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Functional Analysis of the *Lupinus luteus* Cyclophilin Gene Promoter Region in *Lotus japonicus*

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Abstract: Functional analysis of promoter sequences is important to understand the regulation of gene expression. This study aimed to investigate the promoter region of the *Lupinus luteus* cytoplasmic cyclophilin gene (*LlCyP*; AF178458). After bioinformatic analysis, four promoter deletion fragments were fused to the β-glucuronidase reporter gene. We used *Lotus japonicus* as a model plant. After *Agrobacterium rhizogenes* transformation of *L. japonicus*, only the longest promoter region (−1055 bp to ATG) supported the β-glucuronidase expression in root nodule parenchyma. Putative *cis*-elements located between −1055 and −846 bp were subjected to site-directed mutagenesis. Mutations incorporated in the TGATT and AGATT motifs (cytokinin response) abolished *GUS* expression in nodules, but the mutated AAAGAT motif (OSE, organ-specific element) still activated the *GUS* expression in root nodules, mainly in cells surrounding the vascular bundle. Promoter deletion and mutation experiments suggest that *cis*-elements responsible for gene expression in the nodule are located in the region spanning from −1055 to −846 bp. We constructed a deletion fragment, in which the DNA sequence located between −822 and −198 bp was removed (pCYPMG). The promoter region arranged in the pCYPMG supports the expression in the parenchyma of *L. japonicus* nodules, but it is lower than the whole promoter region. The obtained results may be useful for transgene expression in determinate and indeterminate root nodules.

Keywords: *Lupinus luteus*; *Lotus japonicus*; cyclophilin; β-glucuronidase (*GUS*); plant transformation

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1. Introduction

Cyclophilins (CyPs) belong to a family of proteins with peptidyl-prolyl *cis-trans* isomerase activity (PPIase) that are involved in the folding of target proteins. It has been shown that isomerization around Xaa-Pro bonds is one of the most rate-limiting steps in protein folding, and that this process can be accelerated by the PPIase activity of CyPs. Distinct isoforms of cyclophilins are localized in the cytosol [1,2], nucleus [3,4], mitochondria [5], chloroplasts [6], and endoplasmic reticulum [7]. Their functions have been studied in the human immune response because they are receptors for cyclosporine A (CsA), an immunosuppressive drug that is widely used to prevent organ transplant rejection [1,8]. CyPs are also known to play a key role in virus replication in both animal and plant cells [9,10]. Many studies on the function of cyclophilins have demonstrated their role in HIV infection [11–13]. Cyclophilins participate in many cellular processes such as signaling, nucleic acid interactions, mRNA processing and spliceosome assembly, protein degradation, and apoptosis, showing that they are important proteins involved in a wide variety of cellular processes that play crucial roles in development and in the response to various stresses [4,14–19]. In plants, CyPs control transcription and hormone

signaling, and affect plant development and interactions with pathogens [20–22]. It has been shown that some *CyP* transcripts accumulate in response to wounding, heat shock, and low temperature treatment as well as in the meristematic zone of root nodules [23–25]. The mechanisms that determine how cyclophilins contribute to these cellular events are still largely unknown.

Targeted genetic modification of an organism relies on the identification of appropriate promoter sequences that direct the expression of a given gene in the desired place and at the desired time or developmental stage. Tissue/organ-specific promoters are most desirable in biotechnology.

We have used the *Lupinus luteus* cytosolic cyclophilin gene to determine promoter sequences responsible for its gene expression in nodules using a heterologous model system, *Lotus japonicus* (a model legume plant). It would be interesting to find out if the promoter sequences from *Lupinus luteus* would be able to promote expression in nodules of *L. japonicus*. Both plants form different types of nodules during interaction with their symbiotic bacteria. *L. japonicus* forms typical determinate type nodules while *Lupinus luteus* nodules are of indeterminate type. Nodules formed by *Lupinus luteus* are very different from the typical cylindrical shaped nodules formed by legumes such as *Pisum sativum* or *Medicago sativa*. In lupines the nodule primordium is initiated in the subrhizodermal root cortex without the formation of an infection thread in the root hair [26,27].

L. japonicus has been developed as a model plant to study the symbiosis between leguminous plants and rhizobia due to its small genome size, short life cycle, and high seed yield [28,29]. Moreover, *L. japonicus* can be easily transformed using *Agrobacterium rhizogenes* [20,21]. Composite plants, consisting of a wild-type shoot and a transgenic root, are frequently used for functional genomics in legume research [30]. This technique, primary developed for *Medicago truncatula*, allows for the fast and efficient production of transgenic roots which can be efficiently nodulated by symbiotic bacteria. Promoter sequences that are capable of triggering gene expression at a specific location or developmental stage are useful for biotechnology. In this work we analyzed the *LlCyP* promoter to find sequences responsible for its activity in root nodules through using bioinformatic analysis, site-directed mutagenesis, constructing promoter deletion series, and expressing them in a heterologous model system.

