arabidopsis HMGB5 (Launholt et al. 2006) and mammalian HMGB1 (Bonaldi et al. 2003), where the sites contributing to nuclear accumulation are scattered over larger regions of the proteins. At the same time this intramolecular interaction allows that (dependent on the cell type) some of the HMGB2 and HMGB4 proteins are present in the cytoplasm.
As observed in the *Arabidopsis* root, the partitioning of the HMGB2 and HMGB4 proteins into nucleus vs. cytoplasm appears to be cell type-dependent. In mammals, it was reported that the amount of HMGB1 (and HMGB2) found in the cytoplasm is elevated in tissues rich in differentiated cells, while preferential nuclear localization is characteristic of undifferentiated cells (Mosevitski et al. 1989). This is in line with the preferential nuclear localization of HMGB2 and HMGB4 in *Arabidopsis* root meristem cells, when compared to cortical cells. Nucleocytoplasmic partitioning of mammalian HMGB1 is regulated by acetylation of various lysine residues (Bonaldi et al. 2003), but mass spectrometric analyses did not reveal evidence for acetylation of maize HMGB proteins (Stemmer et al. 2002; Stemmer et al. 2003). The nuclear accumulation of insect HMGB proteins is reduced by protein kinase PKC mediated phosphorylation of the basic part of the protein (Wisniewski et al. 1994). For some plant HMGB proteins CK2 mediated phosphorylation of the acidic tail was demonstrated (Stemmer et al. 2002; Stemmer et al. 2003), but in case of HMGB2 mutation of the phosphorylation sites did not markedly affect the nucleocytoplasmic partitioning of the protein, although it modulated the distribution within the nucleus. Since the proportion of HMGB2 and HMGB4 found in the cytoplasm appears to differ between cell types, it is likely that the nuclear import/export rates are modulated, perhaps by a yet to be identified post-translational modification.

The puzzling question that remains is what is the biological role of the chromosomal HMGB2 and HMGB4 proteins that are found in the cytoplasm? The extranuclear/extracellular role of HMGB1 that acts as a specific mediator related to injury and inflammation in mammals (Müller et al. 2004; Yang and Tracey 2010) appears unlikely for plants. For the high levels of cytoplasmic HMGB1/HMGB2 associated with normal mammalian differentiated cell types an inverse correlation was found with increased levels of linker histone H1<sup>0</sup> in nuclei (Mosevitski et al. 1989). Based on the possibility that HMGB proteins and linker histones play a shared role in chromatin and that HMGB proteins confer a more open chromatin structure than H1 (Zlatanova and van Holde 1998), Mosevitski and coworkers proposed that the higher levels of nuclear HMGB proteins in undifferentiated cells reflect the more pronounced chromatin flexibility required for transcriptional dynamics during differentiation processes. Although this may explain the various levels of HMGB proteins in nuclei, it does not really explain the function of some chromosomal HMGB proteins in the cytoplasm of plant cells, while other HMGB types appear to be exclusively nuclear. Since
HMGB proteins are involved in plant responses to abiotic stress conditions (Pedersen and Grasser 2010), the presence of HMGB proteins in the cytosol may allow reacting rapidly on altered environmental conditions by modulating the nucleocytoplasmic distribution these proteins. Analysis of mutant plants lacking the various types of HMGB proteins may provide insight into the potentially different roles of the differentially localized HMGB types.

**MATERIALS AND METHODS**

**Plasmid constructions**

The sequences encoding the various full-length, truncated and chimeric HMG proteins were generated by amplifying the corresponding DNA fragments with *Pfu* DNA polymerase using an *Arabidopsis thaliana* cDNA library as template and the primers listed in Table S1. For expression of the mutant form of HMGB2, in which three Ser codons were replaced by Ala or Asp codons, the coding sequence was generated by overlap extension PCR (Ho et al. 1989). The PCR fragments were cloned into suitable plasmids as detailed below, and plasmid constructions were checked by DNA sequencing. For transient expression of GFP-HMGB fusions in protoplasts, the coding sequences were inserted into plasmids p5´GFP or p3´GFP (Haasen et al. 1999) (providing the expressed protein with an N- or C-terminal green fluorescent protein (GFP) fusion, respectively). For expression of GFP-HMG fusions in stably transformed *Arabidopsis* plants, the GFP-HMG coding sequences were inserted into plasmid pGII0179-35S (Launholt et al. 2006). For analysis of protein dynamics the HMGB coding sequences were inserted in photoactivatable (pa) GFP in plasmid p5´paGFP (Martini et al. 2007). For expression of the wild type and mutant forms of HMGB2 in *E. coli*, the coding sequences were inserted into plasmid pQE9cm (Grasser et al. 1996) providing an N-terminal 6×His-tag. Details of the plasmids generated in this work are summarized in Table S1.

