Control of endoreduplication of trichome by RPT2a, a subunit of 19S proteasome in Arabidopsis

Kaori Sako [1, +], Yuko Maki [1, +], Kumiko K. Imai [2], Takashi Aoyama [2], Derek B. Goto [3], Junji Yamaguchi [1, *]

[1] Faculty of Advanced Life Science and Graduate School of Life Science, Hokkaido University, Kita-ku N10-W8, Sapporo 060-0810, Japan

[2] Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

[3] Creative Research Initiative Sousei, Hokkaido University, Sapporo 001-0021, Japan

Email address:

KS: k-sako@sci.hokudai.ac.jp

YM: y_maki@mail.sci.hokudai.ac.jp

KKI: RXL04266@nifty.com

TA: aoyama@scl.kyoto-u.ac.jp

DBG: derekgoto@me.com

JY: jjyama@sci.hokudai.ac.jp

[*]Corresponding author:

Junji Yamaguchi

Phone & Fax: +81-11-706-2737 E-mail address: jjyama@sci.hokudai.ac.jp

[+]K.S. and Y.M. contributed equally to this work.

Abstract

The ubiquitin/26S proteasome pathway plays a central role in the degradation of short-lived regulatory proteins to control many cellular events. The Arabidopsis knockout mutant *rpt2a*, which contains a defect in the AtRPT2a subunit of the 26S proteasome regulatory particle, showed enlarged leaves caused by increased cell size that correlated with increased ploidy caused by extended endoreduplication. To clarify the role of RPT2a in endoreduplication control, trichome development was genetically examined in further detail. *RHL1* and *GL3* encode proteins that have a role in the positive regulation of endocycle progression in trichomes. *rhl1* mutants are stalled at 8C and have trichomes with only a single branch. The *rpt2a* mutation did not alter the *rhl1* mutant phenotype, and trichomes of double *rpt2a rhl1* mutants resembled that of single *rhl1* mutant. On the other hand, the *rpt2a* mutation suppressed the *gl3* phenotype (stalled at 16C, two trichome branches), and trichomes of the double *rpt2a gl3* mutant resembled that of WT plants. Together, these data suggest that RPT2a functions to negatively regulate endocycle progression following completion of the third endoreduplication step mediated by RHL1 (8C to 16C).

Key words 26S proteasome, Trichome, endoreduplication, *Arabidopsis thaliana*

Introduction

Endoreduplication is a type of cell cycle where nuclear chromosomal DNA replication occurs without cell division and is widespread among eukaryotes, although most common in plants (Nagl 1976; Edgar and Orr-Weaver 2001). In *Arabidopsis thaliana*, the size of mature leaf pavement cells is correlated with their ploidy level, which varies from 2C to 32C due to differences in the number of endoreduplication cycles they have undergone (Melaragno et al. 1993). In Arabidopsis, trichome cells on the surface of aerial tissues are branched, and the number of branches is also correlated with ploidy level (Hulskamp et al. 1999). Arabidopsis trichomes generally display three branches and have a DNA content of 32C through four rounds of endoreduplication (Melarano et al. 1993; Hulskamp et al. 1994; Szymanski and Marks 1998) (Schnittger and Hulskamp 2002).

The 26S proteasome is a multisubunit ATP-dependent protease complex essential for regulated protein turnover in eukaryotes. Conjugation of ubiquitin to proteolytic substrates marks them for degradation by the proteasome. (Hershko and Ciechanover 1998; Glickman and Ciechanover 2002). The 26S proteasome is assembled from two particles: the 20S core particle (CP) and the 19S regulatory particle (RP) (Voges et al. 1999). The RP can be divided further into two subcomplexes, referred to as the base and the lid. The base consists of six AAA-ATPase subunits, RPT1 to RPT6, and non-ATPase subunits, RPN1, RPN2, and RPN10 (Voges et al. 1999; Glickman 2000; Fu et al. 2001). Each proteasome subunit is presumed to have specific functions, but the roles of only a few subunits are known.

The Arabidopsis genome contains two genes, *AtRPT2a* and *AtRPT2b*, which are paralog RPT2 subunits with a difference of only three amino acids in the protein

sequence (Kurepa et al. 2009; Sonoda et al. 2009). We have recently discovered that the *rpt2a* mutant showed a specific phenotype of enlarged leaves caused by increased cell size in correlation to increased ploidy. Detailed analysis revealed that cell expansion is increased in the *rpt2a* mutant by extended endoreduplication at the early stage of leaf development (Sonoda et al. 2009). Trichomes of the *rpt2a* mutant were also larger and had an increased branch number (Sonoda et al. 2009).