2. Materials and Methods

2.1. Fusion of LlCyP Promoter Deletion Fragments to GUS Reporter Gene

Lupinus luteus CyP promoter DNA fragment (pCyP-1055) was analyzed to find potential *cis*-acting elements, by a signal scan search in the PLACE database (A Database of Plant *Cis*-acting Regulatory DNA Elements) (https://www.dna.affrc.go.jp/PLACE/?action=newplace, accessed on 8 January 2007) [31]. The modified promoter-less binary vector pCAMBIA 1391Z (Cambia, Australia) was used to prepare all deletion constructs. This vector contains a promoter-less *GUS* (β-glucuronidase) gene interrupted by a catalase intron (uidAint) which prevents *GUS* expression in bacteria. The *LlCyP* promoter and its deletion derivatives were generated by PCR using specific primers and the *LlCyP* gene promoter as a template. Primers were designed to amplify segments of the *LlCyP* 5′-upstream sequence, from −1055, −845, −430, and −198 bp, upstream from the *LlCyP* translation initiation codon start (Figure 1). These deletion fragments were generated by PCR reactions using four different forward primers (F1, F2, F3, and F4) and a common reverse primer (R) (Figure 1). Each fragment was inserted into the cloning vector pGEM-T Easy (Promega Corporation, Madison, WI, USA). Cloned PCR products were sequenced by the Sanger dideoxy method to confirm their sequences and orientation. The confirmed clones of selected promoter fragments were subcloned into *SalI-NcoI* sites of the modified promoter-less pCAMBIA 1391Z binary vector. This way, we obtained four DNA constructs consisting of an appropriate promoter region connected with *GUS*, named pCyP-1055, pCyP-845, pCyP-430, and pCyP-198. To obtain the pCYPMG construct, the sequence between −822 and −198 bp of the pCyP-1055 was deleted (Figure 1b). This construct was prepared by

PCR using pCyP-1055 as a template and specific primers to amplify the plasmid/insert construct omitting the DNA fragment of the insert, indicated for deletion (Figure 1b). After amplification and phosphorylation (using polynucleotide kinase), resulting constructs were self-ligated and transformed into the *E. coli* competent cells. In all PCR reactions proofreading polymerase (Thermo Scientific Phusion High-Fidelity DNA Polymerase) was used to avoid introducing any other mutations. Plasmids with confirmed sequences were introduced to *A. rhizogenes* (A4TC24 strain) by electroporation with the Gene Pulser II System (Bio-Rad Laboratories, Hercules, CA, USA).

(a)

