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ERECTA-Family Receptor Kinases Regulate Stem Cell Homeostasis via Buffering its Cytokinin Responsiveness in the Shoot Apical Meristem

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Shoot apical meristems (SAMs), which are maintained at the tips of stems, are indeterminate structures and sources of stem cells from which all aerial organs are ultimately derived. Although mechanisms that regulate the homeostasis of the stem cells have been extensively investigated, identification of further unknown regulators should provide better understanding of the regulation. Here, we report that members of the Arabidopsis *ERECTA* (*ER*) receptor kinase family redundantly play a significant role in the regulation of stem cell homeostasis. In wild-type seedlings, the expression of *WUSCHEL* (*WUS*), a central regulator of the stem cell population, is stimulated by cytokinin. Interestingly, however, the SAM morphology and the expression of *CLAVATA3* (*CLV3*), which is expressed in stem cells and therefore serves as a stem cell marker, are relatively stable against cytokinin treatment regardless of increased *WUS* expression. These findings indicate the presence of a mechanism to buffer stem cell homeostasis against an increase in cytokinin. Mutant seedlings lacking all *ER*-family members, which are expressed in the SAM, show an increase in the stem cell population and also the up-regulation of a cytokinin-responsive gene in the SAM. In this mutant, *WUS* expression is stimulated by cytokinin treatment as efficiently as in wild-type plants. However, in contrast to wild-type plants, SAM morphology and *CLV3* expression respond drastically to cytokinin treatment, suggesting that the buffering mechanism to maintain stem cell homeostasis against an increase in cytokinin is severely impaired in this mutant. We suggest that the *ER* family regulates stem cell homeostasis via buffering its cytokinin responsiveness in the SAM.

Keywords: *Arabidopsis thaliana* • Cytokinin • *ERECTA* family • Receptor kinases • Shoot apical meristem • Stem cells.

Abbreviations: ARR, ARABIDOPSIS RESPONSE REGULATOR; CLV3, CLAVATA3; ER, ERECTA; ERL1, ERECTA-LIKE 1; ERL2, ERECTA-LIKE 2; GUS, β-glucuronidase; qRT-PCR, quantitative real-time PCR; SAM, shoot apical meristem; WUS, WUSCHEL.

Introduction

The shoot apical meristem (SAM) plays significant roles in establishment of the plant architecture. The SAM, which is maintained at the tips of stems, is an indeterminate structure and serves as a source of stem cells from which all post-embryonic aerial organs are ultimately derived. Mechanisms that regulate the homeostasis of the stem cells in the SAM have been investigated and some key regulators have been reported (Besnard et al. 2011). Among them, *WUSCHEL* (*WUS*) is expressed at the organizing center just below the stem cell region of the SAM and is required for the maintenance of stem cells (Laux et al. 1996, Mayer et al. 1998). *WUS* activates the expression of *CLAVATA3* (*CLV3*) in stem cells (Fletcher et al. 1999), which encodes a secreted small peptide hormone (Kondo et al. 2006, Ohyama et al. 2009) and also serves as a useful stem cell marker gene (Yadav et al. 2009). The CLV3 peptides in turn repress *WUS* expression (Kondo et al. 2006, Ohyama et al. 2009). This local *WUS–CLV3* feedback loop ensures a constant number of stem cells in the SAM (Brand et al. 2000, Schoof et al. 2000, Betsuyaku et al. 2011). Interestingly, in the inflorescence SAM, this *WUS–CLV3* regulatory circuit was shown to behave relatively stably against fluctuations of the amounts of each component, suggesting the presence of a failsafe mechanism to guarantee stem cell maintenance (Muller et al. 2006, Gordon et al. 2009, Chickarmane et al. 2012). The plant hormone cytokinin is also known to participate in the regulation of stem cell homeostasis by the *WUS–CLV3* circuit (Leibfried et al. 2005, Gordon et al. 2009, Chickarmane et al. 2012). Although cytokinin signaling activates *WUS* expression, cytokinin treatment did not cause the drastic enhancement of SAM size in wild-type inflorescences, suggesting the presence of a mechanism buffering stem cell homeostasis against an increasing amount of cytokinin in the inflorescence SAM (Gordon et al. 2009, Chickarmane et al. 2012). However, although some genes involved in cytokinin signaling pathways have been shown to function in this buffering mechanism (Gordon et al. 2009,

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Chickarmane et al. 2012), information about further unknown participants is still missing. Moreover, it is unknown whether such a buffering mechanism for stem cell homeostasis also exists in the vegetative SAM at the seedling stage.

