

Root hair formation at the root-hypocotyl junction in CPC-LIKE MYB double and triple mutants of *Arabidopsis*

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In *Arabidopsis thaliana*, R3-type MYB genes, *CAPRICE* (*CPC*) and its family of genes including *TRIPTYCHON* (*TRY*), *ENHANCER OF TRY AND CPC1* (*ETC1*), *ETC2* and *CPC-LIKE MYB3* cooperatively regulate epidermal cell differentiation. Root hair formation is greatly reduced by a mutation in *CPC*, and *try* and *etc1* enhance this phenotype. In this study, we demonstrate that *CPC*, *TRY* and *ETC1* are also involved in root hair formation at the root-hypocotyl junction. The *cpc try* and *cpc etc1* double mutants showed a reduced number of root hairs in that area. Additionally, the expression of *ETC1::GUS* was higher near this area. These results suggest that *CPC* family of genes also cooperatively regulates root hair formation at the root-hypocotyl junction in unique ways.

Epidermal cell fate determination, including root hair and trichome differentiation, is a crucial feature of in *Arabidopsis thaliana* development. Molecular genetics studies have revealed that several common regulatory factors are involved in this event. The R2R3-type MYB transcription factor, WEREWOLF (*WER*), induces the expression of the homeodomain-leucine zipper protein, *GLABRA2* (*GL2*), which inhibits root hair differentiation.¹⁻⁴ The other R2R3-type MYB transcription factors, *GLABRA1* and *MYB23*, are homologous to *WER* and are involved in trichome differentiation.⁵⁻⁷ In addition, the basic helix-loop-helix (bHLH) transcription factors *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) inhibit root hair differentiation.⁸ The WD40-repeat protein *TRANSPARENT TESTA GLABRA1* (*TTG1*) also prompts trichome differentiation and inhibits root hair differentiation.^{9,10}

The R3-type MYB transcription factor *CAPRICE* (*CPC*), has been identified as a key regulator of epidermal cell fate determination in *Arabidopsis*.¹¹ Additionally, 6 *CPC*-homologous genes have been identified in the *Arabidopsis* genome: *TRIPTYCHON* (*TRY*), *ENHANCER OF TRY AND CPC1* (*ETC1*), *ENHANCER OF TRY AND CPC2* (*ETC2*), *ENHANCER OF TRY AND CPC3* (*ETC3*)/*CPC LIKE MYB3* (*CPL3*), *TRICHOMELESS1* (*TCL1*) and *TRICHOMELESS2* (*TCL2*)/*CPC LIKE MYB4* (*CPL4*).¹²⁻²⁰ These 7 *CPC* family genes function cooperatively to induce root hair differentiation and inhibit trichome differentiation.

The *CPC* family proteins, *WER*, *GL1*, *TTG1*, *GL3* and *EGL3*, are thought to act as a transcription regulatory complex in the epidermis of *Arabidopsis*.^{18,21-24} This transcriptional

complex regulates *GL2* gene expression in both root hair and trichome differentiation in *Arabidopsis*.^{1,4,9,11,25} The *TTG1-GL3/EGL3-WER* transcriptional complex promotes *GL2* expression to induce a hairless cell differentiation.^{26,27} *CPC* disrupts this transcriptional complex by competing with *WER*, thus negatively regulating the expression of *GL2* and resulting in root hair formation.²⁸ In addition to the root hair formation in the major portion of the root, *CPC* family genes are also involved in root hair formation at the root-hypocotyl junction.¹⁴

In this study, we focused on root hair formation at the root-hypocotyl junction in *CPC-LIKE MYB* family mutants, all of which were in a *Col-0* background. The *cpc* mutant is known to produce a greatly reduced number of root hairs compared with the wild type.¹¹ However, the *cpc* mutant even possessed root hairs at the root-hypocotyl junction, which was similar to the wild type (Fig. 1A). Moreover, the *cpc-2*, *try-29760*, *etc1-1*, *etc2-2* and *cpl3-1* mutants formed root hairs at the root-hypocotyl junction similar to the wild type (*Col-0*) (Fig. 1A). However, *cpc-2 try-29760*, *cpc-2 etc1-1*, *cpc-2 etc2-2* and *cpc-2 cpl3-1* showed reduced number of root hairs compared with the wild type at the root-hypocotyl junction (Fig. 1B). All other double mutants including *etc1-1 try-29760*, *etc1-1 etc2-2*, *etc1-1 cpl3-1*, *etc2-2 try-29760*, *etc2-2 cpl3-1* and *cpl3-1 try-29760* did not show obvious changes in the root-hypocotyl junction (Fig. 1B). Our observation of reduced root hairs in the *cpc-2 try-29760* double mutant is consistent with a previous study by Kirik et al. using the *try-82* (in the *Ler* genetic background) *cpc-1* (in the *WS* genetic background) double mutant.¹⁴ Together, these results suggest that the function of *CPC* is the most prominent among

