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CENTRIN2 Modulates Homologous Recombination and Nucleotide Excision Repair in Arabidopsis [W]

Jean Molinier,[1] Cynthia Ramos,[2] Olivier Fritsch, and Barbara Hohn

Friedrich Miescher-Institute for Biomedical Research, CH-4058 Basel, Switzerland

A genetic screen of a population of *Arabidopsis thaliana* lines exhibiting enhanced somatic homologous recombination yielded a mutant affected in expression of a gene encoding a caltractin-like protein (centrin). The hyperrecombinogenic phenotype could be reproduced using RNA interference (RNAi) technology. Both the original mutant and the RNAi plants exhibited a moderate UV-C sensitivity as well as a reduced efficiency of in vitro repair of UV-damaged DNA. Transcription profiling of the mutant showed that expression of components of the nucleotide excision repair (NER) pathway and of factors involved in other DNA repair processes were significantly changed. Our data suggest an indirect involvement of centrin in recombinational DNA repair via the modulation of the NER pathway. These findings thus point to a novel interconnection between an early step of NER and homologous recombination, which may play a critical role in plant DNA repair.

INTRODUCTION

Plants, because of their lifestyle, have to adapt rapidly to environmental stresses, such as radiation, pathogens, heavy metals, and pollutants, that cause various types of cellular lesions, including membrane oxidation and DNA damage. These stresses can affect cell and genome integrity, leading to irreversible cellular damage that can interfere with plant development. Genetic lesions can be transmitted to the germ line and to the offspring. To preserve genome integrity, DNA repair pathways, such as direct repair (photolyase), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double-strand break (DSB) repair (Puchta and Hohn, 1996; Vonarx et al., 1998; Britt, 1999), have to be activated. The especially harmful DSBs are repaired by two pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ seals DNA ends directly, but this process can lead to sequence alterations. By contrast, HR uses homologous DNA templates for repair and therefore contributes to preserve genome integrity. The choice and the efficiency of one or the other pathway thus critically determine genome stability versus genome flexibility (Puchta and Hohn, 1996). Whereas HR is the preferential mechanism used in yeast (*Saccharomyces cerevisiae*) (Pâques and Haber, 1999), NHEJ seems to be the preferred pathway for repair of DSB damage in plants and mammals (Puchta and Hohn, 1996; Jeggo, 1998; Gorbunova and Levy, 1999). It is still an open debate if this is because of special features of the HR machinery in these higher eukaryotes.

Indirect approaches have been developed to isolate plant genes involved in the HR process. Plant orthologs of some recombination and repair genes were isolated (Klimyuk and Jones, 1997; Doutriaux et al., 1998; Gallego et al., 2000; Hartung and Puchta, 2000; Hays, 2002). Disruption of the *Arabidopsis thaliana Rad50* gene, for instance, leads to elevated somatic homologous recombination frequency (HRF) as compared with the wild type (Gherbi et al., 2001). In addition, Arabidopsis plants lacking photolyase, involved in photoreactivation of UV-induced DNA damage, exhibit an elevated somatic HRF especially after UV-B exposure (Ries et al., 2000). These studies imply that genetic and environmental factors dictate the use of alternative DNA repair pathways. However, it is not known whether other repair pathways are part of the regulatory network that governs the use of NHEJ or HR. To address this question, a genetic screen for Arabidopsis mutants in which the frequency of HR is upregulated was undertaken. Although recombination mutants have been isolated previously, the nature of most of the affected genes has not been studied (Masson and Paszkowski, 1997; Gorbunova et al., 2000; Lucht et al., 2002).

Arabidopsis plants carrying a recombination substrate were mutagenized with an activation tagging T-DNA (Weigel et al., 2000). One of the isolated hyperrecombinogenic mutants was found to be impaired in expression of a gene encoding a caltractin like-protein, centrin. The affected gene seems to be acting as an upstream regulator in the NER pathway because several components of the NER pathway were found transcriptionally changed in the mutant. This points to a switch mechanism relatively high in the hierarchy of DNA repair regulation.

[1] To whom correspondence should be addressed. E-mail jean.molinier@fmi.ch; fax 41 61 697 39 76.

[2] Current address: Scripps Research Institute, Department of Cell Biology, 10550 North Torrey Pines Road, ICND 222, La Jolla, CA 92037.

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.plantcell.org) is: Jean Molinier (jean.molinier@fmi.ch).

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RESULTS

Isolation and Molecular Characterization of Hyperrecombinogenic Mutant

A genetic approach was used to identify genes affecting the frequency of recombinational DNA repair. The employed Arabidopsis recombination reporter line carried an intermolecular recombination substrate (Figure 1). Recombination between the two homologous sequences of a disrupted *uidA* gene leads to the restoration of the functional reporter gene that can be detected using the histochemical β-glucuronidase (GUS) assay. The line IC9, homozygous for the recombination substrate, was mutagenized with an activation tagging T-DNA (Weigel et al., 2000). Out of ~4200 screened T1 plants, 83 showed at least two GUS positive spots on four independent leaves, whereas in the original IC9, such a frequency would occur in only one out of 1000 plants of a population. In the second generation (T2), 21 plants out of the 83 primary candidates still exhibited a high HRF compared with the control plants (data not shown). One particular mutant, P2.4, exhibited in the T2 generation a 36 ± 1 fold increase in HRF compared with the IC9 control plants, confirming the stability of the hyperrecombinogenic phenotype. Sequence analysis of the T-DNA junction revealed that the right border has inserted in chromosome 4 in the coding region of a gene encoding a caltractin like protein, centrin (At4g37010), one nucleotide upstream of the TAA stop codon (Figure 2A). Because one Arabidopsis centrin gene was already annotated, the gene affected in the P2.4 plant was called At*CEN2*.

RT-PCR analysis of a batch of P2.4 T2 plants demonstrated that the mRNA steady state level of the At*CEN2* gene was lower in the P2.4 mutant than in the control plants (Figure 2B). No measurable changes in expression of the surrounding genes (At4g37020 and At4g37000) were detected (data not shown). The T-DNA insertion may have led to either the production of an antisense transcript or the transcription of an aberrant RNA lacking a stop codon that could have resulted in the strong downregulation of the native mRNA. Both possibilities lead to a dominant negative hyperrecombinogenic phenotype, as observed in T1 plants.

