A quantitative hypermorphic CNGC allele confers ectopic calcium flux and impairs cellular development

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The coordinated control of Ca²⁺ signaling is essential for development in eukaryotes. Cyclic nucleotide-gated channel (CNGC) family members mediate Ca²⁺ influx from cellular stores in plants¹-⁴. Here we report the unusual genetic behavior of a quantitative gain-of-function CNGC mutation (brush) in Lotus japonicus resulting in a leaky tetrameric channel. brush resides in a cluster of redundant CNGCs encoding subunits which resemble metazoan voltage-gated potassium (Kv1-Kv4) channels in assembly and gating properties. Plants homozygous for brush are impaired in root development and infection by nitrogen-fixing rhizobia which segregated as a recessive monogenic trait. The brush allele exhibited quantitative behavior since overexpression of the cluster subunits was required to suppress the brush phenotype.

The results reveal a mechanism by which quantitative competition between channel subunits for tetramer assembly can impact the phenotype of the mutation carrier.

The legume-rhizobium symbiosis offers an excellent model system to study the role of Ca²⁺ signaling in eukaryotic cell development. Rhizobia produce lipochitooligosaccharides (LCOs) which stimulate a signal transduction pathway involving oscillations of [Ca²⁺] in the nucleus and perinuclear region of legume root hairs⁵-⁹, leading to rhizobial entry and organ development. The Lotus japonicus mutant brush was previously isolated in a screen for plants
defective in symbiotic cell development\textsuperscript{10}. At 26°C, \textit{brush} roots are stunted and root hair infection threads do not progress into the root cortex, resulting in the formation of non-infected ("empty") nodules. The evidence suggested that the recessive mutation was negatively-interfering with infection thread progression and cell expansion in the root apical meristem. The \textit{brush} mutation (Gifu ecotype) was mapped to the short arm of chromosome 2 at 8.8 cM\textsuperscript{10}, linked to the marker TM0312. Subsequently, a large-scale recombinant screen for fine-mapping was undertaken. In total, 20 of 1148 tested F2 individuals showed recombination events between the flanking markers TM2432 and TM0348 (Figure 1-figure supplement 1). F2 genotyping and subsequent F3 phenotyping refined the target region to 37 kb. One EMS-induced mutation was detected in the first exon of \textit{BRUSH}, a predicted CNGC of unknown function. Because the \textit{brush} mutant phenotype could not be complemented with the genomic region containing \textit{BRUSH} including its native promoter (see below), we searched for additional possible missed mutations. The genome of \textit{brush} was sequenced and aligned with the reference genome. Within the already delineated target interval the mutation in \textit{BRUSH} was confirmed and no additional polymorphisms relative to the Gifu wild-type were detected.

The predicted genomic sequence of \textit{BRUSH} carried a guanine to adenine (G401A) transition in the first exon in \textit{brush} (Figure 1A). Amplification of \textit{brush} cDNA revealed an open-reading frame encoding a protein containing 773 amino acids with an amino acid exchange from glycine to glutamic acid (G134E). The genome of the model plant \textit{Arabidopsis thaliana} contains twenty predicted CNGC genes, which can be classified into four distinct groups (I, II, III, IV)\textsuperscript{11}. Phylogenetic and synteny analysis revealed that \textit{BRUSH} (CNGC.IVA1) is orthologous to the Group IVA members ATCNGC19 and ATCNGC20 (Figure 1B). Similar to other Group IVA CNGCs, \textit{BRUSH} contains a relatively long N-terminal extension followed by six predicted transmembrane domains and a cyclic nucleotide-binding domain (Figure 1C). The \textit{brush} mutation is located in a conserved region previously
identified as a putative sorting signal in Group IVA CNGCs\textsuperscript{12}. Sequencing coupled with gene
prediction of the \textit{brush} target region revealed that \textit{BRUSH} resides in a cluster containing five
\textit{CNGC} loci (Figure 1-figure supplement 2). Analysis of syntenic genomic regions in legumes
and non-legumes revealed that the \textit{CNGC.IVA} cluster expansion occurred early in the legume
lineage and was retained. Transcripts for three of the loci could be amplified (\textit{CNGC.IVA3},
\textit{CNGC.IVA4}, \textit{CNGC.IVA5}) and encode proteins which are closely related to \textit{BRUSH} (Figure
1-figure supplement 3). No transcript was detected for \textit{CNGC.IVA2} which contains a large
transposon insertion in the seventh intron (Figure 2A).

