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Corresponding author:
Chengwei Yang
College of Life Sciences,
South China Normal University,
Guangzhou 510631,
China.
Tel/Fax: 86-20-85210855
E-mail: Yangchw@scnu.edu.cn

Journal research area: Development and Hormone Action
AtMMS21, an SMC5/6 complex subunit, is involved in stem cell niche maintenance and DNA damage responses in *Arabidopsis* roots

Panglian Xu, Dongke Yuan, Ming Liu, Chunxin Li, Yiyang Liu, Shengchun Zhang, Nan Yao, Chengwei Yang*

Guangdong Key Lab of Biotechnology for Plant Development, College of Life Science, South China Normal University, Guangzhou 510631, China (P.X., D.Y., M.L., C.L., Y. L., S.Z., C.Y.); State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510631, China (N.Y.)
Footnotes

1 This work was supported by the National Science Foundation of China (31170269, 30900789, U1201212), Education Department of Guangdong Province (2012CXZD0019) and Guangdong Province Universities and Colleges Pearl River Scholar Funded Scheme (2010).

* Corresponding author; e-mail Yangchw@scnu.edu.cn

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Chengwei Yang (Yangchw@scnu.edu.cn).
ABSTRACT

Plants maintain stem cells in meristems to sustain lifelong growth; these stem cells must have effective DNA damage responses to prevent mutations that can propagate to large parts of the plant. However, the molecular links between stem cell functions and DNA damage responses remain largely unexplored. Here, we report that the SUMO E3 ligase AtMMS21 acts to maintain the root stem cell niche by mediating DNA damage responses in Arabidopsis (Arabidopsis thaliana). Mutation of AtMMS21 causes defects in the root stem cell niche during embryogenesis and post-embryonic stages. AtMMS21 is essential for proper expression of stem cell niche-defining transcription factors. Moreover, mms21-1 mutants are hypersensitive to DNA-damaging agents, have a constitutively increased DNA damage response, and have more DNA double-strand breaks (DSBs) in the roots. Also, mms21-1 mutants exhibit spontaneous cell death within the root stem cell niche, and treatment with DSB-inducing agents increases this cell death, suggesting that AtMMS21 is required to prevent DSB-induced stem cell death. We further show that AtMMS21 functions as a subunit of the SMC5/6 complex, an evolutionarily conserved chromosomal ATPase required for DNA repair. These data reveal that AtMMS21 acts in DSB amelioration and stem cell niche maintenance during Arabidopsis root development.
INTRODUCTION

In plants and animals, small pools of stem cells are maintained as a population of undifferentiated cells that can generate differentiated descendants to sustain growth or replace tissues (Sablowski, 2004). Simple systems, such as the root meristem of Arabidopsis (Arabidopsis thaliana), make excellent experimental models for studying the mechanisms controlling stem cell identity (Nawy et al., 2005). In Arabidopsis roots, quiescent center (QC) cells and the surrounding stem cells together form a stem cell niche (Aida et al., 2004). The root stem cell niche is positioned where activity of the transcription factors SHORT-ROOT (SHR) and SCARECROW (SCR) overlaps with expression of PLETHORA (PLT), which in turn is controlled by an apical–basal gradient of auxin maintained by PIN transporters (Sabatini et al., 2003; Galinha et al., 2007). Downstream of SCR, the Arabidopsis RETINOBLASTOMA-RELATED (RBR) protein and the QC-specific transcription factor WUSCHEL-RELATED HOMEOBOX5 (WOX5) also regulate maintenance of stem cells in plants (Wildwater et al., 2005; Sarkar et al., 2007).

In addition to transcription factors, chromatin factors also govern stem cell self-renewal and differentiation (Orkin and Hchedlinger, 2011). For example, in Arabidopsis, the histone acetyltransferase GCN5 and its cofactor ADA2b are required for root stem cell maintenance through regulation of PLT expression (Kornet and Scheres, 2009). The chromatin remodeling factor PICKLE (PKL) and the PcG protein CURLY LEAF (CLF) antagonistically determine root meristem activity by controlling the expression of stem cell fate genes (Aichinger et al., 2011). Another chromatin factor implicated in root stem cell maintenance is the Chromatin Assembly Factor-1 (CAF-1) complex. Mutation of the FASCIATA (FAS) genes, which encode subunits of the CAF-1 complex, leads to loss of root stem cells, and failure to maintain SCR expression (Kaya et al., 2001). Furthermore, fas mutants show an increased number of DNA double-strand breaks (DSBs) (Endo et al., 2006; Kirik et al., 2006), indicating that the Arabidopsis CAF-1 complex is required for genome stability.

Recent findings reveal that plant stem cells have specialized mechanisms to maintain genomic stability (Sablowski, 2011). Treatments with DNA-damaging agents preferentially kill stem cells in the shoot and root meristem, a response that requires transduction of DNA damage signals by ATAXIA-TELANGIECTASIA MUTATED (ATM),
ATM/RAD3-RELATED (ATR) and SUPPRESSOR OF GAMMA RESPONSE1 (SOG1) (Fulcher and Sablowski, 2009; Furukawa et al., 2010; Sablowski, 2011). In agreement with this, recently characterized mutants involved in DNA repair showed spontaneous death of root stem cells. For example, the accumulation of DNA damage in rad50 and mre11 mutants lead to stem cell death and thus to developmental defects in growing plants (Amiard et al., 2010). MERISTEM DISORGANIZATION 1 (MDO1) is required for the maintenance of stem cells through a reduction in DNA damage (Hashimura and Ueguchi, 2011). These data indicate that protection of genomic stability is an important feature of the plant stem cell niche.

SUMO (small ubiquitin-related modifier) has emerged as a significant regulator of genomic stability (Dou et al., 2011). MMS21 encodes a SUMO E3 ligase that forms a critical component of the STRUCTURAL MAINTENANCE OF CHROMOSOMES (SMC) 5/6 complex, which is an evolutionarily conserved chromosomal ATPase required for cell growth and DNA repair (Duan et al., 2009). The SUMO ligase activity of MMS21 contributes to the roles of the SMC5/6 complex in recombinational repair, collapsed replication fork restart, and rDNA and telomere maintenance, with some relevant substrates having been identified (Potts and Yu, 2005; Zhao and Blobel, 2005). Although protein sumoylation plays important roles in the maintenance of genomic stability from yeast to humans (Dou et al., 2011), it is unknown whether plants possess sumoylation mechanisms that act in the response to DNA damage. In Arabidopsis, AtMMS21/ HIGH PLOIDY2 (HPY2) is a functional SUMO ligase and plays an important role in plant root development (Huang et al., 2009; Ishida et al., 2009), but the precise mechanism of AtMMS21 in regulating root meristem function is unclear.

Here, we show that AtMMS21 regulates root stem cell niche maintenance during embryogenesis and post-embryonic stages. We provide evidence that AtMMS21 is required for stable expression of stem cell niche-defining transcription factors. In addition, AtMMS21 maintains the normal cellular organization of the root stem cell niche by preventing cell death. Mutation of AtMMS21 caused increased DSBs and DSB-inducible gene transcription, showing that AtMMS21 is involved in DNA damage responses during root development. We further demonstrate that AtMMS21 acts as a component of the SMC5/6 complex through its interaction with SMC5, thus revealing critical roles of AtMMS21 in maintaining the root stem cell niche and genome stability by reducing DNA damage.
RESULTS

*mms21-1* mutants show altered cell division and cell differentiation in the root meristem

AtMMS21/HYP2 acts in root meristem development (Huang et al., 2009; Ishida et al., 2009). To investigate the mechanisms by which AtMMS21 affects root growth, we examined the pattern of cell division and cell differentiation in wild-type (WT) and *mms21-1* (T-DNA insertion mutant) roots at different days after germination (DAG). At 5 DAG, *mms21-1* mutants showed shorter roots with smaller meristems (Fig. 1A–D). Time course analysis showed that *mms21-1* meristems reached their maximum size at 1 DAG (Fig. 1D), but WT meristems reached their maximum size at 5 to 7 DAG by a balance of cell division and cell differentiation (Moubayidin et al., 2010). Furthermore, in *mms21-1*, we observed meristem collapse that occurred as the cells of the root meristem differentiated at 7 to 14 DAG, as shown by formation of root hairs and xylem strands in the root tips (Fig. 1F-G). To further establish the role of AtMMS21 in root meristem maintenance, we monitored the expression of markers that express GFP in specific cell types in the root meristems. For example, the *J0571* marker specifically expressed GFP in the endodermis and cortex (Fig. 1H). By contrast, the cell files expressing GFP were not continuous in the *mms21-1* roots (arrowhead in Fig. 1I-J), and the expression often occurred in three layers adjacent to each other (arrow in Fig. 1I). Furthermore, abnormal planes of cell division were often observed in the region of GFP expression (inserts in Fig. 1I). Collectively, these results indicated that AtMMS21 is required for maintaining the pattern of cell division and cell differentiation in the root meristem.