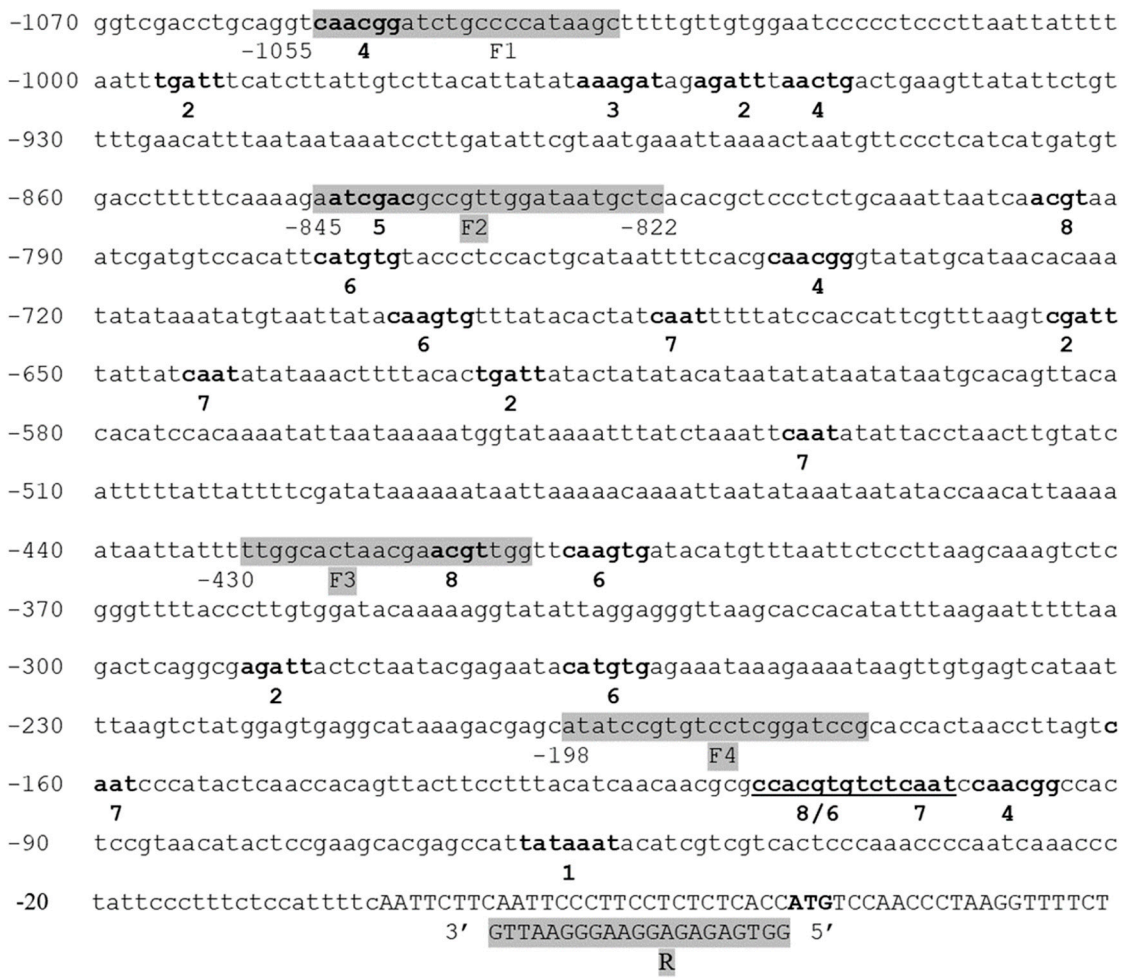

Figure 1. *Cont.*

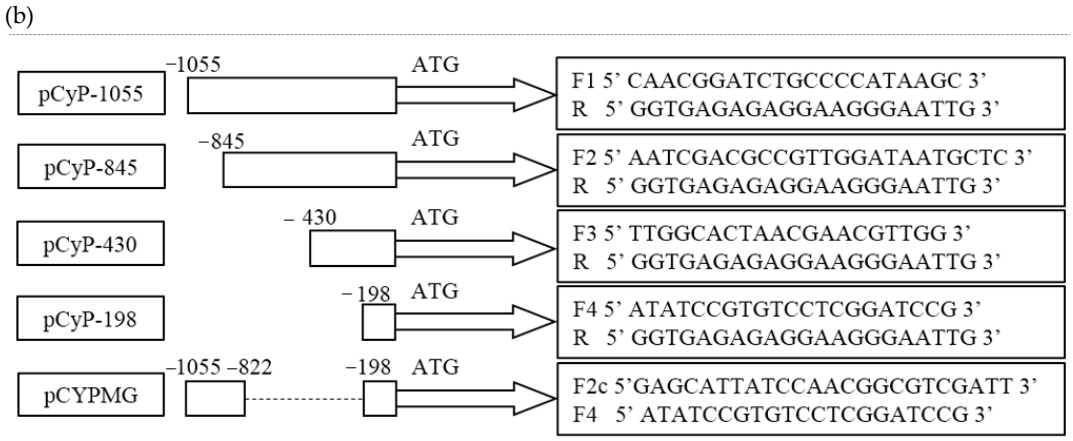

Figure 1. (**a**) Nucleotide sequence of the promoter region of the *LlCyp*. Transcribed sequence is in capital letters. Translational initiation codon is in bold, forward primers are shadowed and the reverse primer is in capitals, shadowed letters. Putative *cis*-acting regulatory motifs (bold) are numbered as follows: 1-TATA box, 2-cytokinin response motif, 3-nodulin-specific element, 4-MYB recognition site, 5-DRE, 6-MYC recognition site, 7-CAAT motif, 8-sequence early responsive to dehydration. Small, bold underlined letters—G-box (5′ CCACGTG 3′) overlapping auxin-responsible consensus sequence (5′ TGTCTC 3′) connected to CAAT box. (**b**) Schematic diagram of *LlCyP* promoter and its deletion constructs. The gene for glucuronidase is indicated as an arrow. Numbering of nucleotides as in (**a**).