The Arabidopsis *ERECTA* (*ER*) family consists of *ER*, *ERECTA-LIKE 1* (*ERL1*) and *ERECTA-LIKE 2* (*ERL2*) (Shpak et al. 2004), and encodes receptor kinases (Torii et al. 1996, Shpak et al. 2004). The *ER*-family members are broadly expressed in various tissues (Yokoyama et al. 1998, Shpak et al. 2004, Uchida et al. 2012) and play roles in diverse aspects of plant development by mediating cell–cell signals that sense and coordinate organ development (Torii et al. 1996, Shpak et al. 2004, Uchida et al. 2012). Although loss-of-function mutations in the *ER* gene do not show obvious defects in SAM regulation (Torii et al. 1996), *er* mutations have been reported to modify meristem defects caused by other mutations (Xu et al. 2003, Qi et al. 2004, Uchida et al. 2011), suggesting that *ER* might play a significant but unknown role in SAM regulation. Also, simultaneous loss of all *ER*-family members confers extreme dwarfism (Shpak et al. 2004), implying the possibility that the *ER*-family members are redundantly involved in SAM function, although this possibility has not been investigated so far.

In this study we report that in seedling plants lacking all *ER*-family members, which are expressed in the SAM, defects in stem cell homeostasis are observed and the regulation of the cytokinin responsiveness in the SAM is severely impaired. We suggest that the *ER* family regulates stem cell homeostasis via buffering of its cytokinin responsiveness in the vegetative SAM.

Results

All *ER*-family members are redundantly involved in SAM regulation

Although *er* mutations do not show obvious defects of SAM regulation, they affect meristem phenotypes caused by other mutations (Xu et al. 2003, Qi et al. 2004, Uchida et al. 2011), suggesting that *ER* might play a significant but unknown role in SAM regulation. We assumed that its two paralogous genes, *ERL1* and *ERL2*, might mask the appearance of potential defects in the SAM of *er* mutants. Therefore, to investigate the involvement of *ER*-family members in SAM regulation, we compared SAM morphologies of multiple mutants of various combinations among *er*-family members (**Fig. 1**). Among the multiple mutants we analyzed, *erl1 erl2*, *er erl1* and *er erl2* double mutants did not exhibit apparent changes in SAM morphology compared with wild-type seedlings (**Fig. 1A–D**). On the other hand, *er erl1 erl2* triple mutant seedlings clearly showed the abnormal SAM morphology (**Fig. 1E**). The SAM of *er erl1 erl2* seedlings appeared to be flat and broadened compared with the wild-type SAM (**Fig. 1A, E**). This observation was supported by examination of longitudinal sections of the SAM (see later **Figs. 3B, D, F, 5G**). Thus, all *ER*-family members are redundantly involved in the regulation of the vegetative SAM. Although the promoter activities of the *ER*-family members were roughly detected at regions including the shoot apex at the seedling stage in a previous study (Shpak et al. 2004), the authors did not examine whether they were active inside the SAM at that stage. When we examined the promoter activities of *ER*, *ERL1* and *ERL2* using *β-glucuronidase* (*GUS*) reporter genes at the seedling stage, all promoters showed strong GUS signals at regions including the shoot apex (**Fig. 2A, C, E**) as reported (Shpak et al. 2004). Our observation of thin plastic sections of the shoot apex regions clearly indicated that GUS signals were detected inside the SAM in all cases (**Fig. 2B, D, F**). These expression patterns of *ER*-family members within the SAM appear consistent with the fact that all *ER*-family members are redundantly involved in SAM regulation (**Fig. 1E**).

Stem cell homeostasis is severely impaired in *er erl1 erl2* seedlings

To analyze the SAM defects of *er erl1 erl2* seedlings further, we examined the expression pattern and level of *WUS*, a central regulator of the stem cell population, and also *CLV3* as a stem cell marker gene. *WUS* expression was observed only in a limited region in the SAM of wild-type plants (**Fig. 3A**) as reported (Mayer et al. 1998), while *er erl1 erl2* plants displayed a slightly broadened expression pattern (**Fig. 3B**) probably because of the broadened shape of the SAM, but the signal intensities of *WUS* transcripts between wild-type and *er erl1 erl2* plants appeared almost comparable (**Fig. 3A, B**). This observation was supported by measurement of the *WUS* transcripts by quantitative real-time PCR (qRT-PCR), which indicated only about a 4-fold increase in the expression in *er erl1 erl2* (**Fig. 3G**). On the other hand, when we examined *CLV3* expression, drastic changes in both the expression pattern and level were observed between wild-type and *er erl1 erl2* seedlings. In wild-type seedlings, *CLV3* was expressed only in several cells at the tip of the SAM (**Fig. 3C**), while in *er erl1 erl2* seedling *CLV3* expression was strongly detected in an increased number of cells (**Fig. 3D**). qRT-PCR indicated a dramatic increase in *CLV3* expression in *er erl1 erl2* seedlings (**Fig. 3H**). This observation was further supported by the examination of the *CLV3* promoter activity using a *GUS* reporter gene (**Fig. 3H**). Thus, stem cell homeostasis is severely impaired in the SAM of *er erl1 erl2* seedlings.