*mms21-1* mutants show defective cellular organization of the root stem cell niche

Our finding that AtMMS21 is crucial for maintaining cell fate in the root meristem prompted us to investigate its possible effect on the cellular organization of the QC and its surrounding stem cells. In WT roots, the QC cells are mitotically inactive and are easily discernible by confocal microscopy (Fig. 2A). However, in *mms21-1*, the pattern of cells in the root tips was disrupted, and the QC could not be identified morphologically (Fig. 2B). Because AtMMS21 is an important regulator of cell cycle progression (Huang et al., 2009; Ishida et al., 2009), we checked whether cell cycle activities were altered in the QC of *mms21-1* roots. For this purpose, we cultured 2-DAG seedlings for 24 h in the presence of 5-ethynyl-2’-deoxyuridine (EdU).
Incorporation of EdU in the nuclei indicates S-phase progression, so EdU can be used to mark cell division in the root meristem (Vanstraelen et al., 2009). After coupling of EdU with the fluorescent substrate Apollo 567, root tips were observed by confocal microscopy. In WT root meristems, most meristematic cells and the QC-surrounding stem cells incorporated EdU, but red fluorescent nuclei were observed only occasionally in the QC cells (marked by pWOX5:GFP), indicating that the QC cells had low mitotic activity (Fig. 2C). By contrast, red fluorescent nuclei were frequently observed in the mms21-1 QC cells (11/15 in mms21-1 versus 2/15 in WT, n = 15) (Fig. 2D). These results indicated that the absence of AtMMS21 induces high mitotic activity of the QC cells, and the irregularly dividing QC cells result in a disordered stem cell niche.

The irregular QC organization of mms2-1 was also clearly observed by the altered expression pattern of the QC-specific marker pWOX5:GFP (Blilou et al., 2005; Sarkar et al., 2007). pWOX5:GFP showed the expected QC-specific expression pattern in WT roots (Fig. 2E). However, the GFP expression domain in the root tips of mms21-1 mutants showed lateral expansion (88%, n = 27) or occasional absence (Fig. 2F-G, data not shown), which again indicated that QC identity was not stably maintained. Consistent with the expansion of WOX5 expression pattern, qRT-PCR analysis also revealed that in mms21-1 roots WOX5 transcript levels increased by nearly two-fold compared with WT roots (Fig. S1D). To further investigate whether the disorganization of the stem cell niche was associated with misspecification of the QC, we monitored the expression of three independent QC-specific β-glucuronidase (GUS) markers (QC25, QC46 and QC184) in mms21-1 mutants. QC25 was expressed in QC cells in WT plants (Fig. 2H), but in mms21-1 roots, the QC25 expression was either absent (33.3%, n = 78) or highly reduced (19.2%, n = 78; Fig. 2I) and diffuse (47.4%, n = 78; Fig. 2J). Similarly, two other QC markers, QC46 and QC184 also showed aberrant expression in the mms21-1 roots (Fig. S1A). These results showed that AtMMS21 is essential for proper QC identity.

To test whether QC function was defective in mms21-1 mutants, we performed Lugol staining using the QC marker line. In WT roots, a layer of columella stem cells (CSCs) is present between the QC and differentiated columella cells contain starch granules (Fig. 2H; Fig. S1A). However, mms21-1 roots showed starch granules at the stem cell position (Fig. 2I-J; Fig. S1A). The mms21-1 roots also showed irregular expression of the CSC marker J2341 and had defective columella layers (Fig. S1B-C), revealing that the stem cell state of CSCs was
disrupted. Furthermore, in the mms21-1 roots, when QC markers were expressed, some GUS-staining cells also accumulated starch granules (Fig. 2J; Fig. S1A), indicating that the cells derived from aberrant QC divisions undergo differentiation, and then fail to execute the QC function of maintaining CSCs in an undifferentiated state. Taken together, these results indicated that AtMMS21 is required for the maintenance of QC organization, identity and function, and thus is essential for root stem cell niche maintenance.

**Mutation of AtMMS21 affects the expression of stem cell niche-defining transcription factors**

Root stem cell niche maintenance depends on the highly specific expression and subcellular localization of the transcriptional regulators SHR and SCR, which provide the radial position of the stem cell niche, and of PLT1 and PLT2, which provide the longitudinal position (Dinneny and Benfey, 2008). To determine whether the stem cell niche defect in the mms21-1 mutants was caused by misregulation of these stem cell niche-defining transcription factors, we examined the expression pattern of these genes in mms21-1 at 5 DAG. We first monitored the expression of pSHR:GFP and the protein localization of pSHR:SHR:GFP in the mms21-1 mutants. The expression region of the SHR transcript in the stele of mms21-1 roots was slightly reduced compared with WT (Fig. 3A-B; Fig. S1D). Also, the expression level of SHR protein was reduced (Fig. 3C-D), and SHR localization, which is in endodermal cells, QC cells and cortical/endodermal stem cells in WT, seemed to be missing in some cell files in mms21-1 roots (arrowhead in Fig. 3D).

SHR expressed in the stele moves into the adjacent cell layer, where it controls SCR transcription and endodermis specification (Cui et al., 2007). Therefore, we next analyzed the effect of AtMMS21 on SCR expression. In WT roots, the pSCR:GFP expressing cells were contiguous and appeared to form a single-cell layer with the endodermal cells, QC cells and cortical/endodermal stem cells (Fig. 3E). By contrast, although the expression of SCR was observed in the corresponding cells in most mms21-1 roots, some root stem cell niche or endodermal cells showed ectopic expression of SCR (arrow in Fig. 3F-G). In extreme cases, SCR expression was abolished in some endodermal cells and stem cell niche, which displayed a discontinuous pattern (arrowhead in Fig. 3G). In addition, to examine the effect of the SHR/SCR pathway on AtMMS21 expression, we analyzed AtMMS21 expression in the shr-1
and scr-1 mutants by qRT-PCR. Results showed that the shr-1 and scr-1 mutations have little
effect on the expression of AtMMS21 (Fig. S2).

PLT1/PLT2 acts in parallel with the SHR/SCR pathway to pattern the root stem cell niche
(Aida et al., 2004). The expression patterns of HPY2/AtMMS21 proteins largely overlap with
those of PLT1 and PLT2 (Ishida et al., 2009); together with our finding that AtMMS21
functions in the maintenance of the root stem cell niche, these results imply that AtMMS21
may have a role in defining PLT expression and/or accumulation. We then analyzed the
transcript levels of PLT1/PLT2 in WT and mms21-1 roots using qRT-PCR. Expression levels of
PLT1 and PLT2 were not significantly reduced in mms21-1 roots (Fig. S1D). Surprisingly, in
mms21-1 root tips, the YFP levels of the translational fusions PLT1pro:PLT1:YFP and
PLT2pro:PLT2:YFP were dramatically reduced or weakly expressed compared with WT (Fig.
3H-M), establishing that loss of AtMMS21 significantly affected the accumulation of PLT
protein in the root meristem. Considering that the correct PLT protein dosage is necessary for
root stem cell maintenance and root formation (Aida et al., 2004; Galinha et al., 2007), our
results revealed that the defective stem cell niche maintenance in the mms21-1 mutants occurs
along with dramatic misregulation of PLT1/2 accumulation.

**mms21-1 mutants show defects in the root stem cell niche from embryogenesis onward**

Root stem cell niches are established during embryogenesis and provide cells for
postembryonic growth (Weigel and Jurgens, 2002; Aida et al., 2004). In postembryonic
mms21-1 roots, the visible defects in stem cell niche organization and marker gene expression
were already observed from 1 DAG onward (Fig. S3). These findings prompted us to
investigate whether AtMMS21 is required for initiation of the stem cell niche during
embryogenesis. The cell division pattern is strictly regulated during WT embryogenesis, but in
the mms21-1 mutants, many types of abnormal embryos with disturbed cellular organization at
the basal embryo pole were frequently observed, starting at the early globular stage (Fig. 4; Fig.
S4A-G). At the late globular stage of WT, the hypophysis had divided asymmetrically,
producing a basal cell and a smaller lens-shaped apical cell (Fig. 4A). By contrast, mms21-1
embryos exhibited abnormal cellular organization of the hypophyseal derivatives (Fig. 4F).
Before the heart stage, nearly 16% of analyzed embryos (n = 31/188) showed aberrant
divisions at the basal pole (Table S2). At the heart stage of WT, the lens-shaped cell undergoes
vertical divisions to generate the QC, while the lower cells divide horizontally to form

columella stem cells and the root cap (Fig. 4B-C). However, 46% of the mms21-1 embryos
examined (62/134) showed irregular planes of cell division in the hypophysis descendants,
leading to disorganization in the basal embryo region where the root stem cell niche initiates
(Fig. 4G-H; Table S2). At later stages of embryo development, aberrant divisions were still
apparent at the root stem cell niche (Fig. 4I), which translates into seedlings without a primary
root in 16% of germinated mms21-1 mutants (n = 35/216; Fig. 7E). Thus, in mms21-1 mutants,
the disturbed cellular organization in the root stem cell niche occurs beginning in
embryogenesis.