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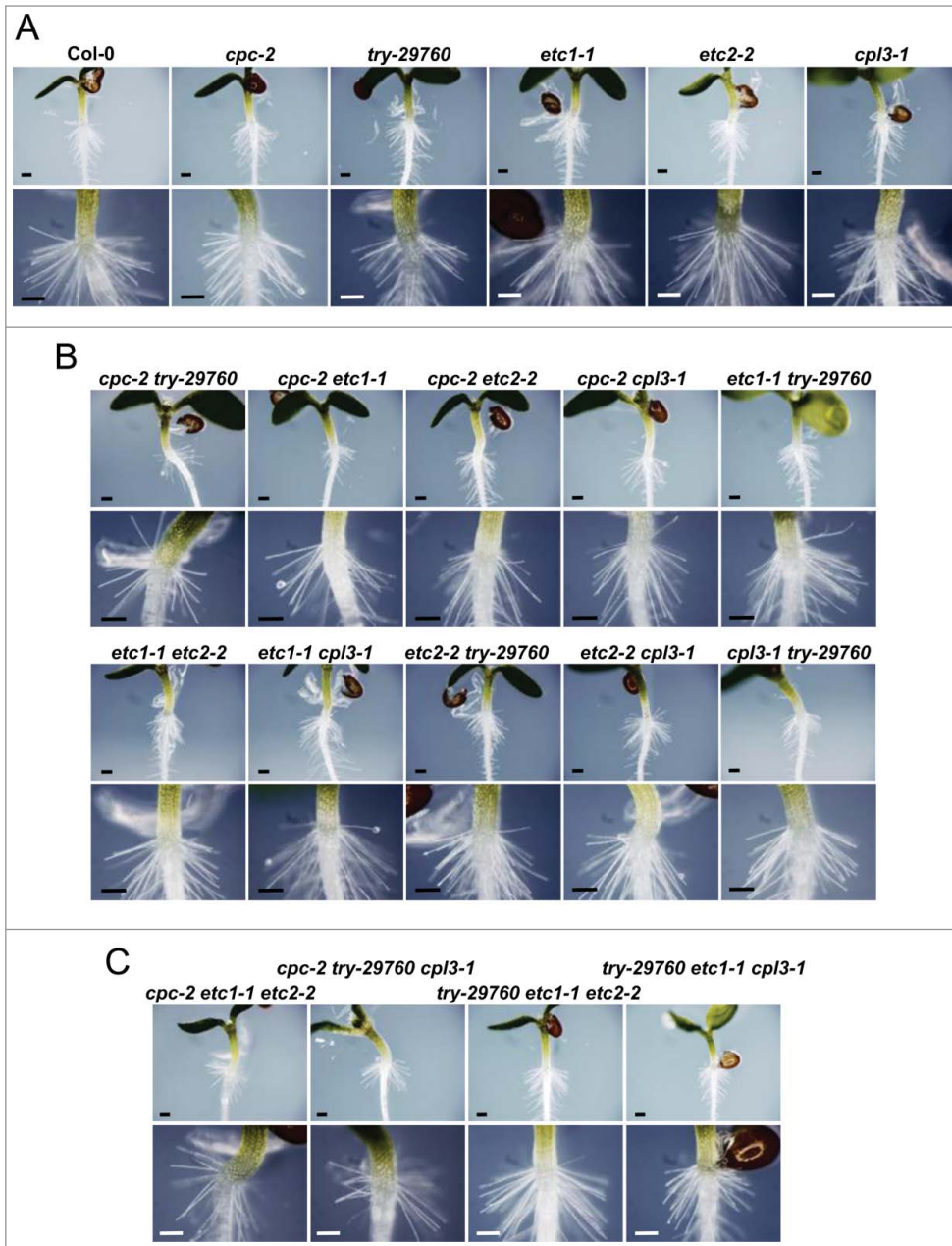