The expression of the cytokinin-responsive *ARR15* is expanded in the SAM of *er erl1 erl2* seedlings

The plant hormone cytokinin is one of the key factors affecting stem cell homeostasis (Leibfried et al. 2005, Gordon et al. 2009, Chickarmane et al. 2012). To examine the involvement of the action of cytokinin in the defects observed in the SAM of *er erl1 erl2* seedlings, we checked the expression pattern of the *type-A ARABIDOPSIS RESPONSE REGULATOR 15* (*ARR15*), which has been reported to be one of the representative cytokinin-responsive genes in the SAM (Leibfried et al. 2005, Zhao et al. 2010). The promoter activity of *ARR15* using a *GUS* reporter gene was strongly detected in the central region of the SAM, but rarely or very weakly in the peripheral regions of the

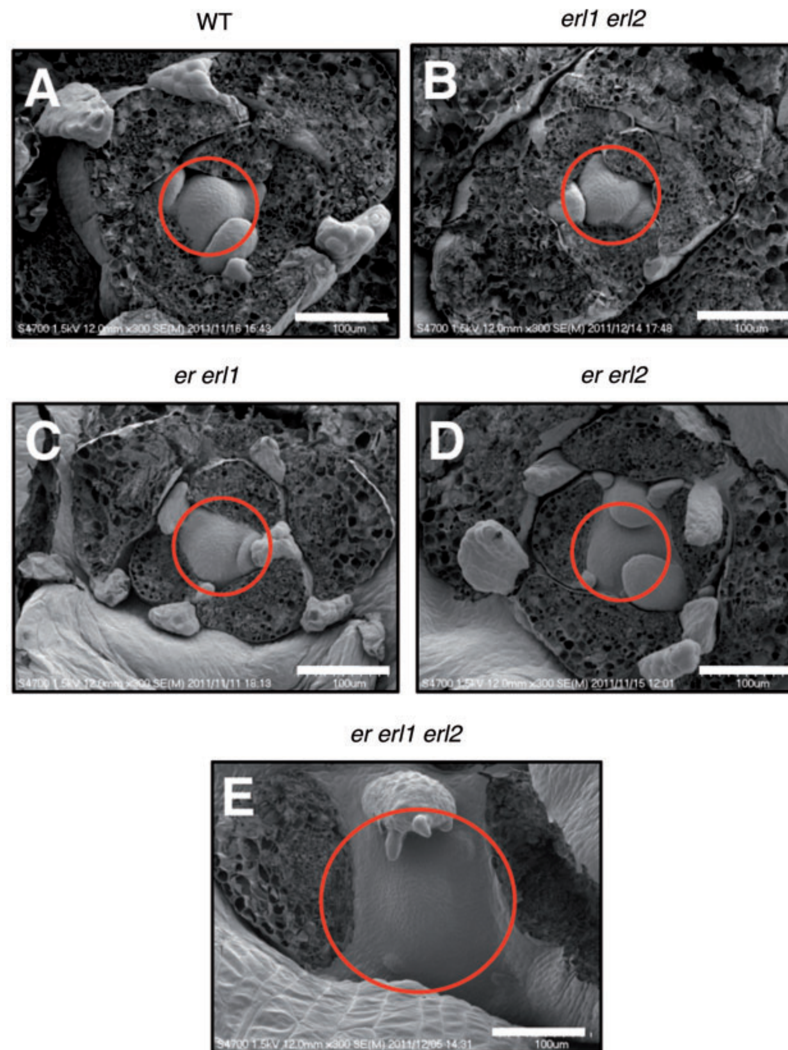

Fig. 1 SAM morphologies of multiple mutants of various combinations among *ER*-family members. The SAMs of 9-day-old seedlings were observed by scanning electron microscopy. Circles indicate the SAMs. Bars = 100 µm.

SAM (**Fig. 4A**). On the other hand, the *ARR15* promoter was active in the entire region of the SAM of *er erl1 erl2* seedlings (**Fig. 4B**). This raised the possibility that the SAM of *er erl1 erl2* seedlings might possess the potential to respond to cytokinin more than the wild-type SAM.

The SAM responds drastically to cytokinin when the activity of the whole *ER* family is attenuated

To examine the above possibility, we compared responses to cytokinin treatment between wild-type and *er erl1 erl2* plants. When wild-type seedlings were treated with cytokinin, the SAM morphology did not obviously change (**Fig. 5A, B**). This observation was supported by the examination of longitudinal sections of the SAM (**Fig. 5C, D**), suggesting that, from the viewpoint of morphology, the vegetative SAM is resistant to an increase in cytokinin in wild-type seedlings. On the other hand, when *er erl1 erl2* seedlings were treated with cytokinin, the SAM morphology was altered dramatically (**Fig. 5E, F**). The originally flat SAM of *er erl1 erl2* seedlings (**Fig. 5E**) rose into an enlarged mound-like structure (**Fig. 5F**) with formation of multiple bumps (**Fig. 5F**, arrowheads), which had the appearance of leaf primordia, at the SAM periphery. Furthermore, longitudinal sectioning (**Fig. 5G, H**) clearly indicated that the mound-like SAM of the cytokinin-treated *er erl1 erl2* seedlings was filled with a very large number of small cells (**Fig. 5H**). These show that the mechanism to buffer the cytokinin responsiveness, which is functional in the wild-type SAM, is severely impaired in the SAM of *er erl1 erl2* seedlings.