To further analyze cell identity in the embryonic stem cell region of mms21-1 mutants, we
examined the expression of QC-specific markers in the embryo. In WT embryos, QC25 was
expressed in the QC precursor cell from the heart-stage (Fig. 4E), and later in the QC cells (Fig.
4K). However, the majority (67%, n = 42) of the mms21-1 embryos showed no detectable
QC25 expression in the corresponding cells at the heart-stage (Fig. 4J). In the mature embryo,
QC25 expression was absent or diffuse (Fig. 4L-M), similar to what was observed in
postembryonic mms21-1 roots. These data indicated that AtMMS21 is essential for proper
specification of the QC.

We also examined the expression of pWOX5:GFP, a QC-specific marker with expression
that is restricted to the QC precursor cell of the heart-stage embryo and, subsequently, becomes
highly localized to the QC in normal embryogenesis (Fig. 4N, P; Fig. S4H) (Haecker et al.,
2004; Sarkar et al., 2007). In line with the altered expression of pWOX5:GFP in postembryonic
mutant roots, in mms21-1 embryos, WOX5 expression was diffuse and expanded to the
adjacent cells (Fig. 4O, Q; Fig. S4H). Maintenance of WOX5 expression depends on the
asymmetric nature of the hypophyseal division (Song et al., 2008); therefore, the diffuse
expression is consistent with our findings that mms21 mutants showed disorganized cell
division in the hypophysis, supporting a role for AtMMS21 in QC organization and correct cell
division patterns of neighboring cells.

Finally, we examined whether the mms21-1 embryo phenotype defects were consistent with
defects in PLT expression, which is necessary for embryonic specification of the root stem cell
(Aida et al., 2004). As observed in postembryonic mms21-1 roots, the expression domains of
PLT1 and PLT2 proteins in the basal embryo of mms21-1 were restricted at heart-stage embryo
compared with WT (Fig. 4S, Fig. S4I). Furthermore, reductions in the expression of PLT1 and
PLT2 were more pronounced in mature embryos (Fig. 4U, Fig. S4J). Collectively, our results
demonstrated that AtMMS21 function is required for the specification and organization of the
root stem cell niche during embryogenesis.

AtMMS21 is required to prevent cell death in the root stem cell niche
Interestingly, we noticed that in several mms21-1 root meristem cells, the cytoplasm was
stained with propidium iodide (PI) (Fig. 3). PI stains the walls of living plant cells and is also
used as a marker for dead cells which have loss of membrane integrity (Truernit and Haseloff,
2008). To investigate the spatial occurrence of cell death, plants were stained at different time
points with PI. At 1 DAG, no PI-stained cells were observed in WT root meristems, but in
mms21-1 roots, several stem cells and the early descendants were stained (Fig. 5A). To confirm
that cells stained with PI are correctly labeled as dead cells, another cell death marker, Sytox
Orange, was used to stain the root tips. As expected, Sytox Orange staining confirmed that the
stem cells and their daughter cells showed cell death in the mms21 roots (Fig. S5B). At 5 DAG,
dead cells in the root meristem of mms21-1 occur in QC cells, surrounding stem cells and the
transiently amplifying region of the vascular tissue, but not in the WT (Fig. 3, Fig. S5D).
Detecting the expression of the QC marker pWOX5:GFP in mms21-1 roots confirmed that the
dead cells were found in the stem cell niche (Fig. 2F-G). Interestingly, the expansion of WOX5
expression and the spatial cell death pattern observed in mms21-1 were similar to phenotypes
observed in WT root meristems after treatment with DNA-damaging agents (Fig. S6B, H)
(Fulcher and Sablowski, 2009; Furukawa et al., 2010).

Given that mms21-1 mutants showed spontaneous cell death even in the absence of
DNA-damaging treatments, we hypothesized that mms21-1 had naturally increased levels of
DNA damage which was sufficient to activate cell death, and that external genotoxic treatment
that increased the cumulative levels of DNA damage would caused more extensive death in the
root meristem. Indeed, after growth for one day in the presence of cisplatin (which forms DNA
cross-links and leads to DSBs), the number of dead cells in mms21-1 root tips increased, and
many dead cells were observed throughout the mms21-1 meristem, but dead cells were not
observed in WT plants (Fig. 5B). After treatment for two days with cisplatin (15 μM), the root
meristems of mms21-1 mutants were not maintained and differentiated; by contrast, only a few
dead cells were found in the WT stem cell niche (Fig. 5B). Thus, absence of AtMMS21 triggered cell death in the stem cell niche and this cell death was associated with DNA damage.

To clarify whether the misregulation of stem cell niche-defining transcription factors is an effect of DNA damage, we examined the expression pattern of these genes after cisplatin treatments. Seedlings were treated with cisplatin (15 μM) for two days treatments until external genotoxic stress was sufficient to induce cell death in the stem cell niche. Although the expansion of WOX5 expression was smaller than observed in mms21-1 mutants, DNA damage have an effect on WOX5 expression (Fig. S6B, H versus Fig. 2F, G). In addition, the expression of SHR, SCR, PLT1 and PLT2 were slightly reduced after cisplatin treatment, which seem like those in the mms21-1 mutants (Fig. S6). Thus, these data indicate that the misregulation of stem cell factors in mms21-1 mutants may be a secondary effect due to DNA damage.

**mms21-1 mutant root tip cells have increased DSBs**

Although experiments in yeast and human cells have demonstrated that MMS21 is required for efficient DSB repair (Potts and Yu, 2005; Zhao and Blobel, 2005), the effect of mms21 mutation on the DNA damage response in plants is unclear. To examine whether the mms21-1 roots had constitutive activation of DNA damage responses, we first measured the expression of the DSB-inducible genes PARP1, BRCA1 and RAD51 by qRT-PCR (Inagaki et al., 2006; Sakamoto et al., 2011). Plants were grown under normal growth conditions and the transcript levels of all three genes were higher in the mms21-1 roots than in the WT plants (Fig. 5C). These data suggested that the mms21-1 root tip cells are exposed to DSBs and showed constitutively activated DNA damage responses, even without additional genotoxic stress.

Next, we directly compared the levels of DSBs in the roots of WT and mms21-1 mutants using comet assays (Menke et al., 2001). Levels of DSBs in WT and mms21-1 roots were measured by the percentage of DNA in the tail of comet in the neutral comet assay. As expected, a higher accumulation of DSBs was observed in mms21-1 root tips than in WT plants under normal conditions (Fig. 5D-E). Taken together, our observations that the mms21-1 mutation upregulated DSB-inducible gene transcription and DSBs, indicated that AtMMS21 is involved in reducing the incidence of DSBs in Arabidopsis roots.
**mms21-1 mutants show increased sensitivity to DNA-damaging treatments**

To examine the sensitivity of the *mms21-1* mutants to DSB-inducing agents, we evaluated the effect of the DNA cross-linking agents cisplatin and MMS, which cause DSBs, on the growth of *mms21-1* and 35S:AtMMS21 plants (Huang et al., 2009). Three DAG seedlings were transferred to medium containing cisplatin (50 μM) or MMS (0.0075%) for 14 days. The *mms21-1* plants treated with DNA-damaging agents did not produce additional true leaves, and these plants died 10 to 14 days after treatments, whereas the WT plants were less affected (Fig. 6A).

We next evaluated the effect of short-term treatment with DNA-damaging agents on the growth of *mms21-1* roots. Three DAG seedlings grown on vertical plates were transferred to medium containing various concentrations of DSB-inducing agents, and incubated for another three days. The more severe inhibitory effects on root elongation were observed in the *mms21-1* mutants compared to WT plants (Fig. 6B-C). We further found that the *mms21-1* root meristems were completely collapsed after exposure to DNA damage, while the application of cisplatin to WT roots phenocopied the phenotypes of *mms21-1* mutants, including smaller meristems, loss of QC activity, disorganized stem cell niches and root caps (Fig. 6D; Fig. S7). These results indicated that the developmental defects in the *mms21* roots were involved in DNA damage responses.

**Arabidopsis MMS21 is a subunit of the SMC5/6 complex**

Animal and yeast MMS21 form a part of the SMC5/6 complex through their interactions with SMC5, and this complex functions in DSB repair (Potts and Yu, 2005; Zhao and Blobel, 2005). Recently, a similar SMC5/6 complex, which also acts in DNA repair, was reported in plant cells (Watanabe et al., 2009). However, non-SMC components of this complex have not been characterized in terms of their interactions with SMC5/6.

