Signal transduction of secretory peptides that regulate leaf epidermal patterning

葉の表皮細胞パターンを調節する分泌ペプチドの情報伝達機構の研究

DISSERTATION

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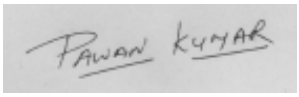

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Contents

Abstract

A stoma consists of two guard cells and a pore between them and regulates gas and water vapour exchange between plant and their environment. During early stages of leaf development, the epidermal cells of leaf primordia known as protodermal cells either terminally differentiate into a pavement cell or become a meristemoid mother cell (MMC). MMC and perhaps its precursor cells are maintained by a basic helix loop helix-type transcription factor, SPEECHLESS (SPCH), which is preferentially present in these cells. MMC divides asymmetrically and produces a small triangular-shaped cell, meristemoid, and its sister cell. Cell lineage initiated by MMC is called the stomatal lineage. The meristemoid eventually becomes the oval shaped guard mother cell (GMC), which divides symmetrically and finally becomes a pair of guard cells of a stoma.

Positioning and density of stomata are regulated by three secretory peptides, EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, and stomagen. *EPF1* is expressed in meristemoid, GMC and young guard cells and controls stomatal patterning through regulation of asymmetric cell division. *EPF2* is expressed in MMC and protodermal cells, and inhibits additional formation of MMC. This feedback loop plays a critical role in regulation of epidermal cell density. *STOMAGEN* is expressed in mesophyll cells and positively controls stomatal density in *Arabidopsis*.

TOO MANY MOUTHS (TMM) encodes a leucine rich repeat receptor-like-protein (RLP) and *ER* family encodes leucine rich repeat receptor-like-kinases (RLK). The loss of function mutants of *tmm* and *er erl1 erl2* show increased in stomatal density with violation of one cell spacing rule. The loss-of function mutants of genes for *YODA* encoding MAP kinase kinase kinase, plants with decreased expression of *MKK4* and *MKK5* encoding MAP kinase kinase, and double mutants of *MPK3* and *MPK6* encoding MAP kinase, stomata are increased in number and are formed as clusters.

Several lines of published evidence have suggested a regulatory pathway as follows. EPF1 and EPF2 are perceived by receptor complexes consisting of TOO MANY MOUTHS,

ERECTA, and ERECTA-LIKE 1 and 2. These receptors activate a mitogen activated protein (MAP) kinase module. MAP kinases phosphorylate and destabilize the transcription factor SPEECHLESS (SPCH), resulting in a decrease in the number of stomatal lineage cells. Stomagen acts antagonistically to EPF1 and EPF2. However, there is no direct evidence that EPF1 and EPF2 activate or that stomagen inactivates the MAP kinase cascade, through which they might regulate the SPCH level. Experimental modulation of these peptides in *Arabidopsis thaliana* would change the number of stomatal lineage cells in developing leaves, which in turn would change the expression of *SPCH*, making the interpretation difficult. Here I reconstructed this signaling pathway in differentiated leaf cells of *Nicotiana benthamiana* to examine signaling without the confounding effect of cell-type change. I found that EPF1 and EPF2 are able to activate the MAP kinase MPK6, and that both EPF1 and EPF2 are able to decrease the SPCH level, whereas stomagen is able to increases it. My data also suggest that EPF1 and EPF2 act through different receptor components. This is first direct report in which *EPF1*, EPF2 and stomagen modulate SPCH level.

Introduction

Stomata are valves on plant epidermis, and their correct placement allows an efficient gas exchange between plants and the atmosphere. The number and placement of stomata are genetically regulated and are also affected by the environment (Hetherington and Woodward 2003; Nadeau and Sack 2002b).

Stomatal development pathway

During early stages of leaf development, the epidermal cells of leaf primordia (known as protodermal cells) either terminally differentiate into pavement cells or become meristemoid mother cells (MMCs). MMCs and perhaps their precursor cells are maintained by a basic helix-loop-helix-type transcription factor SPEECHLESS (SPCH), which is preferentially present in these cells (MacAlister et al. 2007; Pillitteri et al. 2007). Each MMC divides asymmetrically and produces a small triangular-shaped cell (meristemoid) and its sister cell. The cell lineage initiated by the MMC is called the stomatal lineage. Each meristemoid may further undergo one or two rounds of asymmetric cell divisions, known as amplifying divisions, which result in another meristemoid and a sister cell. The meristemoid eventually becomes the guard mother cell (GMC), which divides symmetrically and finally becomes a stoma (Fig. 1). The sister cell may also undergo formative asymmetric cell divisions, creating a satellite meristemoid and its sister cell (Geisler et al. 2000, Bergmann and Sack 2007, Nadeau 2009). The sister cells eventually differentiate into pavement cells. New meristemoids are not formed in contact with the pre-existing stomata, which enforce the "one-cell-spacing" rule, i.e. two stomata are separated by at least one intervening nonstomatal epidermal cell (Geisler et al. 2000; Bergmann and Sack 2007; Nadeau 2009).