To confirm that \textit{brush} carried the causative mutation, the \textit{BRUSH} genomic sequence was
expressed in \textit{brush} transgenic hairy roots driven by its native 2 kb promoter. Surprisingly we
did not observe rescue of either the root or infection thread phenotype (Figure 2-figure
supplement 1A-C). However, when \textit{brush} was transformed with the \textit{BRUSH} genomic
sequence driven by the \textit{L. japonicus} constitutive polyubiquitin promoter, we observed
restoration of the root and infection thread phenotypes, including infected nodules (Figure
2B,C and Figure 2-figure supplement 2A,B). These results suggested that expression level of
\textit{brush} is critical for phenotype manifestation. To further analyze the relationship between
\textit{brush} expression levels and the observed phenotypes, we generated RNA interference (RNAi)
constructs to target the untranslated regions (3’UTR or 5’UTR) of the \textit{brush} transcript in the
\textit{brush} mutant. Transformation of each RNAi construct specifically silenced \textit{brush} (Figure 2D,
Figure 2-figure supplement 3A) and restored rhizobial infection of root cells (Fig. 2E,F). We
then overexpressed the \textit{brush} allele in wild-type Gifu hairy roots to recapitulate the \textit{brush}
phenotype and observed that ectopic overexpression of \textit{brush} impaired hairy root emergence
(Figure 2-figure supplement 3B). Collectively, these results suggest that the expression level
of \textit{brush} is critical for the observed phenotypes and that the phenotypic penetrance of the
allele appears to be dosage-dependent. An EMS mutant (SL1484-1) was then obtained by
TILLING\textsuperscript{13, 14} containing a point mutation (W119stop) early in the \textit{BRUSH} open reading
frame. Analysis of homozygous mutant plants did not reveal any phenotypic root or infection abnormalities after inoculation with rhizobia (Figure 2-figure supplement 3C). The finding that the null mutant of BRUSH is not recapitulating the brush phenotype indicates that brush is an interfering allele and that the loss of BRUSH is compensated by potential redundancy of other CNGCs within the cluster.

To determine if the other CNGC.IVA cluster genes are redundant with respect to BRUSH, we overexpressed CNGC.IVA3, CNGC.IVA4, CNGC.IVA5 in the brush mutant by hairy root transformation. Analysis of transgenic roots revealed that expression of each gene complemented brush, as evidenced by colonized root nodules (Figure 2B,C). Further, we found that overexpression of the predicted Arabidopsis orthologs ATCNGC19, or ATCNGC20 in brush also restored nodulation (Figure 2-figure supplement 4A,B). To assess if each L. japonicus gene is expressed in roots, qPCR was performed before and after inoculation with rhizobia. Transcripts were detected for BRUSH, CNGC.IVA3, CNGC.IVA4, and CNGC.IVA5, which did not show significant changes (<2-fold) after inoculation with the rhizobial symbiont (Figure 2-figure supplement 5A). Spatial expression patterns of promoter:β-glucuronidase (GUS) fusions revealed that BRUSH expression was localized in root hairs and developing nodules after inoculation with rhizobia and that CNGC.IVA3, CNGC.IVA4, and CNGC.IVA5 are expressed in similar domains (Figure 2-figure supplement 5B). Closer investigation of the BRUSHpromoter:GUS activity revealed a lack of expression in roots prior to inoculation and subsequent activity associated with infected root hairs and nodule primordia after inoculation (Figure 2-figure supplement 6). The overlapping expression pattern of the Group IVA CNGCs together with their ability to dampen the brush phenotype indicate that these genes are redundant.

Given that plant CNGCs are anticipated to form both homomeric and heteromeric tetramers\(^\text{15}\), an interaction between BRUSH and redundant CNGCs is conceivable. We initially utilized the yeast split-ubiquitin interaction assay to determine the location of the
BRUSH termini\textsuperscript{16}. The assay utilizes the N-terminal (Nub) and C-terminal (Cub) fragments of yeast ubiquitin (Ubi4)\textsuperscript{17}. Reconstitution of ubiquitin in the cytoplasm leads to proteolytic release of Cub (fused to LexA-VP16) and activation of genetic reporters. We observed that BRUSH-Cub interacted with both NubI-BRUSH and BRUSH-NubI fusions demonstrating that both BRUSH termini are located in the cytoplasm (Figure 3-figure supplement 1). Since voltage-gated ion channel subunits interact via their soluble domains\textsuperscript{18} we focused on the CNGC.IVA soluble termini for yeast two-hybrid interaction assays. We observed a self-interaction for the BRUSH N-terminus (NT) as well as interaction with the NTs of brush, CNGC.IVA3, CNGC.IVA4, CNGC.IVA5 (Figure 3A) along with ATCNGC19 and ATCNGC20 (Figure 2-figure supplement 4C). To further substantiate the yeast interaction, we co-injected full-length subunits into Xenopus laevis oocytes for bimolecular fluorescence complementation (BiFC) assays. Expression of BRUSH-BRUSH, BRUSH-brush, and brush-brush combinations resulted in successful complementation (Figure 3-figure supplement 2A,B). The yeast and oocyte interaction assays demonstrate that CNGC.IVA channels potentially form homo- and hetero-complexes \textit{in vivo}, which may be mediated in part by their NT domains.