The above observation raised a question as to whether the loss of function of the entire *ER* family might confer enhanced cytokinin responsiveness of the whole plant body. To examine this, we checked expression levels of several cytokinin-responsive *type-A ARR* genes (*ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR9*, *ARR15* and *ARR16*) in the whole plant bodies by qRT-PCR. All genes we examined were up-regulated by

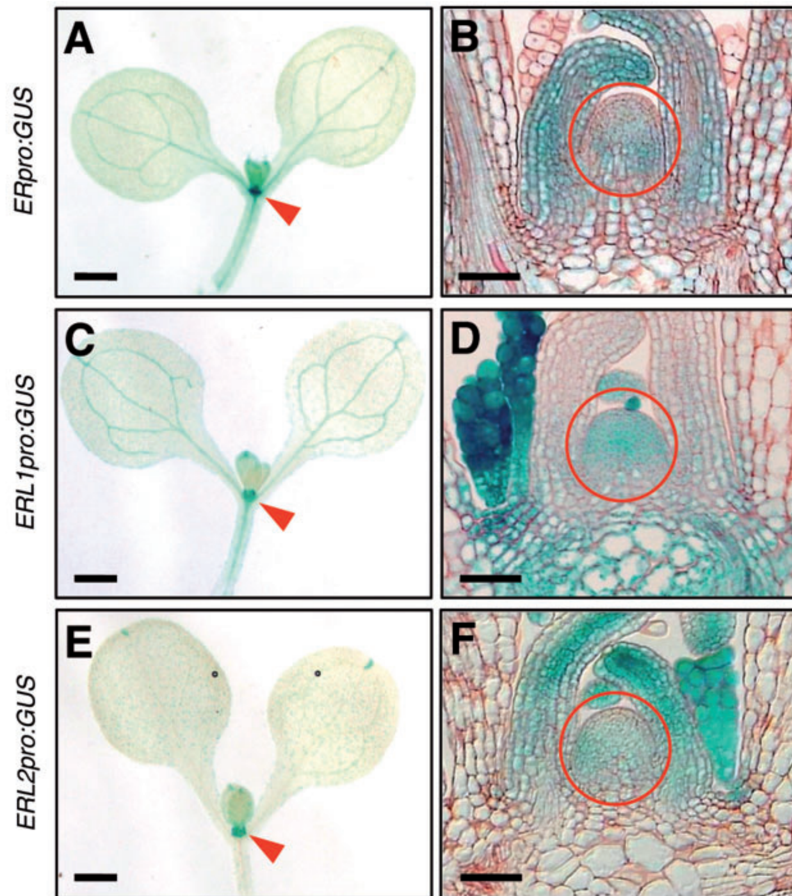

Fig. 2 GUS reporter lines to show promoter activities of *ER*-family members. Nine-day-old seedlings were analyzed. (A, C and E) Whole seedlings. Bars = 1 mm. (B, D and F) Thin plastic sections of the SAMs. Bars = 50 μm. Arrows and circles indicate the shoot apex and the SAMs, respectively.

cytokinin treatment to a similar extent between wild-type and *er erl1 erl2* seedlings (**Supplementary Fig. S1**), indicating that the global cytokinin responsiveness was not obviously altered between wild-type and *er erl1 erl2* plants. Given that the SAM morphology of *er erl1 erl2* seedlings was drastically altered by cytokinin treatment (**Fig. 5F, H**) in contrast to that of wild-type plants, the SAM appears to be a tissue that displays enhanced responsiveness to cytokinin when the activity of the whole *ER* family is attenuated.

Also, as judged by excessive formation of leaf primordium-like structures (**Fig. 5F**, arrowheads) in cytokinin-treated *er erl1 erl2* seedlings and the appearance of cells in the structures (**Fig. 5F**, arrowheads, and **5H**), it was implied that, in addition to homeostasis of the SAM, leaf development might also be affected by cytokinin in *er erl1 erl2* plants. Actually, as shown in **Supplementary Fig. S2**, cytokinin treatment induced excessive formation of apparently abnormal leaves with a squat shape in *er erl1 erl2* plants (**Supplementary Fig. S2C, D**), while cytokinin treatment of wild-type seedlings did not exert significant effects on leaf morphology (**Supplementary Fig. S2A, B**). In cytokinin-treated *er erl1 erl2* seedlings, trichomes were still observed on the malformed leaves (**Supplementary Fig. S2D**), suggesting that their fundamental leaf structure seemed to be maintained. It remains to be investigated whether the SAM of cytokinin-treated *er erl1 erl2* seedlings generates abnormal leaf primordia that secondarily result in malformed leaves or if *er erl1 erl2* leaves show enhanced cytokinin responsiveness during their development.

