Plant pathogenic geminiviruses are composed of small circular ssDNA genomes. They are multiplied in the nuclei of host cells by complementary strand replication (CSR), rolling circle replication (RCR) and recombination-dependent replication (RDR) modes, in the course of which various ssDNA and dsDNA-containing intermediates arise (reviewed by Jeske, 2007, 2009). The replication-initiator protein (Rep) is the only virus-encoded protein essential for replication. Therefore, the diverse DNA amplification and modification processes are conducted largely by host enzymes, and geminiviruses modulate the respective host machinery strongly (reviewed by Jeske, 2007, 2009). The replication-initiator protein (Rep) is the only virus-encoded protein essential for replication. Therefore, the diverse DNA amplification and modification processes are conducted largely by host enzymes, and geminiviruses modulate the respective host machinery strongly (reviewed by Jeske, 2007, 2009). The replication-initiator protein (Rep) is the only virus-encoded

KU80, a key factor for non-homologous end-joining, retards geminivirus multiplication

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KU80 is well-known as a key component of the non-homologous end-joining pathway used to repair DNA double-strand breaks. In addition, the KU80-containing DNA-dependent protein kinase complex in mammals can act as a cytoplasmic sensor for viral DNA to activate innate immune response. We have now, to our knowledge for the first time, demonstrated that the speed of a systemic infection with a plant DNA geminivirus in Arabidopsis thaliana is KU80-dependent. The early emergence of Euphorbia yellow mosaic virus DNA was significantly increased in ku80 knockout mutants compared with wild-type sibling controls. The possible impact of KU80 on geminivirus multiplication by generating non-productive viral DNAs or its role as a pattern-recognition receptor against DNA virus infection is discussed.

For this purpose, the previously characterized mutant line ku80 (FLAG_049H05; supplied by the Versailles Arabidopsis Stock Centre) with abolished KU80 transcription was used (Gallego et al., 2003a, b). The background Wassilewskija ecotype shows slightly earlier flowering than the Columbia ecotype. Homozygous ku80 or wt progeny plants from the segregating parent line were identified by genotyping PCR with different combinations of primers (KU80-LP: 5'-CTT-CAATGCTACCTTTGC-3'; KU80-RP: 5'-GCTTCTG-AGCATTGACTTTGG-3'; Tag5: 5'-CTAAATTGCGT- TTCTATCGAC-3'). For each experiment, ten plants per genotype were inoculated biolistically with EuYMV DNA A (FN435995) and/or DNA B (FN435996) in parallel, and viral DNA accumulation was monitored at 7, 14 and

A supplementary figure and a supplementary table are available with the online Supplementary Material.
21 days post-inoculation (dpi), as described by Richter et al. (2014). Three independent experiments were performed. Viral infection had established at 7 dpi in most individual plant samples, with slight fluctuations between experiments (exemplarily shown for one experiment, Fig. 1a). The main viral DNA forms of ssDNA, covalently closed circular (ccc) DNA and open circular (oc) DNA had emerged with varying intensities among individual samples. Remarkably, the ku80 plants appeared to contain more viral DNA overall than wt plants, ruling out KU80 promoting viral amplification. Statistical analysis of the measured signal densities confirmed a significant difference for each of the viral DNA forms (Fig. 1a, panel) and for all three experiments (Fig. S1, available in the online Supplementary Material). These results support the hypothesis that KU80 might be involved in early pathogen response. Of 16 additional T-DNA insertion lines tested (Table S1), with different inactivated genes involved in recombination, DNA damage tolerance and repair, such as the genes described by Richter et al. (2015), and further unpublished data, none was found to promote geminiviral DNA multiplication. Thus, the behaviour of the ku80 line is, so far, unique.

These significant differences in virus DNA titres declined with progressing infection, as at 14 dpi all samples contained high levels of EuYMV DNAs in approximately similar quantities, regardless of plant genotype (Fig. 1b).

Intriguingly, monomeric lin dsDNA cumulated significantly in ku80 plants at 21 dpi in two out of three experiments (Fig. 1c). Equally, levels of ssDNA or cccDNA were elevated again in ku80 plants in those two experiments. In one experiment, however, no differences in any DNA form were detected. In contrast with the viral DNA titres, onset (at 9–10 dpi) and severity of the symptoms of EuYMV infection did not differ significantly between the plant lines (Fig. 2).

To specify the individual viral DNA forms in closer detail, two-dimensional agarose gel electrophoresis and Southern blot hybridization were employed, as described by Richter et al. (2015). Samples from the first experiment at 7 dpi were pooled to assess whether KU80 directly influences viral replication modes (Fig. 3). In general, intermediates and products of CSR, RCR and RDR were detected in approximately similar amounts for both plant genotypes. This result largely excludes KU80 having a differential impact on any of the viral replication modes. Only the expression of lin dsDNA with a discrete band at monomeric size position as well as the arc of heterogeneous molecules, appeared to be increased slightly in ku80 in comparison with wt plants. The lin dsDNA band has been characterized in detail recently (Paprotka et al., 2015), and was identified as a blunt-ended non-productive intermediate lacking the origin of replication (ori). Heterogeneous lin dsDNA is probably the product of RDR (Jeske et al., 2001; Preiss & Jeske, 2003). Taken together with the occasional accumulation of lin dsDNA at 21 dpi observed in one-dimensional gels (Fig. 1c), the two-dimensional results might serve as a hint that the NHEJ pathway is utilized to join or circularize geminiviral lin dsDNA. However, in the case of the nearly monomeric lin dsDNA, these repair products do not necessarily promote replication, owing to the lack of an ori. The NHEJ pathway is the prevalent pathway for DSB repair in eukaryotic somatic cells. Nevertheless, it can cause deletions or insertions and thus genetic information might be lost (reviewed by Mannuss et al., 2012; Puchta, 2005). Whereas the luxurious inventory of non-coding intervening DNA sequences in the eukaryote genome allows many NHEJ products without changing coding regions, the condensed geminiviral genome will rather suffer from NHEJ-based repair. The consecutive accumulation of aberrant coding regions and RNAs in defective geminiviral DNA can lead to secondary effects in the plant defence cascade. Therefore, abolished KU80-mediated NHEJ in ku80 plants might be advantageous for geminivirus infections by avoiding the accumulation of non-productive, aberrant viral DNAs.

