Marker gene tethering by nucleoporins affects gene expression in plants

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Abbreviations: CCLR, Cell culture lysis reagent; NPC, Nuclear Pore Complex; NE, Nuclear Envelope; SUN, Sad1/ Unc84; CCT, Chromatin Charting; IPTG, Isopropyl β-D-1-thiogalactopyranoside; CDS, Coding Sequence.

In non-plant systems, chromatin association with the nuclear periphery affects gene expression, where interactions with nuclear envelope proteins can repress and interactions with nucleoporins can enhance transcription. In plants, both hetero- and euchromatin can localize at the nuclear periphery, but the effect of proximity to the nuclear periphery on gene expression remains largely unknown. This study explores the putative function of Seh1 and Nup50a nucleoporins on gene expression by using the Lac Operator / Lac Repressor (LacO-LacI) system adapted to Arabidopsis thaliana. We used LacO fused to the luciferase reporter gene (LacO:Luc) to investigate whether binding of the LacO:Luc transgene to nucleoporin:LacI protein fusions alters luciferase expression. Two separate nucleoporin-LacI-YFP fusions were introduced into single insert, homozygous LacO:Luc Arabidopsis plants. Homozygous plants carrying LacO:Luc and a single insert of either Seh1-LacI-YFP or Nup50a-LacI-YFP were tested for luciferase activity and compared to plants containing LacO:Luc only. Seh1-LacI-YFP increased, while Nup50a-LacI-YFP decreased luciferase activity. Seh1-LacI-YFP accumulated at the nuclear periphery as expected, while Nup50a-LacI-YFP was nucleoplasmic and was not selected for further study. Protein and RNA levels of luciferase were quantified by western blotting and RT-qPCR, respectively. Increased luciferase activity in LacO:Luc+Seh1-LacI-YFP plants was correlated with increased luciferase protein and RNA levels. This change of luciferase expression was abolished by disruption of LacI-LacO binding by treating with IPTG in young seedlings, rosette leaves and inflorescences. This study suggests that association with the nuclear periphery is involved in the regulation of gene expression in plants.

Introduction

A large body of evidence from metazoan and yeast research shows that the various components of the nuclear periphery - nuclear envelope (NE), lamina and nuclear pore complexes (NPC) - play crucial roles in organizing the genome and in the regulation of gene expression. Chromatin anchorage at the nuclear periphery affects gene expression and nucleic acid metabolism. Generally, it has been found that association of genes with NE proteins or the lamina causes their repression while localization of genes to the nuclear pores is mainly associated with active transcription. In addition to active genes, RNA processing machinery is also associated with nuclear pores, which enhances or accelerates export of mRNA from the nucleus.3,12,13,20 Nucleoporins involved in regulation of gene expression in metazoans and yeast include Nup98/Nup145N, Sec13, Nup62/Nsp1, Nup153/Nup60, Nup50, TPR/Mlp, Nup96, Nup155/Nup107, Nup93 and Nup88/Nup82. These effects are achieved by either directly interacting with chromatin, for instance binding to the promoter region, or by interacting with components of the transcription and RNA processing machinery12,20; thus the effect of nucleoporins on gene expression may occur at the pore or within the nucleus.