2.2. Construction of LlCyP Promoter Mutants by Site-Directed Mutagenesis

For the promoter site-directed mutagenesis the pCyP-1055 clone was used as a template. We designed pairs of PCR primers back-to-back, so that the entire plasmid would be amplified by the PCR reaction (one of these primers incorporates the desired mutation) (Table S1 Supplementary Material). The PCR reaction creates a linear product whose ends can then be joined (after phosphorylation) with T4 DNA ligase, and the circularized vector is then transformed into *E. coli*. Plasmids with confirmed sequences were introduced to *A. rhizogenes* (A4TC24 strain).

2.3. L. japonicus Transformation and Inoculation of Composite Plants

L. japonicus seedlings were transformed according to the method described by Boisson-Dernier et al. (2001) [30]. *L. japonicus* seeds (Gifu B-129) (provided by Ryo Akashi; Biological Centre in *Lotus japonicus* and *Glycine max*, University of Miyazaki, Japan) were surface sterilized for 10 min in 5% hydrogen peroxide, rinsed several times with distilled water and germinated on the nitrogen-free 0.5x Murashige and Skoog medium (MS, Sigma-Aldrich) in the dark. Seven day-old seedlings were cut about 1.5 cm from the growing root tip and the freshly cut surface was inoculated with *Agrobacterium rhizogenes* carrying the individual DNA construct, grown for 48 h on solid YEB medium (5 g L^{-1} of beef extract, 1 g L^{-1} of yeast extract, 5 g L^{-1} of bacteriological peptone, 5 g L^{-1} of sucrose, and 2 mL L^{-1} of 1 M $MgSO_4$, 1.1% agar) supplemented with rifampicin (100 mg L^{-1}) and kanamycin (50 mg L^{-1}). Inoculated seedlings were grown on 0.5x MS medium at 22 °C in the dark. After three days, seedlings were transferred to 0.5x MS medium supplemented with cefotaxime (200 µg L^{-1}) to eliminate bacteria and were grown at 22 °C under a 16/8 h (day/night) photoperiod, with a light intensity of 200 µmol m^{-2} s^{-1} in the growth chamber. *A. rhizogenes* elimination from the plant culture was continued for two weeks. Agrobacterium-free plants were transferred to pots containing vermiculite and perlite (1:0.5 *v*/*v*) and grown under the same conditions. At the time of planting each plant was inoculated with 0.5 mL of *Mesorhizobium loti* MAFF 303,099 strain suspension. Plants inoculated with *M. loti* were grown at 22 °C under a 16/8 h (day/night) photoperiod and ~65% relative humidity in the growth chamber for five weeks.

2.4. Histochemical GUS Analysis

Histochemical staining of whole roots was performed in *GUS* staining buffer (1 mM X-Gluc 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 20% methanol, 0.1% Tween, 50 mM sodium phosphate pH 7.0, 5 mM DTT, 0.5 mM $K_4Fe(CN)_6 \bullet 3H_2O$, 0.5 mM $K_3Fe(CN)_6$) and incubated overnight at 37 °C. Before sectioning, stained 1.0 cm long root pieces, were first vacuum-infiltrated in FP buffer (2.5% formaldehyde in 50 mM sodium phosphate pH 7.2) and then incubated at 4 °C for 4 h. After washing twice with 50 mM sodium phosphate pH 7.2, fixed tissue samples were dehydrated in an ethanol dilution series, infiltrated with xylene, and embedded in Paraplast Plus (Sigma-Aldrich). After sectioning with a microtome, 10 µm sections were mounted on slides. After Paraplast removal, sections were photographed using a ZEISS Axiovert 200 microscope.

3. Results

3.1. In-Silico Analysis of the LlCyP Promoter Sequence

The DNA genomic region located upstream of the *LlCyP* start codon was searched for possible *cis*-acting elements that could regulate *LlCyP* expression in root nodules. The analysis was performed based on the PLACE database (Figure 1a). Putative TATA (TATAAAT) and a CAAT boxes were found in the region upstream of the predicted translation initiation site (Figure 1a). Both sequences serve as basal promoter elements for the initiation of transcription of eukaryotic genes. Our analysis showed: five ARR1AT motifs (regulatory elements in response to cytokine), five CAATBOX1 elements, six GATA elements (light-dependent and nitrate-dependent control of transcription), four MYB2 consensus elements (YAACKG, found in the promoters of the dehydration- and ABA-responsive genes), and five MYC consensus elements (CANNTG, response to growth and development, as well as response to stresses). There are also other predicted *cis*-regulatory elements: ABRE (ACGTGKC, ABA response element), ARF binding site (TGTCTC, auxin response *cis*-regulatory element), CPBCSPOR (TATTAG, cytokinin-enhanced expression) (Figure 1a) [32–34]. These putative regulatory elements suggest that the promoter region of the *LlCyP* gene may respond to symbiosis signals as well as to a variety of abiotic stress signals.