KAKUTUS (KAK) is a putative HECT-domain E3 ligase that has been identified as a negative regulator of endoreduplication in trichomes. Trichomes in the *kak* knockout mutant have a high branch number due to increase in ploidy levels (Downes et al. 2003; Refy et al. 2003). In the *rpt2akak* double mutant, trichomes showed an additive phenotype of increased branch number and nuclear size, leading to the conclusion that RPT2a and KAKUTUS regulate different steps of the trichome endoreduplication pathway (Sonoda et al. 2009). In order to clarify the regulatory function of RPT2a, we have carried out further detailed genetic analysis of endoreduplication in trichomes. These data revealed that RPT2a is a negative regulator of endoreduplication in trichome cells, likely function after completion of the third endocycle.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (accession Columbia-0) was used as wild type. Seeds of *rpt2a-2*, *gl3, kak, rhl1, cycA2;3(-/-)* mutants were obtained from the ABRC (The Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA; stock number: SALK_005596, SALK_118201 and SALK_117247, CS851356 and SALK_086463,

respectively). The sequences bordering the T-DNA insertion were determined using primer pairs. Seeds of the xVE/mDB-CYCA2;3 have been described previously (Imai et al. 2006).

For plant growth, seeds were surface-sterilized and placed on Murashige and Skoog medium supplemented with 2% sucrose (Germination inducible medium: GIM). After cold treatment for 2 days to synchronize germination, seeds were transfer to 22°C under a 16/8 h light/dark cycle (this time point indicates 0 day after sowing: DAS).

Trichome and flow cytometric analyses

Trichomes were observed using low vacuum scanning electron microscopy (S-3000N; Hitachi High-Technologies Corp.) following the manufacturer's protocol. Trichomes were isolated from 3rd and 4th rosette leaves of 3-week-old plants as described in Zhang and Oppenheimer (2004). The isolated trichomes were washed three times for wash buffer (0.02% Tween-20 and 50 mM Na phosphate buffer (pH 7.0)) and added DAPI staining buffer (1 μg/μl 4', 6-deamidinopphenylindole, 0.02% Tween-20, 0.1 mg/ml *p*-phenylene diamine in Na phosphate buffer (pH 7.0)) for 15 min. The DAPI-stained nuclei were visualized with the confocal laser scanning microscopy (LSM510; Ziess). Leaf ploidy was analyzed using the Ploidy Analyzer PA flow cytometer (Partec) according to the manufacturer's instructions.

Results and Discussion

RPT2a functions in the cyclin dependent endoreduplication pathway

The Arabidopsis *rpt2a* mutant, a knockout of the *AtRPT2a* gene that encodes a

subunit of the 26S proteasome, exhibits pleiotropic phenotypes of enlarged leaves and dysregulation in the meristem function (Ueda et al. 2004; Kurepa et al. 2009; Sonoda et al. 2009). We previously reported that extended endoreduplication in *rpt2a* is associated with the abnormal transcription of cell cycle related genes (Sonoda et al 2009), however, we could not rule out that the extended endoreduplication was due to activation of an unknown endoreduplication pathway. The A-type cyclin CYCA2;3 acts to negatively regulate the succession of endocycles during endoreduplication through suppressing G1/S phase transition (Imai et al. 2006). To confirm that RPT2a does indeed function in the cyclin-dependent pathway, we first tested whether CYCA2;3 is able to restrict the extended endoreduplication in *rpt2a*.

The CYCA2;3 protein contains a D-box domain that is recognized and degraded by the ubiquitin/26S proteasome system. We therefore used transgenic plants containing an estradiol-inducible *mDB-CYCA2;3-GFP* construct, which encodes a full-length CYCA2;3 protein with a mutated D-box fused to GFP. In a wild-type transgenic background, induction of *mDB-CYCA2;3-GFP* resulted in small cotyledon size (Fig. 1e) due to reduced ploidy level (Fig. 1g) compared to the WT (Fig. 1a) and non-induced plants (Fig. 1c). In contrast, induction of *mDB-CYCA2;3-GFP* in a *rpt2a* mutant background (generated by genetic cross) resulted in suppression of the *rpt2a* phenotype and a normal cotyledon size (Fig. 1f) and ploidy level (Fig. 1g) equivalent to that of wild type (Fig 1a) compared to the *rpt2a* (Fig. 1b) and non-induced *rpt2a* mutant (Fig. 1d). These results indicated that the extended endoreduplication in the *rpt2a* is not due to an unknown pathway, but rather due to the general cell cycle pathway.

