

LETTERS

Direct control of shoot meristem activity by a cytokinin-activating enzyme

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The growth of plants depends on continuous function of the meristems. Shoot meristems are responsible for all the post-embryonic aerial organs, such as leaves, stems and flowers¹. It has been assumed that the phytohormone cytokinin has a positive role in shoot meristem function^{2–4}. A severe reduction in the size of meristems in a mutant that is defective in all of its cytokinin receptors has provided compelling evidence that cytokinin is required for meristem activity^{5,6}. Here, we report a novel regulation of meristem activity, which is executed by the meristem-specific activation of cytokinins. The *LONELY GUY (LOG)* gene of rice is required to maintain meristem activity and its loss of function causes premature termination of the shoot meristem. *LOG* encodes a novel cytokinin-activating enzyme that works in the final step of bioactive cytokinin synthesis. Revising the long-held idea of multistep reactions, *LOG* directly converts inactive cytokinin nucleotides to the free-base forms, which are biologically active, by its cytokinin-specific phosphoribohydrolase activity. *LOG* messenger RNA is specifically localized in shoot meristem tips, indicating the activation of cytokinins in a specific developmental domain. We propose the fine-tuning of concentrations and the spatial distribution of bioactive cytokinins by a cytokinin-activating enzyme as a mechanism that regulates meristem activity.

Cytokinins are *N*⁶-substituted adenine derivatives that have a crucial role in many aspects of plant growth and development^{7,8}. Like the other plant hormones, cytokinin activity *in planta* is thought to be controlled by a balance of synthesis, catabolism and inactivating conjugations^{9,10}. Moreover, the regulation of cytokinin signalling further modulates its effects^{11,12}. The spatial and temporal distribution of bioactive cytokinin levels is also strictly controlled in plant development events. Recent studies have shown that cytokinins are synthesized and catalysed at various sites in the plant^{13–15}, but the exact mechanism whereby cytokinin activity is regulated is not entirely clear.

The rice mutant *lonely guy (log)* was identified in a screen for defects in the maintenance of shoot meristems. In addition to a severe reduction of the panicle size and abnormal branching patterns (Fig. 1a), the number of floral organs was also decreased in *log* mutants (Fig. 1b–e), and flowers often contained only one stamen but no pistil (thus ‘*lonely guy*’). The inner floral organs were affected more severely than the outer organs in all allelic mutants examined (Fig. 1e). We view these phenotypes as the result of defects in maintaining meristem activity and have tested this idea by examining the vegetative and reproductive meristems in detail. Although no clear abnormality was observed during vegetative development, the shoot apical meristem (SAM) of *log-1* was smaller than that of wild type (Supplementary Table 1). After transition to the reproductive phase,

a number of meristems that give rise to panicle branches and flowers are generated in the wild-type inflorescence. However, *log* mutant inflorescence meristems and panicle branch meristems abort soon after the production of a few lateral meristems, leading to the production of a small panicle with a reduced number of branches and flowers (Fig. 1a). The wild-type floral meristem continues organ differentiation until it generates a pistil (Fig. 1f), but the *log* mutant