The mechanism to buffer *CLV3* expression against the increased expression of *WUS* by cytokinin treatment is impaired in *er erl1 erl2* seedlings

Previously it was reported that, although cytokinin treatment greatly activated *WUS* expression in the inflorescence SAM, *CLV3* expression was relatively stable against the treatment, indicating that the inflorescence SAM possesses a mechanism to maintain *CLV3* homeostasis regardless of the increased expression of *WUS* by cytokinin treatment (Gordon et al. 2009). Because the morphology of the vegetative SAM of wild-type seedlings displayed a significant resistance to cytokinin treatment (**Fig. 5A–D**), we examined whether a similar buffering system to maintain the *CLV3* homeostasis is functional in the vegetative SAM as well as in the inflorescence SAM. When

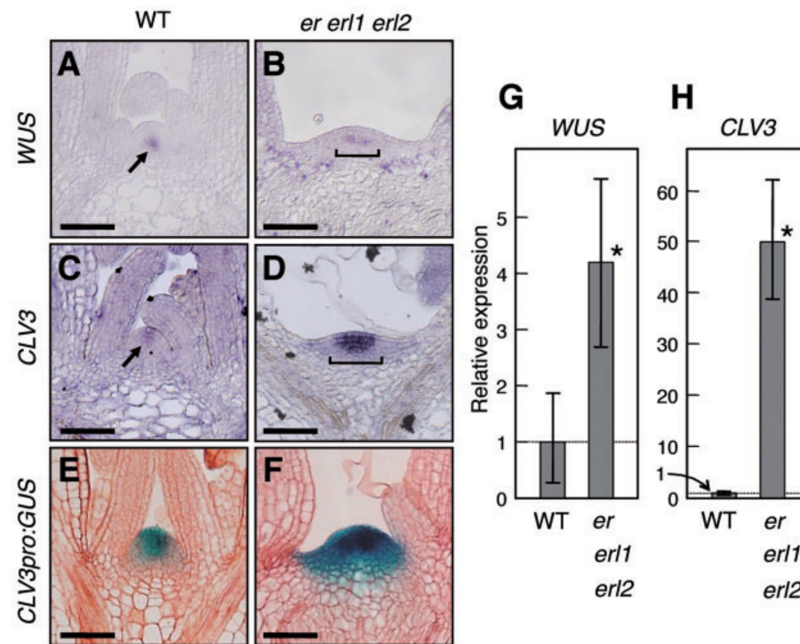

Fig. 3 Expression patterns and levels of *WUS* and *CLV3*. Nine-day-old seedlings were analyzed. (A–D) In situ RNA hybridization. Arrows and brackets indicate signals. Bars = 50 μm. (E and F) *CLV3pro:GUS*. Bars = 50 μm. (G and H) Aerial parts were analyzed by qRT-PCR. The expression levels were normalized with respect to that of *β-TUBULIN* and the normalized values in the wild type were set at 1. Data shown are means of three independent samples (10 individuals were collected as a pool for each sample) with error bars representing the SD. Asterisks indicate significant differences by Student's *t*-test ($P < 0.05$).

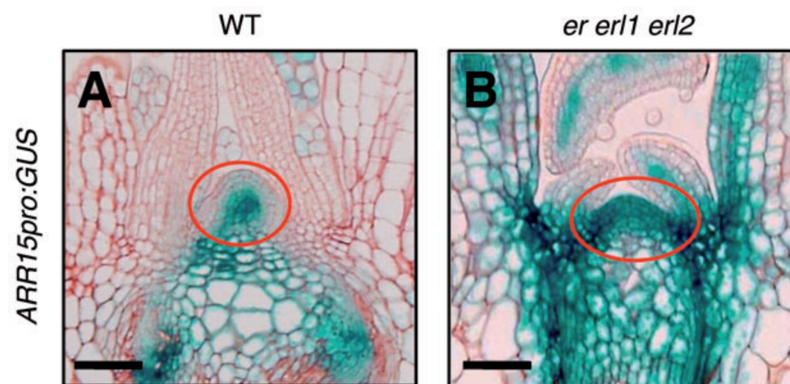

Fig. 4 Expression patterns of *ARR15pro:GUS*. Nine-day-old seedlings were analyzed. Circles indicate the SAMs. Bars = 50 μm.

wild-type seedlings were treated by cytokinin, *WUS* expression was greatly up-regulated (**Fig. 6A**), while *CLV3* expression was not significantly altered (**Fig. 6B**), indicating that the vegetative SAM also possesses the functional buffering mechanism to maintain *CLV3* homeostasis regardless of an increase in *WUS* induced by cytokinin treatment. Next, we examined whether this buffering mechanism is still functional in *er erl1 erl2*. When *er erl1 erl2* seedlings were treated with cytokinin, *WUS* expression was up-regulated similarly to the case of wild-type seedlings (**Fig. 6A**). On the other hand, *CLV3* expression was also greatly up-regulated in *er erl1 erl2* (**Fig. 6B**) in contrast to the case of wild-type plants where *CLV3* expression was relatively stable (**Fig. 6B**). This indicates that the mechanism to buffer *CLV3* homeostasis against an increase of cytokinin is impaired in *er erl1 erl2*.