Phenotype Reproduction

To confirm the link between downregulation of At*CEN2* gene expression and the hyperrecombinogenic phenotype, RNA interference (RNAi) strategy was used. The fourth exon, specific for the At*CEN2* gene, was cloned in inverted repeats into the pOEXhp vector (Figure 3A). The recombination lines IC9, used for the screen, and IC6, another independent line with a similar recombination substrate, were transformed with the pOEXhpCEN construct yielding lines that will be called RNAi plants. HRF was measured in T2 families of eight and six independent transformants of IC9 and IC6, respectively. With a 3- to 12-fold and 3- to 15-fold increase of HRF in IC9 and IC6, respectively, the hyperrecombinogenic phenotype could be reproduced (Figure 3B). In addition, upon treatment with increasing UV-C doses, somatic HRF was still higher in At*CEN2*-deficient plants (RNAi or insertional mutant) compared with the control plants (data not shown). This unambiguously shows that the hyperrecombinogenic phenotype is a consequence of the downregulation of At*CEN2* expression. RT-PCR experiments confirmed the lower At*CEN2* mRNA steady state level in these RNAi plants (Figure 3C). In addition, these data indicate that increased HRFs are independent from genomic location of the recombination substrate because the recombination target sequences were integrated at different genomic positions in the two reporter lines. In addition, At*CEN2*-overexpressing plants did not exhibit increased somatic HRF after UV-C treatment, whereas control plants showed a significant increase after UV-C exposure (data not shown). These results confirm that downregulation of At*CEN2* expression leads to the hyperrecombinogenic phenotype.

Figure 1. Schematic Representation of the Intermolecular Recombination Substrate.

T-DNA of the pGRU'S'G'U' plasmid integrated in the IC9 line, carrying two incomplete partially overlapping *uidA* regions with 1213-bp homologous sequence in direct orientation. Hpt, hygromycin phosphotransferase gene; G, β-glucuronidase gene; LB, left border; P, 35S promoter of *Cauliflower mosaic virus*; RB, right border; T, 35S *Cauliflower mosaic virus* terminator.

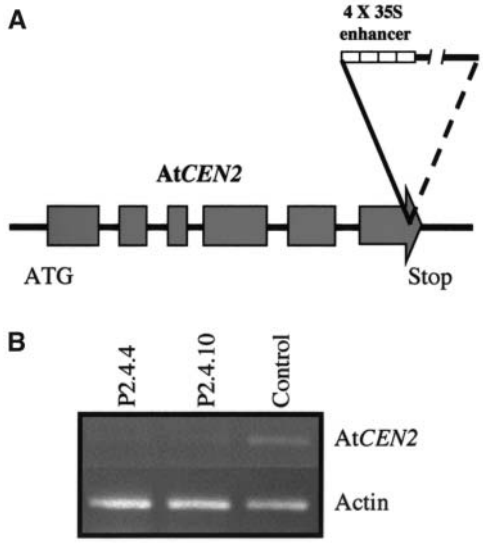

Figure 2. Molecular Analyses of the P2.4 Mutant.

(A) Schematic representation of the insertion site of the mutagenic T-DNA upstream of the stop codon of the At4g37010 gene (At*CEN2*).
(B) Analysis (RT-PCR) of At4g37010 transcript level in IC9 (control) and in two batches of P2.4 T2 plants. Actin 2 primers were used as control for each PCR reaction.

The At*CEN2* Gene Product

The At*CEN2* gene encodes a caltractin-like protein also called centrin, which is a member of the superfamily of the calcium

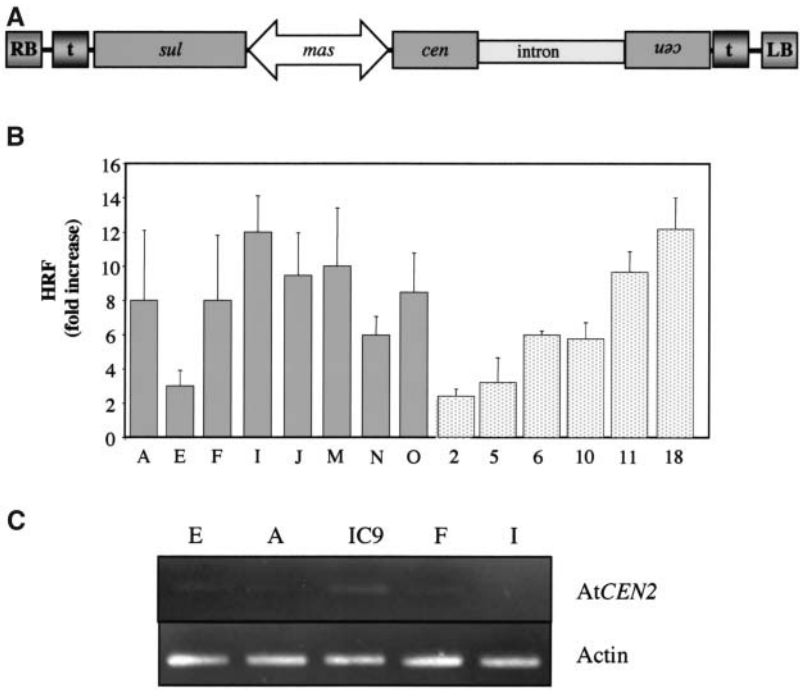

Figure 3. Reproduction of the Hyperrecombinogenic Phenotype by At*CEN2* Downregulation.

(A) Schematic representation of the pOEXhpCEN construct used for downregulation. The fourth exon (156 bp) of the At*CEN2* gene was cloned into the pOEXhp vector in sense and antisense orientations. LB, left border; *mas*, mannopine synthase promoter; RB, right border; *sul*, sulfonamide resistance gene; t, nos terminator.

(B) Enhancements of somatic HRF in two independent lines harboring recombination substrates (IC9, closed bars; IC6, dots). Changes of HRF were calculated relative to the corresponding control (IC9 or IC6 plants). One hundred plants were used per replicate, and experiments were duplicated.