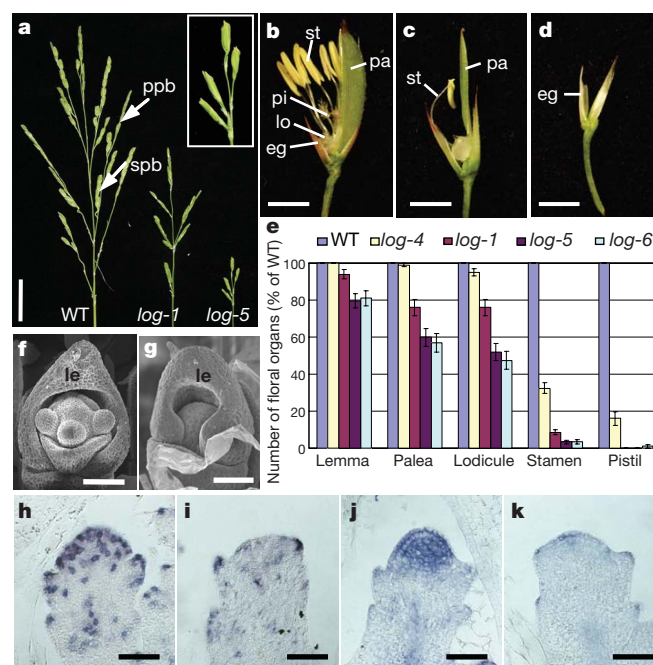


Figure 1 | *log* mutant phenotypes. **a**, Panicles of wild type (left), *log-1* (center) and *log-5* (right). The *log* panicles are small and show altered branching patterns. The inset shows a closer view of the *log-5* panicle. **b**, Wild-type flower. **c**, *log-1* flower with a weak phenotype, which has only one stamen and no pistil. In **b** and **c**, the lemma is removed to show the inner floral organs. **d**, *log-1* flower with a strong phenotype, which has only a pair of empty glumes. **e**, Reduction of the floral organ number in *log* mutants. The reduction in floral organ number in *log* mutants is given as per cent (mean $\pm$ s.d.) of wild type ($n = 100$). **f**, **g**, Scanning electron micrograph of an immature flower of wild type (**f**) and *log-1* (**g**) at the carpel initiation stage. Three stamen primordia are seen in the wild-type flower (**f**). **h–k**, Comparison of marker gene expression in wild type (**h**, **j**) and *log-1* (**i**, **k**); *in situ* hybridization of histone *H4* (**h**, **i**) or *OSH1* (**j**, **k**) expression. ppb, primary panicle branch; spb, secondary panicle branch; eg, empty glume; le, lemma; lo, lodicule; pa, palea; pi, pistil; st, stamen. Scale bars, 3 cm (**a**); 3 mm (**b**, **c**, **d**); and 50 μ m (**f** to **k**).

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floral meristem flattens and stops organ differentiation prematurely, without forming the normal number of floral organs (Fig. 1g). In a wild-type floral meristem, many cells express histone *H4*, accompanying the differentiation of floral organs (Fig. 1h). In contrast, very few *log* mutant cells express histone *H4* (Fig. 1i). *OSH1*, a meristem marker encoding a KNOTTED1-type homeobox (KNOX) protein, is strongly expressed in all wild-type floral meristems¹⁶ (Fig. 1j), whereas it is expressed only in the upper region of the flat meristem of *log*, indicating that few meristematic cells remain (Fig. 1k). These results strongly support the notion that meristem activity is not properly maintained in *log* mutants.

The *LOG* gene was isolated by positional cloning. Fine mapping using 442 mutant *F*₂ plants delimited the gene to a 35 kb region on chromosome 1, in which there were four putative genes (Fig. 2a). Mutations in one of the four predicted genes were found in all six *log* mutant alleles, whereas no mutation was found in any of the other three predicted genes (Fig. 2b; Supplementary Fig. 1). The mutant phenotype of *log-2* was complemented by the introduction of a full-length complementary DNA driven by a 1.5 kb promoter region of *LOG* (Fig. 2c, d). Thus, on the basis of these results, we concluded that the *LOG* gene had been isolated. This gene (LOC_Os01g40630) consists of 7 exons and 6 introns and encodes a polypeptide composed of 242 amino acids (Fig. 2b). There are 10 genes that have high sequence similarity to *LOG* in the rice genome, and *Arabidopsis* has a similar number of homologues (Supplementary Fig. 2). The 11 *LOG* family genes show a variety of expression patterns (Supplementary Fig. 3).

LOG mRNA expression was examined by *in situ* hybridization analysis. *LOG* is expressed weakly in a small area in the upper part of the SAM and axillary meristems of vegetative shoot apices (Fig. 3a–c). Interestingly, the absence of *LOG* transcripts from the site where primordium differentiation for the next leaf will take place was often observed. A higher level of *LOG* expression was repeatedly observed in all meristems of the developing panicle (Fig. 3d), including the panicle branch (Fig. 3e) and floral meristems (Fig. 3f). The strong *LOG* signal is localized in two or three layers of cells at the tip of the meristem and is not observed in the rib domain. To further define where *LOG* is expressed, the mRNA distributions of *LOG*, *OSH1* and *OsCLV3*, a putative *CLAVATA* 3 orthologue, were compared (Supplementary Fig. 4). This comparison clearly indicates that *LOG* expression is restricted to a subset of cells in the meristem, and includes the entire region of presumptive stem cells. Therefore, we propose that *LOG*

expression defines a new region in the meristem and that this region may play a novel part in the maintenance of meristem activity.