Discussion

ER-family receptor kinases redundantly play a significant role in regulation of the SAM

The simultaneous loss of functions of the all *ER*-family members, which were all expressed in the SAM at the seedling stage (**Fig. 2**), caused defects in SAM regulation (**Figs. 1**, **3**, **4**), clearly indicating that *ER*-family members redundantly play a significant role in this regulation. The *ER* family encodes leucine-rich

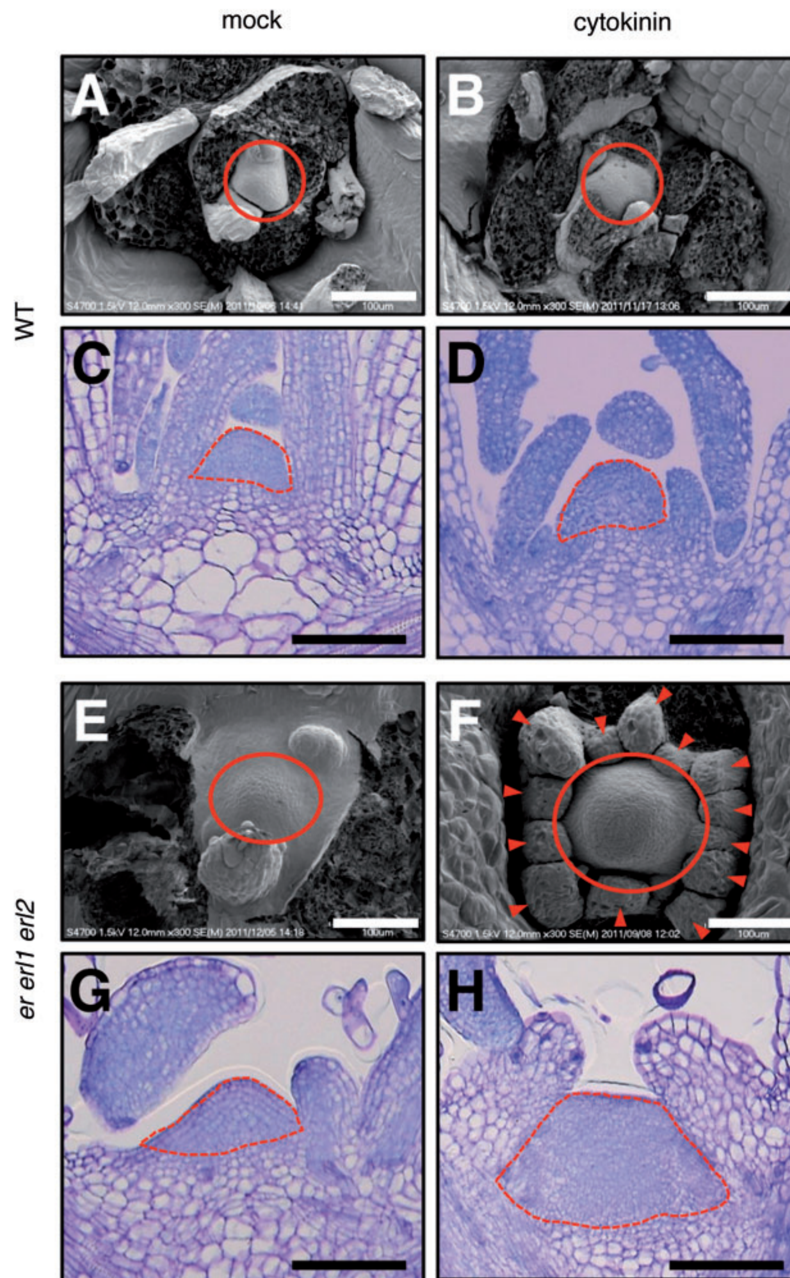

Fig. 5 SAM morphologies with/without cytokinin treatment. The SAMs of 10-day-old seedlings grown on normal or cytokinin-containing media were observed. (A, B, E and F) Observation by scanning electron microscopy. Circles indicate the SAMs. Arrowheads indicate leaf primordium-like structures. Bars = 100 μm. (C, D, G and H) Thin plastic sections. Dotted lines show the SAMs. Bars = 100 μm.

