Molecular and reverse genetic characterization of NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1) genes unravels their function in transcription and nucleotide excision repair in Arabidopsis thaliana

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SUMMARY

Compared with the well-studied biochemical function of NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1) as a histone chaperone in nucleosome assembly/disassembly, the physiological roles of NAP1 remain largely uncharacterized. Here, we define the NAP1 gene family members in Arabidopsis, examine their molecular properties, and use reverse genetics to characterize their biological roles. We show that the four AtNAP1-group proteins can form homodimers and heterodimers, can bind histone H2A, and are localized abundantly in the cytoplasm and weakly in the nucleus at steady state. AtNAP1;4 differs from the others by showing inhibitor-sensitive nucleocytoplasmic shuttling and tissue-specific expression, restricted to root segments and pollen grains. The other three AtNAP1 genes are ubiquitously expressed in plants and the AtNAP1;3 protein is detected as the major isoform in seedlings. We show that disruption of the AtNAP1-group genes does not affect normal plant growth under our laboratory conditions. Interestingly, two allelic triple mutants, Atnap1;1-1 Atnap1;2-1 Atnap1;3-1 and Atnap1;1-1 Atnap1;2-1 Atnap1;3-2, exhibit perturbed genome transcription, and show hypersensitivity to DNA damage caused by UV-C irradiation. We show that AtNAP1;3 binds chromatin, with enrichment at some genes involved in the nucleotide excision repair (NER) pathway, and that the expression of these genes is downregulated in the triple mutants. Taken together, our results highlight conserved and isoform-specific properties of AtNAP1 proteins, and unravel their function in the NER pathway of DNA damage repair.

Keywords: histone chaperone, chromatin, epigenetics, DNA repair, genotoxic stress, Arabidopsis.

INTRODUCTION

In plants, as in other eukaryotes, the basic structural unit of chromatin is the nucleosome. It consists of 146 base pairs (bp) of DNA wrapped around a histone octamer that contains two molecules each of H2A, H2B, H3 and H4 (Luger et al., 1997). The octamer is structured by the H3/H4 tetramer at the center, and the two H2A/H2B dimers attached symmetrically on either side. The H3/H4 tetramer organizes the central portion of nucleosomal DNA, whereas each H2A/H2B dimer contacts about 30 bp on either side. Dynamic processes of nucleosome assembly, disassembly and reassembly occur during transcription, DNA replication, repair and recombination. Histone chaperones are involved in both the deposition of histones during nucleosome assembly and the eviction of histones during nucleosome disassembly. Histone chaperones are also thought to play roles in histone transfer to enzymes using histones as a substrate, in the storage of histone pools during development and in buffering transient histone overload (De Koning et al., 2007).

NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1) represents the primary chaperone of histones H2A and H2B, and is...
with at least AtNAP1;4 being able to shuttle between the cytoplasm and the nucleus. The AtNAP1;4 gene is tissue-specifically expressed in root segments and pollen grains, which contrasts with the ubiquitous expression pattern of the other three AtNAP1 genes. Western blot analysis revealed that AtNAP1;3 is the most abundant isoform of AtNAP1 proteins in young plants. Subcellular fractionation showed that most of AtNAP1;3 is present in the cytoplasm, with only a small fraction being found in the nucleus. To investigate the functions of the three ubiquitously expressed AtNAP1 genes, we obtained loss-of-function mutants of these genes, as well as double and triple mutants. Under our laboratory standard growth conditions, all these mutants showed a wild-type phenotype. Transcriptome analysis revealed that 402 genes are downregulated and 86 genes are upregulated to more than twofold in both Atnap1;1-1 Atnap1;2-1 Atnap1;3-1 and Atnap1;1-1 Atnap1;2-1 Atnap1;3-2 triple mutants, compared with wild-type seedlings. Physiological phenotype tests revealed that the triple mutant seedlings have a decreased capacity of recovery after exposure to UV-C irradiation. Consistently, several genes involved in the nucleotide excision repair (NER) pathway, including AtCEN1 (At3g50360), AtCEN2 (At4g37010), AtXPC (At5g41370 and At5g41360) and AtXPC (At5g16630) (Costa et al., 2001; Molinier et al., 2004; Kunz et al., 2005; Morgante et al., 2005; Liang et al., 2006), are downregulated in the triple mutant seedlings, and a reduced efficiency of the in vitro repair of UV-induced DNA lesions was observed with protein extracts of the triple mutants, compared with wild-type plants. Chromatin immunoprecipitation (ChIP) analyses revealed that AtNAP1;3 binds chromatin with enrichment at AtCEN1, AtCEN2 and AtXPC, compared with ACTIN. Taken together, our results provide important information on AtNAP1 genes, and unravel an essential role for AtNAP1 in the NER pathway.