LOG is annotated as a lysine decarboxylase, which catalyses the decarboxylation of L-lysine to generate cadaverine, a kind of polyamine (<http://rapdb.dna.affrc.go.jp/>). However, we were not able to detect lysine decarboxylase activity with purified recombinant *LOG* protein (data not shown). Genes homologous to *LOG* are found in a wide range of organisms including bacteria, but their functions have not been assigned. Curiously, in some soil phytopathogenic bacteria such as *Agrobacterium rhizogenes*¹⁷ and *Rhodococcus fascians*¹⁸, the *LOG* homologues (*riorf52* and *fas* ORF6, respectively) are located adjacent to the gene for adenosine phosphate-isopentenyltransferase (IPT), which catalyses the initial step of cytokinin synthesis. This led us to pursue the possibility that *LOG* is involved in the control of cytokinin metabolism. The first steps in plant cytokinin synthesis result in the production of *N*⁶-(Δ^2 -isopentenyl)adenine (iP) riboside 5'-tri-, di- or monophosphate (iPRTP, iPRDP or iPRMP, respectively) by IPT; these can then be hydroxylated to *trans*-zeatin (tZ) riboside 5'-tri-, di- or mono-phosphate (tZRTP, tZRDP or tZRMP, respectively) by a cytochrome P450 monooxygenase, CYP735A¹⁰ (Fig. 4a). Active cytokinin species are free-base forms, such as iP and tZ. The current model is that cytokinin nucleotides are converted to active forms in a two-step reaction catalysed by nucleotidase¹⁹ and nucleosidase²⁰, but the responsible genes have not been identified (Fig. 4a). Unexpectedly, we found that *LOG* has phosphoribohydrolase activity, which directly converts a cytokinin nucleotide such as iPRMP and tZRMP to the free-base form with the release of a ribose 5'-monophosphate (Fig. 4b). *LOG* specifically reacts with cytokinin nucleoside 5'-monophosphates, but not with the di- or triphosphate, AMP (Fig. 4c), or any cytokinin ribosides or bases (data not shown). Dephosphoribosylation of the cytokinin nucleotide by *LOG* thus represents a new cytokinin-activating reaction. The *K*_m value of *LOG* for iPRMP is 11.7 μ M, and for tZRMP is 22.0 μ M; specific activity is 5.6 and 4.2 μ mol min⁻¹ mg⁻¹ protein, respectively. Transient expression of *LOG*-GFP shows that *LOG* functions in the cytosol (Fig. 4d; Supplementary Fig. 5). Cytokinin nucleotide phosphoribohydrolase activity has been confirmed for some of the proteins encoded by the *Arabidopsis* *LOG* homologues (T. Kuroha and H.S., unpublished).