We also analyzed branch number of trichomes in mDB-CYCA2;3-GFP induced plants (Fig. 1h and Table 1). However, we could not observe any significant difference

between the induced and non-induced plants in both the WT or *rpt2a* background. These results imply alternative negative regulation system in endoreduplication during trichome development besides the CYCA2;3. Since trichomes for the *CYCA2;3(-/-)* mutant display slightly over-branched phenotypes (Imai et al., 2006), we observed trichomes for the *rpt2a CYCA2;3(-/-)* double mutant. In the double mutant, trichomes showed similar phenotype to those in the *rpt2a* mutant (Fig. 1h, Table 1). These results indicate that deficiency in the *CYCA2;3* gene affects the trichome development to the small extent, since the gene family for *AtCYCA2* shows redundancy.

rpt2a gl3 double mutant recovers normal trichome branching and nuclear size

Examination of DAPI-stained nuclei in *rpt2a* trichomes indicated that *rpt2a* trichomes contain much more DNA than that of WT (Figs. 2a-d). *GLABRA3 (GL3)* encodes an R-like bHLH transcriptional factor that acts as a positive regulator of endoreduplication in trichomes (Hulskamp et al. 1994; Payne et al. 2000). In the *gl3* mutant, progression of the fourth endocycle is blocked, resulting in trichomes with two branches and a ploidy level of 16C (Fig. 2i; Table 1). To further investigate the role of RPT2a in endoreduplication, the *rpt2a* mutant was first crossed with the *gl3* mutant.

In the *rpt2a gl3* double mutant, trichomes mostly showed three branches and normal nuclear size, similar to that of wild type (Figs. 2k, l and Table 1). Relative proportions of trichomes with two, three and four branches in the double mutant was also similar to that in wild type (Fig. 3), suggesting that the *rpt2a* mutation suppressed the *gl3* phenotype. These results indicate that functional GL3 is required for the extended endoreduplication observed in *rpt2a* mutants. Moreover, they also reveal that loss of RPT2a permits progression of endoreduplication through the fourth endocycle, even in

the absence of the GL3 positive regulator of this step.

rpt2a rhl1 double mutant suggests RHL1 acts upstream of RPT2a

The RHL1 protein is a component of topoisomerase VI that functions in the positive regulation of endocycle progression beyond 8C (Sugimoto-Shirasu et al. 2005). The *rhl1* mutant displays trichomes with only one branch, consistent with block of the third endocycle (Figs. 2m, n; Table 1). To determine whether RPT2a may have a role in regulating the third endocycle, the *rpt2a* mutant was crossed with *rhl1*, and trichome phenotype examined in the double *rpt2a rhl1* mutant. Trichomes in the double *rpt2a rhl1* mutant mostly had only a single branch similar to that for the single *rhl1* mutant (Figs. 2o, p; Table 1), revealing that the *rhl1* mutation is able to suppress the *rpt2a* mutant phenotype. This data implied the possibility that RHL1 and RPT2a function at the same regulatory step of trichome endoreduplication.

A regulatory role for RPT2a in trichome endoreduplication

The number of trichome branches is determined by the number of endoreduplication cycles that occur during trichome development. In general, trichome cells proceed through four endocycles, leading to mature trichomes with a DNA content of 32C and three branches. The *rpt2a* mutant has increased branch number and nuclear size in trichomes, caused by extended endoreduplication that leads to a ploidy level up to 64C.

Based on the results of the genetic analyses in this study using *rhl1* and *gl3* mutants, we propose the following hypothetical model for RPT2a function in trichome development (Fig. 4). Topoisomerase VI, which contains RHL1, is essential for progression of at least the third endocycle to increase ploidy level from 8C to 16C.