3.2. Deletion Analysis of LlCyP 5′ Regulatory Region in Composite Plants

Individual promoter deletion fragments were fused to the *GUS* reporter gene, to obtain four DNA constructs: pCyP-198, pCyP-430, pCyP-845, and pCyP-1055 (Figure 1). *A. rhizogenes* cells carrying individual deletion DNA constructs were used to inoculate freshly cut *L. japonicus* seedlings in the root hair emergence zone to induce transgenic hairy roots. The typical transformed phenotype, exhibiting rapid and irregular growth, was observed in nearly 60% of the infected plants after 7 days. *Agrobacterium*-free composite plants were transferred to pots and inoculated with the *M. loti* MAFF 303,099 strain. After five weeks, roots transformed with different constructs were subjected to *GUS* staining. Different patterns of *GUS* activity were observed for individual constructs. For the shortest promoter construct (pCyP-198), high *GUS* activity was observed in the root meristem (MZ), in elongation zones (EZ) and in the quiescent center (CSC) (Figure 2a). Strong *GUS* activity was also observed in the root stele and in the vascular cambium (Figure 2b). The pCyP-198 construct did not promote *GUS* expression in nodules at all (Figure 2c). For the pCyP-430 construct the strongest *GUS* staining was observed in the root meristem zone. In the elongation zone *GUS* staining was observed in the root stele, but in the hair emergence zone (MaZ) it was also detected in the root cortex (Figure 2d). Opposite to the pCyP-198 construct, strong staining was also observed in the root cortex in the zone of maturation (Figure 2e). This construct also did not promote *GUS* expression in nodules (Figure 2f). After transformation with the pCyP-845 construct the highest *GUS* staining was observed in the root cap (Figure 2g). There was no *GUS* expression in root stele, but it was observed in epidermal and root hair cells (Figure 2g,h). The pCyP-845 construct also did not promote *GUS* expression in nodules (Figure 2i). For the pCyP-1055 construct, *GUS* activity was detected in the root meristem zone but not in the root cap region and, as in pCyP-198 and

pCyP-430 constructs, high *GUS* expression was observed in the root stele (Figure 2i–l). For this construct we observed *GUS* staining in nodules, mainly in the parenchyma (Figure 2l).

