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Truncated BAM receptors interfere the apical meristematic activity in a dominant negative manner when ectopically expressed in *Arabidopsis*

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ABSTRACT

Small, secreted signaling peptides that are perceived by receptor-like kinases (RLKs) constitute an important regulatory mechanism in plant organ formation and stem cell maintenance. However, functional redundancy at the level of both ligand and receptor families often makes it difficult to clearly discern the role of individual members by a genetic approach. Here, we show that driven by a constitutive *CaMV 35S* promoter, a truncated BAM protein (BAMΔ) that lacks either the signal peptide (SP) or the cytoplasmic kinase (Ki) domain could cause defective shoot apical meristem (SAM) maintenance, which phenotypically resembled the triple *bam* mutant. Such a dominant-negative effect could also be achieved when the same transgene was driven by the native *AtBAM1* promoter, but not by the *CLV1* promoter. When introduced into a *clv1-4* background, BAMΔ proteins abolished the typical *clv* phenotype by suppressing the transcriptional level of *clv1-4*. In addition to a clear reduction in root length and a decreased number of meristematic cells, the *35S:BAMΔ* transgenic seedlings exhibited considerable resistance to CLE40p- but not to CLV3p-mediated root growth inhibition, implying that BAMs play key roles in the regulation of proximal meristem activity in root through CLE40 peptide. Findings present here not only provide evidence that truncated BAM proteins are strongly dominant negative in regulating apical meristem development but also propose that expression of a truncated version of plant LRR receptor kinase could potentially be used as a powerful tool to reveal its *in vivo* function in signal transduction.

1. Introduction

Plants rely on the activity of the shoot apical meristem (SAM) to postembryonically form the aboveground tissues and organs. Destruction of plant SAM leads to the regeneration of a new functional meristem from the flank of the previous one, demonstrating the large plasticity of the SAM for self-renewal [1]. In *Arabidopsis*, local signaling regulating the population of pluripotent stem cells within the SAM involves the CLAVATA-WUSCHEL (CLV-WUS) signal transduction pathway. Encoding a small signaling peptide secreted from the central zone (CZ), CLV3 negatively regulates the expression of *WUS*, an organizing center (OC)-expressed homeobox transcription factor positively regulating cell proliferation [2–6]. WUS protein limits its own activity by promoting *CLV3* expression, thus establishing a feedback loop to maintain the size of the stem cell niche [7]. In *A. thaliana*, loss-of-function *clv* mutations cause an ectopic accumulation of stem cells and an enlarged shoot meristem due to the unrestricted WUS expression [2,5,6,8,9]. Conversely, overexpression of *CLV3* or mutations in *WUS* results in meristem reduction or even premature termination [3,10] and the organogenesis from the shoot tip is arrested [5].

A Leu-rich repeat Ser/Thr kinase, CLAVATA1 (CLV1), RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2)/TOADSTOOL 2, and a LRR receptor-like protein that lacks a cytoplasmic domain, CLAVATA2 (CLV2), which acts together with a membrane-associated protein kinase, CORYNE (CRN)/SUPPRESSOR OF LLP1 2 (SOL2), are proposed to be responsible for the recognition of CLV3 ligand and to transmit signal *via* WUS in the SAM [11–16]. In addition to them, other receptor kinases also play important roles in CLV-WUS signaling. In 13 subfamilies of *Arabidopsis* LRR-RLKs that were classified according to the organization of LRRs in the extracellular domain [17], BARELY ANY MERISTEM1, 2 and 3 (BAM1, At5g65700; BAM2, At3g49670 and BAM3, At4g20270) form a monophyletic group with CLV1 [18]. In *bam1 bam2* double mutant, a reduction in meristem size is observed and in *bam1 bam2 bam3* triple mutant, a frequent incidence of premature shoot and flower meristem termination is observed [18], thus the developmental role of BAM1 and BAM2 seems antagonistic to that of CLV1 in the SAM. However, the *clv1* null phenotype can be enhanced by an additional mutation in *bam1* or *bam2* [18,19], which raised a controversy over their functions. Binding

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to the CLV3 peptide with similar kinetics [20], BAM1/2 and CLV1 could replace one another's function in cross-complementation experiment, which strongly suggests their common mechanisms in ligand recognition and signal transduction components [18]. Thus, the differences between *CLV1* and *BAM* functions seem to depend on their respective expression locations. It has been suggested that the CLV3-CLV1 signaling cascade normally negatively regulates *BAM* expression and only when CLV1 is mutated, BAM receptors could enter the OC and partly compensate the CLV1 function [21].

CLE-receptor signaling modules are also known to control stem cell maintenance in roots [22–24], where a CLV3 homolog, CLE40, acts as a signal from columella cells (CCs) to restrict the quiescent center (QC)-expressed WUSCHEL-RELATED HOMEOBOX5 (WOX5), a positive regulator of the production of columella stem cells (CSCs) [25–27]. In the RAM, *BAM1* is preferentially expressed in the QC and its surrounding stem cells [24] and is supposed to modulate the cell production by forming heteromeric complexes with RPK2 [14,16,28].

To date, all characterized strong and intermediate *clv1* alleles are dominant negative and *clv1* null alleles develop rather weak phenotypes [29]. Unlike animal receptor kinases such as Tyr kinases (RTK) and transforming growth factor β receptor Ser/Thr kinases, whose truncated isoforms lacking the cytoplasmic kinase domain could act as dominant-negative receptors by blocking the normal activity of the endogenous counterparts [30–32], CLV1 without the kinase domain does not confer a dominant negative effect [33]. Due to frequent co-suppression events, dominant negative approach has not been actively applied in plant RLK studies [34,35] despite of a previous report demonstrating that a truncated *Arabidopsis* LRR-RLK, ERECTA (ER) without the cytoplasmic kinase domain (ΔKi), could confer a dominant negative effect when expressed under the control of its native cis-regulatory elements through interfering with the endogenous ERECTA functions [36].