Likewise, GL3 acts to positive regulate the subsequent fourth endocycle to increase ploidy level from 16C to 32C. Phenotypes of the single and double mutants are consistent with a role for RPT2a in the negative regulation of before fourth endocycle. This would likely involve specific recognition and 26S proteasome-mediated degradation of a protein that promotes endocycle progression. The protein may function the following three possibilities, i.e., 1) interaction with the RHL1, 2) regulation downstream of RHL1 and 3) association with GL3 function. To evaluate these possibilities, further investigations will be needed.

Acknowledgements

We are grateful to Drs. Akira Ikeda, Yutaka Sonoda and Ms. Naoko Yamazaki for discussion and technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (no. 19657013, 19039001, 21025001), in part by the Program for Basic Research Activities for Innovative Bioscience (PROBRAIN) (JY) and Grants-in-Aid for Scientific Research on Priority Area (D.B.G). K.S. acknowledges Research Fellowships from the Japan Society for the Promotion of Science for Young Scientist (2008-2010).

References

Downes BP, Stupar RM, Gingerich DJ, Vierstra RD (2003) The HECT ubiquitin-protein ligase (UPL) family in Arabidopsis: UPL3 has a specific role in trichome development. Plant J 35: 729-742

Edgar BA, Orr-Weaver TL (2001) Endoreplication cell cycles: more for less. Cell 105: 297-306.

El Refy A, Perazza D, Zekraoui L, Valay JG, Bechtold N, Brown S, Hülskamp M, Herzog M, Bonneville JM (2003) The Arabidopsis KAKTUS gene encodes a HECT protein and controls the number of endoreduplication cycles. Mol Genet Genomics 270: 403-414

Fu H, Reis N, Lee Y, Glickman MH, Vierstra RD (2001) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. EMBO J 20: 7096-7107.

Glickman MH (2000) Getting in and out of the proteasome. Semin. Cell Dev Biol 11: 149-158.

Glickman M.H, Ciechanover A (2002) The Ubiquitin-Proteasome Proteolytic Pathway: Destruction for the Sake of Construction. Physiol Rev 82: 373-428.

Hershko A., Ciechanover A (1998) The ubiquitin system. Annu Rev Biochem 67: 425-479.

Hülskamp M, Schnittger A, Folkers U (1999) Pattern formation and cell differentiation: trichomes in Arabidopsis as a genetic model system. Int Rev Cytol 186: 147-178

Hülscamp, M., Miséra, S. and Jürgens, G. (1994) Genetic dissection of trichome cell development in Arabidopsis. Cell 76: 555-566

Imai KK, Ohashi Y, Tsuge T, Yoshizumi T, Matsui M, Oka A, Aoyama T (2006) The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in Arabidopsis endoreduplication. Plant Cell 18: 382-96

Kurepa J, Wang S, Li Y, Zaitlin D, Pierce AJ, Smalle JA (2009) Loss of 26S proteasome function leads to increased cell size and decreased cell number in Arabidopsis shoot organs. Plant Physiol. 150: 178-189.

Melaragno JE, Nehrotra B, Coleman AW (1993) Relationship between endopolyploidy and cell size in epidermal tissue of Arabidopsis. Plant Cell 5: 1661-1668.

Nagl W (1976) DNA endoreduplication and polyteny understood as evolutionary strategies. Nature 261: 614-615.

Payne CT, Zhang F, Lloyd AM (2000) GL3 encodes a bHLH protein that regulates trichome development in arabidopsis through interaction with GL1 and TTG1. Genetics 156:1349-1362

Schnittger A, Hülskamp M (2002) Trichome morphogenesis: a cell-cycle perspective. Philos Trans R Soc Lond B Biol Sci 357:823-826

Smalle J, Kurepa J, Yang P, Babiychuk E, Kushnir S, Durski A, Vierstra RD (2002) Cytokinin growth responses in Arabidopsis involve the 26S proteasome subunit RPN12. Plant Cell 14: 17-32.

Smalle J, Kurepa J, Yang P, Emborg TJ, Babiychuk E, Kushnir S, Vierstra RD (2003) The pleiotropic role of the 26S proteasome subunit RPN10 in Arabidopsis growth and development supports a substrate-specific function in abscisic acid signaling. Plant Cell 15: 965-980.