Figure 1. Root hair formation at the root-hypocotyl junction in *CPC-LIKE MYB* mutants. Root hair formation at the root-hypocotyl junction in 3-day-old seedlings of wild type (Col-0), and *cpc-2*, *try-29760*, *etc1-1*, *etc2-2*, *cpl3-1* single mutants. (A), *cpc-2 try-29760*, *cpc-2 etc1-1*, *cpc-2 etc2-2*, *cpc-2 cpl3-1*, *etc1-1 try-29760*, *etc1-1 etc2-2*, *etc1-1 cpl3-1*, *etc2-2 try-29760*, *etc2-2 cpl3-1* and *cpl3-1 try-29760* double mutants, (B), *cpc-2 etc1-1 etc2-2*, *cpc-2 try-29760 cpl3-1*, *try-29760 etc1-1 etc2-2* and *try-29760 etc1-1 cpl3-1* triple mutants and (C) Scale bars in each photo: 200 μ m.

the *CPC* family of genes for the root hair formation, not only for the major portion of the root, but also at the root-hypocotyl junction. However, a single mutation in *CPC* did not induce apparent differences in root hair density at the root-hypocotyl junction compared with the wild type (Fig. 1A). The *cpc-2 try-29760* and *cpc-2 etc1-1* double mutants were distinguished from the wild type by having a reduced number of root hairs at the

root-hypocotyl junction (Fig. 1B). The combination of 2 mutations, neither of which affected root hair density at the root-hypocotyl junction, reduced the number of root hairs at the root-hypocotyl junction. This phenomenon resembles the regulation of trichome formation by the *CPC* family of genes. The *cpl3 cpc* and *cpl3 etc2* double mutants had more trichomes than each parental line.¹⁸ The *cpc-2 etc1-1 etc2-2* and *cpc-2 try-29760 cpl3-1* triple mutants also showed a clearly reduced number of root hairs at the root-hypocotyl junction compared with the wild type, which was also observed in the *cpc-2 try-29760* and *cpc-2 etc1-1* double mutants (Figs. 1B, C). In contrast, the *try-29760 etc1-1 etc2-2* and *try-29760 etc1-1 cpl3-1* triple mutants did not show remarkable differences compared to the wild type in root hair formation at the root-hypocotyl junction, as the *etc1-1 try-29760* double mutant did (Figs. 1B and 1C). These results also suggest that *CPC* plays the most prominent role in root hair formation at the root-hypocotyl junction among the *CPC* family genes. The *cpc* mutant effect on root hair formation at the root-hypocotyl junction is enhanced by *try* and *etc1*, which suggests that *CPC* plays a leading role in root hair formation at the root-hypocotyl junction, which is associated with *TRY* and *ETC1*. The *etc1-1 try-82 cpc-1* triple mutant phenotype has been reported to be unusual due to its lack of root hairs in the root-hypocotyl junction,¹⁴ which supported our hypothesis that *CPC*, *TRY* and *ETC1* induced root hair formation at the root-hypocotyl junction.

To determine whether *CPC* family genes are expressed at the root-hypocotyl junction, we analyzed the *CPC*, *TRY*, *ETC1*, *ETC2* and *CPL3* promoter-GUS reporter fusion lines. The *CPC::GUS* transgene caused a slight GUS accumulation in the hypocotyl (Fig. 2), which was similar to a previously reported *CPC* gene expression.¹⁴ The *CPC* promoter was also active in the lateral root primordia (Fig. 2, inset in *CPC::GUS*); however, no obvious GUS