In the case of three *BAMs*, all T-DNA or transposon insertion alleles characterized so far were loss-of-function alleles and none of the homozygous single mutants showed obvious phenotypic difference from the wild-type plants [37]. In this work, we constructed a series of truncated BAM receptors and introduced them into *Arabidopsis* plants for reverse genetic analysis. The results showed that BAM proteins lacking either the signal peptide (ΔSP) or the cytoplasmic kinase domain (ΔKi) interfered with the apical meristem function in a dosage- and sequence-dependent manner as a dominant-negative receptor through inhibiting the native BAM functions. Moreover, BAMΔ protein could abolish the contradictory phenotype of *clv1-4* and confer a decreased responsiveness towards the exogenously applied CLE40 peptide. Therefore, in addition to the previously reported ERECTA, the data presented in this work set another example of using domain deletion mutants of plant receptor kinases to generate phenotypes that are missing in single null mutant and to gain new insights into their *in vivo* functions involved in signal transduction pathways coordinating meristem maintenance.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis Columbia-0 (Col-0) seeds were used in this study and *CLV3pro:GUS* [38] line was purchased from the ABRC seed center as N9610. After a 2-d vernalization at 4 °C, surface-sterilized seeds are planted on growth medium containing half strength Murashige and Skoog (MS) basal salts, 1% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) and 1.5% (w/v) agar. For phenotypic analysis of root growth, seeds were germinated and grown on selective media in a vertical position before the root lengths being measured. For *in vitro* peptide application assay, three types of *Arabidopsis* CLE dodeca-peptides (AtCLV3p as RTVPhSGPhDPLHH from AtCLV3, AtCLE40p as RQVPhTGSDPLHH from AtCLE40 and TDIF as HEVPhSGPhNPISN from AtCLE41) were chemically synthesized with a purity of > 90% (Scilight-Peptide, China). *Arabidopsis* seeds were germinated and grown on medium containing 1 μm individual CLE peptide.

2.2. Transgenic constructs and plant transformation

The full-length or partial cDNA fragments of *AtBAM1* were amplified and cloned into *pBI121* vector to generate the *35S:AtBAM1*, *35S:AtBAM1ΔKi* and *35S:AtBAM1ΔSP* constructs, which were introduced into *Agrobacterium tumefaciens* C58 (pMP90) strains using the electroporation method and transformed into plants using the floral dip method [39]. The amino acid sequence of AtBAM1 was also used for tBLASTn search in the poplar genome database. Two putative genes (*PtBAM1* and *PtBAM2*) were amplified by RT-PCR and cloned into pBI121 plasmid. The promoter regions of *CLV1* and *BAM1* were separately amplified from *Arabidopsis* genomic DNA and then replaced the 35S promoter in the *35S:AtBAM1ΔSP* construct to generate the *CLV1pro:AtBAM1ΔSP* and *BAM1pro:AtBAM1ΔSP* constructs. For protein intercellular localization analysis, the full-length and mutant versions of *AtBAM1* was fused in-frame with the green fluorescent protein (*GFP*) at its C-terminal in the binary vector and then co-infiltrated into tobacco leaves with the plasma membrane (PM) marker construct or stably transformed into *Arabidopsis* PM marker line followed by antibiotic selection and seed propagation. Phenotypes of transgenic plants were examined in the T1 generation, and confirmed in the following T2-T3 generations. Oligonucleotides used for cloning and genotyping are listed in Table S1.

2.3. GUS and GFP expression analysis

Staining for the GUS activity in *Arabidopsis* seedlings was performed according to a previous protocol [6]. GUS-stained samples were cleared following the method of Sabatini et al. (1999) and analyzed using a Nikon microscope. For observation of the SAM size, samples were embedded in 6% agarose, cut into 40 μm sections by a vibrating microtome (Leica VT1200) and analyzed by a Nikon microscope. For GFP visualization, transgenic plants were germinated on 1/2 MS medium for 4 days, and tissues were put on slides for observation using a Zeiss 700 confocal microscope. The aquaporin (AtPIP2A) plasma-membrane marker line of *Arabidopsis thaliana* and plasmid pm-rk CD3-1007 fused to mCherry was used for co-localization experiments [40].

2.4. Gene expression analysis

Total RNA was extracted from *Arabidopsis* plants using the EasyPure Plant RNA Kit (TransGen Biotech, China). One μg of total RNA was used to synthesize cDNA with the oligo-$(dT)_{18}$ primer using the EasyScript® First-Strand cDNA Synthesis SuperMix (TransGen, China). Quantitative Real-Time PCR (qRT-PCR) analysis of cDNA was performed on a PikoReal 96 Real-time Thermal Cycler (Thermo Fisher Scientific, Finland) using Real Master Mix (SYBR Green) (NEWBIO, China) and specific primers shown in Table S1. The following thermal cycle condition was used: 95 °C for 2 min, followed by 45 cycles of 95 °C for 20 s and 58 °C for 20 s, 72 °C for 30 s. All reactions were performed in triplicate on three independent pooled samples (10–50 plants per sample). Relative quantification of specific mRNA levels was analyzed using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ methods, normalized using the housekeeping gene *EF1α* and shown in folds of root expression value. Student's *t* test ($P < 0.05$ and $P < 0.01$) was used to determine the significant difference of relative expression level of individual gene among different samples.