While heterochromatic loci such as telomeres and chromocentres are preferentially associated with the nuclear periphery in plants,5,14 it is unknown whether and how the plant NE, lamina and NPC may affect gene expression.17 One reason for this is that until recently only a handful of plant nuclear periphery components were known. Homologues of most of the approximately 60 metazoan and yeast membrane intrinsic NE proteins do not exist in plants,9,14 with the exception of the Sad1/ Unc84 (SUN) domain protein family.9,10 While plants have a structural lamina, BLAST search failed to identify true lamin orthologues of its animal components.2,4,7,8 Plant homologues of nucleoporins were recently identified yet many remain to be functionally characterized.16,22 This study is a first investigation of the effects of plant nucleoporins on gene expression. The nucleoporins AtNup50a and AtSeh1 were chosen because their orthologues in Drosophila melanogaster were previously shown to either enhance (Seh1) or repress (Nup50) gene activity.1 To study changes in gene expression...
expression, we chose the marker gene luciferase used in combination with the LacI-LacO gene tethering system pioneered by Rosin et al.19 Rosin et al.19 created Arabidopsis thaliana Col0 chromatin charting (CCT) plants, which contain a transgene consisting of the bacterial lac operator (LacO) fused in frame to the coding sequence of luciferase (Luc; LacO:Luc transgene). In this study, the lac repressor (LacI) was fused to either AtSeh1 or AtNup50a and expressed in the LacO:Luc plants to tether Luc to either AtSeh1 or AtNup50a via LacI-LacO binding interactions. Changes in Luc expression were examined by luciferase assays, western blot and RT-qPCR.

**Materials and Methods**

**Plasmids and cloning**

The coding sequence (CDS) for LacI was amplified from plJM71 plasmid19 using primers FLacIr (ATGGTTAAATTG-TAACGTTATACGATGGTCGC) and RLacIr (TGGAGTACGATCCACGTCG). DNA was sequenced to ensure mutation-free cloning. Arabidopsis thaliana leaf tissue was used as template to amplify the CDS of Seh1 and Nup50a using the following primers: FSeh1 (ATGGGGAATATCGGGC), RSeh1 (GGAGGCACCTGTATCATC), Lac-Seh1, FNup50a (ATGGGTAACGCACCTGAGC) and RNup50a (AGTACCTGTAAGCAGC) and RNup50a (TGCTGTGAAATATGGTG). Lac-Seh1 and Lac-Nup50a fusions were made by overlapping PCR using primers FNup50aOL (CTCCCAACAGCTACAGTATACGACCGTGATGGTGAG) and RNup50aOL (TAACGCATTATTTTATCATCAACCTGACTGTAGCTGTATGG). Samples were centrifuged for 10 min at 13 000 rpm at 4°C. The protein extract was loaded on a 10% SDS gel and immunoblotted using an anti-luciferase antibody and goat anti-rabbit Cy5 (Jackson Immuno Research). Blots were labeled with anti-luciferase antibody (Sigma) as primary antibody and goat anti-rabbit Cy5 (Jackson Immuno Research) as secondary antibody. Immunoblots were visualized on a FluorChem M (Novex) using a ChemiDoc imaging system (Bio-Rad). Bands were quantified using ImageJ software (National Institutes of Health).

**Luciferase assay**

Fresh or frozen tissue was homogenized using a cooled Tissue Lyser (Qiagen) and tungsten beads. Cell culture lysis reagent (CCLR), part of the Luciferase Assay System (Promega), was used as extraction buffer. The samples were spun down at 4°C, 13 000 rpm for 10 min and the supernatant removed to fresh tubes and kept on ice. Total protein extract was quantified using the RC/DC assay (BioRad). Samples were diluted with CCLR to the same total protein concentration. For each reaction, 100 µl Luciferase Assay Reagent (LAR, Promega) and 20 µl sample were used and for each sample 4 repeat reactions were prepared. A MicroBetaTriLux luminometer (Perkin Elmer) was used to measure luminescence. In each well, luminescence was divided by 5 s. Luminescence counts per second for each sample were converted to a percentage scale using the average LacO:Luc count as 100%. Experiments were repeated with at least 3 biological and 4 technical replicates and analyzed by Student’s t-test using Microsoft Excel.