(C) Expression analysis (RT-PCR) of At*CEN2* transcript level in IC9 (control) and in four independent RNAi T2 plants. Actin 2 primers were used as control for each PCR reaction.

binding EF-hand proteins. These are known to be components of centrosomes and are also involved in cell cycle and DNA repair. AtCEN2 contains four EF-hand calcium binding sites (amino acids: EF-hand I 36 to 48, DIDGSGSIDASEL; EF-hand II 72 to 84, DKNQSGAIDFDEF; EF-hand III 109 to 121, DHDNNGKISPRDI; and EF-hand IV 145 to 157, DRDKDGEVNLEEF; Figure 4). The deduced amino acid sequence of the isolated Arabidopsis centrin was aligned with three human centrins and its orthologs from Arabidopsis and *Nicotiana tabacum*. The N-terminal domain contains the hydrophobic and positively charged residues characteristic for the centrin subfamily (Figure 4). It is important to notice that the centrin mutated in the P2.4 plants contains a predicted ATP/GTP binding site (amino acids 10 to 17, GLKPKGKT), whereas the six other centrins did not show this motif (Figure 4). The At*CEN2* gene product shares 47 and 49% identity with the HsCEN1 and HsCEN2 proteins, respectively. The HsCEN2 has been shown to play a role in the NER pathway responsible for the repair of UV-induced DNA damage (Araki et al., 2001). At*CEN2* mRNA expression was analyzed by RT-PCR in different organs of wild-type C0 plants. Transcripts were detectable in leaves, roots, and at a lower level in stems (data not shown). In flower buds and flowers, At*CEN2* transcripts became detectable only if the number of PCR cycles was increased to 40 (data not shown).

At*CEN2*-Defective Plants Are Mildly UV-C Sensitive

The amino acid identity shared by AtCEN2 and HsCEN2 as well as the increased level of HRF in both P2.4 original mutant and RNAi plants stimulated us to evaluate their sensitivity to different DNA damaging agents. Plants did not exhibit any phenotypic change in absence of treatment or in response to the DSB inducing agent bleomycin, to the alkylating agent MMS, and to the cross-linking agent mitomycin C (data not shown). However, both homozygous P2.4 mutant and RNAi plants exhibited a reduction in growth and a pale-green phenotype after exposure to a dose of 30 Kergs/cm^2 of UV-C, a dose that did not affect the control plants (Figure 5A). By contrast, At*CEN2*-overexpressing plants did not exhibit a significant modification in UV-C tolerance compared with wild-type plants under our experimental conditions (data not shown).

The UV-C sensitivity was also assayed in roots of homozygous P2.4 mutant plants, RNAi plants, and in IC9 control plants. Plants were grown vertically on the agar surface, and roots were irradiated with 10 Kergs/cm^2 of UV-C. Relative root growth (root length treated/root length untreated) in percent was determined per day before and after the UV-C treatment. Growth of roots of the mutant relative to that of the control was transiently reduced to 40% in the IC9 control, to 10% in the original mutant, and to

23% in the RNAi plants (Figure 5B). All lines recovered to the same extent in the following days. This higher transient UV-C sensitivity of both the P2.4 mutant and RNAi plants, compared with control plants, was correlated with a transient increase of the steady state level of At*CEN2* mRNA 2 h after UV-C treatment in wild-type plants (data not shown). On the other hand, the At*CEN1* mRNA steady state level was found to be increased 30 min after exposure to UV-C and bleomycin as well as 2 h after treatment with xylanase, a fungal-derived elicitor (J. Molinier, unpublished data).

Efficiency in Repair of UV-C Damaged DNA

The UV-C sensitive phenotype of both the P2.4 mutant and the RNAi plants would imply a defect in a DNA repair pathway. To test this directly, cell extracts of P2.4, RNAi, and IC9 plants were used in the in vitro DNA repair assay described by Li et al. (2002). This assay measures the efficiency of incorporation of DIG dUTP in a UV-C damaged plasmid in the presence of plant cell extracts. Thereby the efficiency of dark repair of UV-induced DNA damage is evaluated. Cell extracts derived from the mutant plants were less efficient in DIG dUTP incorporation after 1 and 2 h of incubation, suggesting a reduced efficiency in repair of UV-C–induced DNA damage (Figure 6A). Extracts from RNAi plants behaved like the original mutant (data not shown). By contrast, cell extracts derived from centrin-overexpressing plants were more efficient in DIG dUTP incorporation, which could be detected as early as 15 min of incubation (Figure 6B). Taken together, these results show a direct correlation between At*CEN2* expression and the NER efficiency.

Microarray Analysis of the Hyperrecombinogenic Mutant

In an attempt to find a link between the hyperrecombination phenotype, UV-C sensitivity, and the lower efficiency of in vitro repair of UV-damaged DNA, the transcriptome of untreated and UV-C–treated mutant plants was analyzed and compared with that of UV-C–irradiated control plants (IC9 line). Samples were harvested from untreated plants and 2 and 6 h after the treatment with UV-C (6 Kergs/cm^2). The expression profile of mutant plants was compared with the corresponding IC9 control, and genes showing the same change (increase/decrease and at least a ± twofold change) in steady state mRNA levels in the two independent experiments were considered as upregulated and downregulated. First, the hybridization signal for the At*CEN2* gene was called "absent" in the mutant plants during the time course of UV-C treatment, whereas it was called "present" in control plants only 2 h after UV-C treatment (see supplemental data online). The At*CEN1* hybridization signal was called "present" and remained unchanged in both At*cen2* and control plants during the analyzed time course. This result confirms that the mutant was specifically downregulated for At*CEN2* expression.

In total, 138 genes were found to be upregulated, whereas 133 were downregulated in untreated At*cen2* plants compared with the IC9 plants (see supplemental data online). Two hours after UV-C irradiation, 607 and 226 genes, respectively, were upregulated and downregulated in the At*CEN2* mutant plants compared with the control plants. Six hours after the treatment, 104 and 119 genes were still found to be upregulated and downregulated in the At*CEN2*-deficient plants compared with the control plants (see supplemental data online). Among this population of genes showing significant changes in the At*CEN2*-deficient plants, several genes known to be involved in DNA repair exhibited interesting expression profiles. Table 1 lists genes encoding proteins known to be involved in the NER pathway, photoreactivation of UV-induced DNA damage, and in the HR process. The transcript level of the *RAD4* gene involved in the early step of recognition of UV DNA damage is the only