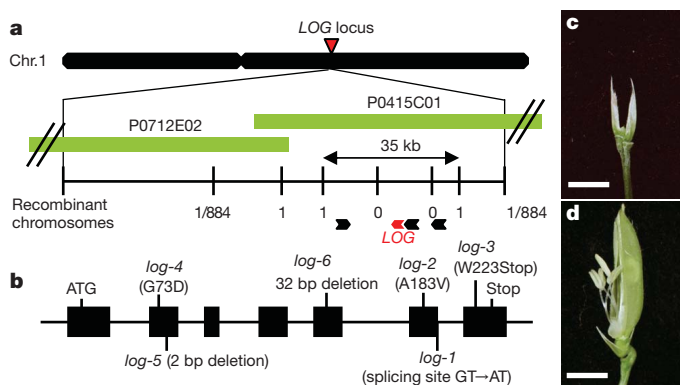


Figure 2 | Positional cloning of *LOG*. **a**, Fine mapping of the *LOG* locus. The region of the *LOG* locus was narrowed within a 35 kb region on chromosome 1 (Chr. 1), which contained 4 predicted genes. **b**, Exon/intron structure of *LOG*. The *LOG* gene contains 7 exons (black box) and 6 introns. Four mutant alleles of the *LOG* gene contain a base substitution that causes an alteration of the splice site (*log-1*), or an amino acid exchange (*log-2* and *-4*), or the generation of a premature stop codon (*log-3*). Two mutant alleles, *log-5* and *log-6*, contain a deletion of 2 and 32 base pairs, respectively. **c**, Complementation test. Defects in *log-2* (**c**) were rescued by the introduction of *LOG* cDNA driven by the *LOG* gene promoter (**d**). Scale bars, 3 mm.

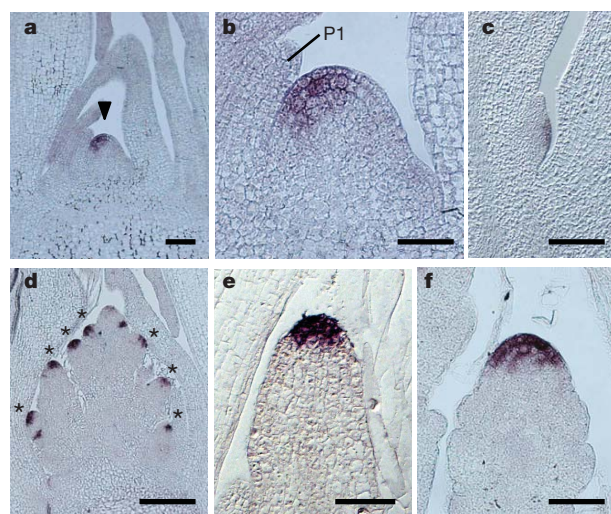


Figure 3 | Expression pattern of *LOG* mRNA. **a, b**, *LOG* expression in the SAM of wild-type meristems. P1 indicates a young leaf at the P1 stage. **c**, *LOG* expression in the axillary meristem. **d**, *LOG* expression in a developing panicle at the primary branch initiation stage. **e, f**, A closer view of *LOG* expression in a primary panicle branch meristem (**e**) and a floral meristem (**f**). The arrowhead in (**a**) indicates the SAM. Asterisks in (**d**) indicate primary or secondary panicle branch meristems. Scale bars, 100 μ m (**a**); 50 μ m (**b, c, e, f**); and 200 μ m (**d**).

The newly identified molecular function of LOG implies that the defects in *log* are very probably caused by the decrease of active cytokinin level in the shoot meristem. However, overall cytokinin concentrations are higher in *log* mutants when the entire inflor-