RESULTS
AtNAP1 proteins can self-bind and bind between each other, but not with NRP1

AtNAP1;1, AtNAP1;2 and AtNAP1;3 share 74–83% identity with each other, and 41–43% identity with AtNAP1;4, but only share <23% identity with NRP1 and NRP2 (for sequence alignments, see Figure S1). The functional NAP1 exists as a stable dimer (Park and Luger, 2006b), and previous studies using pull-down assays revealed that the tobacco proteins Nicta;NAP1;3 and Nicta;NAP1;4 self-bind and bind between each other (Dong et al., 2005). The Arabidopsis proteins NRP1 and NRP2 also self-bind and bind between each other (Zhu et al., 2006). We first investigated direct protein–protein interactions among AtNAP1 proteins using the yeast two-hybrid assay. As shown in Figure 1(a), AtNAP1;1, AtNAP1;2 and AtNAP1;3 can self-bind and bind between each other. It is interesting to note that in spite of its more
diverged sequence, AtNAP1;4 shows a higher binding activity with AtNAP1;1, AtNAP1;2 and AtNAP1;3. As expected for controls, the transcriptional factors ASYMMETRIC LEAVES1 (AS1) and AS2 show a positive binding activity, as has been previously reported by Xu et al. (2003), and two empty vectors or combinations of one empty vector with one AtNAP1-expressing vector (excepting AtNAP1;4) do not show positive results. Because of its autoactivation when cloned in the pGBK7 vector, the self-binding activity of AtNAP1;4 could not be examined in the yeast two-hybrid assay. We further investigated protein–protein interactions in pull-down assays. Total protein extracts from plants expressing YFP-AtNAP1;2, YFP-AtNAP1;3 or YFP-AtNAP1;4 were precipitated by beads coated with 6xHis-AtNAP1;1, 6xHis-AtNAP1;2, 6xHis-AtNAP1;3, 6xHis-AtNAP1;4 or 6xHis-NRP1. As shown in Figure 1(b), YFP-AtNAP1;2, YFP-AtNAP1;3 and YFP-AtNAP1;4 bind 6xHis-AtNAP1;1, 6xHis-AtNAP1;2, 6xHis-AtNAP1;3 and 6xHis-AtNAP1;4, but not 6xHis-NRP1. H2A-YFP was found to bind 6xHis-fused AtNAP1 proteins as well as NRP1 (Figure 1b). Taken together, these results indicate that different AtNAP1 isoforms can form homodimers and heterodimers, but not with NRP1.

**AtNAP1 proteins are abundantly localized in the cytoplasm**

We investigated the subcellular localization of AtNAP1 proteins in living cells by using YFP as a marker of visualization. YFP alone is small enough to diffuse across the nuclear envelope, and is localized in the cytoplasm and the nucleus of transgenic Arabidopsis and tobacco BY2 cells (not shown). YFP-AtNAP1;1, YFP-AtNAP1;2, YFP-AtNAP1;3 and YFP-AtNAP1;4 were all found to be localized primarily in the cytoplasm of transgenic Arabidopsis (Figure 2a–d) and BY2 cells (Figure 2e–h). Treatment with the nuclear export inhibitor leptomycin B (LMB) revealed that YFP-AtNAP1;4 (Figure 2i), but not the other AtNAP1 isoforms (data not shown), shuttles between the cytoplasm and the nucleus in transgenic tobacco BY2 cells.

**AtNAP1;3 is the major isoform present in seedlings, and a small fraction of AtNAP1 proteins can be detected in the nucleus**

In order to facilitate the analysis of endogenous AtNAP1 proteins, we searched for antibodies recognizing AtNAP1 proteins. The antibody produced earlier against Nicta-NAP1;4 (Dong et al., 2005) recognized the recombinant proteins AtNAP1;1, AtNAP1;2 and AtNAP1;3 with similar affinities, but did not cross-react with AtNAP1;4, NRP1 or NRP2 (Figure 3a). Western blot analyses on total protein extracts of 9-day-old Arabidopsis seedlings revealed that AtNAP1;1, AtNAP1;2 and AtNAP1;3 are present at this growth stage (Figure 3b). Comparison of wild-type with mutants (see later for mutant descriptions) indicates that AtNAP1;3 migrates at a higher molecular-weight position, and is present at a much more abundant level in the wild-type extract compared with AtNAP1;1 and AtNAP1;2 (Figure 3b). Both reverse transcription-polymerase chain reaction (RT-PCR) (Zhu et al., 2006) and northern analyses

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**Figure 1.** Protein–protein interactions.