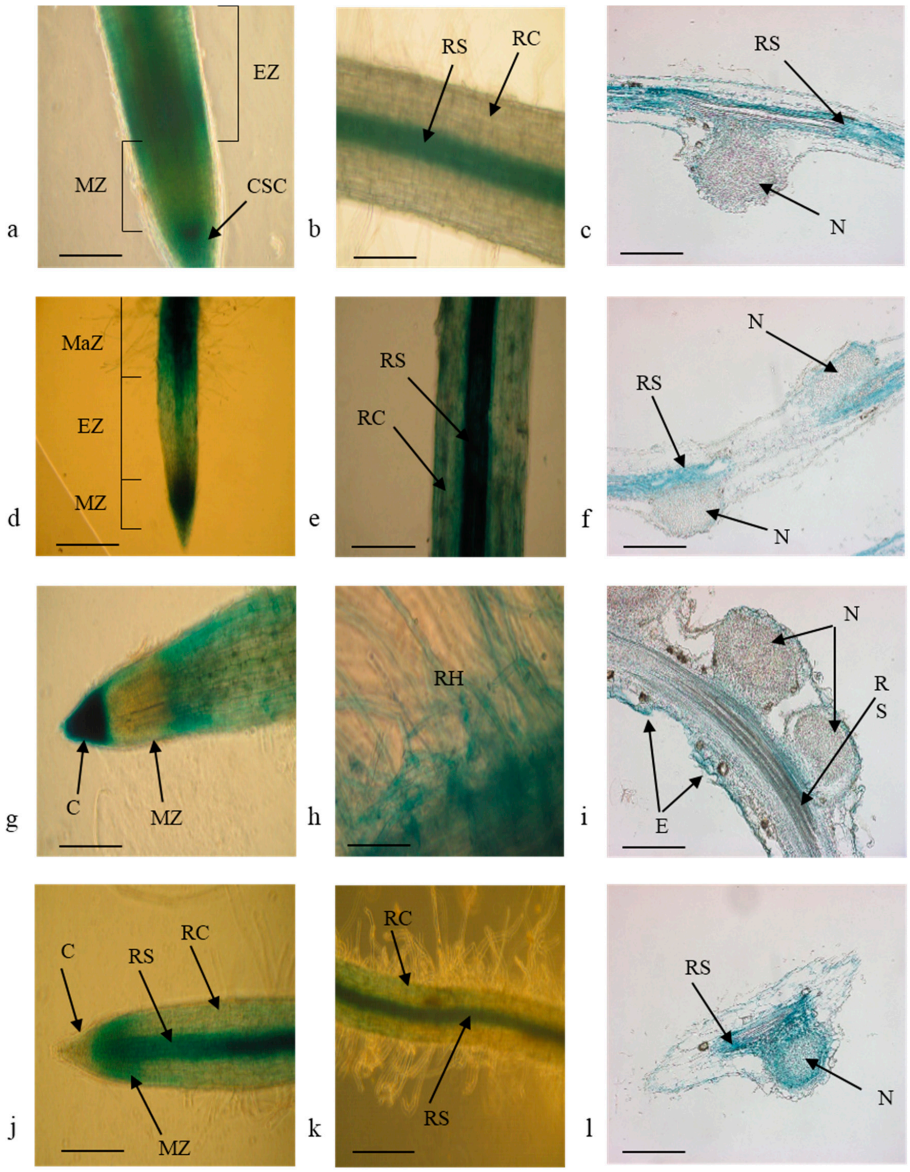

Figure 2. Histochemical localization of β-glucuronidase (*GUS*) activity in roots and nodules of *L. japonicus* transformed with different deletion constructs (composite plants). (**a**–**c**) *L. japonicus* transformed with pCYP-198 construct, (**d**–**f**) *L. japonicus* transformed pCYP-430 construct, (**g**–**i**) *L. japonicus* transformed with pCYP-845 construct and (**j**–**l**) *L. japonicus* transformed with pCYP-1055 construct. CSC—quiescent center, MZ—meristem zone, EZ—elongation zone, RS—root stele, RC—root cortex, N—nodule, MaZ—maturation zone, c—root cap, RH—root hair, E—epidermal cells. Scale bars: **a**, **b**, **d**, **e**, **g**, **j**, and **k** = 100 µm; **c**, **f**, **i**, **h**, and **l** = 50 µm.

3.3. LlCyP Promoter Activity in L. japonicus Nodules

GUS activity was localized in root nodules only in plants transformed with the pCyP-1055 construct, which has a promoter length of −1055 to +1 bp (Figure 2l). These results show that *cis*-acting elements supporting cyclophilin gene expression in nodules are located between −1055 and −846. The 5′ upstream sequence of *LlCyP* was searched for the presence of nodule-specific *cis*-acting regulatory elements. We found the known nodulin motif AAAGAT located in the region between −967 and −962 bp upstream of the start codon (Figure 1) [35]. This motif was characterized as nodule-specific for the "late" nodulin gene expression [36,37]. We also found two motifs which could be recognized by ARR1 proteins

after cytokinin stimulation: AGATT from −959 to −955 bp and TGATT from −996 to −992 bp. Cytokinins play an important role in the nodulation process, and are proposed to function as secondary signals activated after Nod factor perception [38]. Cytokinins trigger cortical cell division and function in nodule organogenesis rather than in the rhizobial infection initiation process. We also found the sequence TAATATAAA localized between −468 and −460 bp, similar to that found in the soybean lbc3 promoter in the SPE region (strong positive element AATATTAAA) [37,39].

The pCyP-845 construct is not able to drive *GUS* expression in nodules. Using site-directed mutagenesis techniques we prepared three DNA constructs with mutations introduced in the putative nodule-specific elements (Table S1 Supplementary Material). In the MUT1 and MUT3 constructs, putative cytokinin responsive elements were converted, TGATT to AGCCA (MUT1) and AGATT to AACCG (MUT3). These changes abolished *GUS* expression in nodules as well as in the root meristem zone (Figure 3a–c,g–i). In the MUT2 construct, the putative nodule-specific element AAAGAT was converted to GCATCG. Surprisingly, this construct supports *GUS* expression in the parenchyma in cells surrounding the vasculature of nodules (Figure 3e,f). To define the minimal number of *cis*-regulatory elements required for the activity of the promoter in nodules, a deletion construct (pCYPMG) was prepared. From the promoter region (pCyP-1055) nucleotides between −822 and −198 bp were deleted (Figure 1b). The pCYPMG supports the expression of the reporter gene in nodules, but the level of expression is much lower than for the pCyP-1055 construct (Figure 2l or Figure 3k,l). These findings suggest that the "deleted" 624 bp long promoter region contains some *cis*-regulatory elements which can enhance the promoter activity in nodules (Figure 1a).