To characterize their channel properties, we injected \textit{Xenopus} oocytes with either \textit{BRUSH} or \textit{brush} for two-electrode voltage clamping. Expression was confirmed for both BRUSH-YFP and brush-YFP by confocal microscopy (Figure 3B,C). Oocytes expressing BRUSH (Figure 3B,D) or BRUSH-YFP (Figure 3-figure supplement 2C,E) failed to yield significant inward currents at negative voltages in the presence of up to 30 mM CaCl\textsubscript{2}. In contrast, under the same experimental conditions, oocytes expressing brush (Figure 3C,D) or brush-YFP (Figure 3-figure supplement 2D,E) produced voltage- and time-dependent inward currents at negative voltages. The currents were evident starting from 15 mM external CaCl\textsubscript{2} and increased in a dose-dependent manner with the external CaCl\textsubscript{2} concentration (Figure 3E). Exchange of Ca\textsuperscript{2+} as a charge carrier to K\textsuperscript{+} abolished the voltage-dependent inward currents.
mediated by brush-YFP (Figure 3-figure supplement 3D,F), indicative of a hyperpolarization-activated Ca\(^{2+}\)-permeable channel.

Since an N-terminal missense mutation leads to activation of brush, the conserved CNGC.IVA cytoplasmic domain may mediate channel gating (Figure 4A). The expression of brush alone in oocytes induces Ca\(^{2+}\) influx, therefore assembly of a brush homocomplex leads to deregulated activity (Figure 4B). As brush is recessive we speculate that brush is mainly positioned in silent heterotetrameric complexes in the heterozygous state, but assembles into a population of homocomplexes in homozygous plants triggering the phenotype (Figure 4C).

Given that brush is expressed in root hairs and nodule primordia after inoculation with rhizobia and that Ca\(^{2+}\) spiking in brush is intact\(^{10}\), the deregulated Ca\(^{2+}\) influx activity may impair rhizobial infection progression by interfering with downstream signaling events.

brush is a rare recessive gain-of-function missense mutation and exhibits an unusual quantitative genetic behavior. We pinpointed the CNGC tetrameric complex in combination with the expanded gene family as the causative factors for the unusual genetics. Although definitive evidence demonstrating that plant CNGCs form tetramers is required, their inclusion in the superfamily of voltage-gated ion channels predicts they will form such complexes. In support of this conclusion, the recent cryo-electron microscopy structures of the TAX-4 CNG channel from Caenorhabditis elegans\(^{19}\), the prokaryotic LliK CNG channel from Leptospira licerasiae\(^{20}\), and the human hyperpolarization-activated cyclic nucleotide-gated (HCN1) channel\(^{21}\) all disclose a tetrameric assembly. Therefore, we speculate that quantitative competition amongst redundant subunits for tetramer inclusion clarifies why a BRUSH null is phenotypically wild type and why overexpression is required to suppress the brush phenotype.

Expression of brush in oocytes revealed that the mutation renders the channel permeable to Ca\(^{2+}\) influx under hyperpolarizing conditions. Evidence obtained from Arabidopsis\(^{2, 22, 23}\), Medicago truncatula\(^{1}\), and the moss Physcomitrella patens\(^{24}\) CNGCs also supports the inward
rectification of Ca\(^{2+}\) by plant CNGCs, while numerous physiological studies have implicated CNGCs as being intimately linked to Ca\(^{2+}\)\(^4, 25-27\). Although brush was impermeable to K\(^+\) in our assay, evidence exists that some plant CNGCs are permeable to other cations (K\(^+\) and Na\(^+\)) in heterologous systems\(^28-31\). Collectively, the evidence demonstrates that plant CNGCs inwardly rectify cations.

Our results demonstrate that plant CNGC.IVAs may share more in common with metazoan Kv1-Kv4 K\(^+\) channels relative to typical mammalian CNGs. Similar to BRUSH, Kv1-Kv4 channel gating and subunit interactions are mediated by an N-terminal T1 domain\(^18, 32\). In contrast, human CNGs assemble via C-terminal interactions and are gated by binding of cyclic nucleotides\(^18, 33\). In addition to CNGCs, plants contain a family of shaker-type K\(^+\) channels with cyclic nucleotide-binding domains. Similar to BRUSH, these channels are not gated by cyclic nucleotides, but instead are regulated by voltage and relative ion concentrations\(^34\). Since plant CNGCs have been difficult to assess in heterologous systems\(^15\), the discovery that a single residue substitution in a conserved domain is sufficient for activation represents a significant advance towards understanding their regulation.