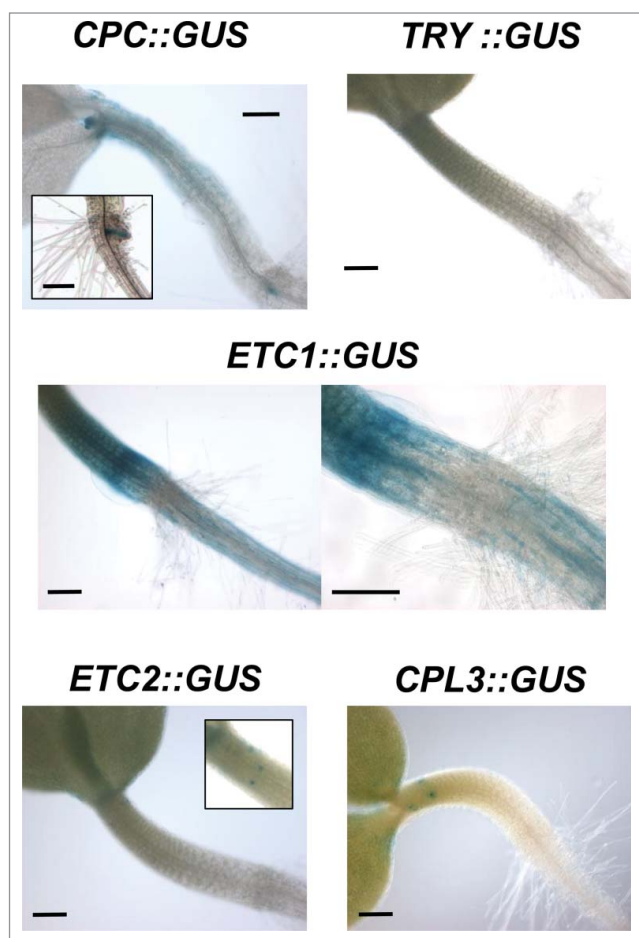


Figure 2. Promoter activity of *CPC*-LIKE MYB genes at the root-hypocotyl junction in 3-day-old *Arabidopsis* seedlings of *CPC::GUS*, *TRY::GUS*, *ETC1::GUS*, *ETC2::GUS* and *CPL3::GUS* lines. Inset in *CPC::GUS* shows the *CPC* promoter is not active at the root-hypocotyl junction, although it is active in the lateral root primordia. Inset in *ETC2::GUS* shows that the *ETC2* promoter is active in the hypocotyl guard cells. Scale bars: 200 μm .

expression was observed at the root-hypocotyl junction (Fig. 2). No significant GUS expression was detected in the *TRY::GUS* hypocotyl, as previously reported.¹⁴ In addition, no TRY activity was observed at the root-hypocotyl junction (Fig. 2). The *ETC2::GUS* and *CPL3::GUS* transgene caused a GUS accumulation in the hypocotyl guard cells (Fig. 2), which is consistent with previous reports.^{15,18} *ETC2* and *CPL3* promoter activity was not observed at the root-hypocotyl junction (Fig. 2). In conclusion, no significant GUS expression was detected at the root-hypocotyl junction in any of the promoter-GUS transgenic lines that we have examined so far. Only the *ETC1::GUS* transgene caused a strong GUS accumulation near the region of the root-hypocotyl junction (Fig. 2). These results suggest that ETC1 is the most abundant protein at the root-hypocotyl junction among those of the CPC family. Although mutant analyses revealed the involvement of *CPC*, *TRY* and *ETC1* in root hair formation at the

root-hypocotyl junction, *CPC::GUS* and *TRY::GUS* expressions were hardly observed near the root-hypocotyl junction. CPC is the most important gene for root hair formation at the root-hypocotyl junction according to mutant analysis (Fig. 1), but *CPC* expression was not detected in this area (Fig. 2). There are 2 possibilities why *CPC* promotes root hair formation more than *ETC1* does at the root-hypocotyl junction. First, the CPC protein may have a more appropriate structure to promote root hair formation at the root-hypocotyl junction than ETC1 does, even if the *CPC* expression is at an undetectable level in this area. For instance, there are some amino acid differences between CPC and ETC1 along their entire sequences.¹⁴ Second, we observed the expressions of the *CPC* family genes in the 3-day-old seedlings (Fig. 2). There is another possibility that expressions of the *CPC* family genes at earlier stages may be important for root hair induction at the root-hypocotyl junction.