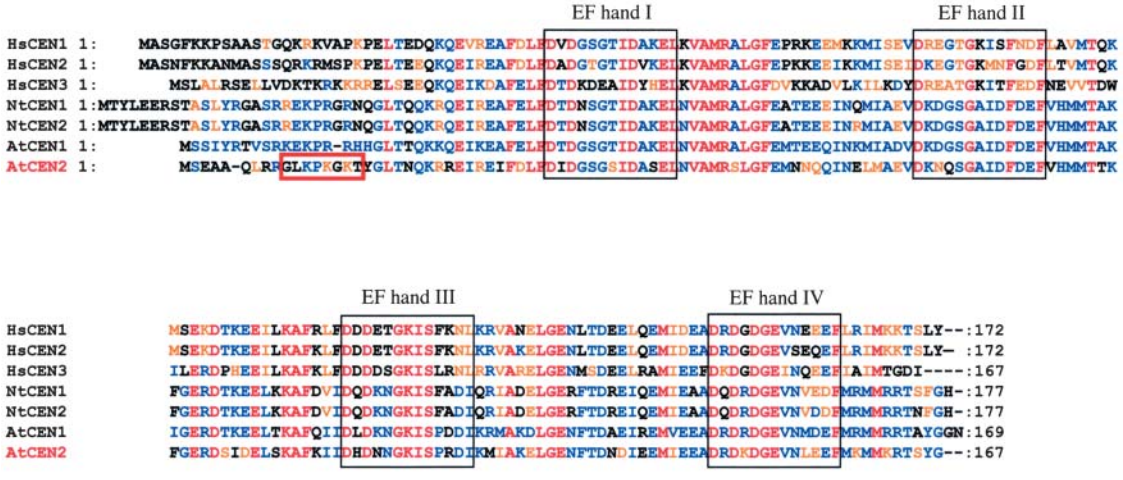

Figure 4. Sequence Alignment of At*CEN2* Gene Product with Six Other Centrins.

Comparison of the amino acid sequence of AtCEN2 from Arabidopsis to the three centrin isoforms from human (Hs), to the two isoforms from tobacco (Nt), and to one AtCEN1 from Arabidopsis (At). Identical amino acids are in red, similar amino acids are in orange, and amino acids sharing 30% conservation are in blue. The ATP/GTP binding domain is highlighted in a red rectangle, and the four EF-hand calcium binding sites are denoted by black rectangles.

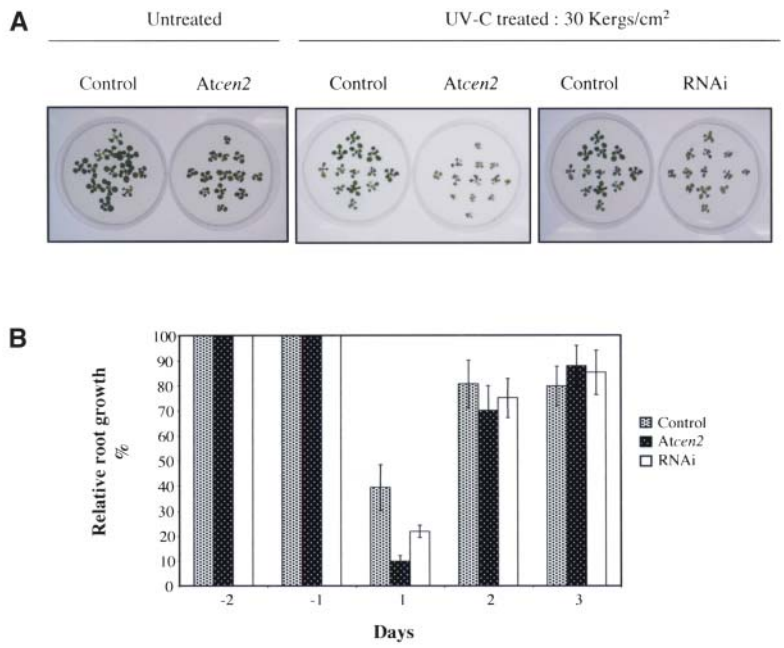

Figure 5. UV-C Sensitivity Assays.

(A) One-week-old mutant (At*cen2*), RNAi (pOEXhpCENA), and control plants (IC9) were exposed to a sublethal UV-C dose (30 Kergs/cm^2). The phenotype was evaluated 1 week after exposure.

(B) Root-growth assay. Three-day-old mutant (At*cen2*), RNAi (pOEXhpCENA), and control plants (IC9) were exposed to 10 Kergs/cm^2 of UV-C. Root growth was measured 2 d before (days −2 and −1) and 3 d after irradiation (days 1, 2, and 3). Root growth was calculated relative to the corresponding untreated plants. Ten plants per replicate were used, and experiments were triplicated.

component of the NER pathway found to be downregulated 2 h after UV-C exposure (Table 1). Most genes encoding components involved in damage recognition (one *RAD23* and *TFB1*), chromatin remodeling (*RAD3* and *RAD25*), dual 5′ incision (*RAD1*), and sealing (*ligase I*) were found to be upregulated in the untreated as well as UV-C–treated centrin mutant (Table 1). A gene encoding a protein involved in photoreactivation (*uvr3* photolyase) was found to be downregulated in the untreated centrin mutant, whereas genes involved, directly or indirectly, in DSB repair (*RAD51C* and *ATM*) were found to be upregulated in the centrin mutant (Table 1).

By contrast, steady state levels of mRNA of other genes encoding repair proteins known to be involved in DSB repair, such as *RAD50*, *RAD51*, *RAD54*, and *Ligase IV*, did not exhibit significant changes in the mutant under our conditions (data not shown). Interestingly, the *MIM* gene encoding a Structural Maintenance of Chromosome-like (SMC) protein was found to be upregulated in the At*CEN2* mutant (Table 1).

DISCUSSION

Genetic changes that may occur in somatic plant cells can eventually be passed to the progeny through meiosis because plants produce gametes from somatic lineages (Puchta and Hohn, 1996). Thus, repair mechanisms must be activated and controlled to prevent genetic aberrations to be transmitted to the progeny. As a consequence, it is expected that links between different repair pathways exist to efficiently maintain genome stability.

In both yeast and mammals, several examples exist for interconnections between repair pathways; when factors involved in the NER or the MMR pathways were deregulated, recombinational DNA repair was shown to be affected. A direct involvement of RAD1 (NER) or MSH2 (MMR) in the HR process has been demonstrated (Liang et al., 1998; Pâques and Haber, 1999; Elliott and Jasin, 2001; Villemure et al., 2003). Deregulation of an upstream step of the BER pathway in yeast has been documented to indirectly influence recombinational DNA repair (Hendricks et al., 2002). In bacteria, yeast, and mammals, a combination of NER and HR was shown to be required for inter/intrastrand cross-link repair; inter/intrastrand cross-link removal is performed by the NER machinery, whereas the resulting secondary repair substrates, DSBs, can be repaired by HR (Cole and Sinden, 1975; Jachymczyk et al., 1981; De Silva et al., 2000).