repeat receptor-like kinases in a subfamily of transmembrane-type signaling receptors (Torii et al. 1996, Shpak et al. 2004), and it has been inferred that ER-family proteins function in receptor complexes through associations with multiple partners (Shpak et al. 2003). Although such partners are assumed for regulation of stomatal development (Nadeau and Sack 2002, Lee et al. 2012) in which the ER family has also been reported to be involved (Shpak et al. 2005), information about interacting partners of the ER family in SAM regulation is completely lacking. Moreover, although some ligands for ER-family receptors in other types of developmental regulation have been reported (Hara et al. 2007, Hara et al. 2009, Hunt and Gray 2009, Abrash and Bergmann 2010, Kondo et al. 2010, Sugano et al. 2010, Abrash et al. 2011, Uchida et al. 2012), such ligands for SAM regulation are still unknown. Thus, identification of interacting partners for the receptor complexes and the ligands would be important for further understanding of SAM regulation by the ER-family receptor kinases. Also analysis of intracellular signaling pathways downstream of the ER family would be an interesting future issue.

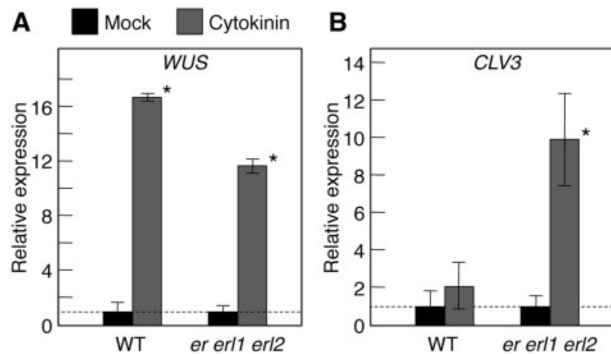

Fig. 6 Expression levels of *WUS* and *CLV3* with/without cytokinin treatment. Aerial parts of 10-day-old seedlings grown on normal or cytokinin-containing media were analyzed by qRT-PCR. The expression levels were normalized with respect to that of *β-TUBULIN* and the normalized values without cytokinin treatment were set at 1. Data shown are means of four independent samples (10 individuals were collected as a pool for each sample) with error bars representing the SD. Asterisks indicate significant differences by Student's *t*-test ($P < 0.05$).

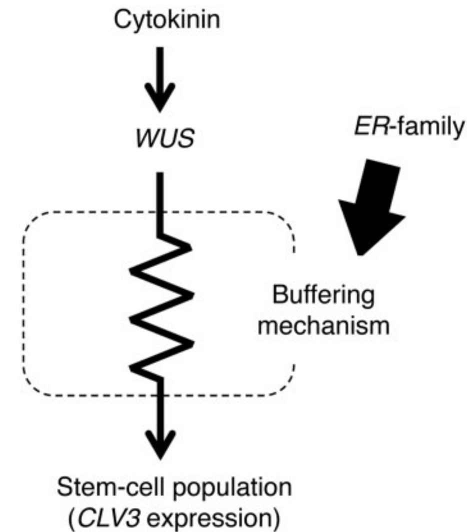

Fig. 7 Brief illustration of the proposed system to buffer stem cell homeostasis against an increase of cytokinin. See text for the explanation.

The *ER* family regulates stem cell homeostasis via buffering of its cytokinin responsiveness in the vegetative SAM

Our results indicate that when the activity of the whole *ER* family is attenuated, the SAM can no longer buffer cytokinin responsiveness to maintain SAM homeostasis (**Figs. 5**, **6**). *WUS* expression was greatly stimulated by cytokinin treatment (**Fig. 6A**) both in the wild-type and in *er erl1 erl2* plants, while responses of the stem cell marker *CLV3* differed greatly between them (**Fig. 6B**); wild-type seedlings did not show a significant increase in *CLV3*, while *er erl1 erl2* seedlings did. These findings suggest that the buffering mechanism to maintain SAM homeostasis could be located downstream of the *WUS* function and upstream of the stem cell regulation marked by *CLV3* expression, and that the *ER* family is involved in the buffering mechanism (**Fig. 7**). Further investigation of this mechanism would be an important future interest. To that end, we will discuss a few points in the next section.

Candidate factors that could be integrated into the mechanism to buffer SAM homeostasis involving the *ER* family

We observed that the activity of the *ARR15* promoter was expanded in the SAM of *er erl1 erl2* seedlings (**Fig. 4B**). *ARR15* is considered to be a cytokinin-responsive gene (D'Agostino et al. 2000), while its gene products, ARR15 proteins, are known to serve as a negative factor of cytokinin signaling for negative feedback regulation (Kiba et al. 2003). Therefore, although the SAM of *er erl1 erl2* seedlings responds drastically to cytokinin (**Figs. 5**, **6**), it is likely that the negative regulation of cytokinin signaling by ARR15 proteins is still functional even in *er erl1 erl2* plants. Because this negative effect of ARR15 proteins is reported to play a significant role in stem cell maintenance (Leibfried et al. 2005), it could serve as one of modifiers of the buffering ability of the system in which the *ER* family is involved.