(a) Yeast two-hybrid assay. Construct combinations of a pGADT7-based and a pGBK7-based expression vector were co-transformed into the yeast strain AH109. A dilution \( \times 10 \) series of yeast cells were plated onto media lacking leucine and tryptophan (−LT), or onto media lacking leucine, tryptophan and adenine (−LTA). The growth of yeast cells on −LTA plates indicates positive protein–protein interactions.

(b) Pull-down assay. Total protein extracts of transgenic Arabidopsis plants expressing YFP-AtNAP1;2, YFP-AtNAP1;3, YFP-AtNAP1;4 or H2A-YFP were incubated with beads coated with 6xHis-AtNAP1;1, 6xHis-AtNAP1;2, 6xHis-AtNAP1;3, 6xHis-AtNAP1;4 or 6xHis-NRP1, and the pull-down fractions were analyzed by Western blot using a polyclonal anti-GFP antibody (which cross-reacts with YFP). The input was loaded as the control.
(not shown) indicate that although the AtNAP1;4 transcript level is barely detectable (see below for the specific expression of AtNAP1;4), the transcript levels of AtNAP1;1, AtNAP1;2 and AtNAP1;3 are similarly high. It is thus likely that a post-transcriptional mechanism differentially regulates the protein synthesis and/or stability of AtNAP1;3, compared with AtNAP1;1 and AtNAP1;2.

We further examined the cytoplasmic and nuclear distribution of AtNAP1 proteins by subcellular fractionation. As shown in Figure 3(c), the AtNAP1 proteins (mostly AtNAP1;3) are found in the total and cytoplasmic fractions, and are also found in the nuclear fraction, but at a much lower level. The controls, dimethyl-histone H4 lysine 4 (H3K4m2) as a nuclear marker and tubulin as a cytoplasmic marker, yield the expected results (Figure 3c), thereby validating our fractionation. These results are consistent with previous observations on the predominant cytoplasmic localization of AtNAP1 proteins, and provide additional information by showing that small quantities of AtNAP1 proteins are present in the nucleus. The detection of AtNAP1 proteins in the nucleus confirms their proposed histone chaperone activities in nucleosome assembly/disassembly.

**AtNAP1;4 is tissue-specifically expressed in root segments and pollen grains**

Previous RT-PCR analysis revealed that AtNAP1;1, AtNAP1;2 and AtNAP1;3 genes are ubiquitously expressed in all material examined, including seedlings, roots, stems and young flower buds, whereas the expression of AtNAP1;4 was undetectable using up to 30 cycles of amplification (Zhu et al., 2006). To test the possibility that AtNAP1;4 is expressed in a low number of cells of specific types, we fused the β-glucuronidase (GUS) cDNA to the genomic clone of AtNAP1;4 (Figure 4a). The clone contained the exons and
introns, as well as 619 bp of the upstream sequences from the predicted translational start site of the AtNAP1;4 gene. Consistent with the RT-PCR study, AtNAP1;4::AtNAP1;4-GUS activity was undetectable in most plant tissues examined, except the root segment covering the apical end of the differentiation zone, the elongation zone of the root (Figure 4b) and the mature pollen within the anthers of open

Figure 3. Western blot analyses of AtNAP1 proteins.
(a) Antibody quality test: 0.1-μg portions of recombinant proteins 6xHis-AtNAP1;1, 6xHis-AtNAP1;2, 6xHis-AtNAP1;3, 6xHis-AtNAP1;4, 6xHis-NRP1 and 6xHis-NRP2 were analyzed. The upper panel shows Coomassie staining of the gel, and the lower panel shows Western blot results using the antibody initially raised against Nicta;NAP1;4. Note that the antibody cross-reacts with AtNAP1;1, AtNAP1;2 and AtNAP1;3, but not with AtNAP1;4, NRP1 and NRP2.
(b) Detection of AtNAP1 proteins in wild-type (Col) and mutant plants. A total of 10 μg of total protein extracts from 9-day-old seedlings were analyzed using the same antibody as described in (a); the solid arrowhead indicates the position of AtNAP1;3; the open arrowhead indicates the position of AtNAP1;1 and AtNAP1;2, which migrate closely together; and the asterisk indicates an unknown protein that shows a weak unspecific signal that is found in all samples.
(c) Subcellular fractionation of 9-day-old wild-type (Col) and the m123-1 mutant seedlings: 10 μg of total proteins and the corresponding equivalent cytoplasmic and nuclear fractions were analyzed. AtNAP1 was detected using the antibody described above. H3K4m2, specifically present in the nucleus, and tubulin, specifically present in the cytoplasm, serve as fractionation quality controls.