To examine their *in vivo* protein interaction we first tested whether AtSMC5 and AtMMS21 colocalize in Arabidopsis protoplasts. When *CFP-AtMMS21* was coexpressed with *YFP-AtSMC5*, predominantly nuclear signals were observed, along with a low signal in the cytosol (Fig. 7A). We next used yeast two-hybrid assays to analyze the direct physical interaction of AtMMS21 with AtSMC5. We found that AtMMS21 interacted with AtSMC5 in yeast (Fig. 7B). This interaction was confirmed using Bimolecular Fluorescence
Complementation (BiFC) in plant cells. When BiFC constructs of *pSAT6–nEYFP:MMS21* and *pSAT6–cEYFP:SMC5* were cotransformed into onion epidermal cells, YFP fluorescence was observed in the nucleus, whereas the controls showed only a background signal (Fig. 7C), indicating that MMS21 physically interacts with SMC5 in plant cells. Therefore, Arabidopsis MMS21 is likely the ortholog of the yeast/human MMS21 and is a subunit of the SMC5/6 complex.

Additionally, we examined the phenotypic similarities between *mms21-1* and *smc5-2* mutants. A previous study showed that heterozygotes for a T-DNA insertion in the Arabidopsis *SMC5* gene did not yield homozygous mutants, and siliques of heterozygous plants contained 25% shrunken seeds, indicating that *SMC5* is essential for seed development (Watanabe et al., 2009). These prompted us to speculate that *SMC5* might function in embryogenesis. Similar to *mms21-1* mutants, aberrant embryos with abnormal cell division planes were observed in *SMC5/smc5-2* siliques (27%, n = 58/218; Fig. 7D). These aberrant divisions were apparent at the basal pole where the root meristem initiates and resulted in seedlings without a main root in 9% (16/188) of germinated *SMC5/smc5-2* mutants (Fig. 7E). Thus, *mms21-1* and *smc5-2* mutants exhibited strikingly similar phenotypes, including abnormal embryogenesis and hypersensitivity to DNA damage (the present study and (Watanabe et al., 2009), suggesting that MMS21 and SMC5 likely function in common or similar pathways to maintain genomic stability and embryo development in plants.

**DISCUSSION**

*AtMMS21* is required for maintenance of the root stem cell niche

*AtMMS21* encodes a SUMO E3 ligase involved in root development (Huang et al., 2009; Ishida et al., 2009); further pursuing the implications of this result, here we demonstrated that *AtMMS21* acts in regulating stem cell niche maintenance during root development. The fate of stem cells surrounding the QC can be used as a readout of the QC’s organizing activity (Gonzalez-Garcia et al., 2011). By measuring the expression of QC-specific markers and the differentiation of stem cells, we provided two types of evidence supporting the requirement for *AtMMS21* in the maintenance of root stem cell niche. On the one hand, the irregular QC
organization (observed by microscopy and \textit{pWOX5:GFP} expression), the aberrant expression of QC-specific markers (\textit{QC25}, \textit{QC46}, \textit{QC184}), and the mitotic activation of QC cells all indicated that \textit{AtMMS21} is essential for proper organization and identity of the QC (Fig. 2; Fig. S1). On the other hand, the appearance of starch granules in the region of the QC and columella stem cells, together with defective columella layers and ground tissue indicated that QC function and the cell fate of root stem cells are also not maintained properly in the \textit{mms21-1} roots, leading to disorganized meristem pattern and ultimately to short roots (Fig. 1-2; Fig. S1). Hence, our observations confirmed that \textit{AtMMS21} acts in stem cell niches to regulate correct root meristem patterning and function. The stem cell niche is established early in embryogenesis (Weigel and Jurgens, 2002), and the master regulators of root development such as SHR/SCR and PLT1/2 affect the establishment of the root stem cell niche during embryonic patterning formation (Di Laurenzio et al., 1996; Aida et al., 2004). Similar to \textit{shr}, \textit{scr} and \textit{plt1plt2} mutants, disorganized cell division in the hypophysis region and the defective expression of QC-specific markers were observed in \textit{mms21-1} embryos (Fig. 4; Fig. S4), indicating that \textit{AtMMS21} is required for initiation of the stem cell niche during embryogenesis. Taken together, our data indicate that \textit{AtMMS21} is important for the maintenance of root stem cell niches, both during embryogenesis and post-embryonic stages.

In mammals, the pluripotency transcription factors Oct4, Nanog and Sox2 regulate the fate of embryonic stem cells (Orkin and Hochedlinger, 2011). Although the activities of Oct4 and Sox2 are regulated by sumoylation (Hietakangas et al., 2006; Wei et al., 2007; Van Hoof et al., 2009), it is unclear whether this sumoylation is mediated by SUMO E3 ligase. In plants, root stem cell niches are also regulated by pluripotency transcription factors, including PLT1, PLT2, SHR, SCR and WOX5 (Sablowski, 2011). In the \textit{mms21-1} roots, the normal restricted spatial expression pattern of \textit{WOX5/SHR/SCR} was partially lost, ectopic expression of \textit{WOX5/SCR} in adjacent cells was observed and protein levels of PLT1/2 were severely reduced (Fig. 3). In light of the finding that MMS21 is required for chromatin organization during cell division in budding yeast (Rai et al., 2011), \textit{AtMMS21} might be targeted to chromatin through its interaction with the SMC5/6 complex and function to ensure the stable epigenetic state of the chromosomes, which is required for the stable expression of pluripotency transcription factors within the stem cell niche (Fig. 3; Fig. S3). Intriguingly, PLT1/2 positively regulates the expression and/or accumulation of \textit{AtMMS21/HPY2} proteins in the root meristem (Ishida et al.,
Therefore, it will be interesting to examine whether a feedback loop exists in which PLT1/2 modulates AtMMS21 accumulation, and AtMMS21 influences the accumulation of PLT proteins to maintain pluripotency in the Arabidopsis stem cell niche. Similar to such a scenario, a regulatory loop exists between chromatin factors and stem cell transcription factors in mammals (Loh et al., 2007; Orkin and Hochedlinger, 2011). It is worth noting that MMS21 has yeast, plant and animal orthologs, suggesting that this mechanism is ancient and conserved. Hence, our results provide an important clue for further investigation of whether the animal MMS21 also plays a role in the maintenance of stem cell niches.

AtMMS21 functions in reducing DNA damage

The protection of stem cells against DNA damage is crucial for normal development, but little is known about the interplay between DNA damage responses and stem cell niche maintenance (Hashimura and Ueguchi, 2011). Here we provided three pieces of evidence showing that AtMMS21 plays a crucial role in the maintenance of stem cell niches through a reduction in DNA damage. First, the mms21-1 mutants showed clear cell death in root stem cells and their daughters. Also, external genotoxic treatment caused more extensive death in mms21-1 root meristems (Fig. 5A-B), suggesting that mutation of AtMMS21 caused increased DNA damage in the cells. In support of this, two recent reports have shown that Arabidopsis root stem cells and their descendants undergo cell death upon DNA damage (Fulcher and Sablowski, 2009; Furukawa et al., 2010). Second, even under normal conditions, a higher accumulation of DSBs was observed in root tips of mms21-1 than in those of WT plants. Consistent with this, several genes known to be associated with increased DSB damage were induced in the roots of mms21-1 (Fig. 5C-E). Third, the mms21-1 mutants exhibited increased sensitivity to the effects of DSB-inducing agents on both root elongation and meristem activity (Fig. 6). Therefore, a possible explanation for the growth defects observed in mms21-1 mutants is that stem cell death increased as a consequence of the accumulation of unrepaired DSBs. This idea is consistent with previous reports that defects in DNA repair genes (e.g. MDO1, MRE11, FAS, BRU1/MGO3/TSK and TEB) result in similar phenotypes, including elevated levels of DSBs, hypersensitivity to DNA-damaging agents, stem cell death and misexpression of stem cell regulators, thus causing defective meristem structure with fasciated stems and short roots (Kaya et al., 2001; Bundock and Hooykaas, 2002; Takeda et al., 2004; Inagaki et al., 2006;
Amiard et al., 2010; Hashimura and Ueguchi, 2011). All of these developmental defects were also observed in the mms21-1 mutants (the present study and (Huang et al., 2009; Ishida et al., 2009), suggesting that AtMMS21 is important for root stem cell niche maintenance through the reduction of DSBs.