Another important aspect of negative feedback regulation of cytokinin signaling is down-regulation of cytokinin biosynthesis enzyme genes, which is induced by activation of cytokinin signaling (Miyawaki et al. 2004). The *LONELY GUY* (*LOG*) family encodes enzymes to catalyze the final activation steps to produce active cytokinins (Kurakawa et al. 2007, Kamada-Nobusada and Sakakibara 2009, Kuroha et al. 2009, Tokunaga et al. 2012). Interestingly, a recent paper reported that, among Arabidopsis *LOG*-family members, regulation of *LOG4* expression is integrated into the *WUS*–*CLV3* regulatory circuit (Chickarmane et al. 2012). Actually, when we examined the *LOG4* expression levels by qRT-PCR, they were decreased in *er erl1 erl2* (**Supplementary Fig. S3**). Thus, it would be an attractive scenario that the *ER* family regulates stem cell homeostasis via the regulation of *LOG4* expression. In addition, it was reported that *LOG1* and *LOG7* are also expressed in the SAM (Kuroha et al. 2009, Yadav et al. 2009) and that *LOG7* in particular plays a pivotal role in the maintenance of the SAM (Tokunaga et al. 2012). It might be possible that *LOG1* and/or *LOG7* are also integrated into the *WUS*–*CLV3* regulatory circuit and involved in the buffering system where *ER*-family members exert their functions.

Recently it has been reported that a cross-talk between cytokinin signaling and auxin signaling plays a significant role in maintenance of the stem cell population (Zhao et al. 2010). Interestingly, the pathways triggered by both hormones directly converge on the promoters of *ARR7* and *ARR15* (Zhao et al. 2010), which negatively regulate cytokinin signaling; auxin represses their expression, while cytokinin activates it.

Although it remains to be shown fully how this cytokinin–auxin cross-talk is integrated into the *WUS*–*CLV3* regulatory circuit, it is possible that auxin signaling is also a candidate component of the buffering system in which the *ER* family is involved.

Although the detailed molecular mechanisms as to how the *ER* family contributes to the buffering system to maintain stem cell homeostasis are still largely unknown, we believe that our findings provide a cue to investigate a novel aspect of the regulation of the SAM.

Materials and Methods

Plant materials and growth conditions

The wild-type accession of *Arabidopsis thaliana* used in this study was Columbia (Col). *er-105*, *erl1-2* and *erl2-1* mutants were reported previously (Torii et al. 1996, Shpak et al. 2004). Promoter–*GUS* reporter lines of the *ER* family (Shpak et al. 2004), *CLV3* (Brand et al. 2002) and *ARR15* (Zhao et al. 2010) were also described previously.

Surface-sterilized seeds were plated on Murashige and Skoog medium and then transferred to a growth room at 22°C under continuous white light. For cytokinin treatment, plants were grown on media containing 5 μM 6-benzylaminopurine (BAP).

Quantitative RT-PCR

RNA isolation, synthesis of first-strand cDNA and real-time PCR were performed as previously described (Uchida et al. 2011). Primers used for real-time PCR were as follows; *WUS*, gcgatgcttatctggaacat and cttccagatggcaccactac; *CLV3*, aaagtgaatgggttggagca and tcatgtagtcctaaacccttcgt; *ARR4*, gtcatcgagagattgcttcgt and acgccatccactatctaccg; *ARR5*, tcagagaacatcttgcctcgt and atttcacaggcttcaataagaaatc; *ARR6*, gaacattttgcctcgtattgatag and cgagagttttaccggcttca; *ARR7*, tcatctgagaacatcttacctcgt and ttcaccggtttcaacaagaat; *ARR8*, acgttcctgcaagaatctcc and ggtttcaacttggtaagatcagc; *ARR9*, acgttcctgcaagaatcagc and cagccaatcttactggtttcaa; *ARR15*, gagaacatacaacctcgtatagaacaa and gctaatttcaccggttttagca; *ARR16*, atggatgtggtggcttatga and tctaagcgcattctctgctg; and *LOG4*, gtttgatgggtttggtttcg and caccggtcaactctctaggc.

Histology

The procedures of GUS staining were previously described (Uchida et al. 2007). To make plastic sections, materials were fixed in formalin/acetic acid/alcohol (FAA) and then embedded in Technovit 7100 resin as previously described (Uchida et al. 2012). Sections of 4 μm were stained with 0.02% toluidine blue or 0.04% neutral red.

In situ RNA hybridization

In situ hybridization was performed according to Nahar et al. (2001). Templates for transcription of *WUS* and *CLV3* antisense probes were described in Hamada et al. (2000) and Kinoshita et al. (2010), respectively.

Supplementary data

Supplementary data are available at PCP online.

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