However, connections between different repair pathways have not been thoroughly studied in plants. Therefore, a genetic screen aimed at the isolation of Arabidopsis mutants with enhanced levels of somatic HR was employed to identify new components required for either HR or balancing of DNA repair pathways.

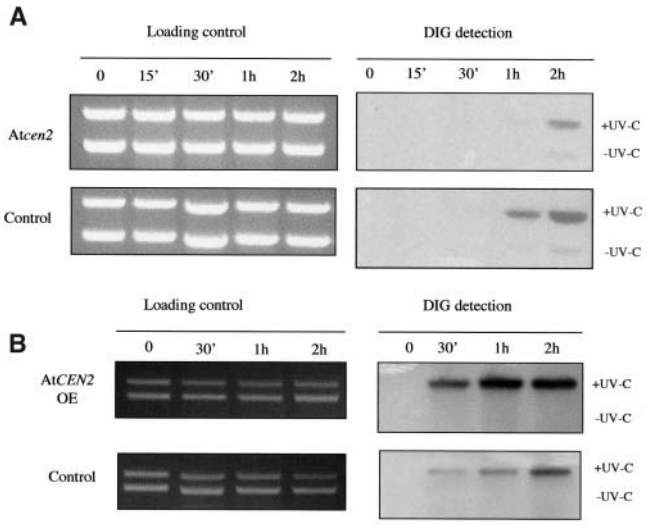

Figure 6. In Vitro DNA Repair Assay of UV-C–Damaged Plasmid.

Cell extracts (25 μg) from mutant (At*cen2*) and control (IC9) plants **(A)** and from centrin overexpressor and control (wild-type) plants **(B)** were incubated with UV-C–damaged (UV-C–treated pGEX, +UV-C) and control (untreated pBKS, -UV-C) plasmids in the presence of DIG dUTP. Incorporation was evaluated during a time course.

Hyperrecombinogenic At*cen2* Plants Exhibit a Reduced Efficiency of Repair of UV Damage

Somatic HR frequency of a mutant was increased by >35-fold. The isolated hyperrecombinogenic mutant exhibited a reduced mRNA steady state level of a gene encoding a caltractin-like protein, centrin. Centrin is a ubiquitous protein component of centrosomes and mitotic spindle poles (Salisbury, 1995) and is a member of the highly conserved superfamily of calcium binding EF-hand proteins. Centrin was discovered in the flagellar apparatus of the unicellular algae *Chlamydomonas reinhardtii* and was shown to be responsible for the contraction of the calcium-sensitive structures (Huang et al., 1988; Salisbury et al., 1988). The yeast centrin homolog Cdc31p is essential for cell viability by contributing to cell integrity/morphology and spindle pole body duplication (Spang et al., 1993). In plants, two tobacco centrins (*Nt*CEN1 and *Nt*CEN2) have been shown to be associated with microsomes but not with the microtubule organizing centers (Stoppin-Mellet et al., 1999), whereas a centrin gene (*ap3.3a*) was found to be induced upon inoculation of Arabidopsis plants by bacteria (Cordeiro et al., 1998). The Arabidopsis centrin protein AtCEN2 shares 49% identity with human centrin 2 (HsCEN2), which has been shown to stabilize xeroderma pigmentosum group C (XPC) in cooperation with HR23B. This led to efficient stimulation of the NER in vitro (Araki et al., 2001). However, in this study, a biological link between the NER process with other repair pathways, such as recombinational DNA, has not been established.

NER is a DNA repair pathway that contributes to global genome repair and that can eliminate most DNA lesions, specifically photochemical lesions such as cyclobutane pyrimidine dimers and (6-4)-photoproducts (Sancar, 1996). XPC plays a role in the first step of the NER by recognizing the UV-induced DNA damage. In our study, cell extracts prepared from plants that are downregulated in At*CEN2* expression were found to be less efficient in dark repair of UV-induced DNA damage, whereas extracts from overexpressing plants showed an elevated capacity of repair of such lesions. In addition, At*cen2* plants showed elevated sensitivity to UV-C irradiation as compared with the control plants. These observations thus point to an involvement of AtCEN2 in the repair of UV-C–induced DNA damage. Apart from CEN2, orthologs for the two other components of the human XPC complex (RAD4 and RAD23) also exist in Arabidopsis (for review, see Hays, 2002). The reduced amounts of AtCEN2 may reduce the stability of a plant RAD4-RAD23 complex leading to a defect in recognition of UV DNA damage. In parallel to this lower CEN2 amount, the mRNA steady state level of one of the RAD23 encoding genes was found to be increased in the mutant plant. This may lead to a higher RAD23 protein content that may in part compensate for the loss of CEN2 by stabilizing the recognition complex. Although the regulation of the activity of the Arabidopsis XPC factor AtRAD4 is expected to be on the protein level, the At*RAD4* mRNA steady state level is also lower in the mutant 2 h after UV-C irradiation. These results suggest that in this mutant, the whole recognition step of UV-induced damage is affected at different levels, the outcome of which is a reduced efficiency of NER as well as UV-C sensitivity. Therefore, the accumulation of unrepaired lesions and nicks may lead to the formation of secondary damage, such as DSBs that may influence the use of HR (Figure 7). In addition, this suggests that a novel link between an early step of NER and HR exists.

Why Do At*cen2* Plants Exhibit a Hyperrecombinogenic Phenotype?

To explain the link between downregulation of expression of the NER protein CEN2 and enhancement of HR, several not mutually exclusive scenarios can be considered: (1) the accumulation of an increase level of DNA lesions provides more substrates for recombinational DNA repair, (2) the use of common regulatory pathways (checkpoints) activates downstream components of repair processes, and (3) common effectors (enzymes/chromatin remodeling factors) are in operation that specifically help in the recombinational DNA repair process. A functional genomic approach was used to identify possible contributions of the above-mentioned hypothetical links to the hyperrecombinogenic phenotype of the At*CEN2*-deficient plants.