Figure 4. Tissue-specific expression of AtNAP1;4.
(a) Gene structures of AtNAP1;4::AtNAP1;4-GUS. The gray box with white arrow represents the promoter, dark boxes represent exons, lines represent introns, and the last exon is fused in frame with the reporter GUS, as indicated.
(b–d) Histochemical analysis of AtNAP1;4::AtNAP1;4-GUS expression in a 5-day-old seedling (b), in a flower (c) and in an anther (d).
flowers (Figure 4c,d). This tissue-specific expression of AtNAP1;4::AtNAP1;4-GUS was reproducibly observed in eight independent transgenic lines. Re-examination by RT-PCR revealed that AtNAP1;4 transcripts were detectable from wild-type inflorescences using 41 cycles of amplification (data not shown).

Production and characterization of the loss-of-function of Atnap1 mutants

To investigate the function of AtNAP1, we obtained T-DNA insertion lines from the Arabidopsis Biological Resource Center (ABRC, http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). One T-DNA insertion mutant allele each was identified for AtNAP1;1, AtNAP1;2 and AtNAP1;4, and two independent mutant alleles were identified for AtNAP1;3 (Figure 5a). In all of these insertion lines, the T-DNA segregated as a single locus. Homozygous (hereafter called mutant) plants were obtained for each of these T-DNA insertion lines by self-pollination. For convenience, the mutants Atnap1;1-1, Atnap1;2-1, Atnap1;3-1, Atnap1;3-2 and Atnap1;4 were named m1, m2, m3-1, m3-2 and m4, respectively. RT-PCR analysis revealed that the full-length transcripts of the corresponding genes were undetectable in the mutant plants (Figure 5b), indicating that T-DNA insertions caused the loss of function of the AtNAP1 genes. None of the mutants showed detectable growth modifications. The increased growth of leaves previously reported for Atnap1;1-1 (Galichet and Gruissem, 2006) was not reproduced under our in vitro culture and glasshouse growth conditions. Considering their overlapping expression pattern and their high conservation of protein sequences and properties, it is likely that AtNAP1 genes have redundant functions. Because AtNAP1;4 differs from the other three AtNAP1 genes and shows a tissue-specific expression, we subsequently focused our work on m1, m2, m3-1 and m3-2 to obtain double and triple mutants to investigate overlapping functions. Through crosses between different mutants, selfing segregation and PCR genotyping (see Figure S2), we obtained the double mutants Atnap1;1-1 Atnap1;2-1 (m12), Atnap1;1-1 Atnap1;3-1 (m13-1), Atnap1;1-1 Atnap1;3-2 (m13-2), Atnap1;2-1 Atnap1;3-1 (m23-1) and Atnap1;2-1 Atnap1;3-2 (m23-2), as well as the triple mutants Atnap1;1-1 Atnap1;2-1 Atnap1;3-1 (m123-1) and Atnap1;1-1 Atnap1;2-1 Atnap1;3-2 (m123-2). Remarkably, all these double and triple mutants show a wild-type phenotype (Figure 5c for m123-1; data not shown for other mutants). RT-PCR analyses confirmed that the corresponding AtNAP1 genes were knocked down in the mutants (Figure 5b). Western blot analysis also confirmed that the corresponding AtNAP1 proteins were undetectable in the mutants (Figure 3b). In addition, we noticed that AtNAP1;3 levels were elevated in m1, m2 and m12, and that AtNAP1;1/AtNAP1;2 levels were elevated in m3-1 compared with wild-type seedlings (Figure 3b), suggesting a homeostatic compensation mechanism. Nonetheless, AtNAP1;4 transcripts were undetectable in the seedlings of all mutants (including m123-1 and m123-2) and the wild-type by RT-PCR, using up to 41 cycles of amplification. This last observation did not support the assumption that AtNAP1;4 could be ectopically expressed, which would compensate for the loss of function of other AtNAP1 proteins in the mutants.

Perturbation of gene expression in Atnap1 mutants

Plants with loss-of-function of AtNAP1 genes grow in a macroscopically similar manner to wild-type plants under our laboratory in vitro culture and glasshouse growth conditions.
et al. (2006), is downregulated by more than twofold in the AtXPD genes examined, including fold in the shown), and also revealed a downregulation of about 1.5-fold in the 123-1 mutant compared with the wild type (Figure 6b). The NER pathway gene AtXPC (Kunz et al., 2005) was among the downregulated genes found in the m123-2 mutant, but not in the m123-1 mutant (Table S1). RT-PCR confirmed the downregulation of AtXPC in the m123-2 mutant (not shown), and also revealed a downregulation of about 1.5-fold in the m123-1 mutant (Figure 6b). In contrast, the other genes examined, including AtXPD, AtXPB, AtXPG and AtRAD23-1, which are potentially involved in the NER pathway (Kunz et al., 2005), as well as AtRAD51, AtRAD54 and AtATM, which are involved in the double-strand break (DSB) DNA repair pathway (Garcia et al., 2003; Li et al., 2004; Osakabe et al., 2006), did not show major changes of expression in the mutant (Figure 6b). This is also consistent with transcriptome data. Analysis of gene expression in response to UV treatment revealed that expression of AtCEN1, AtCEN2 and to a lesser degree AtXPB is induced by UV in both the wild type and the m123-1 mutant, albeit at a weaker level in the mutant (Figure 6c). It indicates that AtNAP1 proteins are dispensable for initiation, but have an expression enhancer role for these genes.