Sonoda Y, Sako K, Maki Y, Yamazaki N, Yamamoto H, Ikeda A, Yamaguchi J (2009) Regulation of leaf organ size by the Arabidopsis RPT2a 19S proteasome subunit. Plant J 60. 68-78

Sugimoto-Shirasu K, Roberts K (2003) "Big it up": endoreduplication and cell-size control in plants. Curr Opin Plant Biol 6: 544-553.

Sugimoto-Shirasu K, Roberts GR, Stacey NJ, McCann MC, Maxwell A, Roberts K (2005) RHL1 is an essential component of the plant DNA topoisomerase VI complex and is required for ploidy-dependent cell growth. Proc Natl Acad Sci USA 102: 18736-41

Szymanski DB, Marks MD. (1998) GLABROUS1 overexpression and TRIPTYCHON alter the cell cycle and trichome cell fate in Arabidopsis. Plant Cell 10: 2047-2062

Ueda M, Matsui K, Ishiguro S, Sano R, Wada T, Paponov I, Palme K, Okada K (2004) The *HALTED ROOT* gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of *Arabidopsis* meristems. Development 131. 2101-2111.

Voges, D, Zwickl P, Baumeister W (1999) The 26S proteasome: a molecular machine designed for

controlled proteolysis. Annu Rev Biochem 68: 1015-1068

Zhang X, Oppenheimer DG (2004) A simple and efficient method for isolating trichomes for downstream analyses. Plant Cell Physiol 45: 221-224.

Figure legends

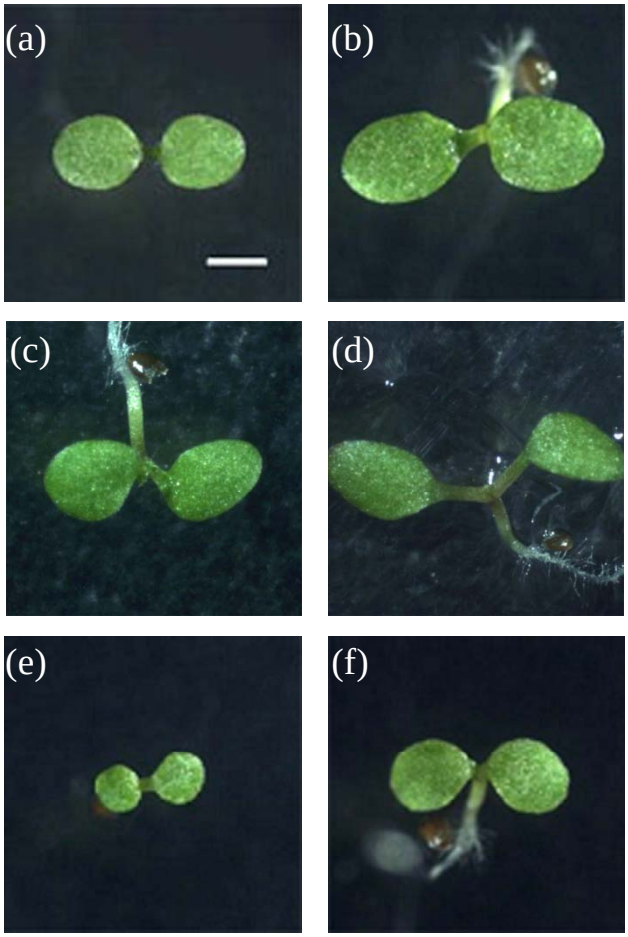
Fig. 1 Ploidy analysis and cotyledon size of WT and *rpt2a* plants expressing mDB-CYCA2;3-GFP. Anatomy (**a**-**f**) in cotyledons of WT (**a**), *rpt2a* (**b**), non-induced mDB-CYCA2;3-GFP in WT (**c**) and in *rpt2a* (**d**), induced mDB-CYCA2;3-GFP in WT (**e**) and in *rpt2a* background (**f**). Scale bars: 1 mm. (**g**) Distributions for ploidy in cotyledons of WT and *rpt2a* plants expressing mDB-CYCA2;3-GFP. (**h**) Distributions for branching numbers in trichomes of WT and *rpt2a* plants expressing mDB-CYCA2;3-GFP, CYCA2;3(-/-) and *rpt2a CYCA2;3(-/-)* double mutant. Cotyledons from seedling grown for 6 DAS on GIM agar medium containing β-estradiol (10 μM) were subjected to flow cytometry.