**Methods**

**Plant Material and transformations**

*Lotus japonicus* Gifu (wild-type, accession B-129)\(^35\), Miyakojima (accession MG-20)\(^36\) and *brush* (EMS mutant SL0979-2, Gifu)\(^14\) plants were used. The *BRUSH* TILLING line SL1484-1 was obtained from the *L. japonicus* TILLING facility (John Innes Centre, Norwich, UK). The seed bag numbers of critical lines are listed in Supplemental File 1C. Seeds were scarified with sandpaper, sterilized for 10 minutes in 4\% sodium hypochlorite, and imbibed overnight in sterile water at 4°C. Hairy roots were generated using the *Agrobacterium rhizogenes* strain AR1193\(^37\). Nodulation experiments were carried out by inoculating plants grown in pots or week jars containing a sand-vermiculite mixture and Fähraeus\(^38\) media with
Mesorhizobium loti MAFF303099 expressing DsRed\textsuperscript{39}. Hairy roots were stained for GUS as described previously\textsuperscript{40}. Plants were cultivated in growth cabinets at 22°C (16 h light/8 h dark). All complementation and GUS experiments were carried out a minimum of three times and displayed similar results. Crossings were performed as described previously\textsuperscript{41}. Primers and plasmids used for all experiments are listed in Supplementary File 1A and Supplementary Figure 1B, respectively.

**Map-based cloning of the brush mutation**

F2 plants from a cross between *brush* and MG-20 were used for fine mapping using SSR markers as described\textsuperscript{42}. Primer sequences were obtained from the Kazusa DNA Research Institute website (http://www.kazusa.or.jp/lotus/markerdb_index.html). The region was further refined using identified SNPs. The *brush* target interval between TM2432 and SNP3 (approximately 103 kb) was sequenced by Sanger sequencing. The *brush* genome was also reassembled after next-generation sequencing to identify mutant-specific polymorphisms. Nuclear DNA (see below) of *brush* seedlings was subjected to next-generation sequencing at Eurofins MWG, Germany, using an Illumina HiSeq 2000 (Illumina, USA) with a read length of 2 x 100 bp. Genes in the *brush* target region were annotated after sequencing using Genscan\textsuperscript{43} and Artemis\textsuperscript{44}. CLC Genomics Workbench (CLC bio, Denmark) was used to analyze the sequencing data.

**Nuclear DNA extraction for next-generation sequencing**

Four-week-old *brush* seedlings were grown in the dark for two days before leaf material was harvested and ground in liquid nitrogen. Approximately 2 g of powder was transferred to a 50 ml Falcon tube and dissolved in 20 ml ice-cold HB buffer (10 mM Tris, 80 mM KCl, 10 mM EDTA, 1 mM spermine, 1mM spermidine, 0.5 M sucrose, 0.5 % triton X-100, 0.15% β-mercaptoethanol, pH 9.4 with NaOH) by gentle shaking on ice. The solution was filtered through two layers of Miracloth (Calbiochem, Merck, Germany). The flow-through was transferred to a 15 ml Falcon tube and the nuclei were pelleted at 4°C by centrifugation (1800
x g) for 15 min. The pellet was washed two times by resuspension in HB buffer and centrifugation (4°C, 1800 x g for 5 min), resuspended in 500 μl CTAB buffer (55 mM cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, pH 8), and incubated at 60°C for 30 min. 500 μl chloroform:isoamylalcohol (24:1) was added and mixed by inverting the tube several times. After a centrifugation step at 8000 x g (4°C) for 10 min, the upper phase was transferred to a new tube. 5 μl of RNase (10 mg/ml stock concentration) was added and incubated at 37°C for 30 min. 0.6 volumes ice-cold isopropanol was added and mixed by inverting the tube several times. The nuclear DNA was then precipitated at -20°C overnight and spun down for 10 min at 16,000 x g and 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol and a centrifugation step for 10 min at 16,000 rpm and 4°C. The pellet was air-dried and resuspended in 55 μl TE.

**Yeast Two-Hybrid and Split-Ubiquitin Assays**

Yeast two-hybrid interaction assays were conducted with the haploid yeast strain AH109 (Clontech). Split ubiquitin interaction assays were carried out in the haploid strain THY.AP4. THY.AP4 and plasmids for split-ubiquitin were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). Plasmids used for both interaction assays are shown in Supplementary File 1B. Bait and prey plasmids were introduced via double transformation using the lithium acetate method and selected on media lacking leucine and tryptophan (-LW). The interacting protein pair of CCaMK and CYCLOPS was used as a control. Positive transformants were restreaked on -LW, then used to inoculate overnight cultures in liquid -LW media. Overnight cultures were diluted to OD$_{600}$ of 0.5 in sterile water and diluted 10-fold. 5 μl was spotted on -LW or solid media lacking leucine, tryptophan, adenine, and histidine (-LWAH). Yeast plates were incubated at 28°C for 3-5 days. All interaction assays were independently conducted a minimum of three times.