**AtNAP1 triple mutants show defects in UV-damage repair**

The downregulation of some genes involved in the NER pathway prompted us to investigate the physiological phenotype of AtNAP1 triple mutants in response to DNA damages. We tested plant growth in the presence of bleomycin and hydroxyurea, inhibitors of the DSB repair pathway and DNA synthesis, respectively. No significant difference was observed between m123-1 and wild-type plants in response to these inhibitors (data not shown). Interestingly, compared with the wild type, both m123-1 and m123-2 triple mutants exhibit a more pronounced reduction in growth and pale-green phenotype in response to UV-C damage (Figure 7a). The recovery rates of m123-1 and m123-2 were much lower than that of the wild type after UV-C treatment (Figure 7b).
We further investigated the efficiency of DNA repair by cell extracts of wild-type, \textit{m123-1} and \textit{m123-2} mutant plants using a previously described \textit{in vitro} assay (Li \textit{et al.}, 2002). In this assay, the DNA repair efficiency is evaluated by incorporation of DIG-labeled dUTP in a UV-damaged plasmid DNA. As shown in Figure 7c, cell extracts prepared from the mutant plants show a less efficient DNA repair efficiency than that from the wild-type plants (Figure 7c). Taken together, our data indicate that \textit{AtNAP1} genes play important roles in the NER pathway, which is involved in the repair of DNA lesions caused by UV-C.

Using ChIP assays, we investigated the chromatin-binding activity of \textit{AtNAP1;3} at DNA repair genes. ChIP fractions from the \textit{m12} and \textit{m123-1} mutant plants were analyzed by quantitative PCR using gene-specific primers (see Table S2 for primer information). The values were normalized by input so as to minimize the variation introduced by the variable efficiency of the primers, then the values obtained in \textit{m12} (in which \textit{AtNAP1;3} is present) were subtracted from those obtained in \textit{m123-2} (in which \textit{AtNAP1;3} is absent) so as to enhance the specificity of detection of \textit{AtNAP1;3}, and finally the relative binding activity at different regions within the promoters (\textit{P}) and exons (\textit{E}) of the genes were referenced against that at \textit{AtACT2-P}, which is given a value of 1 (Figure 7d). The binding activity of \textit{AtNAP1;3} at \textit{AtCEN1-E}, \textit{AtRAD51}, \textit{AtRAD54} and \textit{AtATM} was found to be similar to that at \textit{AtACT2}, whereas the binding activities at \textit{AtCEN1-P}, \textit{AtCEN2} and \textit{AtXPB1} were about 1.3–1.9-fold higher. This higher binding activity correlates with the downregulation of \textit{AtCEN1}, \textit{AtCEN2} and \textit{AtXPB1} found in the triple mutants \textit{m123-1} and \textit{m123-2}, thus suggesting that \textit{AtNAP1} proteins are positive regulators of expression of these NER-pathway genes.

**DISCUSSION**

\textit{Arabidopsis} has four \textit{NAP1}-group genes and two \textit{NRP} genes. Although \textit{AtNAP1} and \textit{NRP} proteins can all interact with H2A/H2B, our study reveals differences between the \textit{AtNAP1} and \textit{NRP} groups of proteins at molecular, cellular and functional levels. \textit{NRP1} and \textit{NRP2} were previously reported to form homodimeric and heterodimeric com-
plexes (Zhu et al., 2006). Here, we show that the four AtNAP1 proteins can form homodimeric and heterodimeric complexes, but they cannot bind NRP1, suggesting that the AtNAP1 and NRP proteins form group-specific complexes. Whereas YFP-fused proteins of NRP1 and NRP2 are localized in the nucleus (Zhu et al., 2006), YFP-fused AtNAP1 proteins are primarily localized in the cytoplasm. YFP-AtNAP1:4 can shuttle between the nucleus and the cytoplasm, and a small fraction of AtNAP1:3 was found in the nuclear fraction. It appears that mechanisms exist to avoid overload of AtNAP1 proteins in the nucleus: an overload might interfere with nucleosome dynamics, causing genome dysfunction. Whereas AtNAP1:4 shows specific expression in pollen and root segments, the other three AtNAP1 genes are abundantly expressed in all plant organs examined. Furthermore, post-transcriptional mechanism(s) differentially regulate the product levels of the latter genes in such a way that AtNAP1:3 becomes the major isoform of AtNAP1 proteins. Finally, an analysis of Atnap1 mutant plants revealed the compensatory homeostatic regulation of AtNAP1 protein levels. Together, these observations support the idea that the nature of the multigene family provides AtNAP1 with more flexibility to cope with the developmental and growth adaptation requirements of plants.