Discovery of new bioactive secretory peptides

To identify new peptide mediators that regulate plant development, Hara et al. 2007 overexpressed 153 genes that encode peptide/polypeptide (<150 amino acids) with a secretory signal sequence. Through this screen, they identified EPIDERMAL PATTERNING FACTORs (EPFs) which affects epidermal patterning. Three important genes, *EPF1*, *EPF2* and *STOMAGEN/EPFL9* (*EPF-LIKE 9*) were discovered. The *EPF* family contains 11 genes whose amino acids sequence is evolutionary conserved. Among them *EPF1* is the first reported gene that regulates stomatal positioning by controlling asymmetric cell division. *EPF2* negatively control whereas *STOMAGEN* positively controls stomatal density.

Structure of the EPF family peptides

EPF family genes encode 102-172 amino acid polypeptides with a secretory signal sequence at the N-terminal end and six conserved cysteine residues at their C-terminal end. Out of three peptides, the NMR structure of the stomagen has been resolved (Ohki et al. 2011). The stomagen peptide has six cysteine residues that form three disulfide bonds. These six cysteine residue are conserved in all members of *EPF* family members. The stomagen peptide is composed of a loop and scaffold. Deletion of these three disulfide bonds introduces unfolding and inactivity. The stomagen peptide structure also suggested that the loop confers the functional specificity of EPFs and the scaffold is structurally required for their activity.

EPF1, EPF2 and stomagen peptides have different biological functions

Stomatal placement and number are regulated by peptides that mediate intercellular communication, EPF1 (Hara et al. 2007), EPF2 (Hara et al. 2009; Hunt and Gray 2009), and stomagen/EPFL9 (Hunt et al. 2010; Kondo et al. 2010; Sugano et al. 2010; Ohki et al. 2011). *EPF1* is expressed in meristemoids, GMCs and young guard cells, whereas *EPF2* is

expressed in MMCs and their early descendants (Fig. 2). *EPF1* controls stomatal positioning through regulation of asymmetric cell division, whereas *EPF2* inhibits the formation of additional MMCs. Both *EPF1* and *EPF2* force the stomatal lineage stem cells to differentiate into pavement cells (Hara et al. 2007; Hara et al. 2009). EPF2 is a key molecule in the negative feedback loop that limits the number of stomatal lineage (Hara et al. 2009). *STOMAGEN* is expressed in mesophyll cells and their precursors, and acts on protodermal cells to increase the number of stomata (Hunt et al. 2010; Kondo et al. 2010; Sugano et al. 2010)

TMM and *ER* family genes

Several lines of genetic evidence have suggested that EPF1 and EPF2 are perceived by receptor complexes consisting of TOO MANY MOUTHS, ERECTA, and ERECTA-LIKE 1 and 2. *TMM* encodes a leucine-rich-repeat (LRR) containing receptor-like-protein (RLP). The predicted protein contains 10 uninterrupted plant-type LRRs and a putative COOH terminal, trans-membrane domain (Nadeau and Sack 2002a). TMM lacks a cytoplasmic kinase domain thus is similar to LRR-RLPs. The recessive *too many mouths* (*tmm*) mutation randomizes plane of asymmetric cell division and thus producing clustering of stomata. The expression of *TMM* is found in meristemoids, guard mother cells, stomata and neighbor cells (cells next to stomata, meristemoids and GMCs). This localization in neighbor cells is consistent with phenotypic data, suggesting that TMM function in an intercellular signaling pathway in which neighbor cells receives cue about the position of adjacent stoma or precursors. Later Hara et al. 2007 discovered this positional signaling molecule is the EPF1 peptide.

Arabidopsis ER and its functional paralogues *ERL1* and *ERL2* show synergistic interaction in promoting above-ground organ growth (Shapak et al. 2003, Shapak et al. 2004). These three genes encode LRR receptor-like kinases (RLKs). Complete loss of function of all three

ER-family genes, results in plant epidermis show clustered stomata. Together, the ER-family LRR-RLKs act as negative regulators of stomatal development. The expression of *ER*, *ERL1* and *ERL2* were observed in protodermal cells of leaf primordia. The expression of *ER* diminished to below detectable levels before epidermal cell differentiated. *ERL1* and *ERL2* showed strong expression in stomatal lineage cells (meristemoid, GMC, young guard cells). The expression pattern of *ER* family genes and *TMM* were similar. The complex genetic interaction of *TMM* and *ER*-family genes suggest that their products work in combination to determine stomatal lineage cells (Abrash et al. 2010; Abrash et al. 2011; Shapak et al. 2005). Overexpression of EPF1 and EPF2 decreases the number of stomata when overexpressed in wild-type Arabidopsis but not in the *tmm* and *er erl1 erl2* mutants (Hara et al. 2007; Hara et al. 2009). Similarly, application of stomagen increases the number of stomata of wild-type but not of the *tmm* and *spch* mutants (Kondo et al. 2010; Sugano et al. 2010).