**Clone preparation for Xenopus oocyte experiments**
BRUSH and brush coding sequences were cloned for Xenopus expression with a custom Golden Gate strategy using a modified backbone obtained from the Standard European Vector Architecture 2.0 database. The backbone (with flanking bacterial transcriptional terminators) was derived from pSEVA191 and was chosen to alleviate toxicity issues uncovered while cloning CNGC.IVA sequences into pUC-based Golden Gate backbones and pGEMHE. A ccdB cassette compatible with Golden Gate cloning was amplified and inserted into the AvrII/Sacl sites of pSEVA191 to create the LII backbone pSEVA191 1-2. The coding sequences of BRUSH and brush were then combined in a Bsai cut-ligation with custom made modules containing the T7 promoter as well as the 5’UTR and 3’UTR sequences of β-globin mRNA (amplified from pEMHE). The same backbone was used to express the constructs for BiFC analysis, where LI Golden Gate B-C or D-E parts encoding for the N-terminal (VN) or C-terminal (VC) portions of mVenus were inserted.

**Functional analysis in Xenopus laevis oocytes**

BRUSH and brush capped mRNA synthesis, oocyte injection, and voltage-clamp recordings were performed as described. cRNA was synthesized (mMESSAGE mMACHINE T7 Transcription Kit, ThermoFisher) and oocytes were injected (General Valve Picospritzer III, Parker Hannifin Corp.) with approximately 25 ng cRNA or with RNase-free water as a control. Injected oocytes were stored at 18 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5mM HEPES, 10 mM sorbitol, pH 7.4 with NaOH) adjusted to 220 mOsm/L with sorbitol and supplemented with 25 µg/ml gentamycin until use. Measurements were recorded 2 to 3 days after injection using the two-electrode voltage-clamp technique with a Turbo Tec-10Cx amplifier (NPI electronic GmbH). During two-electrode voltage clamp measurements, oocytes were constantly perfused with bath solution composed of 30 mM CaCl₂, 10 mM MES-Tris pH 7.4, adjusted to 220 mOsm/L with mannitol and supplemented with either 100 µM 8-Bromo-cAMP (Sigma) or 100 µM 8-CPT-cAMP.
(BioLog). For analysis of channel permeabilities, CaCl$_2$ was exchanged as indicated in the figure legends with 5 mM CaCl$_2$, 15 mM CaCl$_2$, or 60 mM KCl. Starting from a holding potential of -40 mV, voltage steps from +60 to -160 mV in 20 mV decrements were applied (PatchMaster, HEKA Electronics Inc.). For localization, YFP was fused to the C-terminus of BRUSH or brush. Oocytes were imaged by confocal microscopy 2 to 3 days after injection with BRUSH-YFP and brush-YFP cRNA (Leica TCS SP5, excitation: 488 nm, detection: 525-575 nm) to confirm expression. The same protocol was used for BiFC experiments, except that 25 ng of each cRNA was co-injected prior to imaging.

**Gene Expression Analysis**

For analysis of gene expression after rhizobial inoculation, *Lotus japonicus* Gifu seeds were germinated and grown on half-strength B5 agar plates for fourteen days. Six plants were planted per week jar containing sand/vermiculite with Fåhraeus media. After seven days root tissue from a single jar was collected and pooled (represents a biological replicate) for the Day 0 time point. *Mesorhizobium loti* MAFF303099 expressing DsRed was added to the remaining jars and tissue was collected in the same manner after twelve days. To analyze gene expression after RNAi, positive hairy roots were isolated from individual plants six weeks after inoculation with *Mesorhizobium loti* MAFF303099 DsRed. For both experiments, root tissue was ground in liquid nitrogen and RNA was extracted with a Spectrum Plant Total RNA Kit (Sigma). Genomic DNA was removed using a Turbo DNA-free Kit (Ambion) and total RNA (1 µg for the time course and 200 ng for RNAi) was used for cDNA synthesis with Superscript III (ThermoFisher). cDNA was then checked for genomic DNA contamination by PCR. Expression of *CNGC.IVA* cluster genes after rhizobia inoculation was analyzed by qPCR using SYBR Select Master Mix (Applied Biosystems) with a CFX96 Real-time PCR machine. *brush* expression after RNAi was analyzed by qPCR using mi-real-time EvaGreen Master Mix (Metabion) with a QuantStudio 5 Real-Time PCR System (ThermoFisher). In both cases, the plotted data point for each biological replicate represents the mean of three
technical replicates. The relative expression was calculated with the $2^{\Delta\Delta CT}$ method\textsuperscript{54} using eEF-1A\textalpha (GenBank: BP045727) as the reference.