**Production of recombinant HMGB2 proteins**

The *E. coli* M15 strain was transformed with the pQE9 expression vectors and the recombinant wild type and mutant HMGB2 proteins were purified by three-step column chromatography (metal-chelate, cation exchange, anion exchange) as previously described (Grasser et al. 1996). Purified proteins were checked by SDS-PAGE and MALDI-TOF mass spectrometry.
**CK2-mediated phosphorylation of HMGB2**

Recombinant wild type and mutant HMGB2 were phosphorylated *in vitro* using recombinant maize CK2α in the presence of [γ-32P]ATP as previously described (Stemmer et al. 2002). The samples were separated by SDS-PAGE in 18% SDS-polyacrylamide gels and phosphorylated proteins were visualized with a Cyclone storage phosphorimager (Canberra Packard).

**Immunoblot analysis**

An antiserum against purified recombinant *Arabidopsis* HMGB2 was produced by commercial immunization and tested as previously described (Launholt et al. 2006). The antiserum against *Arabidopsis* HMGB1 was previously described (Launholt et al. 2006; Lildballe et al. 2008). For immunoblot analyses, proteins were extracted from ~3 g of green tissue using 2% (w/v) trichloroacetic acid, as described previously (Grasser et al. 1996). The protein extracts were analyzed by SDS-PAGE and Coomassie staining to check the quality of the extracts. Immunodetection of the HMGB proteins was performed as previously described (Launholt et al. 2006).

**Indirect immunofluorescence**

Immunodetection of HMGB1 and HMGB2 in root tip cells of *Arabidopsis* seedlings was performed using HMGB1/HMGB2-specific antisera as previously described (Launholt et al. 2006).

**Plant growth and Agrobacterium-mediated transformation**

*Arabidopsis thaliana* (Col-0) and transgenic plant lines were grown in soil in a phytochamber at 22°C and 16h light per day as previously described (Lolas et al. 2010). The pGII0179 plasmids and the plasmid pSOUP (Hellens et al. 2000) were used to co-transform the *Agrobacterium tumefaciens* strain pGV3101 by electroporation. Transformed *Agrobacterium* cells were used to transform *Arabidopsis thaliana* plants of the ecotype Col-0 employing the floral dip method (Clough and Bent 1998). Plants growing on the selective medium were transferred to soil and isolated genomic DNA was tested by PCR for the presence of the transgene (Lolas et al. 2010).
Transient protoplast transformation assays with GFP fusion constructs
Protoplasts of dark-grown tobacco BY-2 cells were transiently transformed using PEG-mediated transformation as previously described (Merkle et al. 1996). Excitation of GFP was performed with a standard UV light source and fluorescein isothiocyanate filters. For CLSM, samples were directly examined under oil with a 63× objective and a DM RBE TCS4D microscope (Leica) equipped with an argon-krypton laser (excitation at 488 nm, beam splitter at 510 nm, filter at 515 nm) using Leica Scanware. Analysis of the localization of the different GFP fusion proteins was performed in three independent experiments, representing each approximately 60 - 80 transformed protoplasts.

Microscopic analysis of protein localization in Arabidopsis root
Transgenic seedlings expressing GFP-HMGA and –HMGB fusions were grown on MS medium (Murashige and Skoog 1962) at long day conditions at ~21°C in a plant incubator (Percival Scientific). Four-day old seedlings were fixed in fixing solution (4,5% (w/v) paraformaldehyde, 50 mM phosphate buffer pH 7.2, 150 mM NaCl) prior to analysis by CLSM using a 510 Meta instrument (Zeiss) with Zeiss LSM Image browser software using the argon laser (settings: pinhole 394 µm and filter BP 505-550).

Monitoring of intracellular protein dynamics using paGFP
The analysis of real time protein mobility in transiently transformed tobacco BY-2 protoplasts expressing HMGB proteins fused to paGFP using multifocal two-photon laser scanning microscopy was performed as previously described (Martini et al. 2007).

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Table 1. The plasmid constructions and primers used in this work.

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Figure Legends

Figure 1. Detection of *Arabidopsis* HMGB2 by immunoblotting and immunofluorescence. A, Coomassie stain of a protein extract of Col-0 seedlings and recombinant HMGB1 and HMGB2 proteins separated by SDS-PAGE in 18% polyacrylamide gels (left panel). Immunoblot analysis of Col-0 protein extract and recombinant HMGB1/HMGB2 using an HMGB1 antiserum (middle panel) and an HMGB2 antiserum (right panel). Comparable amounts of Col-0 extract and of the recombinant proteins were used for the two immunoblots. Migration positions of marker proteins are given. Due to the 6xHis-tag the recombinant HMGB1 and HMGB2 proteins displayed a slightly reduced electrophoretic mobility, when compared to the corresponding proteins of the Col-0 extract. B, Indirect immunofluorescence analysis of Col-0 root tip cells using HMGB1 (top panels) and HMGB2 (bottom panels) antisera. For comparison a DAPI stain is shown, staining the nuclear DNA (left panels). The immunofluorescence images (middle panels) and the merge with the DAPI stain (right panels) are shown. Dividing cells are indicated by arrows.