The expression profile of At*CEN2*-defective plants showed that the mRNA steady state levels of several factors involved in DNA repair processes were affected. (1) Untreated At*cen2* plants showed a lower mRNA steady state level of photolyase (*uvr3*), which is involved in direct repair by photoreactivation of UV-induced DNA damage (Britt, 1999). The downregulation of the main component of this direct repair pathway, as found in the mutant, correlates with the results obtained by Ries et al. (2000), who observed that photolyase-deficient Arabidopsis plants exhibited a higher somatic HRF in both absence and presence of UV-B irradiation. Thus, a decrease of photolyase transcripts,

Table 1. Expression Profiles of Arabidopsis Components of the NER, Direct Repair, and Recombination Pathways in the Centrin Mutant

Repair Factor	Affymetrix Probe Set Name	MIPS Access Number	Repair Pathway	Role	Time after UV-C Exposure 0	2 h	6 h
RAD 4 [XPC]	250105_at	At5g16630	NER	Recognition	0.8	*0.4*	0.8
	261352_at	At1g79650		Stabilizes the	1.1	1.1	0.9
RAD 23	262708_at	At1g16190	NER	recognition	NC	NC	0.9
[HHRad23]	258499_at	At3g02540		complex	0.7	0.9	1.0
	249512_at	At5g38470			**2.1**	**2.3**	1.2
TFBI [p62]	264548_at	At1g55680	NER	TFIIH	**4.1**	1.2	1.0
RAD 25 [XPB]	249307_s_at	At5g41370	NER	Chromatin remodeling	**4.2**	**2.2**	**2.7**
RAD 3 [XPD]	264356_at	At1g03190	NER	Chromatin remodeling	**2.3**	0.7	1.5
RAD 1 [XPF]	249292_at	At5g41150	NER	5′ Incision with Rad10	**2.6**	**2.2**	**2.6**
Ligase 1	256374_at	At1g66730	NER	Sealing after repair synthesis	NC	**7.1**	1.0
Photolyase *uvr3*	258227_at	At3g15620	Direct repair	Photoreactivation	*0.4*	NC	NC
ATM	252350_at	At3g48190	Rec	Signaling	**2.9**	NC	0.9
RAD51C	245417_at	At2g45280	Rec	Strand exchange	NC	**2.7**	NC
MIM	247528_at	At5g61460	Rec	Chromatin structure	**10.8**	1.5	NC

Expression profiles of some Arabidopsis components of the NER, direct repair (photoreactivation), and recombination (Rec) pathways in the centrin mutant before (time point 0) and after (time points 2 h and 6 h) exposure to UV-C irradiation (6 Kergs/cm^2). For each time point, the expression level (average difference) was compared to the corresponding control. Human orthologs are indicated in brackets. Changes are expressed as fold change. NC, not calculated (absent flag in one of the replicates); upregulation is shown in bold; downregulation is shown in italics.

combined with the lower NER efficiency, may lead to the formation of more DSBs that can serve as substrates for the HR process (Figure 7).

Surprisingly the At*RAD1* gene, encoding an endonuclease, which together with Rad10p (ERCC1) is involved in the 5′ incision of the UV damage, was found to be upregulated in the At*cen2* plants compared with the control. These results are in apparent conflict with the work of Fidantsef et al. (2000) and Li et al. (2002) who have shown that *RAD1*-deficient Arabidopsis plants exhibited higher UV-C sensitivity and reduced efficiency of dark repair of UV-induced DNA damage. Therefore, our data suggest that the increased load of damage, because of downregulation of photolyase and to lower NER efficency, may be responsible for the increased *Rad1* mRNA level. However, this higher *RAD1* transcipt level is not directly responsible for the reduced NER efficiency; it rather points to an activity in another step of the DNA repair process. On the other hand, a direct role for the Arabidopsis Rad1p endonuclease in HR has been recently shown; *rad1* mutant plants exhibited a reduced level of removal of nonhomologous overhangs compared with wild-type plants (Dubest et al., 2002). Thus, the higher *RAD1* mRNA steady state level in the centrin mutant may contribute to the higher HRF by an efficient removal of nonhomologous tails during the recombination process. Nevertheless, it cannot be excluded that the upregulation of At*RAD1* also contributes to an increased amount of nicks. Those, because of mechanical stress or metabolic activities, would then lead to the accumulation of DSB, which have to be repaired by the HR pathway (Figure 7).

A role of components of other repair pathways, such as BER, in the HR process or in the formation of secondary substrate cannot be excluded (Hendricks et al., 2002). However, our profiling experiments did not reveal any significant changes in expression of genes known to be involved in the BER process.

(2) Regulatory factors controlling DNA repair pathways could also contribute to the hyperrecombinogenic phenotype. At*cen2* plants exhibit an elevated At*ATM* expression compared with the control plants. ATM is known in mammals to be involved in cell cycle checkpoints and also in the regulation of DNA repair activities and apoptosis (for review, see Kastan and Lim, 2000). In Arabidopsis, Garcia et al. (2003) have shown that *ATM* mutants were impaired in expression of At*RAD51*, At*Parp-1*, and At*LigIV*. They suggested that At*ATM* is essential for the transcriptional response to DSB. It could be envisaged that in the At*cen2* mutant, exhibiting a higher At*ATM* mRNA steady state level, several components of DNA repair pathways may be transcriptionally induced or activated via this signaling pathway (Figure 7).

(3) Also, chromatin structure plays a crucial role in DNA repair (Ura and Hayes, 2002). Components involved in chromatin dynamics during the NER process, such as the two DNA helicases RAD25 and RAD3, were transcriptionally upregulated in At*cen2* plants. This may contribute to facilitate the accessibility of homologous templates for recombinational DNA repair, although information on their direct involvement in the HR process is not available. In addition, the At*MIM* gene, coding for a protein with close homology to an SMC-like protein, was found to be highly upregulated in the centrin mutant. Arabidopsis plants defective in At*MIM* expression exhibited a higher sensitivity to DNA damaging agents and a reduction in intrachromosomal recombination (Mengiste et al., 1999). Conversely, overexpression of the At*MIM* gene stimulated intrachromosomal recombination (Hanin et al., 2000). Our data corroborate the previous