**Bioinformatics and Statistics**

*Arabidopsis thaliana* protein sequences were obtained from TAIR. A multiple sequence alignment was generated using MUSCLE in CLC Main Workbench (CLC bio, Denmark). A Maximum Likelihood phylogenetic tree was calculated using UPGMA (100 bootstrap iterations were performed). One-way ANOVA statistical analysis of data followed by a post-hoc Tukey’s multiple comparisons test and t-tests were calculated using GraphPad Prism.

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**References:**


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Figure 1. brush contains a point mutation in CNGC.IVA1 (BRUSH). (A) Schematic of the intron-exon structure of BRUSH highlighting the brush mutation in the first exon (red asterisk). (B) Phylogenetic tree of BRUSH (red node end) in relation to Arabidopsis thaliana CNGC proteins. BRUSH is orthologous to the Group IVA members ATCNGC19 and ATCNGC20. (C) Overview of the BRUSH protein domain structure highlighting the conserved Group IVA domain (CD, green), transmembrane domains (T, light blue), putative filter region (F, orange), and the predicted cyclic nucleotide-binding domain (CNBD, purple). Shown below is the CD sequence in brush (G134E mutation, asterisk) relative to BRUSH, ATCNGC19, and ATCNGC20 and the Group IVA CNGC consensus. Numbers at the branch points in (B) indicate the percentage bootstrap values (100 iterations) for the inferred tree. Scale bar in (B) indicates the number of amino acid substitutions per site.

Figure 1-figure supplement 1. Map-based cloning of the brush mutation on chromosome 2. Overview of contigs CM0435 and CM0312 (Lotus japonicus genome v2.5) on the short arm of chromosome two surrounding the brush target region. The relative location of SSR and SNP markers used for mapping are indicated on the contigs. The brush mutation was previously linked to TM0312 by rough mapping. The red asterisk indicates the position of the EMS-induced mutation found in brush. SSR markers were analyzed by fluorescent PCRs and SNPs were genotyped by sequencing.

Figure 1-figure supplement 2. Syntenic chromosomal locations of CNGC.IVA genes in selected species. L. japonicus contains five CNGC.IVA genes in a cluster on chromosome two. The CNGC.IVA cluster is bordered by genes encoding for β-amylase and glutamate decarboxylase. Other legumes such as Glycine max (soybean) and Medicago truncatula also contain a cluster of CNGCIVA genes with the same anchor genes. In contrast, two non-legume plant species of the Rosaceae family (Fragaria vesca and Prunus persica) contain a single CNGCIVA copy in association with the glutamate decarboxylase anchor. Also shown is chromosome three of Arabidopsis thaliana which contains two CNGCIVA copies. The slash between CNGCIVAc and CNGCIVAf on Medicago chromosome six represents a gap of 480 kb.

Figure 1-figure supplement 3. Protein sequence comparison of Group IVA CNGCs from Lotus japonicus. Protein sequence alignment of BRUSH (CNGC.IVA1), CNGC.IVA3, CNGC.IVA4, and CNGC.IVA5. Residues identical to BRUSH are shown as dots and gaps are shown as dashes. Shown is the Group IVA CNGC conserved domain (CD, green), brush mutation (red asterisk), predicted transmembrane domains (TM, light blue), putative selectivity filter (F, orange) in the pore region, and the cyclic nucleotide-binding domain (CNBD, purple). CNGC.IVA3 (81%), CNGC.IVA4 (65%), and CNGC.IVA5 (72%) show high sequence identity to BRUSH.

Figure 2. Genetic complementation of brush. (A) Genomic region surrounding BRUSH on L. japonicus chromosome 2 showing a cluster of five CNGC.IVA genes. CNGC.IVA2 contains a large transposon insertion (shown as a gap). (B) Complementation assay of brush roots by overexpression (ubiquitin promoter, UBQ) of the four expressed CNGC.IVA cluster members (bright field, top panel). The presence of red fluorescent nodules (arrow) colonized by Mesorhizobium loti expressing DsRED (lower panel) indicates successful complementation. (C) Number of nodules per transformed plant from (B) (n=10 for all constructs). (D) Quantitative PCR analysis of brush transcript levels after RNAi targeting either the 5’UTR or 3’ UTR of brush. The normalized fold expression of brush is shown relative to empty vector control roots (n=6 for all constructs). (E) Complementation analysis of brush expressing RNAi fragments targeting either the 5’UTR or 3’UTR of brush in the brush mutant. Panels are the same as (B). (F) Number of nodules per transformed plant (n=10 for all constructs) from (E). Roots for both complementation experiments were observed six weeks after inoculation with rhizobia. Scale bars in (B) and (E) represent 1 mm. Letters in (C), (D), and (F) indicate different statistical groups (ANOVA followed by Tukey’s HSD test, F(4, 45)= 10.86 (C), F(2, 15)=20.82 (D), F(2, 27)=22.72 (F), p-value < 0.001).
Figure 2-figure supplement 1. Expression of the BRUSH native genomic sequence in brush. (A) Complementation assay in brush by transformation of BRUSH230pro-Brush genomic or empty vector relative to Gifu wild type. No infected nodules were observed in brush roots expressing the BRUSH genomic sequence. Shown are DsRED fluorescence of rhizobia (middle panel) and GFP fluorescence (lower panel) for transformation control. Composite plants were sown in Weck jars, cultivated at 26°C, and inoculated with M. loti expressing DsRed. Roots were analyzed four weeks after inoculation. (B) Box plot showing the number of nodules per transformed plant (n=8) from (A). (C) Close-up of transformed roots. Scale bars in (A) represent 2 mm, 0.2 mm in (C). Different letters in (B) indicate different statistical groups (ANOVA followed by Tukey’s HSD test, F(2, 21)= 33.60, p-value < 0.001).