Although the precise mechanism by which Arabidopsis MMS21 ameliorates DNA damage (Fig. 5-6) is unclear, animal and yeast studies provide numerous possible explanations. Experiments in yeast and human cells have demonstrated that MMS21 plays crucial roles in facilitating DSB repair, collapsed replication fork restart, and telomere elongation by homologous recombination (HR) (Duan et al., 2009). Although knowledge of the role of the SMC5/6 complex in plants is still limited, a recent study in Arabidopsis indicated that the SMC5/6 complex enhances sister chromatid alignment after DNA damage and thereby facilitates correct DSB repair via HR between sister chromatids (Watanabe et al., 2009). It is likely that AtMMS21 acts in the meristem to efficiently repair DSBs by HR, which is consistent with the finding that DSBs are preferentially repaired by HR between sister chromatids in meristematic cells (Watanabe et al., 2009). Detailed analysis of HR frequency and chromatid behaviors in mms21-1 might help further our understanding of AtMMS21 function in DSB repair and meristem maintenance. In this respect, meristematic cells afflicted by DSBs are faced with the choice of inducing stem cells and their early descendants to die preferentially, or delaying cell division to repair the damage, and endocycles are thought to be an alternative strategy protecting against DSBs (Fulcher and Sablowski, 2009; Adachi et al., 2011). Therefore, in line with the premature onset of endocycles (Ishida et al., 2009) and the early differentiation of root meristematic cells (Fig. 1), we propose that AtMMS21 is important for the prevention of cell death, early differentiation and premature endocycle onset through a reduction in DSBs (Fig. 8). Additionally, unlike the known SUMO E3, SIZ1, which functions as single proteins, yeast and mammalian MMS21/NSE2 is a conserved subunit of the SMC5/6 complex, which associates with the long coiled-coil region of SMC5 and regulates SMC5/6 function (Duan et al., 2009). Here, we demonstrated that Arabidopsis MMS21 physically interacts with SMC5 (Fig. 7), suggesting that the mechanism of MMS21 in DNA repair may represent a conserved function in different organisms. Furthermore, the phenotypic overlap between mutants of AtMMS21 and AtSMC5 suggests a functional overlap between these genes in the maintenance of genomic integrity and the specification of stem cell niches during
embryogenesis.

CONCLUSION

The present study uncovers a cellular mechanism to explain how AtMMS21 defines the stem cell niche and ameliorates DSBs (Fig. 8). AtMMS21 encodes a SUMO E3 ligase expressed in the root meristem (Huang et al., 2009; Ishida et al., 2009), where AtMMS21 mediates the expression levels and patterns of stem cell niche-defining transcription factors; these transcription factors in turn regulate root stem cell niche maintenance. Moreover, AtMMS21 acts as a subunit of the SMC5/6 complex, and is essential for the role of the SMC5/6 complex in the reduction of DNA damage. In this regard, AtMMS21 maintains root stem cell survival by ameliorating DSB damage, and thus ensures normal structure and function of the root stem cell niche. Although this explanation has not yet provided definitive evidence for the connection between DSB responses and stem cell niche maintenance, it confirms that AtMMS21 is required for the maintenance of stem cells by reducing DNA damage. In this context, a significant direction for future research will be to identify AtMMS21 target proteins and elucidate the molecular mechanism of AtMMS21 in the maintenance of stem cells and genomic stability, which will help our understanding of how sumoylation control developmental process and genomic stability in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The mms21-1 mutant and 35S:AtMMS21 Arabidopsis (Arabidopsis thaliana, Columbia-0 ecotype) were isolated as described previously (Huang et al., 2009). The following marker lines and mutants were used: QC25, QC46 and QC184 (Sabatini et al., 2003); smc5-2 (Watanabe et al., 2009); pWOX5:GFP (Blilou et al., 2005); pSHR:GFP (Helariutta et al., 2000); pSHR:SHR:GFP (Nakajima et al., 2001); pSCR:GFP (Wysocka-Diller et al., 2000); PLT1pro:PLT1:YFP and PLT2pro:PLT2:YFP (Galinha et al., 2007); shr-1 (Benfey et al., 1993); scr-1 (Di Laurenzio et al., 1996); J1092, J0571 and J2341 were from the Haseloff enhancer trap GFP line collection (http://www.plantsci.cam.ac.uk/Haseloff).
Seeds were surface-sterilized for 2 minutes in 75% ethanol, followed by 5 minutes in 1% NaClO solution and rinsed five times with sterile water, plated on Murashige and Skoog (MS) medium with 1% sucrose and 0.8% agar, and then stratified at 4°C in the dark for 2 days. Plants were grown under long-day conditions (16 hours light/8 hours dark) at 22°C in a phytotron.

**Root Meristem Size Analysis**

Seeds were germinated and grown on vertically oriented plates from 1 to 14 days. Roots were examined at different days after germination (DAG) depending on the experiment. Approximately 30 to 50 seedlings were examined in at least three independent experiments, which gave similar results. Roots were mounted in chloral hydrate and then root meristem size was determined by counting the number of cortex cells in a file extending from the QC to the first elongated cell, which was excluded (Perilli and Sabatini, 2010).

**Marker Gene Expression Analysis**

The markers QC25, QC46, QC184, J1092, J0571, J2341, pWOX5:GFP, pSHR:GFP, pSHR:SHR:GFP, pSCR:GFP, PLT1pro:PLT1:YFP, PLT2pro:PLT2:YFP were crossed to the mms21-1 mutants. Homozygous plants for both mms21-1 and marker genes were obtained from F2 populations, and analyzed in the next generations.

For quantitative real-time PCR, root tips of 5 DAG seedlings were harvested, and total RNA was extracted using the Plant Easy Spin RNA Miniprep Kit (BioMIGA). RNA was treated with DNaseI and reverse transcribed using a PrimeScript RT reagent kit (Takara). After the RT reaction, the cDNA template was subjected to PCR reactions using SYBR Premix Ex Taq (Perfect Real Time) (Takara). Quantitative RT-PCR was performed using three replicates and ACTIN2 (At3g18780) as a reference gene. Real-time measurements of PCR product accumulation were carried out using a 7500 Fast Real-Time PCR system (Applied Biosystems). Data presented are the averages from three biological replicates with SD (Inagaki et al., 2006). The statistical significance was evaluated by Student’s t-test. The primer sets are listed in Table S3.

**Histological Analysis**
Columella root cap cells specifically accumulate starch granules that are visualized by Lugol staining. Roots were incubated in Lugol solution for 5 minutes, washed in water once, and then mounted in HCG solution (chloroacetaldehyde:water:glycerol = 8:3:1) for 10 to 20 min before microscopy (Zhou et al., 2010).

Histochemical analysis of β-glucuronidase (GUS) activity in enhancer trap lines used as QC markers was performed according to the described method (Stahl et al., 2009) with some modifications. GUS stock solution [0.05 M NaPO₄ buffer (pH 7.0), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 10 mM X-glucuronide] was made as described previously (Stahl et al., 2009). The seedling roots were stained in a 3 in 10 dilution of the stock solution at 37°C for 8 to 10 hours, rinsed with water and mounting in HCG solution for microscopy.

For confocal laser imaging of roots, cell walls were labeled with propidium iodide (PI) as described (Truernit and Haseloff, 2008). Roots were counterstained with 10 μg/mL PI (Sigma) for 1 minute, washed once in distilled water and mounted in water for confocal microscopy. Another cell death marker, Sytox orange (250 nM, Invitrogen) was also used to stain the roots for 5 min before imaging.

The EdU incorporation assay was performed as previously described (Vanstraelen et al., 2009; Zhou et al., 2010) with slight modifications. Two-DAG Seedlings were grown in liquid MS containing 10 μM EdU (Cell-Light EdU Apollo 567 In Vitro Imaging Kit, RiboBio) for 24 hours, and were fixed in 4% formaldehyde in PBS (pH 7.4) for 30 minutes. Root tips of seedlings were harvested, and were then washed in 3% BSA in PBS for 5 min, treated with 0.5% Triton in PBS for 20 minutes, and washed again. Coupling of EdU to Apollo 567 was performed according to the manufacturer’s instructions, and samples were imaged using confocal microscopy.

Microscopy

Seedling roots and ovules were cleared and mounted in HCG solution and Hoyer’s solution, respectively. Samples were captured using Nomarski optics on a Leica DM2500 with a DFC420 digital camera (Leica), and processed with Leica Application Suite software. Confocal images were taken using a Zeiss LSM 710 laser scanning microscope with the following excitation (Ex) and emission (Em) wavelengths (Ex/Em): 561 nm/ 591 to 635 nm for
PI, 543 nm/580 to 610 nm for Sytox Orange, 488 nm/505 to 530 nm for GFP, 514 nm/530 to 600 nm for YFP, 458 nm/475-525 nm for CFP, and 550 nm/565 nm for EdU Apollo. At least 15 to 30 seedlings or embryos were examined, and three independent experiments were performed. The seedling roots on MS medium were photographed using the SONY DSC-H50 camera. Images were processed with Photoshop CS4 software (Adobe, USA).