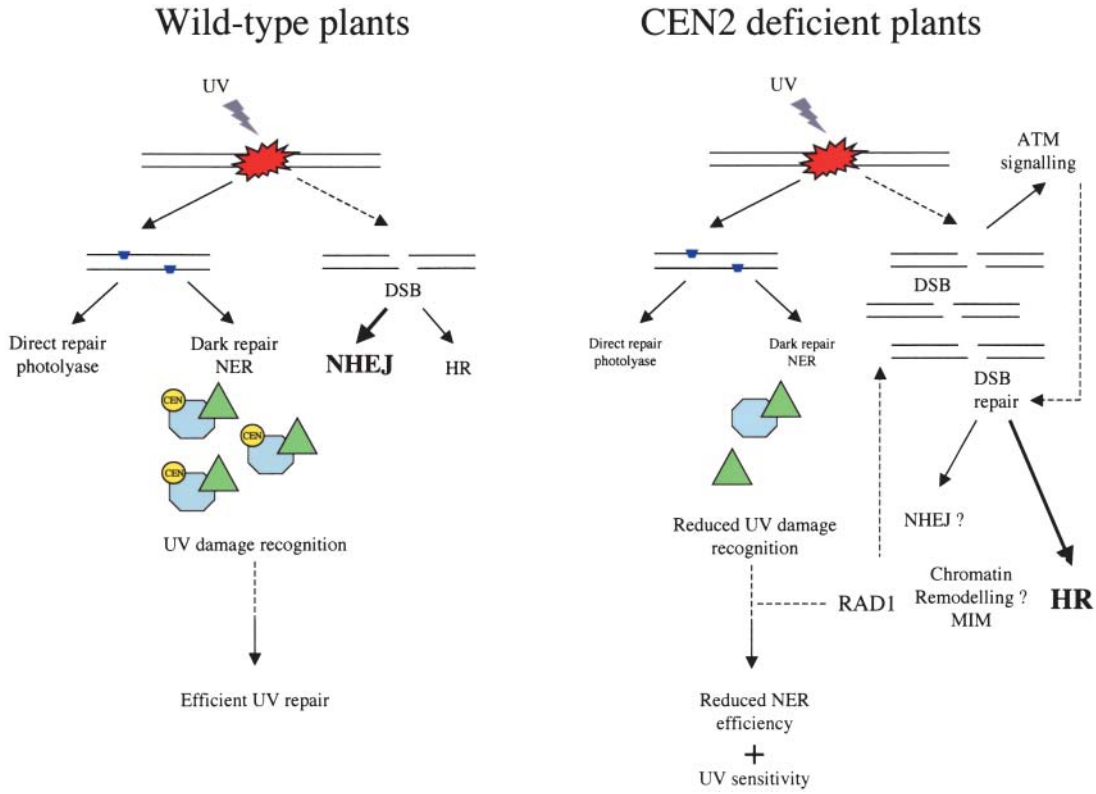

Figure 7. A Model Explaining the Hyperrecombinogenic Phenotype of At*CEN2*-Deficient Plants.

In response to UV irradiation, cyclobutyl pyrimidine dimers, (6-4)-photoproducts (blue trapezium) are formed. In addition, DNA DSBs are introduced. Different repair processes are activated. In At*CEN2* wild-type plants, DNA photolyase contributes efficiently to the direct repair of UV damage, whereas NER contributes to the dark repair of this damage. For the recognition step of the UV lesion, a complex consisting of RAD4-RAD23 (blue hexagon-green triangle, respectively) is stabilized by CEN2. DNA DSBs can be formed as the result of an extra nick in a single stranded region because of mechanical stress of a chromosome. These DSBs are mainly repaired by the NHEJ (bold), and recombinational DNA repair (HR) remains a minor pathway (lower case). In the At*cen2*-deficient plants, the lower mRNA steady state level of photolyase (lower case) could contribute to a lower efficiency of direct repair of UV damage. In addition, the lack of CEN2 as well as the reduced amount of RAD4 may reduce the stability/efficiency of the recognition complex involved in the first step of the NER pathway. Therefore, the reduced efficiency of the two major UV repair pathways may lead to the accumulation of DSBs. Repair of these is taken over by HR (bold). Concomitantly, the DSB repair process could be activated by the ATM signaling pathway. MIM may also contribute to remodel chromatin during the HR process.

studies on the role of At*MIM* in the recombinational repair process (Figure 7).

In conclusion, our results strengthen the idea that a link between DNA repair pathways exists in plants. Genetic defects of DNA repair pathways, such as photoreactivation, DSB repair, or NER, were shown to lead to activation of the HR pathway (Gherbi et al., 2001; Ries et al., 2000; this study). Critical points for this regulation may include connections between upstream recognition processes, competition of repair factors for substrates, and the state of chromatin at the damaged sites (Figure 7).

METHODS

Plant Material and Activation Tagging Plasmid

The homozygous *Arabidopsis thaliana* IC9 line (ecotype Columbia) carrying a single copy of the intermolecular recombination substrate GRU'S'G'U' was used as reporter line for the genetic screen. The pGRU'S'G'U' T-DNA carries a nonfunctional chimeric *uidA* gene containing different deletions (derived from plasmid pGUS 23; Tinland et al., 1994). The resulting recombination substrate plasmid pGRU'S'G'U' carried the partially overlapping *uidA* region with 1213-bp homologous sequence in direct orientation on its T-DNA (Molinier et al., 2004; Figure 1). The activation tagging plasmid pSKI015 (Weigel et al., 2000), containing four repetitions of the 35S enhancer close to the right border and the Basta resistance gene as selectable marker, was used as mutagenic T-DNA.

Growth Conditions

For in vitro culture, plants were germinated on GM medium (MS salts [Duchefa, Haarlem, The Netherlands], 1% sucrose, and 0.8% agar, pH 5.8) in the presence or absence of a selectable agent. Plants were grown in a culture chamber under a 16-h/8-h photoperiod (20°C/16°C). For soil cultured plants, seeds were sown (20/pot) and put at 4°C in the dark for 3 d. The pots were transferred to a phytotron kept under a regime of

a 16-h/8-h photoperiod (20°C/16°C; 70% humidity). At the four leaf developmental stage, plants were individualized and grown for further experiments.

Screening for Hyperrecombination Phenotype

The pSKI015 plasmid was mobilized into *Agrobacterium tumefaciens* and used to transform the homozygous IC9 line employing the in planta transformation method (Bechtold and Pelletier, 1998). The progeny of the transformed IC9 plants was germinated on soil, and Basta-resistant plants were isolated. Three to four leaves of 4181 3-week-old individual primary transformants (T1) were harvested, and histochemical GUS assay was performed (Jefferson et al., 1987). The number of GUS+ spots (number of HR events) per plant was determined. Plants having at least two GUS+ spots on two independent leaves were considered as putative mutants. T1 plants were selfed, and plants of the T2 generation (>30 plants) were analyzed for the recombination frequency (average number of GUS+ spots per plant in a population of >30 plant siblings). Experiments were at least duplicated. The P2.4 plant showed a significant increase of HRF and was considered as a hyperrecombination mutant.