Fig. 2 Analysis of genetic crosses between *rpt2a* and endoreduplication mutants. Morphology and fluorescence images (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**) and confocal images (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**) of DAPI-stained leaf trichomes. Scale bars: 100 μm. (**a**, **b**), WT; (**c**, **d**), *rpt2a*; (**e f**), *kak*; (**g**, **h**), *rpt2a kak* double mutant; (**i**, **j**), *gl3*; (**k**, **l**), *rpt2a gl3* double mutant; (**m**. **n**), *rhl1*; (**o**, **p**), *rpt2a rhl1* double mutant.

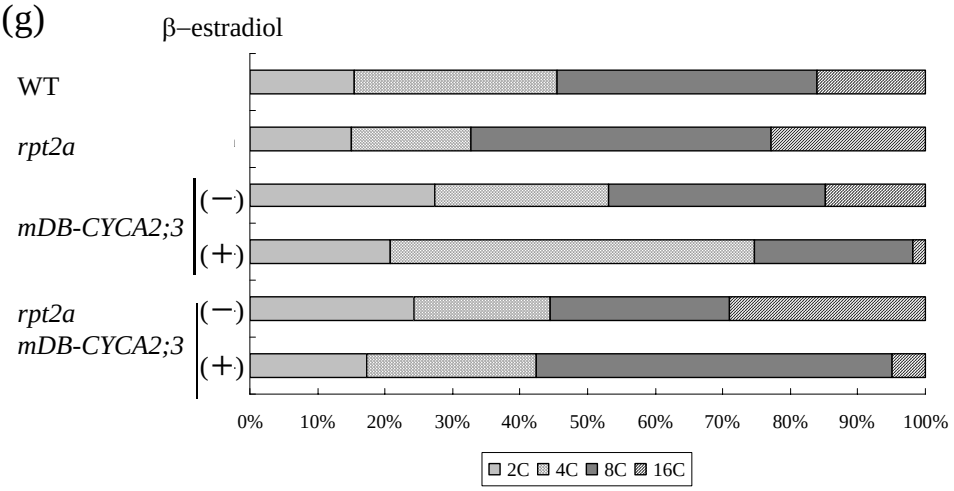
Fig. 3 Comparison of trichome branch numbers in endoreduplication mutants. Relative proportions of trichome branch numbers on the adaxial surface of 3-week-old third and fourth leaves in WT, *rpt2a*, *gl3*, *rpt2a gl3* double mutant, *kak*, *rpt2a kak* double mutant, *rhl1*, and *rpt2a rhl1* double mutant.

Fig. 4 Proposed model of physiological role of RPT2a in controlling endoreduplication during trichome development. In this model, RPT2a negatively

regulates the third endocycle (8C to 16C) and/or the fourth endocycle (16C to 32C).


(a)
(b)
(c)
(d)
(e)
(f)

(g)


β−estradiol
WT
rpt2a
mDB-CYCA2;3
(−)
(+)
rpt2a
mDB-CYCA2;3
(−)
(+)
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
2C 4C 8C 16C

(h)