Figure 2-figure supplement 2. Complementation of brush by overexpression of BRUSH. (A) Nodulation complementation assay of brush by hairy root transformation of an empty vector control or UBQpro-Brush genomic construct relative to wild type. Infected nodules were observed in brush roots constitutively expressing the BRUSH genomic sequence. Shown are bright field images of roots and DsRED fluorescence of rhizobia. (B) Rhizobia remain entrapped in infection pockets in brush root hairs (left image, arrow). Overexpression of BRUSH restores infection of root hairs (middle) leading to infected nodule cells (right). The root hair images are composites of bright field and DsRED fluorescence. Plants with hairy roots were sown in Weck jars, cultivated at 26°C, and inoculated with M. loti expressing DsRed. Roots were analyzed four weeks after inoculation. Scale bars represent 0.5 mm in the top three panels of (A), lower panel 2 mm. Scale bars in (B) represent 15 µm.

Figure 2-figure supplement 3. RNAi off-target controls and phenotypes associated with either overexpression of brush or a null BRUSH allele. (A) qPCR of CNGC.IVA3, CNGC.IVA4, and CNGC.IVA5 in hairy roots transformed with an empty vector control (control) or an RNAi fragment targeting the 5′UTR (5′RNAi) or 3′UTR (3′RNAi) of brush in the brush mutant. The normalized fold expression is shown relative to the average expression level in empty vector control roots (n=6 for all constructs). (B) Hairy root formation efficiency of Gifu wild-type plants transformed with a T-DNA containing either UBQpro-Brush genomic or UBQpro-brush genomic relative to empty vector control. Numbers on the columns indicate the number of plants with GFP-positive hairy roots per total number of transformed plants. (C) A BRUSH TILLING line (SL1484-1) with a G357A exchange leading to a stop codon (W119Stop) was isolated. Nodule formation in homozygous SL1484-1 plants (n=3) was not impaired relative to wild-type (n=8). Plants were cultivated at 26°C and inoculated with M. loti MAFF 2kbpro. DsRed. Nodules were counted two weeks after inoculation. Letters in (A,C) indicate statistical groups. (A) ANOVA followed by Tukey’s HSD test; F(8,45)=0.9405, p-value > 0.05, (B) t-test, p-value > 0.05.

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voltage relations (n=4) in the presence of 30 mM CaCl₂ (closed circles) and 60 mM KCl (open circles) ±
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List of Supplementary and Source Data Files

- Supplementary File 1. Oligonucleotides used in this study.
- Supplementary File 2. DNA plasmids used in this study.
- Supplementary File 3. List of key plant material used in this study.