**SDS-PAGE and immunoblotting**

Proteins were extracted as described for the luciferase assay. Ice cold acetone was added and proteins precipitated for 2h at −20°C. Samples were centrifuged for 10min at 13 000 rpm at 4°C, the supernatant removed and the pellet dried. The dried pellet was resuspended in Laemml buffer 11 and stored at −20°C. The protein extract was loaded on a 10% SDS gel and proteins separated by SDS-PAGE. Proteins were blotted onto nitrocellulose membrane and blocked over night at 4°C in 5% milk PBS (Jackson Immuno Research). Blots were labeled with anti-luciferase antibody (Sigma) as primary antibody and goat anti-rabbit Cy5 (Jackson Immuno Research). Blots were visualized on a FluorChem M (Novex) using a ChemiDoc imaging system (Bio-Rad). Bands were quantified using ImageJ software (National Institutes of Health).
Immunoresearch) as secondary antibody. Antibody labeling was imaged using a ChemiDoc transluminator (BioRad). Images were analyzed using ImageJ to measure the band intensity. Western blots were repeated more than 3 times and data presented for one blot is representative of these experiments. Band intensities were normalized onto a percentage scale by using the average LacO:Luc band intensity as 100%. Data was analyzed using Microsoft Excel.

**RT-qPCR**

Tissue was ground in liquid nitrogen with a mortar and pestle and stored at -80°C. RNA was extracted using a Nucleospin RNA extraction kit (Macherey-Nagel) following manufacturer’s recommendations. rDNase digestion was performed in solution after elution of RNA and a Nucleospin RNA Clean-up kit (Macherey-Nagel) was used to re-purify the RNA. RNA was quantified with a nanodrop spectrophotometer. The RT reactions were performed with V1LO cDNA synthesis kit (Invitrogen) according to the manual (using typically 100ng RNA in 10μl reaction volume). cDNA was diluted fold10- and checked by PCR using GAPC primers to assess contamination with genomic DNA and consistency in samples. For the qPCR, SYBR Green (Applied Biosystems) was used according to manufacturer’s recommendations in an ABI 7500 qPCR cycler, with standard thermal profile. Primer pairs were tested to ensure high and similar amplification efficiencies. LUC expression (primers FLUC1 GGCGTTAATCAGAGAGGCGA and RLUC1 TGGCCTCTGCTATGAACGCGC) was normalized to the expression of OTC (AT1G75330, primers OTC_qRT-F TGAAGGGA-CAAAGGTCTGTATTT and OTC_qRT-R CGCAGA-CAAGTGGAATGGA). Seh1-LacI-YFP (no LacO:Luc) and wild-type samples were used to control for unspecific LUC amplification or sample contamination. All samples were run in triplicate. For comparing LUC expression in tethered versus non-tethered lines, data from 5 RT-qPCR experiments were combined yielding a total of 8 biological replicates per line (one extreme outlier in LacO:Luc samples was removed from analysis). For the IPTG experiment, 3 biological replicates were used. Relative LUC RNA levels were calculated using the ddCt method.21 Data analysis and Student’s t-tests were performed using Microsoft Excel.

**Results**

**Nucleoporin-LacI tethering transgenes alters LacO:luciferase activity**

Over 200 CCT lines were created by,19 which contain a single copy of the LacO:Luc transgene randomly inserted in the genome. In some of these lines, luciferase expression, as inferred by luciferase activity assays, was higher than in others indicating the positioning of the transgene was affecting luciferase expression.19 For this study, we chose CCT lines with medium luciferase expression levels to measure a putative increase and/or decrease in luciferase activity. These were transformed with either p35S:Seh1-LacI-YFP or p35S:Nup50a-LacI-YFP. Ten day old homozygous, single insert seedlings were used to measure luciferase activity and therefore detect whether the presence of either Seh1-LacI-YFP or Nup50a-LacI-YFP caused any changes in luciferase levels. Luminescence counts per second were used as
measure for luciferase activity and were normalized to a percentage scale, where luminescence in the non-tethered LacO:Luc line was set at 100%. Co-expression of Seh1-LacI-YFP caused a significant increase in luciferase activity (227.7 ± 22.2%; p < 0.01), while co-expression of Nup50a-LacI-YFP caused a significant decrease in luciferase activity (17.1 ± 0.5%; p < 0.01; Fig. 1).