DNA-damaging Treatments and Comet Assay

For DNA-damaging sensitivity assay, seedlings at three DAG were transplanted onto the surface of MS agar plates containing methyl-methane sulfonate (MMS) or cisplatin (Sigma) and marked to show the initial positions of their root tips. They were incubated vertically for an additional 14 days or three days, and the lengths of the newly elongated primary roots were determined using Digimizer 4.2 (http://www.digimizer.com). Root growth was measured and expressed as a percentage of the average length of roots on control plates as described previously (Inagaki et al., 2006). Root meristem size and QC activity were measured as described above. To investigate whether the defective meristem and stem cell niche phenotype of mms21-1 mutants is a DSB stress effect, different concentrations of cisplatin and MMS were tested and the lowest concentration that gave apparent phenotypes was used. Results presented are averages of 15 to 25 seedlings. Root growth experiments were repeated three times with similar results.

DSBs were measured using comet assays with the N/N protocol as described (Menke et al., 2001). Root tips of 5 DAG seedlings were harvested, and prepared as described (Menke et al., 2001). Images of SYBR Green I–stained comets were captured using a fluorescence microscope (DM2500, Leica). DNA damage was calculated by averaging the values for the percentage of DNA in tails from three individual slides, scoring 80 comets per slide (Takahashi et al., 2010). The comet analysis was performed using CASP software (http://www.casp.of.pl/). The statistical significance was evaluated by Student’s t-test.

Protein Colocalization and Interaction between AtMMS21 and AtSMC5

For protein colocalization analysis, the coding sequence of AtMMS21 was amplified and fused in a modified Bluescript pSK vector containing 35Spro:CFP (Tao et al., 2005), and the coding sequence of AtSMC5 was amplified and fused in the modified Bluescript pSK vector.
containing 35S\textsubscript{pro:}YFP (Tao et al., 2005). Primers are listed in Table S3. This resulted in the CFP-AtMMS21 and YFP-AtSMC5 constructs, which were co-transfected into Arabidopsis protoplasts. Arabidopsis mesophyll protoplasts were prepared and transfected as described previously (Yoo et al., 2007). The fluorescence signals in transfected protoplasts were examined by confocal microscopy.

For yeast two-hybrid analysis, the coding sequence of AtMMS21 was amplified and fused to the GAL4 activation domain in the pGADT7 vector (Clontech), and the coding sequence of AtSMC5 was amplified and fused to the GAL4 DNA binding domain in the pGBKT7 vector (Clontech). Primers are listed in Table S3. The bait and prey constructs were transformed into the yeast strain AH109 (Clontech). The interaction between pAD-T and pBD-53 was used as the positive control and pAD-T and pBD-Lam were used as the negative control. Co-transformed yeast strains were selected on synthetic defined SD/–Leu/–Trp medium. Protein–protein interactions were tested using selective medium SD/–Leu/–Trp/–His and supplied with 5 mM 3AT (3-Amino-1,2,4-Triazol, Clontech). β-galactosidase activity was measured to assay for the interaction between AtMMS21 and AtSMC5 in a GAL4 two-hybrid system according to the manufacturer’s protocol (Clontech).

For bimolecular fluorescence complementation (BiFC) analysis, the full-length cDNA of AtMMS21 was amplified and fused with the N-terminal part of the YFP coding sequence to give rise to the plasmid pSAT6\textsubscript{N}-nEYFP\textsubscript{N1}-AtMMS21, and the full-length cDNAs of AtSMC5 was amplified and fused with the C-terminal part of YFP to result in the plasmid pSAT6\textsubscript{C}-cEYFP\textsubscript{N1}-AtSMC5. Primers used for plasmid construction are listed in Table S3. Vector combinations (samples and controls) were used and BiFC assays were performed as described (Walter et al., 2004; Huang et al., 2009). YFP fluorescence after bombardment of onion epidermal cells was visualized by confocal microscopy.
Supplemental Data

Supplemental Figure S1. Marker gene expression in mms21-1 roots.

Supplemental Figure S2. The shr-1 and scr-1 mutants show little effect on the expression of AtMMS21.

Supplemental Figure S3. Maintenance of the stem cell niche is defective in mms21-1 roots from 1 DAG.

Supplemental Figure S4. Defects in the stem cell niche in mms21-1 embryos.

Supplemental Figure S5. Sytox Orange staining of mms21-1 mutant shows dead cells in the root meristems.

Supplemental Figure S6. Effects of DNA-damaging treatments on the expression of stem cell niche-defining genes.

Supplemental Figure S7. The root meristem of mms21-1 mutants shows increased sensitivity to DNA-damaging treatments.

Supplemental Table S1. AtMMS21 is required for root stem cell niche maintenance.

Supplemental Table S2. Frequency of embryos exhibiting defective basal region among the progenies of mms21-1 mutants.

Supplemental Table S3. Primers used in this study.

ACKNOWLEDGMENTS

We thank Philip Benfey (Duke University), Ben Scheres (Utrecht University), Lieven De Veylder (Ghent University), A.B. Britt (University of California), Lizhen Tao (South China Agricultural University) and ABRC (Ohio State University) for kindly providing seeds used in this study.
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**FIGURE LEGENDS**

**Figure 1.** Pattern of cell division and cell differentiation is defective in the *mms21-1* root meristem. A, Phenotype of WT and *mms21-1* seedlings at 5 DAG. Bar = 1 cm. B and C, Root tips of WT and *mms21-1* at 5 DAG. The QC is marked in red, and the QC is surrounded by stem cells: endodermal/cortical stem cells (green), vascular stem cells (yellow) and columella stem cells (blue). White and black arrowheads indicate the QC and the first elongated cortex cell, respectively. Bar = 50 μm. D, Root meristem cell number of the WT and *mms21-1* from 1 to 14 DAG. Data shown are average ± SD (n = 30), and asterisks indicate significant differences compared with control plants (P < 0.005; Student’s *t*-test). E and F, Root tips of WT and *mms21-1* at 9 DAG. Arrow and arrowhead in (F) indicate root hair and mature protoxylem cells, respectively. Bar = 100 μm. G, Meristem collapse frequency at different time points for WT and *mms21-1*. Values are mean ± SD (n = 20) of three biological replicates. H to J, Expression of ground tissue marker *J0571* in the WT and *mms21-1* at 5 DAG. The region with altered cell division plane is magnified in the inset (I). The arrowhead and arrow indicate discontinuous expression and ectopic expression of *J0571*, respectively. Bar = 20 μm.

**Figure 2.** AtMMS21 is essential for QC organization, identity and function. A and B, Longitudinal view of the stem cell niche of WT and *mms21-1* roots at 5 DAG stained with PI showing cells of the QC (arrowhead) and columella stem cells (arrow). C and D, EdU incorporation assays in WT and *mms21-1* root meristems. The QC cells are marked with green fluorescence of *pWOX5:GFP* and EdU positive nuclei are marked by red fluorescence. WT QC cells do not have red fluorescent nuclei, indicating that the QC is not mitotically active; by contrast, red fluorescent EdU positive nuclei in the *mms21-1* QC cells indicate a state of active division (arrow). E to F, Expression pattern of *pWOX5:GFP* in the WT and *mms21-1* at 5 DAG. H to J, Double staining of *QC25:GUS* marker and starch granules (purple) in 5 DAG WT and *mms21-1*. The arrowhead and arrow in WT indicate QC and columella initials, respectively. Arrows in *mms21-1* indicate cells in the region of the QC and columella stem cells. Bar = 20 μm in (A-G); 50 μm in (H-J).

**Figure 3.** Mutation of AtMMS21 caused misexpression of stem cell niche-defining transcription factors. Expression pattern of stem cell marker genes in the roots of WT and
mms21-1 at 5 DAG. A and B, pSHR:GFP; C and D, pSHR:SHR:GFP; E to G, pSCR:GFP; H to J, PLT1pro:PLT1:YFP; K to M, PLT2pro:PLT2:YFP. The insets in (A-D) show the expression of SHR, observed in the GFP channel. The arrowhead in panel (D) indicates SHR localization was missing in some cell files in the mms21-1 roots. The arrowhead and arrow in panel (F, G) indicate discontinuous expression and ectopic expression of pSCR:GFP, respectively. Bar =20 μm.

Figure 4. mms21-1 shows defects in the root stem cell niche during embryogenesis. A to D, WT embryos in late globular (A), early heart (B), middle heart (C) and cotyledon (D) stages. F to I, mms21-1 mutants embryos at stages equivalent to (A-D). Arrows point to aberrant cell divisions in the basal embryo domain of mms21-1 mutants. E and J, QC25 expression in heart stage embryos of WT and mms21-1. K to M, QC25 expression in mature stage embryos of WT and mms21-1. N to Q, pWOX5:GFP expression in mature stage embryos of WT and mms21-1 (P shows the outlined area in N, and Q shows the outlined area in O, observed in the GFP channel). R to U, Expression patterns of PLT2pro:PLT2:YFP in WT and mms21-1 embryos. Bar =20 μm.