Although the functional redundancy of different AtNAP1 genes could explain the wild-type phenotype of single and double mutants, before our study the triple mutants, m123-1 and m123-2, were not predicted to exhibit a normal growth phenotype. Our findings indicate that AtNAP1 is dispensable for plant growth, and suggests that in the absence of AtNAP1, H2A/H2B might be chaperoned by other type(s) of factor(s). The first candidates for such factors are NRP1 and NRP2, which were previously characterized as H2A/H2B chaperones (Zhu et al., 2006). Otherwise, FACILITATES CHROMATIN TRANSCRIPTION (FACT) and NUCLEOLIN could also fulfill H2A/H2B chaperone activities in Arabidopsis. Similar to those in AtNAP1 and NRP proteins, long acidic stretches are found in the Spt16 subunit of FACT (Duroux et al., 2004) and in NUCLEOLIN (Kojima et al., 2007; Petricka and Nelson, 2007; Pontvianne et al., 2007). The human FACT possesses intrinsic histone H2A/H2B chaperone activity, which depends on the acidic region of Spt16 (Belotserkovskaya et al., 2003). It has been shown that the acidic region of human NUCLEOLIN is required for its chaperone activity of H2A/H2B (Angelov et al., 2006). Furthermore, the chromatin-binding properties of the Arabidopsis FACT (Duroux et al., 2004) and chromatin disorganization observed in the Atnuc-L1 mutant (Pontvianne et al., 2007) are consistent with the proposed histone chaperone activity of FACT and NUCLEOLIN. We hypothesize that these distinct types of H2A/H2B chaperones (AtNAP1, NRP s, FACT and NUCLEOLIN) could be used to selectively modulate the dynamics of specific chromatin domains, and to provide overlapping functions to safeguard genome activity.

In spite of their normal growth phenotype, m123-1 and m123-2 mutants show perturbed gene expression. The perturbed genes include some genes that are potentially involved in DNA damage repair, protein phosphorylation/dephosphorylation and hormone responses (see Table S1), which suggests substantial roles for AtNAP1 in physiological processes. In this study, we focused on DNA damage repair, and showed that AtNAP1 is involved in the NER pathway.

The NER pathway is crucial, and contributes to global genome repair to eliminate most DNA lesions, particularly photochemical lesions such as the cyclobutane pyrimidine dimer and the pyrimidine (6–4) pyrimidine dimer (Sancar, 1996). In our study, cell extracts prepared from m123-1 and m123-2 mutant plants show less efficiency in the repair of UV-induced DNA lesions, compared with extracts from wild-type plants. In addition, both mutants showed lower recovery rates of plant growth after UV-C damage, compared with the wild type. Several genes involved in the NER pathway, including AtCEN1, AtCEN2, AtXPB and AtXPC, were found to be downregulated in both m123-1 and m123-2 mutants. AtCEN1 and AtCEN2 proteins are 65% identical, and both belong to the CENTRIN family of EF-hand calcium binding proteins. They are involved in microtubule organization and DNA repair (Molinier et al., 2004; Liang et al., 2006; Azimzadeh et al., 2008). The human CENTRIN2 (HsCEN2) is considered to act at the initial step in the recognition of DNA lesions, and the human XPC–XPB complex acts at a second step as a helicase in unwinding the DNA encompassing the lesion (Kunz et al., 2005). AtCEN2 is highly similar to HsCEN2 in sequence and in domain organization (Molinier et al., 2004), and forms a protein complex in Arabidopsis similar to that in humans (Liang et al., 2006). Arabidopsis harbors a duplication of the XPB ortholog, AtXPB1 and AtXPB2, and the proteins encoded by the duplicated genes show 95% amino acid identity (Costa et al., 2001; Morgante et al., 2005). Our gene expression analysis covers both AtXPB1 and AtXPB2. Consistent with functioning in the NER pathway, the AtCEN2 protein was shown to act in DNA repair both in vitro and in vivo (Molinier et al., 2004; Liang et al., 2006), and the knock-down of either AtCEN2 (Molinier et al., 2004) or AtXPB1 (Costa et al., 2001) resulted in an increased sensitivity of plants to DNA damage. Our data corroborate the previous studies on the role of AtCEN2 and AtXPB in DNA repair, and suggest that the downregulation of these NER-pathway genes caused the UV-sensitive mutant phenotypes in m123-1 and m123-2. AtNAP1;3 binds chromatin with enrichment at AtXPB, AtCEN1 and AtCEN2, indicating that AtNAP1 proteins may function as transcriptional activators of these NER-pathway genes. Histone chaperones could also be directly involved in the displacement or exchange of histones occurring during the process of NER (Dinant et al., 2008). Such a role for NAP1-family proteins is, however, currently unknown.
In conclusion, we have shown that the four AtNAP1-group proteins have both conserved as well as isoform-specific properties. Our reverse-genetic approach unequivocally showed that AtNAP1-deficient plants can grow normally under laboratory conditions, but become hypersensitive to DNA damage caused by UV irradiation. We propose that AtNAP1 may function as a modulator of genome transcription in plant responses to environmental stress.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All A. thaliana alleles were derived from the Columbia ecotype. Atnap1;1, Atnap1;2, Atnap1;3 and Atnap1;4 alleles were obtained from SALK_013610, SALK_131746, SAIL_373_H11, SAIL_84_B01 and BX290796 of T-DNA insertion lines from the ABRC. Different combinations of double and triple mutants were obtained in our laboratory by genetic crossing (see Figure S1). In vitro plant culture was performed on agar-solidified MS medium obtained in our laboratory by genetic crossing (see Figure S1). The resulting resin was used in the pull-down assays, according to a previously described protocol (Dong et al., 2005). Total protein extracts from about 10 g of 2-week-old transgenic Arabidopsis plants expressing YFP-AtNAP1;2, YFP-AtNAP1;3, YFP-AtNAP1;4 or H2A-YFP were each distributed equally to the aforementioned five recombinant protein-coated resins. A quarter of each pull-down fraction was used for Western blot analysis.