Confocal microscopy was used to observe the localization of the nucleoporin-LacI-YFP tethers. Seh1-LacI-YFP was mainly accumulated at the nuclear periphery in root cells, lower epidermal cotyledon cells and trichomes (Figs. 2A–C). On the other hand, Nup50a-LacI-YFP did not localize to the nuclear periphery but instead was nucleoplasmic in all tissues observed (Fig. 2D and E). These subcellular localizations are similar to those reported by and indicate that the LacI moiety does not affect the proper targeting and localization of the 2 nucleoporins. The differing subcellular localisations of the Seh1-LacI-YFP tether and the Nup50a-LacI-YFP tether may partly explain the differing luciferase levels in the 2 lines. In an attempt to investigate further the possible enhancement of gene expression mediated by the NPC, we decided to focus on the Seh1-LacI-YFP tether. The enhanced expression of luciferase in the presence of Seh1-LacI-YFP tether was further confirmed in F3 isogenic lines derived from initial transgenic plants co-expressing LacO:Luc and Seh1-LacI-YFP (data not shown).

Seh1-LacI-YFP increases LacO:Luc transcription

Having found an increase in luciferase activity in the LacO:Luc+Seh1-LacI-YFP line, we wanted to establish whether this was correlated with an increased luc expression at the RNA and protein levels. RT-qPCR was used to compare luc expression levels in the line with no Seh1-LacI-YFP (LacO:Luc only) with the line co-expressing the Seh1-LacI-YFP tether (LacO:Luc+Seh1-LacI-YFP). LUC RNA levels were calculated relative to LacO:Luc. The presence of the Seh1-LacI-YFP tether increased levels of luciferase mRNA by nearly fold-2 (1.9 ± 0.18) compared to LacO:Luc only (P < 0.001) indicating that luc transcription was upregulated (Fig. 3A). In addition to increased luciferase mRNA, the presence of the Seh1-LacI-YFP tether also significantly increased the level of luciferase protein – 190 ± 7% compared to LacO:Luc luciferase protein levels of 100% (p = 0.001; Fig. 3B). Therefore, our results suggest that increased luciferase mRNA levels lead to increased luciferase protein levels, which in turn result in increased luciferase activity (Fig. 1). Hence, measuring luciferase activity in this system is an appropriate indicator for luc expression levels.

In order to test whether the enhanced expression of Luc resulted from specific binding of LacI to LacO, we applied isopropyl b-D-1-thiogalactopyranoside (IPTG), which reduces the affinity of LacI for LacO 1000-fold15,18 used IPTG treatment followed by RT-qPCR to assay the disruption of specific binding of GFP-LacI to LacO in tobacco. Using both RT-qPCR and western blotting, we measured RNA and protein levels 20h post mock-treatment or
100 mM IPTG treatment. While IPTG treatment neither affected RNA nor protein levels in the non-tethered LacO:Luc line, it did cause a significant decrease (p=0.037) in the Seh1-LacI-YFP tethered line (Fig. 4). Thus we conclude that specific interaction was occurring between LacI and LacO in our system.

**Increased luciferase activity in other tissues**

Expression of luciferase in non-tethered LacO:Luc and tethered LacO:Luc+Seh1-LacI-YFP lines was previously established in 7 d old whole seedling extracts. To investigate whether the LacI-LacO tethering effect is present in other plant tissues, we measured luciferase activity in adult rosette leaves, stems, inflorescences and seeds. This time, luminescence of LacO:Luc in leaf tissue was set to 100%. In both leaves and inflorescences, luciferase activity was high and increased significantly when the Seh1-LacI-YFP tether was co-expressed (p<0.001; Fig. 5). In leaf tissue, luciferase levels in LacO:Luc+Seh1-LacI-YFP were 209 ± 3%, higher than in LacO:Luc leaves (100 ± 1%). In inflorescences, in LacO:Luc+Seh1-LacI-YFP luciferase activity levels were 152 ± 1%, higher than in LacO:Luc (72 ± 1%). In stems and seeds, however, luciferase activity was very low – 7.4 ± 0.4% in LacO:Luc and 6.4 ± 0.7% in LacO:Luc+Seh1-LacI-YFP in stems and 5.5 ± 0.4% in LacO:Luc and 7.9 ± 0.5% in LacO:Luc+Seh1-LacI-YFP in seeds. In fact, expression levels in stem and seed
samples were similar to the negative control Seh1-LacI-YFP only (9.5 ± 0.5%; data not shown) indicating these are background levels and that luciferase does not appear to be expressed in these tissues, likely as a result of gene silencing or degradation of the product.