In this study, we demonstrated that *CPC*, *TRY* and *ETC1* act in a cooperative manner to induce root hair formation at the root-hypocotyl junction, and *CPC* has a pivotal role in this function. In addition, we show that *ETC1* mRNA may be abundant near the root-hypocotyl junction because *ETC1::GUS* expression was detected there. There is evidence from genetic studies that *CPC* is the most important gene for the formation of root hairs at the root-hypocotyl junction. The expression of *ETC1* is higher at this area, which reflects the functional differences among the CPC family genes.

The plant materials used in this study have been described previously and included the *cpc-2*,²⁹ *cpl3-1*,¹⁸ *try-29760*, *etc1-1*, and *etc2-2* mutants.³⁰ All mutants were carried on the Col-0 background. The selection of the double and triple mutants of *cpc*, *try*, *etc1*, *etc2*, and *cpl3* has also been previously described.¹⁸ Seeds were surface-sterilized and sown on the surface of 1.5% agar plates using a method described previously,³¹ and grown for the observation of seedling phenotypes. Seeded plates were held at 4°C for 2 days and then incubated at 22°C for 3 days under continuous white light (50–100 $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Phenotypes of 3-day-old seedlings of the wild type (Col-0), *cpc-2*, *try-29760*, *etc1-1*, *etc2-2*, *cpl3-1*, *cpc-2 try-29760*, *cpc-2 etc1-1*, *cpc-2 etc2-2*, *cpc-2 cpl3-1*, *etc1-1 try-29760*, *etc1-1 etc2-2*, *etc1-1 cpl3*, *etc2-2 try-29760*, *etc2-2 cpl3-1*, *cpl3-1 try-29760*, *cpc-2 etc1-1 etc2-2*, *cpc-2 try-29760 cpl3-1*, *try-29760 etc1-1 etc2-2* and *try-29760 etc1-1 cpl3-1* mutants were observed using a Leica MZ16FA stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Images were recorded using a high-sensitivity CCD color camera system (Keyence VB 7010, Osaka, Japan).