Figure 2. Detection of HMGB1 and HMGB5 in the nucleus, while HMGB2/3 and HMGB4 are observed both in nucleus and cytoplasm. Protoplasts prepared from BY-2 tobacco cell suspension cultures were transformed with plasmids driving the expression of the indicated GFP fusion proteins (see top panel for overview). The observed nucleocytoplasmic distribution is also summarized in the top panel (N, nuclear accumulation; N+C, localization in nucleus and cytoplasm; N>C, indicating the tendency of higher fluorescence in the nucleus relative to cytoplasm) based on inspecting 60-80 transformed protoplasts each. A, GFP-HMGB1, B, GFP-HMGB5, C, GFP-HMGB2, D, HMGB2-GFP, E, GFP-HMGB3, F, HMGB3-GFP, G, GFP-HMGB4, H, HMGB4-GFP. The left part of each panel represents an image of a protoplast at normal exposure, while the right part shows an overexposed image of the same protoplast (except in G, where instead of the overexposed image, the corresponding bright field image is shown). Fluorescence intensities in CLSM images of transformed protoplasts are displayed using a false colour palette. Low fluorescence signals are indicated by red colour, and progressively stronger signals are indicated by orange over yellow to white. Blue indicates top scale signals/over-exposure. The size bar equals 10 μm.
**Figure 3.** Nucleocytoplasmic distribution of HMGA, HMGB5, HMGB2 and HMGB4 fused to GFP in stably transformed *Arabidopsis* seedlings. The analyzed plants expressed GFP-HMGA (A-C), GFP-HMGB5 (D-F), GFP-HMGB2 (G-I) and GFP-HMGB4 (J-L). Detection of GFP fluorescence by CLSM of roots tips of 3-day old transgenic seedlings, expressing the indicated GFP constructs. The left images each show the GFP fluorescence and the right panels each show an overlay with the corresponding bright field image. The top images show overviews of the intact root tips, while close-up views of squeeze-preps of meristematic cells (middle images) and of cortical cells (bottom images) are shown. The scale bars represent 10 or 50 µm.

**Figure 4.** Nucleocytoplasmic distribution of truncated and chimeric HMGB proteins in comparison to full-length proteins. BY-2 protoplasts were transformed with plasmids driving the expression of the indicated GFP fusion proteins (see top panel for overview). For truncated and chimeric HMGB proteins the origin of the domains (incl. amino acid positions) is represented in the scheme. The observed nucleocytoplasmic distribution is also summarized in the top panel (N, nuclear accumulation; N+C, localization in nucleus and cytoplasm; N>C, indicating the tendency of higher fluorescence in the nucleus relative to cytoplasm) based on inspecting 60-80 transformed protoplasts each. A-N, representative images of the GFP fluorescence of the indicated GFP-HMGB proteins. Fluorescence intensities in CLSM images are displayed using a false colour palette (cf. Fig. 2). The size bar equals 10 µm.

**Figure 5.** Role of CK2-mediated phosphorylation on the subcellular localization of HMGB2. A, Alignment of the acidic C-terminal domains of *Arabidopsis* HMGB proteins in comparison to maize HMGB1. The serine residues known to be phosphorylated by CK2 in maize HMGB1 (Stemmer et al. 2002) are indicated by arrows. These sites are essentially conserved in *Arabidopsis* HMGB2/3 and HMGB4, but not in HMGB1 and HMGB5. B, Phosphorylation assays using maize CK2α and radiolabelled ATP. Phosphorylation reactions without addition of substrate protein, or using HMGB2 or mutated (S→A) HMGB2 (in which the three serine residues within the acidic tail were changed to alanines) as substrate proteins. The phosphorylation reactions were terminated at the indicated times, separated by SDS-PAGE in 18% polyacrylamide gels and analyzed using a phosphorimager. The migration positions of marker proteins are given as well as the migration of HMGB2. C-H, subcellular localization of wild type and mutant HMGB2. BY-2 protoplasts were transformed with plasmids driving the expression of the indicated GFP fusion proteins. The above-
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**Figure 6.** HMGB2 and HMGB4 show re-localization from the nucleus to the cytoplasm while HMGB1 remains nuclear. Up to five selected fluorescence images of BY-2 protoplasts are shown that expressed paGFP-HMGB2 (A-E), paGFP-HMGB4 (F-K), or paGFP-HMGB1 (L-N). O shows a bright-field image of the protoplast shown in (L-N). Pictures were taken before (0 s), during (50 s to 250 s), and after (300 s) photoactivation of paGFP in the nuclei at the time points indicated. The regions of interest (ROI), where photoactivation was performed in (A-N), were located within the nuclei that are indicated by dashed lines. Before photoactivation, the average fluorescence intensity was barely detectable. Shortly after onset of photoactivation (performed with a two photon LSM during 250 s using fs laser bursts (covering an area of 7x8 µm with 64 parallel laser foci, at 4 mW at 800 nm per focus), strong fluorescent signals were detected and the dynamics of photoactivated paGFP was monitored continuously in each case. Fluorescence intensity panels and scale bars are shown to the left of each row. In P-Y, photoactivation was performed in the cytoplasm. Five selected fluorescence images of BY-2 protoplasts are shown that expressed paGFP-HMGB2 (P-T) or paGFP-HMGB4 (U-Y). The ROI is indicated by a dashed line, the nucleus is indicated by a dotted line.
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Fig. 3, Pedersen et al.
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