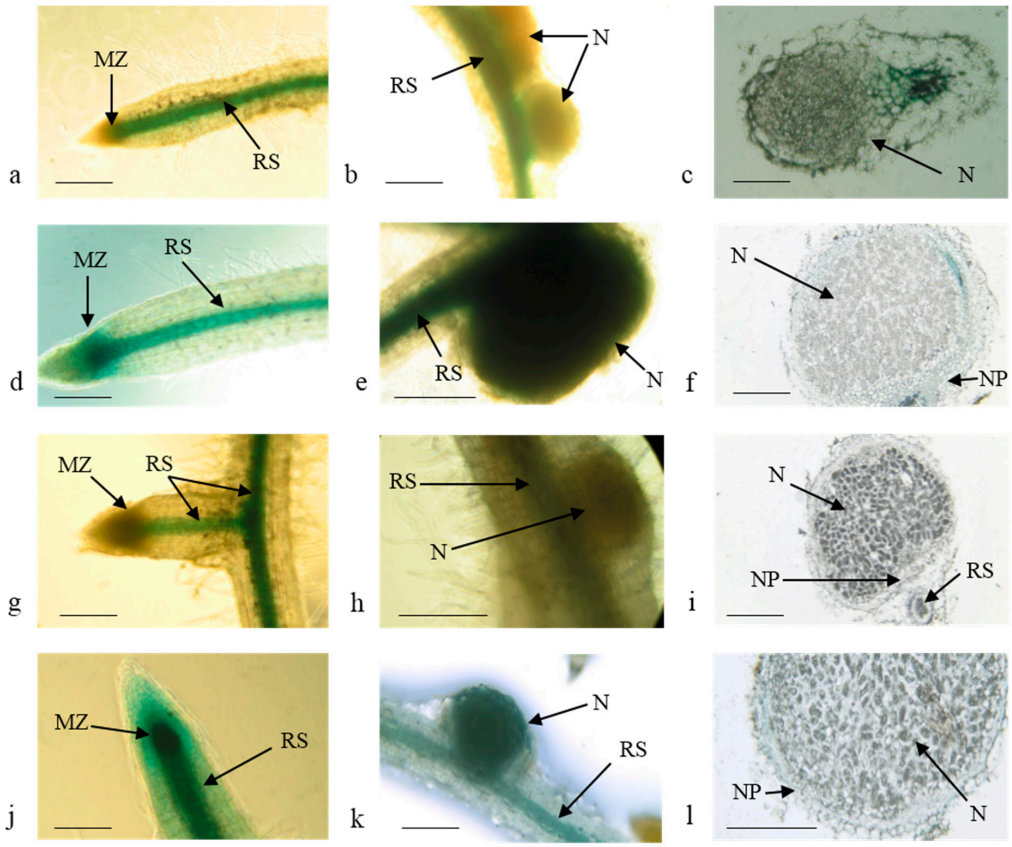

Figure 3. Histochemical localization of β-glucuronidase (*GUS*) activity in roots and nodules in composite plants of *L. japonicas* transformed with mutant constructs. (**a**–**c**) Mut1; (**d**–**f**) Mut2; (**g**–**i**) Mut3; (**j**–**l**) pCYPMG. MZ—meristem zone, RS—root stele, N—nodule, NP—nodule parenchyma. Scale bars: **a**, **b**, **d**, **e**, **g**, **h**, **j**, and **k** = 100 μm; **c**, **f**, **i**, and **l** = 50 μm.

4. Discussion

The best characterized *cis*-acting regulatory elements, controlling gene expression in nodules, are AAAGAT and CTCTT [37]. These two motifs are usually located approximately 300 bp from the start codon in the nodulin promoter, but sometimes the location of these elements is different, as in the *ENOD40* gene located over 2000 bp upstream of the ATG codon [40]. In the promoter of the *LlCyP* gene, we found that the AAAGAT motif is located far upstream of the translation start site (−967 to −962 bp) (Figure 1a) and the longest construct (pCyP-1055) supports *GUS* activity in nodules in composite *L. japonicus* plants after *M. loti* inoculation (Figure 2l). In the nearest proximity to that nodulin specific element, we found two motifs that can be recognized by ARR1 proteins after cytokinin stimulation: AGATT from −959 to −955 bp and TGATT from −996 to −992 bp (Figure 1a). As cytokinins play an important role in the nodulation process, such an integration of *cis*-regulatory elements may promote the expression of the *LlCyP* gene in nodules. In the 5′ upstream sequence of *LlCyP* we also found the sequence TAATATAAA located between −468 and −460, which is similar to the motif called strong positive element (SPE—AATATTAAA). However, the pCyP-845 construct did not mediate *GUS* expression in the *L. japonicus* nodules (Figure 2i).