The promoter::GUS plants used in this study have been described previously.¹⁸ Three-day-old seedlings of *CPC::GUS*, *TRY::GUS*, *ETC1::GUS*, *ETC2::GUS* and *CPL3::GUS* were immersed X-Gluc solution containing 1.0 mM XGluc (5-bromo-4-chloro-3-indolyl- β -glucuronide), 1.0 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 1.0 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 100 mM NaPi (pH 7.0), 100 mM EDTA and 0.1% Triton X-100. *CPC::GUS*, *TRY::GUS*, *ETC2::GUS* and *CPL3::GUS* seedlings were incubated at 37°C for 6 hours, and *ETC1::GUS* seedlings were incubated at 37°C for 2 and one-half hours.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Lee MM, Schiefelbein J, WEREWOLF, a MYB-related protein in Arabidopsis, is a position-dependent regulator of epidermal cell patterning. *Cell* 1999; 99:473-83; PMID:10589676; [http://dx.doi.org/10.1016/S0092-8674\(00\)81536-6](http://dx.doi.org/10.1016/S0092-8674(00)81536-6)
- Masucci JD, Rerie WG, Foreman DR, Zhang M, Galway ME, Marks MD, Schiefelbein JW. The homeobox gene GLABRA2 is required for position-dependent cell differentiation in the root epidermis of Arabidopsis thaliana. *Development* 1996; 122:1253-60; PMID:8620852
- Di Cristina M, Sessa G, Dolan L, Linstead P, Baima S, Ruberti I, Morelli G. The Arabidopsis Athb-10 (GLABRA2) is an HD-Zip protein required for regulation of root hair development. *Plant J* 1996; 10:393-402; PMID:8811855; <http://dx.doi.org/10.1046/j.1365-313X.1996.10030393.x>
- Rerie WG, Feldmann KA, Marks MD. The GLABRA2 gene encodes a homeo domain protein required for normal trichome development in Arabidopsis. *Genes Dev* 1994; 8:1388-99; PMID:7926739; <http://dx.doi.org/10.1101/gad.8.12.1388>
- Larkin JC, Oppenheimer DG, Pollock S, Marks MD. Arabidopsis GLABROUS1 Gene Requires Downstream Sequences for Function. *Plant Cell* 1993; 5:1739-48; PMID:12271054; <http://dx.doi.org/10.1105/tpc.5.12.1739>
- Oppenheimer DG, Herman PL, Sivakumaran S, Esch J, Marks MD. A myb gene required for leaf trichome differentiation in Arabidopsis is expressed in stipules. *Cell* 1991; 67:483-93; PMID:1934056; [http://dx.doi.org/10.1016/0092-8674\(91\)90523-2](http://dx.doi.org/10.1016/0092-8674(91)90523-2)
- Kirik V, Schnittger A, Radchuk V, Adler K, Hulskamp M, Baumlein H. Ectopic expression of the Arabidopsis AtMYB23 gene induces differentiation of trichome cells. *Dev Biol* 2001; 235:366-77; PMID:11437443; <http://dx.doi.org/10.1006/dbio.2001.0287>
- Bernhardt C, Lee MM, Gonzalez A, Zhang F, Lloyd A, Schiefelbein J. The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root. *Development* 2003; 130:6431-9; PMID:14627722; <http://dx.doi.org/10.1242/dev.00880>
- Galway ME, Masucci JD, Lloyd AM, Walbot V, Davis RW, Schiefelbein JW. The TTG gene is required to specify epidermal cell fate and cell patterning in the Arabidopsis root. *Dev Biol* 1994; 166:740-54; PMID:7813791; <http://dx.doi.org/10.1006/dbio.1994.1352>
- Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, Esch JJ, Marks MD, Gray JC. The TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis, encodes a WD40 repeat protein. *Plant Cell* 1999; 11:1337-50; PMID:10402433; <http://dx.doi.org/10.1105/tpc.11.7.1337>
- Wada T, Tachibana T, Shimura Y, Okada K. Epidermal cell differentiation in Arabidopsis determined by a Myb homolog, CPC. *Science* 1997; 277:1113-6; PMID:9262483; <http://dx.doi.org/10.1126/science.277.5329.1113>
- Esch JJ, Chen MA, Hillestad M, Marks MD. Comparison of TRY and the closely related At1g01380 gene in controlling Arabidopsis trichome patterning. *Plant J* 2004; 40:860-9; PMID:15584952; <http://dx.doi.org/10.1111/j.1365-313X.2004.02259.x>
- Gan L, Xia K, Chen JG, Wang S. Functional Characterization of TRICHOMELESS2, a New Single-Repeat R3 MYB Transcription Factor in the Regulation of Trichome Patterning in Arabidopsis. *BMC Plant Biol* 2011; 11:176; PMID:22168948; <http://dx.doi.org/10.1186/1471-2229-11-176>
- Kirik V, Simon M, Hulskamp M, Schiefelbein J. The ENHANCER OF TRY AND CPC1 gene acts redundantly with TRIPTYCHON and CAPRICE in trichome and root hair cell patterning in Arabidopsis. *Dev Biol* 2004; 268:506-13; PMID:15063185; <http://dx.doi.org/10.1016/j.ydbio.2003.12.037>