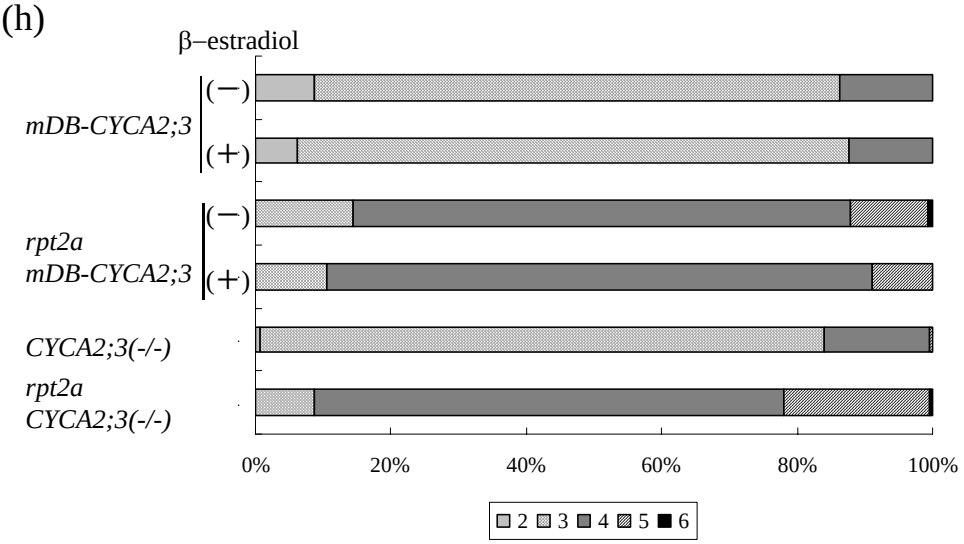

β−estradiol
mDB-CYCA2;3
(−)
(+)
rpt2a
mDB-CYCA2;3
(−)
(+)
CYCA2;3(-/-)
rpt2a
CYCA2;3(-/-)
0%
20%
40%
60%
80%
100%
2 3 4 5 6

Fig. 1

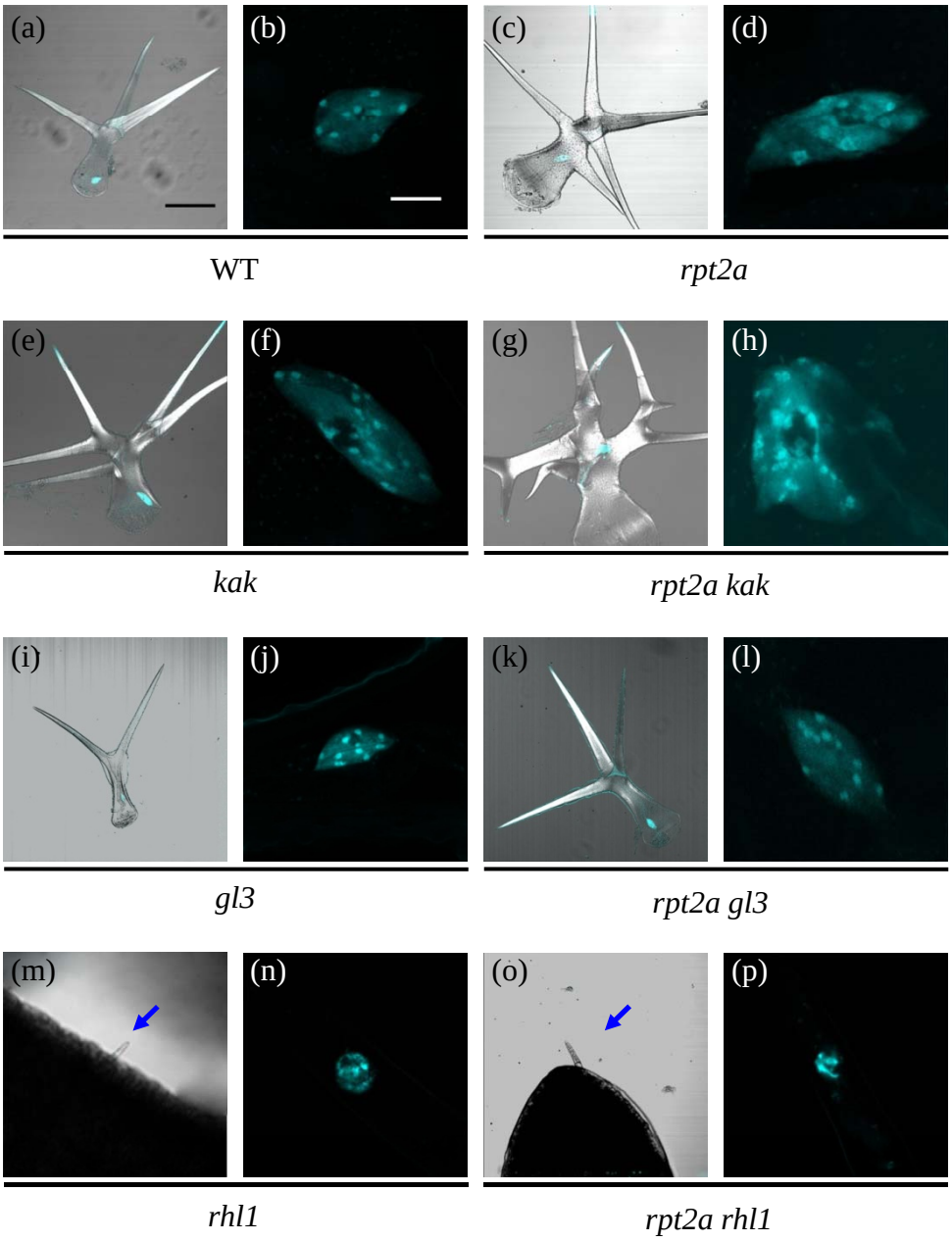

(a)
(b)
(c)
(d)
WT
rpt2a
(e)
(f)
(g)
(h)
kak
rpt2a kak
(i)
(j)
(k)
(l)
gl3
rpt2a gl3
(m)
(n)
(o)
(p)
rhl1
rpt2a rhl1