To address the functional significance of the individual *cis*-regulatory elements present in the *LlCyP* promoter, we evaluated the effects of targeted nucleotide substitutions in pCyP-1055 on the promoter activity. A representative example of each category of mutations incorporated is provided in Figure 3. Mutations incorporated in the cytokine responsive elements (AGATT and TGATT) abolish reporter gene expression in nodules, but not in roots (Figure 3a,b,g,h). Mutations incorporated in the OSE element (AAAGAT) did not affect expression of the reporter gene in nodules, however it was lower than for the pCyP-1055 construct (Figure 2l or Figure 3e,f) which indicates that there are other *cis*-regulatory elements that are responsible for the gene expression in nodules. These findings suggest that *cis*-acting elements interacting with proteins in response to hormones are particularly important for the gene expression in nodules.

L. japonicus and *Lupinus luteus* produce different types of nodules: determinate and indeterminate, respectively. In situ hybridization in *Lupinus luteus* nodules with the antisense cyclophilin probe had the highest signal in the meristematic zone [23]. In contrast to indeterminate nodules, the determinate nodules do not have a strictly defined meristematic zone [41,42]. The activity of meristem in the determinate-type nodule is observed during early nodule development and its location is poorly characterized [42,43]. Nodulation involves very important cell processes and enhanced expression of certain plant genes, which are referred to as early (their protein products are involved in the infection process) and late nodulin genes (their protein products are responsible for nodule function) [44,45]. Many of these genes encode hydroxyproline-rich glycoproteins (HRGP) and proline-rich proteins (PRPs) [46]. Some of them are cell wall components affecting nodule formation and function. The activity of *LlCyP* promoter in nodules is probably connected with the need for a large amount of cyclophilins that carry out isomerization around Xaa-Pro bonds, in the process called protein folding enabling HRGPs and PRPs to attain their native structure [23]. After histochemical staining of nodules from *L. japonicus* transformed with the pCyP-1055 construct, the *GUS* activity was detected in nodule parenchyma (Figure 2l). This suggests that despite the differences in nodule type, the promoter region of the *LlCyP* cyclophilin gene contains sequences which activate gene expression in both kinds of nodule. The promoter region in the pCyP-1055 construct contains cytokinin- and auxin-responsive elements.

The experiment with the promoter deletion (pCYPMG), indicates that the constructed promoter region contains all *cis*-acting elements required to maintain the expression of the reporter gene in nodules. This region contains: TATAAAT box, CAAT motif, MYB and MYC recognition sites (from ATG to −198 bp), cytokinin response motifs and nodulin specific element (from −1055 to −822 bp of the original promoter sequence). However, the gene expression from this "minimal" promoter is not restricted to root nodules since *GUS* staining is also observed in the meristem zone of root tips and root stele (Figure 3j).

Serial deletion analyses are a powerful method to evaluate the specificity and capability of promoters. In plant biotechnology, the main aim is to improve crops by introducing genes conferring a desired phenotype. Tissue/organ-specific promoters have become the focus of plant genetic engineering. Some plant promoters were analyzed for their capability to trigger gene expression in a specific manner according to the developmental stages [47]. Crop legumes can form symbiotic interactions with nitrogen-fixing bacteria in response to nitrogen deficiency. These symbiotic relationships play a significant role in improving soil fertility. The benefits gained from growing legume crops can be maximized by using high-yielding varieties that create conditions for efficient assimilation of nitrogen by symbiotic soil bacteria. The "minimal" promoter reported in this work can be used in experiments to trigger gene expression in the root nodules, e.g., in *L. japonicus.* The creation of "minimal" promoters, required for a certain expression, is important for biotechnology. In this work, we have shown that our promoter constructed in pCYPMG, presents opportunities for application in targeted expression systems. Further work to improve the capabilities of the promoter should be carried out.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11050435/s1, Table S1: Primers used for site directed mutagenesis.

Author Contributions: Author contribution statement: K.N. and P.N. conceived and designated research, K.N. and P.O. conducted experiments, M.S. prepared tissue sectioning, K.N. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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