Molecular Analysis of the P2.4 Hyperrecombinogenic Mutant

Genomic DNA of the P2.4 T1 plant was prepared using the DNeasy extraction kit (Qiagen, Valencia, CA). The right border–genomic DNA junction was analyzed by plasmid rescue as described by Weigel et al. (2000). The effect of the activation tagging T-DNA on the neighboring gene was determined by RT-PCR. Total RNA from T2 P2.4 plants was prepared using the RNeasy extraction kit (Qiagen). The reverse transcription was performed using 3 μg of total RNAs, oligo(dT) primer, and the reverse transcription kit (Amersham, Piscataway, NJ). The PCR reaction was performed in 50 μL of reaction mixture containing 1 μL of the RT reaction mixture, 1.25 units of ExTaq (Takara, Shiga, Japan), 1.5 mM $MgCl_2$, 200 μM of each dNTP, and 2 μM of gene-specific primers. The centrin-specific primers (MIPS access number At4g37010) were CEN958 (5′-CAAATAAACGAATTGATGGCAG-3′) and CEN 3′ (5′-CCATTACTAATTGATTTATACTTAGC-3′). Initial denaturation was done at 94°C for 1 min, and then amplification was done for 25 to 35 cycles with a denaturation time of 1 min at 94°C, followed by annealing for 1 min at 58°C and extension for 30 s at 72°C. Different dilutions of the RT reaction mixture and number of PCR cycles were used to confirm that the PCR amplifications were within the linear range.

Reproduction of the Hyperrecombinogenic Phenotype

For RNAi technology, the pOEXhp vector driving expression of double-stranded RNA was constructed. The fourth exon (156 bp) specific for this centrin gene (MIPS access number At4g37010) was cloned into the pOEXhp vector between the *Nco*I-*Xho*I sites in sense orientation and the *Bsr*GI-*Avr*II sites in antisense orientation. The resulting pOEXhpCEN plasmid was mobilized into Agrobacterium and used to transform the IC9 plants carrying the intermolecular recombination substrate. HRF was measured in the T2 generation in a batch of >60 plants. Experiments were at least duplicated. The same experiment was conducted using another reporter line carrying the same intermolecular recombination substrate but at an independent insertion site (IC6 line). HRF was measured in 2-week-old plants (at least 50 plants per replicate). The steady state level of centrin mRNA in these RNAi plants was determined by RT-PCR as described in the previous paragraph.

Centrin Overexpression

The pOEX4 vector driving expression of the At*CEN2* cDNA under the control of the mannopine synthase constitutive promoter was constructed. The cDNA of the At*CEN2* gene was amplified by RT-PCR from total RNA prepared from leaves and cloned into the pOEX4 vector between the *Nco*I-*Avr*II sites. The resulting pOEX4CEN plasmid was mobilized into Agrobacterium and used to transform Arabidopsis wild-type plants. At*CEN2* overexpression was verified by RT-PCR UV-C treatment.

To evaluate the UV-C (254 nm) sensitivity, 1-week-old in vitro germinated IC9, homozygous P2.4, and RNAi plants were irradiated with different doses of UV-C (0, 10, 30, 50, or 70 Kergs/cm^2) using a Mineralight-Lamp (UV-Products, San Gabriel, CA). Plants were immediately returned to the growth chamber. The phenotype was observed 1 week later. Twelve plants per replicate were used; the experiment was duplicated.

For the root-growth assay, 3-d-old in vitro germinated IC9, homozygous P2.4, and RNAi plants were transferred to square plates containing GM medium and grown vertically for an additional day. Root length was measured before UV-C treatment (10 Kergs/cm^2) and the following 3 d (days 1, 2, and 3). The relative root growth was calculated: (root length treated/root length untreated) × 100. Ten plants per replicate were used. Experiments were triplicated.

In Vitro Repair Assay

The in vitro repair assay according the method described by Li et al. (2002) was performed on cell extracts from the homozygous P2.4 mutant, RNAi plants, overexpressing plants, and from both the IC9 line and wild-type plants as control. The pGEX and pBSK plasmids were linearized and purified as described by Li et al. (2002). The pGEX-linearized plasmid was UV-C damaged (450 J/m^2) using the Stratalinker (Stratagene, La Jolla, CA). Twenty-five micrograms of protein extracts were used per time point and mixed with 300 ng of UV-C–damaged plasmid (pGEX) and 300 ng of non-UV-C–damaged plasmid (pBSK) as control. The reaction was stopped 15 min, 30 min, 1 h, or 2 h after incubation. Plasmids were purified using the gel extraction kit (Qiagen). The agarose gel and DIG detection procedures were performed as described by Li et al. (2002).

Microarray Analysis

Seven-day-old homozygous At*cen2* and IC9 (control) in vitro germinated plants were transferred to large Petri dishes (160-mm diameter) containing GM medium to a density of 1 plant/cm^2 and grown for an additional week. To reduce biological variation, nonirradiated control plants were also moved to the chamber where the UV-C treatment (6 Kergs/cm^2) was performed and immediately returned to the growth chamber. Experiments were duplicated and performed at the same time of the day to exclude biological variations because of the influence of the circadian clock. Whole plants, including the roots, were harvested before and 2 and 6 h after treatment. Twenty to thirty plants per time point, originating from different plates, were randomly collected and immediately frozen in liquid nitrogen. Total RNA was prepared from treated and control plants using the plant RNeasy extraction kit (Qiagen). Fifteen micrograms of total RNA were processed, labeled, and hybridized to the ATH1 Affymetrix GeneChips (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Data analysis was performed using GeneSpring 5 software (Silicon Genetics, Redwood City, CA). The expression profile, each in duplicate, of At*cen2* plants was compared with that of the corresponding IC9 plants (At*cen2* versus IC9) for each time point. Genes of the At*CEN2* mutant plants exhibiting the same change, increase and decrease, in expression level in each replicate were considered as upregulated or downregulated, respectively. In addition, a threshold of ± twofold change was used for producing the final list of upregulated and downregulated genes in the At*cen2* plants.

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