Western blot

Proteins were separated by SDS-PAGE, and transferred to an Immobilon-P polyvinylidene difluoride transfer membrane (Millipore, http://www.millipore.com). The membrane was blotted with specific antibodies. The antibodies used in this study are: the anti-GFP rabbit polyclonal antibody (Molecular Probes, now part of Invitrogen, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) at a dilution of 1:5000, the anti-Nicta; NAP1;4 mice polyclonal antibodies (Dong et al., 2005) at a dilution of 1:2000, the anti-dimethyl H3K4 (cat. no. 07-030; Millipore) at a dilution of 1:2000, the anti-α-tubulin monoclonal antibody (cat. no. T6199; Sigma-Aldrich, http://www.sigmaaldrich.com) at a dilution of 1:10 000.

Microscopy

The epifluorescence and differential interference contrast images were taken using a confocal laser scanning microscope (model LSM510; Zeiss, http://www.zeiss.com).

Subcellular protein fractionation

For subcellular fractionation, about 2 g of 9-day-old seedlings were infiltrated with extraction buffer [400 mM sucrose, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 5.3, 10 mM NaCl, 5 mM MgCl2, 5 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM DTT and 1× complete protease inhibitor] and ground in 9 ml of the same buffer. After passage through a 100-μm filter, the lysate was layered on top of a discontinuous gradient consisting of successive 4-ml layers of iodixanol 15% and 45% in the extraction buffer. The discontinuous gradient was centrifuged at 1500 for 15 min at 4°C. The cytoplasmic fraction was obtained from the top of the 15% iodixanol layer, and the nuclei fraction was obtained from the 15/45% iodixanol interface. The pure nuclei were washed three times with the extraction buffer. A 10-mg portion of whole-cell extract and the corresponding equivalent cytoplasmic and nuclear fractions were used for Western blot analysis.

GUS-reporter construction and enzyme activity assay

The genomic fragment of AtNAP1;4 was PCR amplified using primers N4-Pro-5’ and N4-Pro-3’ (see Table S2), and was then cloned into the HindIII–Smal restriction sites of vector pBI101, resulting in pAtNAP1;4::AtNAP1;4-GUS. This construct contains a 619-bp upstream section of the predicted translational start site, and all exons and introns of AtNAP1;4, and is fused in frame with the reporter GUS at the last exon. The construct was introduced into Arabidopsis plants by Agrobacterium-mediated transformation. A histochemical GUS enzyme activity assay was performed as described by Yu et al. (2003). The essential results were reproducibly obtained from more than 10 plants for each transgenic line.

RT-PCR

Total RNA was prepared using the TRizol kit according to the manufacturer’s instructions (Invitrogen). Semiquantitative RT-PCR

The resulting resin was used in the pull-down assays, according to a previously described protocol (Dong et al., 2005). Total protein extracts from about 10 g of 2-week-old transgenic Arabidopsis plants expressing YFP-AtNAP1;2, YFP-AtNAP1;3, YFP-AtNAP1;4 or H2A-YFP were each distributed equally to the aforementioned five recombinant protein-coated resins. A quarter of each pull-down fraction was used for Western blot analysis.