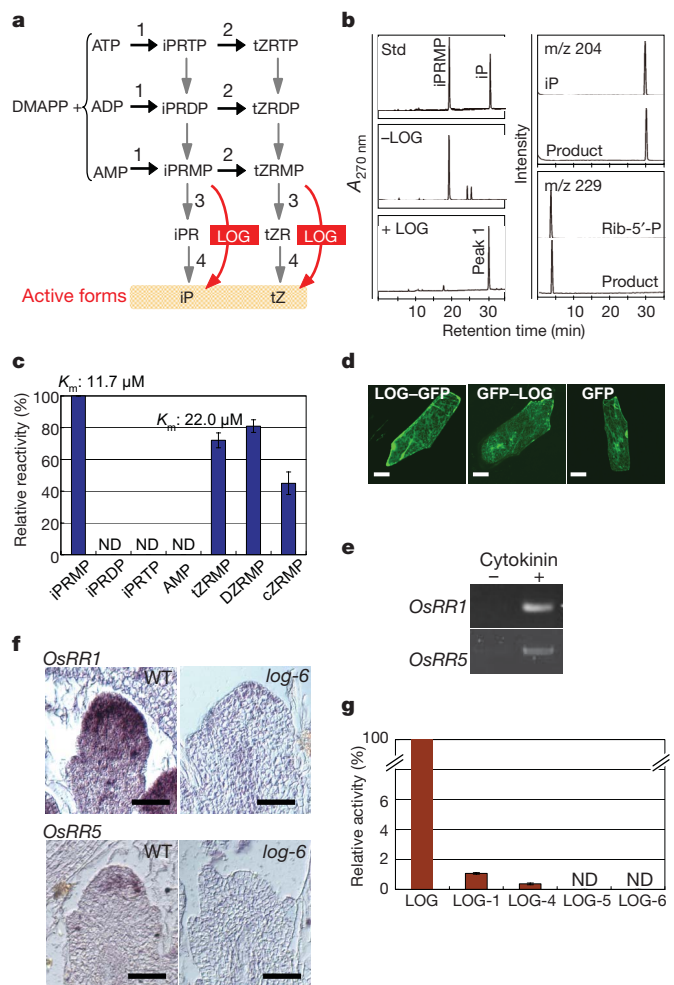


Figure 4 | Enzymatic function of LOG as a cytokinin nucleoside 5'-monophosphate phosphoribohydrolase. **a**, Schematic representation of cytokinin biosynthesis and activating pathway. DMAPP, dimethylallyl diphosphate; iPR, iP riboside; tZR, tZ riboside. 1, IPT; 2, CYP735A; 3, nucleosidase; 4, nucleosidase. Black arrows indicate reactions whose genes for the enzyme are identified, whereas grey arrows indicate that the genes are not identified. **b**, Detection and identification of reaction products. Standards (Std) of iPRMP and iP, reaction products of iPRMP without LOG (–LOG) and with LOG (+LOG) were separated by HPLC (in the left panels). Standards of iP and ribose 5'-monophosphate (Rib-5'-P) and the reaction products in +LOG (product) are analysed by liquid chromatography/mass spectrometry monitoring at mass-to-charge ratio (*m/z*) 204 for iP, and at 229 for Rib-5'-P (in the right panels). Mass spectra of the iP and peak 1 are shown in Supplementary Fig. 6. **c**, Substrate specificity of the iP for various nucleotides. DZMP, dihydrozeatin riboside 5'-monophosphate; cZMP, *cis*-zeatin riboside 5'-monophosphate. The K_m values for iPRMP and tZMP are shown above the bars. **d**, Analysis of subcellular location of LOG protein. Chimeric constructs containing *CaMV35S::LOG-GFP* (LOG-GFP), *CaMV35S::GFP-LOG* (GFP-LOG) or *CaMV35S::GFP* (GFP) were transiently expressed in onion epidermal cells. **e**, Cytokinin responsiveness of *OsRR1* and *OsRR5* in the shoot meristem was analysed by PCR with reverse transcription (RT-PCR). RNA was isolated from shoot apices collected from plants that were treated with 10 μ M benzyl aminopurine (BAP) for 60 min. **f**, *In situ* hybridization analysis of *OsRR1* and *OsRR5* expression in floral meristems of wild type (WT) and *log-6*. **g**, Enzymatic activity of LOG mutant proteins. Recombinant enzyme activity was assayed with iPRMP, and the activities are relative to LOG as 100. The data shown in **c** and **g** are means with s.d. ($n = 3$). ND, not detected. Scale bars, 50 μ m (**d**); and 50 μ m (**f**).

escence is measured, presumably owing to feedback regulation (data not shown). The determination of local cytokinin contents in the tip of the shoot meristem, where LOG is expressed (Fig. 3), is practically impossible due to the small number of LOG-expressing cells. Thus, as an alternative way to examine the *in vivo* function of LOG, we examined transcript levels of the cytokinin-inducible *RESPONSE REGULATOR*²¹ genes (*OsRR1* and *OsRR5*) in the meristem. The great reduction of *OsRR1* and *OsRR5* mRNAs in the floral meristem of *log-6* supports the argument that active cytokinin levels are lower in shoot meristems of *log* mutants (Fig. 4e, f). Furthermore, we asked whether there is a correlation between the LOG activity and phenotype severity in the *log* mutants (Fig. 4g). Residual activity was detected in LOG-1 and LOG-4, whose mutant alleles confer relatively weak phenotypes, but no activity was observed in LOG-5 and LOG-6, which are encoded by severe phenotype alleles (Figs 1e, 4g). These findings strongly support our notion that the *log* phenotype is the result of a malfunction of cytokinin activation in shoot meristems.