- Kirik V, Simon M, Wester K, Schiefelbein J, Hulskamp M. ENHANCER of TRY and CPC 2 (ETC2) reveals redundancy in the region-specific control of trichome development of Arabidopsis. *Plant Mol Biol* 2004; 55:389-98; PMID:15604688; <http://dx.doi.org/10.1007/s11103-004-0893-8>
- Schellmann S, Schnittger A, Kirik V, Wada T, Okada K, Beermann A, Thumfahrt J, Jürgens G, Hülkamp M. TRIPTYCHON and CAPRICE mediate lateral inhibition during trichome and root hair patterning in Arabidopsis. *EMBO J* 2002; 21:5036-46; PMID:12356720; <http://dx.doi.org/10.1093/emboj/cdf524>
- Simon M, Lee MM, Lin Y, Gish L, Schiefelbein J. Distinct and overlapping roles of single-repeat MYB genes in root epidermal patterning. *Dev Biol* 2007; 311:566-78; PMID:17931617; <http://dx.doi.org/10.1016/j.ydbio.2007.09.001>
- Tominaga R, Iwata M, Sano R, Inoue K, Okada K, Wada T. Arabidopsis CAPRICE-LIKE MYB 3 (CPL3) controls endoreduplication and flowering development in addition to trichome and root hair formation. *Development* 2008; 135:1335-45; PMID:18305006; <http://dx.doi.org/10.1242/dev.017947>
- Wang S, Kwak SH, Zeng Q, Ellis BE, Chen XY, Schiefelbein J, Chen JG. TRICHOMELESS1 regulates trichome patterning by suppressing GLABRA1 in Arabidopsis. *Development* 2007; 134:3873-82; PMID:17933793; <http://dx.doi.org/10.1242/dev.009597>
- Tominaga-Wada R, Nukumizu Y. Expression Analysis of an R3-Type MYB Transcription Factor CPC-LIKE MYB4 (TRICHOMELESS2) and CPL4-Related Transcripts in Arabidopsis. *Int J Mol Sci* 2012; 13:3478-91; PMID:22489163; <http://dx.doi.org/10.3390/ijms13033478>
- Bernhardt C, Lee MM, Gonzalez A, Zhang F, Lloyd A, Schiefelbein J. The bHLH genes GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) specify epidermal cell fate in the Arabidopsis root. *Development* 2003; 130:6431-9; PMID:14627722; <http://dx.doi.org/10.1242/dev.00880>
- Esch JJ, Chen M, Sanders M, Hillestad M, Ndkium S, Idelkope B, Neizer J, Marks MD. A contradictory GLABRA3 allele helps define gene interactions controlling trichome development in Arabidopsis. *Development* 2003; 130:5885-94; PMID:14561633; <http://dx.doi.org/10.1242/dev.00812>
- Zhang F, Gonzalez A, Zhao MZ, Payne CT, Lloyd A. A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. *Development* 2003; 130:4859-69; PMID:12917293; <http://dx.doi.org/10.1242/dev.00681>
- Payne CT, Zhang F, Lloyd AM. GL3 encodes a bHLH protein that regulates trichome development in Arabidopsis through interaction with GL1 and TTG1. *Genetics* 2000; 156:1349-62; PMID:11063707
- Bernhardt C, Zhao M, Gonzalez A, Lloyd A, Schiefelbein J. The bHLH genes GL3 and EGL3 participate in an intercellular regulatory circuit that controls cell patterning in the Arabidopsis root epidermis. *Development* 2005; 132:291-8; PMID:15590742; <http://dx.doi.org/10.1242/dev.01565>
- Ishida T, Kurata T, Okada K, Wada T. A genetic regulatory network in the development of trichomes and root hairs. *Annu Rev Plant Biol* 2008; 59:365-86; PMID:18257710; <http://dx.doi.org/10.1146/annurev.arplant.59.032607.092949>
- Tominaga-Wada R, Ishida T, Wada T. New insights into the mechanism of development of Arabidopsis root hairs and trichomes. *Int Rev Cell Mol Biol* 2011; 286:67-106; PMID:21199780; <http://dx.doi.org/10.1016/B978-0-12-385859-7.00002-1>
- Tominaga-Wada R, Wada T. Regulation of root hair cell differentiation by R3 MYB transcription factors in tomato and Arabidopsis. *Frontiers in plant science* 2014; 5:91; PMID:24659995; <http://dx.doi.org/10.3389/fpls.2014.00091>
- Kurata T, Ishida T, Kawabata-Awai C, Noguchi M, Hattori S, Sano R, Nagasaka R, Tominaga R, Koshino-Kimura Y, Kato T, et al. Cell-to-cell movement of the CAPRICE protein in Arabidopsis root epidermal cell differentiation. *Development* 2005; 132:5387-98; PMID:16291794; <http://dx.doi.org/10.1242/dev.02139>
- Tominaga-Wada R, Nukumizu Y, Wada T. Flowering is delayed by mutations in homologous genes CAPRICE and TRIPTYCHON in the early flowering Arabidopsis cpl3 mutant. *J Plant Physiol* 2013; 170:1466-8; PMID:23796522; <http://dx.doi.org/10.1016/j.jplph.2013.05.013>
- Okada K, Shimura Y. Reversible Root Tip Rotation in Arabidopsis Seedlings Induced by Obstacle-Touching Stimulus. *Science* 1990; 250:274-6; PMID:17797309; <http://dx.doi.org/10.1126/science.250.4978.274>