Lee et al. 2012 performed binding experiments and suggested that both EPF1 and EPF2 bind to receptor kinases ERECTA (ER) and ER-LIKE 1 (ERL1), whereas EPF2 also binds to the receptor-like protein TOO MANY MOUTHS (TMM). They also identified that ER family proteins form receptor homomers and hetrodimers in vivo. On the other hand TMM associates with the ER family but not with itself. Their results suggested ER family as primary receptors for EPF1 and EPF2 and TMM works as signal modulator. They claimed that EPF2-ERECTA and EPF1-ERL1 as a ligand- receptor pairs specifying two steps of stomatal development: initiation and spacing divisions. Lee et al. 2012 did not included ERL2 and stomagen in their experiments.

MAP kinase cascade

Mitogen activated protein kinase (MAPK) cascades are three kinase signalling modules that are evolutionary conserved in eukaryotes (Ichimura et al. 2002). They play important

functions in regulating both stress responses and plant growth and development. MAP kinase signaling networks are positive regulators of environmental stress induced stomatal closure and negative regulator of stomatal development (Bergmann et al. 2004a, Wang et al. 2007, Neill et al. 2008).

The MAP kinase module consisting of YODA (YDA), MKK4/MKK5 and MPK3/MPK6 plays an important role in stomatal development, and YDA functions as a MAP kinase kinase kinase, MKK4/MKK5 are MAP kinase kinases, and MPK3/MPK6 are MAP kinases. MPK6 is a major MAP kinase functioning in stomatal-lineage precursors (Lampard et al. 2008). Several mutants with perturbed functions of components of this module are available, such as loss-of-function mutants of *YDA* (Bergmann et al. 2004), plants with decreased expression of *MKK4* and *MKK5* (Wang et al. 2007), and double mutants of *MPK3* and *MPK6* (Wang et al. 2007). In all these mutants, the number of stomata is increased, and stomata form clusters.

SPCH **is required for asymmetric cell division**

SPEECHLESS (SPCH) is a basic helix-loop-helix (bHLH) type transcriptional factor. The homozygous *spch* mutant plant lacks asymmetric cell divisions that generate GMC. *SPCH* is expressed in stomatal lineage cells (MMCs and meristemoids). The overexpression of *SPCH* induces extra cell divisions in cells that do not normally divide but without increase in stomatal density (Macalister et al. 2007). They also suggested that *spch-1* mutation is epistatic to any mutation that affects divisions late in the stomatal lineage. The *spch-1* mutation is epistatic to *yda*, *tmm* and *er* all three mutants, with respect to stomatal phenotype, suggesting that *SPCH* acts downstream of these genes. *SPCH* is necessary and sufficient for asymmetric cell division that establishes stomatal lineage (Macalister et al. 2007).

MPK3 and MPK6 phosphorylate and destabilize SPCH

The MAP kinase cascade implicated in stomatal development (*YDA, MKK4/MKK5* and *MPK3/MPK6*) are broadly expressed and involved in multiple activities. Lampard et al. 2008 showed that SPCH is a substrate of MPK3 and MPK6. The *spch* loss-of-function phenotype is strikingly similar to that caused by constitutive activation of MAPK pathway components *YDA, MKK4/MKK5*. They showed that SPCH but not its paralogs, MUTE and FAMA, could be phosphorylated by both MPK3 and MPK6. Overexpression of a constitutive-active form of *YDA* did not change transcription of *SPCH*, but rather limits the production or abundance of SPCH protein.

***SPCH*-paralogues genes control distinct steps of stomatal development**

SPCH has two paralogues genes *MUTE* and *FAMA*. The expression of *MUTE* was strongly observed in meristemoids and at lower levels in GMC and guard cells. Overexpression of *MUTE* converts entire leaf epidermis in to stomata. The loss of function of *MUTE* makes meristemoids abort after excessive asymmetric cell divisions and fail to differentiate stomata. MUTE is a key switch gene for meristemoid to acquire GMC identity.