- Figure 1-source data 1. Figure 1B source data.
- Figure 2-source data 1. Figure 2C source data.
- Figure 2-source data 2. Figure 2D source data.
- Figure 2-source data 3. Figure 2F source data.
- Figure 2-source data 4. Figure 2- figure supplement 1B Source Data.
- Figure 2-source data 5. Figure 2- figure supplement 3A,C Source Data.
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- Figure 3-source data 1. Figure 3D Source Data.
- Figure 3-source data 2. Figure 3E Source Data.
- Figure 3-source data 3. Figure 3- figure supplement 2E Source Data.
- Figure 3-source data 4. Figure 3- figure supplement 2F Source Data.
**Figure 1.** *brush* contains a point mutation in *CNGC.IVA1 (BRUSH).* (A) Schematic of the intron-exon structure of *BRUSH* highlighting the *brush* mutation in the first exon (red asterisk). (B) Phylogenetic tree of *BRUSH* (red node end) in relation to *Arabidopsis thaliana* CNGC proteins. *BRUSH* is orthologous to the Group IVA members ATCNGC19 and ATCNGC20. (C) Overview of the *BRUSH* protein domain structure highlighting the conserved Group IVA domain (CD, green), transmembrane domains (T, light blue), putative filter region (F, orange), and the predicted cyclic nucleotide-binding domain (CNBD, purple). Shown below is the CD sequence in *brush* (G134E mutation, asterisk) relative to *BRUSH*, ATCNGC19, and ATCNGC20 and the Group IVA CNGC consensus. Numbers at the branch points in (B) indicate the percentage bootstrap values (100 iterations) for the inferred tree. Scale bar in (B) indicates the number of amino acid substitutions per site.
Figure 1-figure supplement 1. Map-based cloning of the brush mutation on chromosome 2. Overview of contigs CM0435 and CM0312 (Lotus japonicus genome v2.5) on the short arm of chromosome two surrounding the brush target region. The relative location of SSR and SNP markers used for mapping are indicated on the contigs. The brush mutation was previously linked to TM0312 by rough mapping. The red asterisk indicates the position of the EMS-induced mutation found in brush. SSR markers were analyzed by fluorescent PCRs and SNPs were genotyped by sequencing.
Figure 1-figure supplement 2. Syntenic chromosomal locations of CNGC.IVA genes in selected species. L. japonicus contains five CNGC.IVA genes in a cluster on chromosome two. The CNGC.IVA cluster is bordered by genes encoding for β-amylase and glutamate decarboxylase. Other legumes such as Glycine max (soybean) and Medicago truncatula also contain a cluster of CNGCIVA genes with the same anchor genes. In contrast, two non-legume plant species of the Rosaceae family (Fragaria vesca and Prunus persica) contain a single CNGCIVA copy in association with the glutamate decarboxylase anchor. Also shown is chromosome three of Arabidopsis thaliana which contains two CNGCIVA copies. The slash between CNGCIVA and CNGCIVAf on Medicago chromosome six represents a gap of 480 kb.
**Figure 1-figure supplement 3. Protein sequence comparison of Group IVA CNGCs from *Lotus japonicus*:** Protein sequence alignment of BRUSH (CNGC.IVA1), CNGC.IVA3, CNGC.IVA4, and CNGC.IVA5. Residues identical to BRUSH are shown as dots and gaps are shown as dashes. Shown is the Group IVA CNGC conserved domain (CD, green), brush mutation (red asterisk), predicted transmembrane domains (TM, light blue), putative selectivity filter (F, orange) in the pore region, and the cyclic nucleotide-binding domain (CNBD, purple). CNGC.IVA3 (81%), CNGC.IVA4 (65%), and CNGC.IVA5 (72%) show high sequence identity to BRUSH.
Figure 2. Genetic complementation of brush. (A) Genomic region surrounding BRUSH on L. japonicus chromosome 2 showing a cluster of five CNGC.IVA genes. CNGC.IVA2 contains a large transposon insertion (shown as a gap). (B) Complementation assay of brush roots by overexpression (ubiquitin promoter, UBQ) of the four expressed CNGC.IVA cluster members (bright field, top panel). The presence of red fluorescent nodules (arrow) colonized by Mesorhizobium loti expressing DsRED (lower panel) indicates successful complementation. (C) Number of nodules per transformed plant from (B) (n=10 for all constructs). (D) Quantitative PCR analysis of brush transcript levels after RNAi targeting either the 5’UTR or 3’ UTR of brush. The normalized fold expression of brush is shown relative to empty vector control roots (n=6 for all constructs). (E) Complementation analysis of brush expressing RNAi fragments targeting either the 5’UTR or 3’UTR of brush in the brush mutant. Panels are the same as (B). (F) Number of nodules per transformed plant (n=10 for all constructs) from (E). Roots for both complementation experiments were observed six weeks after inoculation with rhizobia. Scale bars in (B) and (E) represent 1 mm. Letters in (C), (D), and (F) indicate different statistical groups (ANOVA followed by Tukey’s HSD test, F(4, 45)= 10.86 (C), F(2, 15)=20.82 (D), F(2, 27)=22.72 (F), p-value < 0.001).
Figure 2-figure supplement 1. Expression of the BRUSH native genomic sequence in brush. (A) Complementation assay in brush by transformation of BRUSH\textsubscript{2kbpro:BRUSH} genomic or empty vector relative to Gifu wild type. No infected nodules were observed in brush roots expressing the BRUSH genomic sequence. Shown are DsRED fluorescence of rhizobia (middle panel) and GFP fluorescence (lower panel) for transformation control. Composite plants were sown in Wecj jars, cultivated at 26°C, and inoculated with *M. loti* expressing DsRed. Roots were analyzed four weeks after inoculation. (B) Box plot showing the number of nodules per transformed plant (n=8) from (A). (C) Close-up of transformed roots. Scale bars in (A) represent 2 mm, 0.2 mm in (C). Different letters in (B) indicate different statistical groups (ANOVA followed by Tukey’s HSD test, F\textsubscript{2, 21}= 33.60, p-value < 0.001).
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