In *Arabidopsis*, two types of homeobox genes, *KNOX* and *WUSCHEL* (*WUS*), function in independent and complementary pathways to establish and maintain shoot meristems^{22,23}. The two pathways have direct links with cytokinins. *WUS*, expressed in the small number of cells within the shoot meristem, represses the type-A *RESPONSE REGULATOR* genes²⁴, which are the primary targets of cytokinin signal transduction¹². *KNOX* proteins activate cytokinin biosynthesis in the SAM through the induction of *IPT* genes in *Arabidopsis*^{25,26} and rice²⁷. These findings indicate that modulation of cytokinin activity in the meristem is one of the important functions of the genetic networks controlling shoot meristem activity. Here, we have demonstrated another way in which the activity of cytokinin is regulated in the meristem through the fine-tuning of biologically active cytokinin levels. It is currently not possible to rule out the occurrence and physiological importance of a two-step reaction for cytokinin activation. In any case, the LOG-dependent direct pathway would be dominant at least in the shoot meristem. Although cytokinin functions as both a local paracrine signal and a long-distance signal^{10,28}, our results indicate that in the shoot meristem it functions as a local paracrine signal rather than as a long-distance signal transported from other organs. Cell-specific conversion from inactive to an active form at the site where cytokinin activity is required could be an efficient way to fully control cytokinin function, and would provide a mechanism for preventing cytokinin action in tissues or at times where it is not needed. Future analyses will determine how LOG function is incorporated into the overall network controlling cytokinin biosynthesis and shoot meristem activity.

METHODS

Detailed methods are described in Supplementary Information.

Plants and mapping. *log-1* (cv. Taichung65) was generated by ethylmethane sulphonate chemical mutagenesis. *log-2*, *log-3*, and *log-6* (cv. Nipponbare) were found among a population of tissue-culture-derived plants. *log-4* and *log-5* (cv. Taichung65) were generated by *N*-methyl-*N*-nitrosourea chemical mutagenesis. The *LOG* locus was mapped using 442 mutant *F*₂ plants from a cross between *log-1* and wild type (Kasalath).

In situ hybridization. PCR-amplified cDNAs were cloned into the pGEM-T easy vector (Promega), linearized and used to make digoxigenin-labelled anti-sense probes. Tissue fixation and *in situ* hybridization were carried out according to Kouchi *et al.*²⁹.

Recombinant enzymes. The coding regions of *LOG*, *log-1*, *log-4*, *log-5* or *log-6* were ligated into pCOLD1 (Takara) to express His-tagged recombinant proteins. BL21 (DE3) harbouring pG-Tf2 (Takara) was used as the *Escherichia coli* host.

Enzyme assays. Enzyme activity of LOG as a cytokinin nucleoside 5'-monophosphate phosphoribohydrolase was measured by incubating the enzyme with 50 μ M substrate at 30 °C for the appropriate period. The reaction products were separated and monitored as described previously³⁰.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Genbank accession numbers are as follows: *LOG* (AK071695), LOC_Os01g51210 (AK061091), LOC_Os05g46360 (AK068633), LOC_Os03g49050 (AC123974), LOC_Os09g37540 (AK062595), LOC_Os05g51390 (AK242659), LOC_Os03g64070 (AK061341), LOC_Os03g01880 (AK099538), LOC_Os10g33900 (AK108805), LOC_Os02g41770 (AP005000), LOC_Os04g43840 (AK069293), At2g28305 (NM_128389), At2g35990 (NM_129158), At2g37210 (NM_129277), At3g53450 (NM_115205), AT4G35190 (NM_119685), At5g03270 (NM_120405), At5g06300 (NM_120713), At5g11950 (NM_121233) and At5g26140 (NM_122515). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to J. K. (akyozuka@mail.ecc.u-tokyo.ac.jp) or H.S. (sakaki@riken.jp).