FAMA (*FMA*) is named after the goddess of rumor. The expression of *FMA* is observed in guard mother cells and is necessary and sufficient to promote guard cell identity. *FAMA* regulates a critical switch between divisions and differentiations. Like *FLP/MYB88, FAMA* is required for halting divisions at the end of stomatal lineage, but in addition *FAMA* has instructive role in promoting guard cell fate (Ito et al. 2006). The expression of *MUTE* and *FAMA* is dependent on *SPCH* and results suggested that they work together to maintain stomatal lineage.

FOUR LIPS (FLP) and MYB88 are partially redundant R2R3 MYB type transcriptional factors, which are required for progression from meristemoids to guard cells. In *flp-1* mutants,

stomata often consist of three to four guard cells, as if the GMCs continued to divide (Yang and Sacks, 1995; Lai et al. 2005). *MYB88* has no mutant phenotype alone but enhances the proliferation defect in *flp-1* GMCs (Lai et al. 2005).

Three closely related basic-helix-loop-helix (bHLH) transcriptional factors SPCH, MUTE, FAMA are responsible for formation of MMC, GMC and guard cells respectively (Macalister et al. 2007, Pilliteri et al. 2007a). However it is not known what mechanisms coordinates their action. Kanaoka et al. 2008 identified two other bHLH proteins ICE1/SCREAM (SCRM) and SCREAM2 which function together with SPCH, MUTE and FAMA via the formation of hetrodimer and are required for identity of early stomatal lineage cells. *SCRM* is *INDUCER OF CBF EXPRESSION1*, a master regulator of freezing tolerance, thus implicating potential link between the transcriptional regulation of environmental adaptation and development in plants. The important genes involved in stomatal development pathway are shown in table 1.

Biological functions of other *EPF* family genes *EPFL4, EPF5* and *EPFL6*

Three members of the *EPF* gene family, *EPF1*, *EPF2* and *STOMAGEN*/*EPFL9*, have clear role in stomatal development. The fourth member of the family, *EPFL6/CHALLAH* (*CHAL*), however exhibits different behaviors than *EPF1*, *EPF2* and *STOMAGEN*. *CHAL* was initially identified in a screen for suppressors of *tmm* tissue specific phenotypes (Abrash et al. 2010). The other two genes *EPFL4* and *EPFL5* do not require *TMM* and also function more effectively in *tmm* mutants. These three genes require *ER* family genes to function. *EPFL4-6* (also known as *CHALLAH* family) forms a distinct subgroup of *EPFLs*. *CHALLAH* family members are highly expressed in internal tissues, especially in hypocotyls and stems, and appear to function as growth (but not normally as stomatal) regulators (Abrash et al. 2011, Uchida et al. 2012).

The *Arabidopsis ER* gene promotes inflorescence elongation: *er* plants exhibit characteristics, compact inflorescence with short internodes and pedicels. Uchida et al. 2012 clearly showed that proper inflorescence architecture in *Arabidopsis* can be specified by inter-tissue-layer communication between the phloem and endodermis mediated by ER and its signaling ligands EPFL4 and EPFL6.

A putative model for stomatal development

The genetic evidence suggested a plausible model: EPF1 and EPF2 are perceived by receptor complexes consisting of TOO MANY MOUTHS, ERECTA, and ERECTA-LIKE 1 and 2. These receptors activate a mitogen activated protein (MAP) kinase module. MAP kinases phosphorylate and destabilize the transcription factor SPCH, resulting in a decrease in the number of stomatal lineage cells. The stomagen peptide acts antagonistically to EPF1 and EPF2 (Fig. 3). However, there is no direct evidence that EPF1 and EPF2 activate or that stomagen inactivates the MAP kinase cascade, through which they might regulate the SPCH level.

Aim of this study: An important problem is that above-mentioned ligand-receptor-MAP kinase-SPCH signal transduction pathway has not been proven directly. I aimed to obtain biochemical evidence for whether EPF1, EPF2, and stomagen modulate the activity of the MAP kinase cascade and SPCH protein levels. I also aimed to know what receptor components are required to transduce the information of these three signaling peptides. Constitutive overexpression of *EPF1*, *EPF2* or *STOMAGEN* by making transgenic Arabidopsis plants would not be informative because it would affect the number of early stomatal lineage cells, which in turn secondarily change *SPCH* expression levels and MAP kinase activation levels. To circumvent this problem, I expressed SPCH and signaling

components in mature leaves of *Nicotiana benthamiana*, and examined their roles in regulating the SPCH level.