Figure 5. mms21-1 roots show a stem-cell death phenotype and elevated levels of DSBs. A, Root stem cells and their early descendants stained with PI in the mms21-1 roots at 1 DAG, indicating cell death. B, PI staining of WT (top) and mms21-1 (bottom) after treatment with 15 μM cisplatin for 0 hour (left), 24 hours (center) and 48 hours (right). Bar =20 μm. C, Real-time RT-PCR analysis of the expression of DSB-inducible genes in WT and mms21-1 roots. Data are means ± SD of three biological repeats. Asterisks indicate significant differences compared with control plants (P < 0.005; Student’s t-test). D, Representative examples of nuclei seen in the comet assay from the WT (top) and mms21-1 (bottom) roots. E, DNA damage as measured by the percentage of DNA in the tail of nuclei in comet assays for WT and mms21-1 roots. The mean value of more than 200 nuclei is shown with SD bars. The asterisk indicates a statistically significant difference according to Student’s t-test (P < 0.01).

Figure 6. mms21-1 roots show increased sensitivity to DNA-damaging agents. A, Phenotypes of WT and mms21-1 grown on MS (top) and medium containing 50 μM cisplatin (middle) or
0.075% MMS (bottom). 3-DAG seedlings were transplanted onto medium for 14 days. Bar = 1 cm. B and C, Sensitivity of root growth to DNA-damaging agents. 3-DAG seedlings were transplanted onto medium containing various concentrations of MMS or cisplatin for three days. Relative root elongation is the ratio of root elongation in medium containing the DNA-damaging agents to root elongation in control medium. Each point represents the average of the results from 20 to 30 seedlings, and error bars represent SD. Asterisks indicate significant differences (P < 0.01; Student’s t-test) relative to WT. D, Effects of cisplatin on the root meristem and the expression of QC25:GUS in WT (top) and mms21-1 (bottom). Treatments as described in (B and C). Bar = 50 μm.

Figure 7. Arabidopsis MMS21 interacts with SMC5. A, AtMMS21 colocalizes with AtSMC5 predominantly in the nucleus. Arabidopsis protoplasts were cotransfected with CFP-AtMMS21 and YFP-AtSMC5. CFP/YFP signals were visualized by confocal microscopy using different channels. Bar = 10 μm. B, AtMMS21 interacts with AtSMC5 in yeast two-hybrid assay. Yeast cells were cotransformed with: pBD-53+pAD-T (positive control); pBD-Lam+pAD-T (negative control); pADMMS2+pGBK7; pGADT7+pBDSMC5; pADMMS21+pBDSMC5. Blue colonies in an X-Gal assay show an interaction in vivo between AtMMS21 and AtSMC5. C, BiFC analysis of the interaction between AtMMS21 and AtSMC5 in onion epidermal cells by confocal microscopy. Images were acquired in the YFP and differential interference contrast (DIC) channels, respectively, and then merged together. Cell nuclei were stained with DAPI, showing a strong interaction between AtMMS21 and AtSMC5 in the nucleus. D and E, Functional similarity between mms21-1 and smc5-2 mutants. As in mms2-1 mutants (see Fig. 4 and Fig. S4 for mms21-1 embryos), the embryos of SMC5/smc5-2 siliques showed aberrant cell plate formation in the basal embryo region from the early stages of embryogenesis (D), and likely give rise to seedlings germinating without a main root (E). Bar = 20 μm in (D); 1 cm in (E).

Figure 8. A model for AtMMS21 functions in stem cell niche maintenance and DNA damage responses in the root meristem. Subunit composition of SMC5/6 complexes in Arabidopsis based on the model of Watanabe et al. (2009), SMC5-SMC6 heterodimers associate with the δ-kleisin NSE4. Here we demonstrate that AtMMS21 is an additional subunit of the SMC5/6
complex via direct binding of SMC5. AtMMS21 controls two important processes influencing the root stem cell niche maintenance. First, AtMMS21 is essential for proper identity of QC and stem cells by stable expression of the stem cell niche-defining transcription factors within the niches. Ishida et al. (2009) showed that AtMMS21/HPY2 functions downstream of PLT1/2; we therefore suggest that a feedback loop exists between AtMMS21 and PLT1/2 in maintaining the root stem cell niche. Second, AtMMS21 is required to prevent cell death, early differentiation, premature endocycle onset and misregulation of stem cell factors in the root meristem through a reduction in DSBs. Current knowledge about AtMMS21 highlights that AtMMS21-mediated regulation of the balance between cell division, cell endocycle, cell differentiation and cell death is critical for the normal cellular organization and function of the root meristem. QC: quiescent center; SCN: stem cell niche; SCs: stem cells.

Supplemental Figure S1. Marker gene expression in mms21-1 roots. A, Double staining of QC markers (QC46:GUS, QC184:GUS) and starch granules in WT and mms21-1 at 5 DAG. Bar = 50 μm. B, Expression of marker J2341 was expanded to more than several cell layers in mms21-1. C, Root cap marker J1092 was expressed in the defective columella layers of mms21-1 roots. D, Real-time RT-PCR analysis of the expression of root stem cell marker genes in mms21-1 roots. Data presented are mean values of three biological repeats ±SD. Asterisks indicate significant differences (P < 0.05; Student’s t-test) relative to WT. Bar = 20 μm in (B-C).

Supplemental Figure S2. The shr-1 and scr-1 mutants show little effect on the expression of AtMMS21. Relative expression levels of AtMMS21 were detected by qRT-PCR in the shr-1 seedlings and scr-1 roots compared with WT at 5 DAG. Data presented are mean values of three biological repeats ±SD.

Supplemental Figure S3. Maintenance of the stem cell niche is defective in mms21-1 roots from 1 DAG. A and B, Double staining of QC markers (QC25:GUS, QC46:GUS) and starch granules in WT and mms21-1 at 1 DAG. The arrowhead and arrow in WT indicate QC and columella initials, respectively. Arrows in mms21-1 indicate cells in the region of QC and columella stem cells. Bar = 100 μm. C-G, Expression pattern of pWOX5:GFP (C), pSHR:GFP...
(D), pSCR:GFP (E) and PLT1pro:PLT1:YFP (F), PLT2pro:PLT2:YFP (G) in the WT and mms21-1 at 1 DAG. Bar = 20 μm.

**Supplemental Figure S4.** Defects in the stem cell niche in mms21-1 embryos. A and E, WT embryos at early globular stage (A) and globular stage (E). B-D and F-G, Defective mms21-1 embryos at early globular stage (B-D) and globular stage (F-G), respectively. H, pWOX5:GFP expression in heart stage embryo of WT and mms21-1. I and J, Expression pattern of PLT1pro:PLT1:YFP in WT and mms21-1 embryo. Bar = 20 μm.

**Supplemental Figure S5.** Sytox Orange staining of mms21-1 mutants shows dead cells in the root meristems. Root tips of WT (A, C) and mms21-1 (B, D) were stained with Sytox Orange which stains dead cells at 1 DAG (A, B) and 5 DAG (C, D). Arrows indicate the dead cells. Bar = 50 μm.

**Supplemental Figure S6.** Effects of DNA-damaging treatments on the expression of stem cell niche-defining genes. 3-DAG seedlings were transplanted onto medium containing 15 μM cisplatin for one or two days. A-F and M-P, Confocal images of pWOX5:GFP (A, B), pSHR:GFP (C, D), pSCR:GFP (E, F), pPLT1:PLT1:YFP (M, N) and pPLT1:PLT1:YFP (O, P) seedlings which were treated with 0 μM (A, C, E, M, O) or 15 μM cisplatin (B, D, F, N, P) for one day. G-L and Q-T, Confocal images of pWOX5:GFP (G, H), pSHR:GFP (I, J), pSCR:GFP (K, L), pPLT1:PLT1:YFP (Q, R) and pPLT1:PLT1:YFP (S, T) seedlings which were treated with 0 μM (G, I, K, Q, S) or 15 μM cisplatin (H, J, L, R, T) for two days. Arrowheads and arrows indicate the QC and dead cells, respectively. Bar = 50 μm.

**Supplemental Figure S7.** The root meristem of mms21-1 mutants exhibits increased sensitivity to DNA-damaging treatments. Effects of DNA-damaging agents on the root meristem of WT (top) and mms21-1 mutants (bottom). 3-DAG root tips of WT and mms21-1 were treated either with 0 μM cisplatin/MMS (left), 15 μM cisplatin (center), or 0.005% MMS (right) for 3 days. Bar = 100 μm.