Discussion

In the chromatin charting lines created by,\textsuperscript{19} a LacO:Luc transgene is randomly inserted within the chromosomes causing variations in the expression level of the marker gene luciferase. We took the opportunity of this variable level of expression to select lines in which the luciferase is expressed at a moderate level in order to demonstrate that tethering of the LacO:Luc transgene to a nucleoporin via LacI-LacO interactions may change the expression levels of luciferase. While nucleoplasmic Nup50a appears to repress marker gene expression, peripherally localized Seh1 enhances expression. As suggested by,\textsuperscript{19} sub-nuclear localization of the LacI tether is likely to change the localization of the transgene from random distribution to the site where the tether is localized. Thus, in the case of Seh1-LacI-YFP tether, the LacO:Luc transgene is also likely present at the nuclear periphery (Fig. 2). That the changes in expression levels are due to LacI-LacO mediated tethering of the nucleoporin to the transgene is further suggested by disruption of the tethering with IPTG. Increased expression as a result of interaction with LacI-YFP unbound to Seh1 is unlikely as this is not seen with the NUP50a constructs or in other experiments with both N- and C- constructs including the construct YFP-Seh1-LacI and in other lines (data not shown). Luciferase levels at the mRNA, protein and enzyme activity level increased significantly in the presence of the Seh1-LacI-YFP tether but dropped significantly when the LacI-LacO tethering was disrupted with IPTG. While the data presented clearly indicates an effect of Seh1-LacI-YFP tethering on luciferase expression, we were unable to obtain data demonstrating that this occurs at the NPC and thus other explanations (for instance that Seh1 has transcriptional activator activity) cannot be ruled out. Nucleoporins have been shown to be active in altered gene expression both in the nucleoplasm and at the nuclear periphery.\textsuperscript{12,20} It proved technologically impossible to establish the location of the tether using 3-D FISH.

Interestingly, drosophila orthologues of Seh1 and Nup50a, DmSec13 and DmNup50, were shown to enhance gene expression.\textsuperscript{1,20} The enhancing effect observed here for Seh1 may suggest that some similarities in nucleoporin effect on gene expression exist across different kingdoms. The enhancing effects of DmSec13 and DmNup50 were due to direct interactions of the native protein with chromatin.\textsuperscript{1,20} Whether native
Arabidopsis Seh1 and Nup50a interact with chromatin, or other components involved in gene regulation, and how they affect expression of native genes, remains to be identified. In addition, while it is clear that nucleoporin tethering via LacI-LacO affects marker gene expression, the underlying molecular pathways need to be investigated. So far reverse genetics, proteomic marker gene expression, the underlying molecular pathways need to be identified. In addition, components involved in gene regulation, and how they affect Arabidopsis Seh1 and Nup50a interact with chromatin, or other components being identified, this opens an exciting area of future investigations and we continue to explore the biological significance of chromatin tethering in plants.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Figure 5. Seh1-LacI-YFP effect on luciferase activity in various tissues. Luciferase activity in rosette leaves, stems, inflorescences and seeds in the absence (black) or presence (gray) of the Seh1-Lac-Luc tether. Luciferase activity increased from 100 ± 1% to 209 ± 3% (p < 0.001) in leaf and from 72 ± 1% to 152 ± 1% (p < 0.001) in inflorescence tissue, and remained similar in stems (7.4 ± 0.4% and 6.4 ± 0.7%) and seeds (5.5 ± 0.4 and 7.9 ± 0.5%) (p > 0.05). Average ± sem.
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