Western blot

Proteins were separated by SDS-PAGE, and transferred to an Immobilon-P polyvinylidene difluoride transfer membrane (Millipore, http://www.millipore.com). The membrane was blotted with specific antibodies. The antibodies used in this study are: the anti-GFP rabbit polyclonal antibody (Molecular Probes, now part of Invitrogen, http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) at a dilution of 1:5000, the anti-Nicta; NAP1;4 mice polyclonal antibodies (Dong et al., 2005) at a dilution of 1:2000, the anti-dimethyl H3K4 (cat. no. 07-030; Millipore) at a dilution of 1:2000, the anti-α-tubulin monoclonal antibody (cat. no. T6199; Sigma-Aldrich, http://www.sigmaaldrich.com) at a dilution of 1:10 000.

Microscopy

The epifluorescence and differential interference contrast images were taken using a confocal laser scanning microscope (model LSM510; Zeiss, http://www.zeiss.com).

Subcellular protein fractionation

For subcellular fractionation, about 2 g of 9-day-old seedlings were infiltrated with extraction buffer [400 mM sucrose, 10 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 5.3, 10 mM NaCl, 5 mM MgCl2, 5 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.1 mM DTT and 1× complete protease inhibitor] and ground in 9 ml of the same buffer. After passage through a 100-μm filter, the lysate was layered on top of a discontinuous gradient consisting of successive 4-ml layers of iodixanol 15% and 45% in the extraction buffer. The discontinuous gradient was centrifuged at 1500 for 15 min at 4°C. The cytoplasmic fraction was obtained from the top of the 15% iodixanol layer, and the nuclei fraction was obtained from the 15/45% iodixanol interface. The pure nuclei were washed three times with the extraction buffer. A 10-mg portion of whole-cell extract and the corresponding equivalent cytoplasmic and nuclear fractions were used for Western blot analysis.

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RT-PCR

Total RNA was prepared using the TRizol kit according to the manufacturer’s instructions (Invitrogen). Semiquantitative RT-PCR
was performed using Impron-II reverse transcriptase (Promega, http://www.promega.com) and quantitative RT-PCR with the kit from Takara (http://www.takara-bio.com). The gene-specific primers used in the PCR analysis are presented in Table S2.

Microarray

Six-day-old homozygous m123-1, m123-2 and wild-type Col (control) seedlings were harvested, and total RNA was prepared using the TRizol kit (Invitrogen). The microarray analyses were performed by using Affymetrix gene chips at Shanghai Huaguan Biochip (http://www.hgbiochip.com/english.html). Data analysis was performed using GENESPRING 5 software (Silicon Genetics, now part of Agilent, http://www.agilent.com). The expression profiles, each in duplicate, of m123-1 and m123-2 plants were compared with those of wild-type Col plants (m123-1 versus Col and m123-2 versus Col). Genes of m123-1 and m123-2 mutants exhibiting the same increase or decrease in expression level in each replicate were considered as upregulated or downregulated, respectively. In addition, a threshold of twofold or greater change was used for producing the final lists of upregulated and downregulated genes in m123-1 and m123-2.

UV treatment and in vitro DNA repair assay

To evaluate the plant response to UV, 8-day-old in vitro germinated plants were irradiated with a dose of 3750 J m⁻² of UV-C (254 nm) using a Mineralight-Lamp (UVP, http://www.uvp.com) three times every 24 h. Plants were immediately returned to the growth chamber after each exposure. The phenotype was observed 13 days after the last UV treatment. For analysis of gene expression in response to UV treatment, 8-day-old seedlings were irradiated three times with 500 J m⁻² UV-C, and then incubated for recovery in a growth chamber for 2 h before RNA isolation.

The in vitro DNA repair assay was performed according to the method described by Li et al. (2002). The linearized pGEX plasmid was treated by UV-C (450 J m⁻²) using the Stratalinker (Stratagene, http://www.stratagene.com) to increase the DNA damage. We used 25 μg of plant protein extracts per time point mixed with 300 ng of UV-C-treated pGEX, together with 300 ng of linearized pBSK plasmid, which was not pre-treated by UV-C. Incorporation of DIG-labeled 2'-deoxyuridine-5'-triphosphate (dUTP) in the plasmids was detected as previously described by Li et al. (2002).

ChIP analysis

About 2 g of 6-day-old seedlings were used for ChIP analysis, which was performed as described by Zhao et al. (2005). The gene-specific primers used in the ChIP analysis are presented in Table S2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of amino acid sequences of Arabidopsis NAP1-family proteins.

Figure S2. A schematic representation of procedures for obtaining the double and triple Atnap1 mutants.

Table S1. List of differentially expressed genes found in the triple Atnap1 mutants m123-1 and m123-2.

Table S2. List of primers used in this study.

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REFERENCES