Fig. 2

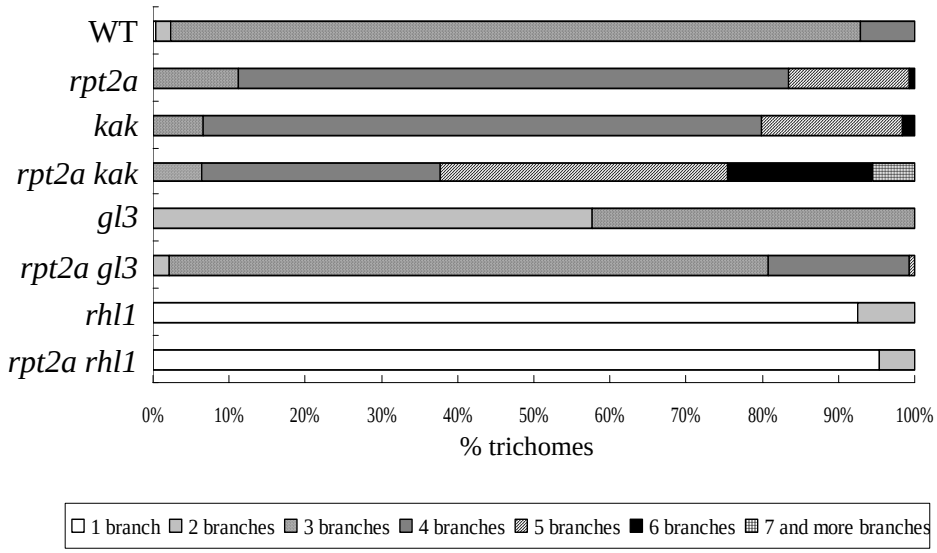

WT
rpt2a
kak
rpt2a kak
gl3
rpt2a gl3
rhl1
rpt2a rhl1
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
% trichomes
1 branch
2 branches
3 branches
4 branches
5 branches
6 branches
7 and more branches

Fig. 3

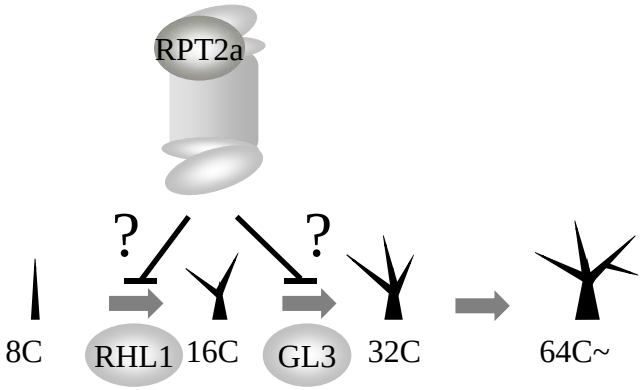


Fig. 4

Table 1
Analysis of leaf trichome branch number

	Branch number									
	1	2	3	4	5	6	7	8	9	Total
WT	2	9	447	35						493
rpt2a			51	331	73	3				458
kak			33	363	92	8				496
rpt2a kak			23	112	135	68	17	2	1	358
gl3	6	18	137							323
rpt2a gl3		6	222	52	2					282
kak gl3		5	255	76	8					344
rhl1	98	8								106
rp2a rhl1	41	2								43
mDB-CYCA2;3 (β-estradiol -)		27	242	43						312
mDB-CYCA2;3 (β-estradiol +)		24	313	48						385
rp2a mDB-CYCA2;3 (β-estradiol -)			55	279	43	3				380
rp2a mDB-CYCA2;3 (β-estradiol +)			48	369	41					458
CYCA2;3(-/-)		3	355	66	2					426
rpt2a CYCA2;3(-/-)			33	261	81	2				377