Results

Validation of the experimental approach

To validate the suitability of transient gene expression in *N. benthamiana* for my purpose, I first confirmed the expression of all *GFP* constructs (*EPF1, EPF2, TMM, ER, ERL1, ERL2* and *SPCH*) and *TMM-TdTomato* in mature *N. benthamiana* leaves by confocal microscopy (Fig. 5) and by Western blotting (for GFP constructs, EPF2-Myc and ERL2-HA; Fig. 6). Hereafter, I omit "GFP" in construct/protein descriptions. I detected GFP in nucleus, cell margin and cytoplasm (Fig. 5A), EPF1, EPF2 and stomagen in the apoplast (Fig. 5B–D), receptor-like components (TMM-TdTomato, TMM, ER, ERL1 and ERL2) at the cell margins (Fig. 5E–I, F′–I′), and the transcription factor SPCH in the nuclei (Fig. 5J, J′). Bands of expected molecular weight were detected for all GFP fusion proteins (Fig. 6), although cleavage products of fusion proteins were also detected.

I planned to use the levels of recombinant SPCH (as detected by Western blotting) as a read-out in my experiments. A drawback of the transient expression system is that transformation efficiency sometimes varies. To compare SPCH produced in different samples, I used normalization to phosphinothricin acetyltransferase (PAT), encoded by the same plasmid as SPCH (Fig. 7). Control experiments showed that PAT was not produced in *Agrobacterium tumefaciens,* but was only expressed in *Agrobacterium*-infiltrated *N. benthamiana* (Fig. 8).

EPF1, EPF2, STOMAGEN and *TMM* did not affect *SPCH* transcript levels

I used the constitutive CaMV 35S promoter to express all genes. As I studied SPCH protein degradation, it was important to ensure the absence of variability at the transcript level. Therefore, in parallel with immunoblotting analysis, I examined the effect of *EPF1, EPF2, STOMAGEN* and *TMM* on *SPCH* transcript levels. The *SPCH* transcript level was

unchanged by the expression of any one of them (Fig. 9).

The receptor components TMM, ER, ERL1 and ERL2 increase SPCH levels

Unexpectedly, expression of receptor components *TMM*, *ER*, *ERL1* and *ERL2* with *SPCH* increased SPCH protein levels (Fig. 10). It is possible that receptor components expressed without ligands interferes with endogenous MAP kinase pathway of *N. benthamiana.* It is also possible that the receptor components expressed without ligand acts as dominant negative for tobacco endogenous receptor and increased SPCH protein levels.

Stomagen increases SPCH protein levels

SPCH was co-transformed with *STOMAGEN* and one or two of *TMM*, *ER*, *ERL1*, *ERL2*; and SPCH protein levels were examined (Fig. 11 and Fig. 16-17). *STOMAGEN* alone increased the SPCH level, indicating that differentiated leaf cells of *N. benthamiana* have a machinery that can perceive stomagen. Co-expression of *TMM* and *STOMAGEN* further increased SPCH levels (Fig. 11). The mature bio active stomagen peptide was gifted by Kondo et al. 2010 and also synthesized as described by them. The purification and refolding (Fig. 12 and Fig. 13) of 45 amino acids stomagen was checked with control stomagen peptide. The stomagen peptide found bioactive when applied to *Arabidopsis thaliana* (Fig. 14). Then co-infiltration of the stomagen peptide, the mature form, was effective to increase SPCH-GFP signal intensity in *N. benthamiana* leaf epidermal cells (Fig. 15A). This increase in SPCH-GFP signal was confirmed by Western blotting, which show increased in SPCH protein levels (Fig. 15B). *STOMAGEN* did not further increase the SPCH level when co-transformed with *ER* or *ERL1* alone (Fig. 16A). However, *STOMAGEN* co-transformed with *ER* and *TMM* or with *ERL1* and *TMM* increased the level of SPCH (Fig. 16B). I next examined the effect of *ERL2* on *STOMAGEN*-mediated increase in SPCH levels.

STOMAGEN did not further increase the SPCH level when *TMM* and *ERL2* were co-transformed (Fig. 17). Taken together, a putative model is proposed in which TMM and ER or TMM and ERL1 work together to perceive stomagen but ERL2 do not have any role in this stomagen mediated increase in SPCH protein levels (Fig. 18).

EPF1 decreases SPCH protein level

I examined the effect of *EPF1* on the SPCH protein levels. *EPF1* expression with or without *TMM* decreased the SPCH protein level (Fig. 19), indicating that differentiated leaf cells of *N. benthamiana* possess a machinery to perceive not only stomagen, but also EPF1. However, *EPF1* co-expression with *ER* or *ERL1* did not change the SPCH level (Fig. 20A). I reasoned that, in the absence of overexpressed TMM, ER and ERL1 might either sequester EPF1 or inhibit the MAPK cascade, and thereby inhibit SPCH degradation. *EPF1* decreased the SPCH level in the presence of *ER* and *TMM*, *ERL1* and *TMM* (Fig. 20B), or *ERL2* and *TMM* (Fig. 21) combinations. These results provide evidence that either of ER, ERL1 and ERL2, in combination with TMM, can mediate the EPF1 signal. Taken together, a plausible model would be that TMM functions with ER, ERL1 and ERL2 to transduce EPF1 signal and decreased SPCH protein levels (Fig. 22).