Further on, the head start of early systemic infection observed in ku80 plants might indicate that the KU70/KU80 complex in plants acts as a viral DNA sensor similar to the Ku80-containing DNA-PK complex in mammals. Since the KU70/KU80 heterodimer binds only to lin dsDNA (Tamura et al., 2002), geminivirus infection would be detected after the first rounds of RDR producing linear dsDNA forms. This impact might be perceived more effectively during early infection, when viral DNA levels are still low and not yet in signal saturation. However, no homologue of DNA-PKcs has been identified in plants, which is responsible for the downstream, IRF-3 mediated response in mammals. Therefore, further investigations are necessary to elucidate the respective signal transduction pathway or the mechanism behind the observed KU80-mediated antiviral effect.

A further promising hint comes from the functional interaction between KU70/KU80 and the Werner-like exonuclease (WEX, syn. WRNexo) in A. thaliana, which stimulates the exonuclease activity of WEX (Li et al., 2005). WEX is homologous to the 3′-5′ exonuclease domain of the human Werner protein (Plchova et al., 2003), a member of the RECQ helicase family. RECQ helicases are crucial for genome stability and control DSB repair by resolving or disrupting replicative and recombinogenic DNA intermediates in the 3′ to 5′ direction (reviewed by Hartung & Puchta, 2006; Knoll & Puchta, 2011). The plant RECQ2 helicase disrupts displacement loops (D-loops) of homologous recombination intermediates and interacts with WEX. Thus, both plant proteins together probably reconstitute the function of the single human Werner protein in trans (Hartung et al., 2000; Kobbe et al., 2008). Consequently, KU70/KU80, WEX and RECQ2 combined might operate in a manner that counteracts homologous recombination and shifts repair modes to NHEJ with the detrimental effects for viral genomes mentioned previously.
Fig. 1. Emergence of EuYMV DNA forms in ku80 and wt A. thaliana plants at (a) 7, (b) 14 and (c) 21 dpi Total nucleic acids (500 ng each) from ten inoculated plants per genotype were separated in 1.4 % agarose gels in the presence of 5 μg ethidium bromide ml⁻¹. Genomic plant DNA (pDNA) is shown as loading control. One plant per genotype was inoculated with EuYMV DNA B alone as mock control (m). Virus DNA was visualized by Southern blot hybridisation with DIG-labelled full-length DNA A probes of EuYMV, an anti-DIG alkaline phosphatase-conjugated antibody and CSPD, as described by Richter et al., (2015). Hybridisation standards with 1, 10 and 100 pg of linear dsDNA are indicated. Viral DNA forms are multimeric (>1 x; mult), open circular (oc), double-stranded linear (lin), covalently closed circular (ccc), linear and circular single-
Moreover, WEX has homology to RNase D proteins and was shown to be required for post-transcriptional gene silencing (PTGS) in plants, though the exact underlying mechanism remains uncertain (Glazov et al., 2003).

Correspondingly, the *Caenorhabditis elegans* orthologue of the Werner protein (MUT-7) exhibits RNase D homology and is essentially involved in transposon silencing and RNA interference in general (Ketting et al., 1999; Tops et al., 2005). In this context, Trinks et al. (2005) have identified a putative 3'-5' exonuclease in *A. thaliana* with homology to WEX whose transcription was substantially upregulated upon geminiviral AC2 protein expression. This protein was called WEL-1 (Werner exonuclease-like 1), is different from the Werner enzymes discussed, and showed only limited preservation of the conserved motifs of other Werner-like exonucleases. It has been suggested that WEL-1 plays a role in geminivirus-induced silencing suppression by interfering with WEX's function in PTGS.
This might further indicate a certain relevance of WEX-mediated PTGS during geminivirus infections.

The nuclear functions of KU70/KU80 in NHEJ are well described. However, both KU70 and KU80 localize to the nucleus as well as to the cytoplasm in *A. thaliana* (Tamura et al., 2002). The cytoplasmic localization has not yet been associated with any functional relevance. PTGS in the cytoplasm is a well-known mechanism with antiviral impact in plants (reviewed by Szittya & Burgyán, 2013; Zvereva & Pooggin, 2012), and could be a further indication of a connection between the observed antiviral effect of KU80, the cytoplasmic localization of KU70/KU80 and PTGS via the interaction with WEX. KU70/KU80 could be a novel intracellular PRR against viral DNA in plants (for a review on plant PRR, see Zipfel, 2014) and might fulfil a similar role of binding and detecting extranuclear viral linear DNA to the DNA-PK complex in mammals.

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