**Supplemental Table S1.** AtMMS21 is required for root stem cell niche maintenance.
Supplemental Table S2. Frequency of embryos exhibiting defective basal region among the progenies of mns21-1 mutants.

Supplemental Table S3. Primers used in this study.
Figure 1. Pattern of cell division and cell differentiation is defective in the mms21-1 root meristem. A, Phenotype of WT and mms21-1 seedlings at 5 DAG. Bar = 1 cm. B and C, Root tips of WT and mms21-1 at 5 DAG. The QC is marked in red, and the QC is surrounded by stem cells: endodermal/cortical stem cells (green), vascular stem cells (yellow) and columella stem cells (blue). White and black arrowheads indicate the QC and the first elongated cortex cell, respectively. Bar = 50 μm. D, Root meristem cell number of the WT and mms21-1 from 1 to 14 DAG. Data shown are average ± SD (n = 30), and asterisks indicate significant differences compared with control plants (P < 0.005; Student’s t-test). E and F, Root tips of WT and mms21-1 at 9 DAG. Arrow and arrowhead in (F) indicate root hair and mature protoxylem cells, respectively. Bar = 100 μm. G, Meristem collapse frequency at different time points for WT and mms21-1. Values are mean ± SD (n = 20) of three biological replicates. H to J, Expression of ground tissue marker J0571 in the WT and mms21-1 at 5 DAG. The region with altered cell division plane is magnified in the inset (J). The yellow and red arrowheads indicate discontinuous expression and ectopic expression of J0571, respectively. Bar = 20 μm.
Figure 2. AtMMS21 is essential for QC organization, identity and function. A and B, Longitudinal view of the stem cell niche of WT and mms21-1 roots at 5 DAG stained with PI showing cells of the QC (arrowhead) and columella stem cells (arrow). C and D, EdU incorporation assays in WT and mms21-1 root meristems. The QC cells are marked with green fluorescence of $pWOX5:GFP$ and EdU positive nuclei are marked by red fluorescence. WT QC cells do not have red fluorescent nuclei, indicating that the QC is not mitotically active; by contrast, red fluorescent EdU positive nuclei in the mms21-1 QC cells indicate a state of active division (arrow). E to F, Expression pattern of $pWOX5:GFP$ in the WT and mms21-1 at 5 DAG. H to J, Double staining of QC25:GUS marker and starch granules (purple) in 5 DAG WT and mms21-1. The arrowhead and arrow in WT indicate QC and columella initials, respectively. Arrows in mms21-1 indicate cells in the region of the QC and columella stem cells. Bar = 20 μm in (A-G); 50 μm in (H-J).
Figure 3. Mutation of AtMMS21 caused misexpression of stem cell niche-defining transcription factors. Expression pattern of stem cell marker genes in the roots of WT and mms21-1 at 5 DAG. A and B, pSHR:GFP; C and D, pSHR:SHR:GFP; E to G, pSCR:GFP; H to J, PLT1pro:PLT1:YFP; K to M, PLT2pro:PLT2:YFP. The insets in (A-D) show the expression of SHR, observed in the GFP channel. The arrowhead in panel (D) indicates SHR localization was missing in some cell files in the mms21-1 roots. The arrowhead and arrow in panel (F, G) indicate discontinuous expression and ectopic expression of pSCR:GFP, respectively. Bar = 20 μm.
Figure 4. *mms21-1* shows defects in the root stem cell niche during embryogenesis. A to D, WT embryos in late globular (A), early heart (B), middle heart (C) and cotyledon (D) stages. F to I, *mms21-1* mutants embryos at stages equivalent to (A-D). Arrows point to aberrant cell divisions in the basal embryo domain of *mms21-1* mutants. E and J, *QC25* expression in heart stage embryos of WT and *mms21-1*. K to M, *QC25* expression in mature stage embryos of WT and *mms21-1*. N to Q, *pWOX5:GFP* expression in mature stage embryos of WT and *mms21-1* (P shows the outlined area in N, and Q shows the outlined area in O, observed in the GFP channel). R to U, Expression patterns of *PLT2:YFP* in WT and *mms21-1* embryos. Bar =20 μm.
Figure 5. *mms21-1* roots show a stem-cell death phenotype and elevated levels of DSBs. 

A, Root stem cells and their early descendants stained with PI in the *mms21-1* roots at 1 DAG, indicating cell death. B, PI staining of WT (top) and *mms21-1* (bottom) after treatment with 15 μM cisplatin for 0 hour (left), 24 hours (center) and 48 hours (right). Bar =20 μm. C, Real-time RT-PCR analysis of the expression of DSB-inducible genes in WT and *mms21-1* roots. Data are means ± SD of three biological repeats. Asterisks indicate significant differences compared with control plants (P < 0.005; Student’s t-test). D, Representative examples of nuclei seen in the comet assay from the WT (top) and *mms21-1* (bottom) roots. E, DNA damage as measured by the percentage of DNA in the tail of comet assays for WT and *mms21-1* roots. The mean value of more than 200 nuclei is shown with SD bars. The asterisk indicates a statistically significant difference according to Student’s t-test (P < 0.01).
Figure 6. *mms21-1* roots show increased sensitivity to DNA-damaging agents. A, Phenotypes of WT and *mms21-1* grown on MS (top) and medium containing 50 μM cisplatin (middle) or 0.075% MMS (bottom). 3-DAG seedlings were transplanted onto medium for 14 days. Bar = 1 cm. B and C, Sensitivity of root growth to DNA-damaging agents. 3-DAG seedlings were transplanted onto medium containing various concentrations of MMS or cisplatin for three days. Relative root elongation is the ratio of root elongation in medium containing the DNA-damaging agents to root elongation in control medium. Each point represents the average of the results from 20 to 30 seedlings, and error bars represent SD. Asterisks indicate significant differences (P < 0.01; Student's t-test). D, Effect of cisplatin on the root meristem and the expression of *QC25:GUS* in WT (top) and *mms21-1* (bottom). Treatments as described in (B and C). Bar = 50 μm.
Figure 7. Arabidopsis MMS21 interacts with SMC5. A, AtMMS21 colocalizes with AtSMC5 predominantly in the nucleus. Arabidopsis protoplasts were cotransfected with CFP-AtMMS21 and YFP-AtSMC5. CFP/YFP signals were visualized by confocal microscopy using different channels. Bar = 10 μm. B, AtMMS21 interacts with AtSMC5 in yeast two-hybrid assay. Yeast cells were cotransformed with: pBD-53+pAD-T (positive control); pBD-Lam+pAD-T (negative control); pADMMS21+pGBK7; pGADT7+pBDSMC5; pADMMS21+pBDSMC5. Blue colonies in an X-Gal assay show an interaction in vivo between AtMMS21 and AtSMC5. C, BiFC analysis of the interaction between AtMMS21 and AtSMC5 in onion epidermal cells by confocal microscopy. Images were acquired in the YFP and differential interference contrast (DIC) channels, respectively, and then merged together. Cell nuclei were stained with DAPI, showing a strong interaction between AtMMS21 and AtSMC5 in the nucleus. D and E, Functional similarity between mms21-1 and smc5-2 mutants. As in mms2-1 mutants (see Fig. 4 and Fig. S4 for mms21-1 embryos), the embryos of SMC5/smcb5-2 siliques showed aberrant cell plate formation in the basal embryo region from 14 DAF to 16 DAF. These embryos likely give rise to seedlings germinating without a main root (E). Bar = 20 μm in (D); 1 cm in (E).
Figure 8. A model for AtMMS21 functions in stem cell niche maintenance and DNA damage responses in the root meristem. Subunit composition of SMC5/6 complexes in Arabidopsis based on the model of Watanabe et al. (2009), SMC5-SMC6 heterodimers associate with the δ-kleisin NSE4. Here we demonstrate that AtMMS21 is an additional subunit of the SMC5/6 complex via direct binding of SMC5. AtMMS21 controls two important processes influencing the root stem cell niche maintenance. First, AtMMS21 is essential for proper identity of QC and stem cells by stable expression of the stem cell niche-defining transcription factors within the niches. Ishida et al. (2009) showed that AtMMS21/HPY2 functions downstream of PLT1/2; we therefore suggest that a feedback loop exists between AtMMS21 and PLT1/2 in maintaining the root stem cell niche. Second, AtMMS21 is required to prevent cell death, early differentiation, premature endocycle onset and misregulation of stem cell factors in the root meristem through a reduction in DSBs. Current knowledge about AtMMS21 highlights that AtMMS21-mediated regulation of the balance between cell division, cell endocycle, cell differentiation and cell death is critical for the normal cellular organization and function of the root meristem. QC: quiescent center; SCN: stem cell niche; SCs: stem cells.