EPF2* decreases the SPCH protein level only in the presence of *ERL1* (or *ERL2*) and *TMM

EPF2 alone or in the presence of *TMM* did not affect the SPCH protein level (Fig. 23). *EPF2* with either *ER* or *ERL1* or *ERL2* also had no effect on SPCH level (Fig. 24A, Fig. 26A and Fig. 27A). EPF2 with ER and TMM did not affect the SPCH level. SPCH level decreased when EPF2 was co-transformed with *ERL1* and *TMM* (Fig. 24B and Fig. 26B), or with *ERL2* and *TMM* (Fig. 25 and Fig. 27B). These results may reflect the ligand-receptor specificity.

EPF2-EGFP signals were not clearly detected in Western blotting but I see clear effect of peptide. Possibly undetectable amount of EPF2 was effective. Because EPF2-EGFP was not clearly detected in Western blotting so I again performed experiments with EPF2-Myc and obtained same results (Fig. 26 and Fig. 27). These results represent the ligand-receptor specificity. I proposed a putative mode in which EPF2 decreased SPCH protein levels only in the presence of TMM and ERL1 or TMM and ERL2 (Fig. 28).

EPF1 and *EPF2* activate a MAP kinase

SPCH can be a substrate of MPK3 and MPK6 (Lampard et al. 2008). I examined whether EPF1 or EPF2 affect MPK6 activation, which is a major MAP kinase functioning in stomatal-lineage precursors. I immunoprecipitated MPK6-FLAG with anti-FLAG antibody. Then I detected its active (phosphorylated) form by Western blotting with anti-phospho-specific ERK1/2 antibody and detected total MPK6 with anti-FLAG antibody. In the presence of *TMM*, *EPF1* (but not *EPF2* or *STOMAGEN*) increased the phosphorylated form of MPK6, suggesting that EPF1 activated MPK6 (Fig. 29).

Next, I examined the effect of *EPF1*, *EPF2* and *STOMAGEN* on MPK6 activation in the presence of *ER* and *TMM*, and found that only *EPF1* activated MPK6 (Fig. 30). This is consistent with the data that *EPF1* but not *EPF2* decreases the SPCH level in the presence of *ER* and *TMM* (Fig. 24B and Fig. 26B). I also examined the role of *ERL1*. Both *EPF1* and *EPF2* activated MAP kinase in the presence of *TMM* and *ERL1* (Fig. 31). This is consistent with the data that EPF2 decrease SPCH protein levels in the presence of *TMM* and *ERL1* (Fig. 25 and Fig. 27). *STOMAGEN* did not affect the activity of MPK6.

YDA and constitutive active form of *YDA* decreased SPCH protein levels

Constitutive active form of *YDA* (*CA-YDA*) does not prevent transcription of *SPCH*, but

rather limits the production or abundance of SPCH protein (Lampard et al 2008). I here also checked the effect of *YDA* and *CA-YDA* on SPCH protein level and found both decreased SPCH protein level (Fig. 32). The *CA-YDA* was more effective than *YDA*. My results suggested that *YDA* is also effective to decrease SPCH protein levels.

Approach for direct interaction of ligand-receptor pairs specifying stomatal patterning

EPF2 binds with TMM, ER and ERL1, and EPF1 binds with ER and ERL1, in pull-down assays of *N. benthamiana* extracts and in vitro using a sensor chip (Lee et al. 2012). For use in sensor chip experiments, they used recombinant EPF1 and EPF2 expressed in tobacco. The binding of EPF1, EPF2 and stomagen with ERL2 has not been tested yet. The binding of stomagen with TMM, ER and ERL1 also not reported yet. The determination of specific dissociation constant (K_d) of stomagen, EPF1 and EPF2 with putative receptor components TMM, ER, ERL1 and ERL2 will help, to identify detailed specific function of these receptor components. For this purpose the mature stomagen, EPF1 and EPF2 (thought to be mature peptide) with and without N-terminal FITC conjugated peptides were ordered to a company. I received stomagen, EPF2, FITC-stomagen, FITC-EPF1 peptides but company (Gen Script USA Inc) failed to synthesized EPF1 and FITC-EPF2. The purified stomagen labelled with non radioactive iodine was degraded (Fig. 33 and Fig. 34). The other purified and refolded peptides EPF2, FITC-stomagen, FITC-EPF1 were biologically inactive (data not shown). I used another approach to get biologically active recombinant proteins, in which *EPF1-EGFP*, *EPF2-EGFP* and *STOMAGEN-EGFP* were overexpressed in Tobacco BY2 cells (Fig. 35) but unfortunately proteins were biologically inactive. I also overexpressed *EPF1*, *EPF2* and *STOMAGEN* in bacterial cells and purified using His tag (Fig. 36) but unfortunately purified recombinant proteins were biologically inactive (data not shown). After using these approaches I failed to detect direct ligand-receptor binding.

Discussion

In last few years many secretory peptides were discovered and their importance in plant development and defence against pathogen has been reported. Stomata development is a complex process and regulated by defensin like cysteine-rich secretory EPIDERMAL PATTERNING FACTOR (EPF) peptides. EPF family contains 11 genes (Hara et al. 2009) and out of these only the known positive regulator is stomagen and negative regulators are EPF1 and EPF2 peptides for regulation of stomatal placement and density.

In this study, I reconstructed the signaling pathway of early stomatal lineage cells. First I observed the effect of receptor components on SPCH protein level and found each one increased SPCH protein level (Fig. 10). The possible explanation for this unexpected data is that *Arabidopsis* receptor components *TMM, ER, ERL1,* and *ERL2* act as dominant negative for Tobacco receptors. In normal case Tobacco endogenous machinery decrease SPCH protein level and expression of *Arabidopsis* receptor component disrupt this machinery by acting as dominant negative. It is also possible that *EPF1* orthologous gene present in Tobacco which decreases SPCH protein and expression of receptor components disrupt this pathway by making improper receptor combinations or disrupt MAP Kinase activity. Next I checked effect of secretory peptides one-by-one on SPCH protein level.

I have demonstrated that *STOMAGEN* increases the SPCH protein level, and that this effect is particularly clear when *STOMAGEN* is co-expressed with *TMM* and *ER* (or *ERL1*). This is consistent with the genetic evidence that the effect of stomagen depends on TMM (Kondo et al. 2010; Sugano et al. 2010) and SPCH (Sugano et al. 2010), and supports the idea that stomagen is perceived by receptor complexes containing TMM and ER family receptors, and increases the level of SPCH.

I have also found that *EPF1* decreases the SPCH level in the presence of *TMM*. Endogenous E*R*-family orthologs could form receptor complexes with TMM in differentiated

N. benthamiana cells. This is likely because, although *ER* is preferentially expressed in young shoot tissues, it is also expressed in mature *Arabidopsis* leaves (ArrayExpress accession number E-AFMX-9). Although *ER* or *ERL1* in the absence of *TMM* were not sufficient in mediating the function of *EPF1*, *EPF1* decreased the SPCH level in the presence of *TMM*.

Although *EPF2* was also able to decrease the SPCH level, the requirements for the *EPF2* effect were different from those of *EPF1*. *EPF2* decreased the SPCH level only in the presence of *TMM* and *ERL1* (or *ERL2*), whereas the *TMM* and *ER* combination was ineffective. In *Arabidopsis*, overexpression of *EPF2* under control of the constitutive CaMV 35*S* promoter inhibits formation of MMCs, whereas similarly overexpressed *EPF1* does not. However, EPF1 overexpression inhibits formation of meristemoids (Hara et al. 2009). My results indicate that EPF1 and EPF2 require different receptor components, and therefore may explain the differences in their effects. Pillitteri et al. (2011) examined gene expression profiles in the *scrm-D mute* double mutant, in which most epidermal cells are early stomatal lineage cells. I re-analyzed these data (Fig. 41). *EPF2* and *TMM* (which are expressed in early stomatal lineage cells) are highly expressed in this mutant. *ERL1* and *ERL2*, but not ER, were also up-regulated. This suggests that *ERL1* and *ERL2*, but not *ER*, are preferentially expressed in early stomatal lineage cells. The fact that *EPF2* acts at an earlier stage than *EPF1* (Hara et al. 2009), and the EPF2 preference for ERL1 and ERL2 (this study), are in accordance with the specific expression of *ERL1* and *ERL2* in early stomatal lineage cells. Although EPF1 and EPF2 can bind to both ER and ERL1 (Lee et al. 2012), they may differ in the mechanisms of receptor activation.

Lee et al. (2012) concluded that ER, but not ERL1, is critical for the function of EPF2 because a loss-of-function mutant expressing a dominant-negative form of *ER* has an increased number of stomata, similar to the *epf2* mutant. This is in contrast to an earlier report that the *erl1* and *erl2* mutants, but not the *er* mutant, have increased numbers of stomata

(Shpak et al. 2005). Our results are consistent with the data of Shpak et al. (2005). Careful examination of the effects of *EPF2* overexpression in plants carrying mutations in one or two of the *ER*-family receptors and *STOMAGEN* would clarify this discrepancy.

MAP kinases MPK3 and MPK6 can phosphorylate and destabilize SPCH (Lampard et al. 2008). Several lines of genetic evidence have suggested that EPF1 and EPF2 signal through a MAPK cascade, in which the MAP kinases MPK3 and MPK6 play important roles. Here I provide direct evidence that EPF1 activates MPK6, but I was unable to detect *EPF2*-mediated increase in MPK6 phosphorylation in the presence of *TMM* and *ER* (Fig. 30). The effect of *EPF2* on the SPCH level was clear only in the presence of *TMM* and *ERL1* but not in the presence of *TMM* and *ER*. This is consistent with the result that EPF2 activates MPK6 in the presence of both TMM and ERL1 (Fig. 31). Although I need to further investigate how MAP kinases are regulated by different combinations of ligand/receptor components, my current study provides evidence that peptide mediators regulate SPCH levels through a MAP kinase cascade, thus regulating cell identity.

The MAP kinases, MPK3 and MPK6 can phsphorylate SPCH but not other two closely related bHLH transcriptional factors MUTE and FAMA and destabilized it (Lampard et al. 2008). Because of this I only checked change in SPCH protein levels with respect to MAP kinase. Lampard et al. 2008 also reported that *CA-YDA* did not change transcription but degraded SPCH protein in *A. thaliana*. I here found that *YDA* and *CA-YDA* decrease SPCH protein in *N. benthamiana.*

My results suggest a putative model for stomagen signal transduction in which stomagen increased SPCH protein levels and their by regulates specific cell fates (Fig. 30). Most probably the stomagen peptide is perceived by TMM+ER or TMM+ERL1 receptor components on protodermal cells, MMCs and meristemoids and increase SPCH protein levels their by positively regulates stomatal development (Fig. 37). The stomagen peptide cannot act

through TMM and ERL2 to increase SPCH protein level. The stomagen peptide mediated change in cell fate is not possible to determine in Tobacco because differentiated leaf cells were used for experiments.

Results suggest a putative model for EPF1 signal transduction in which EPF1 activates MAP kinase and decreased SPCH protein levels (Fig. 38). EPF1 requires TMM+ER, TMM+ERL1 and TMM+ERL2 to act on meristemoid cells and decrease SPCH protein level, their by stop meristemoids to further enter in to stomatal lineage pathway. The overexpression of *EPF1* decreased stomatal density and *epf1* mutant plants show increased stomatal density with stomatal clusters. This decrease in SPCH protein levels cannot regulate plane of asymmetric cell division because SPCH is a transcriptional factor and transcriptional factor could not regulate plane of division. EPF1 may regulate stomatal positioning by using unknown factor.

EPF2 requires specific receptor components to decrease SPCH protein level. In a putative model, EPF2 needs TMM+ERL1 and TMM+ERL2 to act on protodermal cells and MMCs to decrease SPCH protein levels (Fig. 39). Most probably EPF2 mediated decrease in SPCH protein levels affect cell fate of protodermal cell and MMCs, results in convert them to pavement cells.

Taken all results together, a complex putative model is hypothesized in which *EPF1* and *EPF2* decreased whereas *STOMAGEN* increased SPCH protein levels through regulation of MAP kinase cascade activity and their by regulate specific cell fates in *Arabidopsis* (Fig. 40). In this model most probably stomagen competes with EPF2 at TMM+ERL1 receptor component, on protodermal cells and MMCs. The binding of peptides decides whether protodermal cells and MMCs enter in stomatal lineage pathway or form pavement cells. The stomagen peptide also competes with EPF1 at TMM+ER and TMM+ERL1, outside of meristemoids. This competition at receptor components decide whether stomatal lineage

pathway continue or form pavement cells. In simple words stomagen and EPF2 cannot compete at TMM+ERL2 and TMM+ER receptor combinations. I got one inconsistent result that stomagen cannot increase SPCH by TMM and ERL2 receptor component because stomagen could not compete with EPF1 and EPF2 at this point. The loss-of-function phenotype of *STOMAGEN* by gene silencing using artificial micro RNA had reduced stomatal density (Sugano et al. 2010). The decrease in stomatal density is not severe like MAP Kinase cascade mutants; so it is also possible that some unknown positive regulator compete with EPF1 and EPF2 at this point. However the function of the stomagen peptide, should be checked by peptide treatment in *er erl1* double mutant.

The binding experiments by Lee et al. 2012 did not included ERL2 and stomagen. My tentative model also included ERL2 but Lee et al. 2012 reported that ERL2 have negligible function in stomata development. All the binding experiments of EPF1 and EPF2 were performed individually with TMM, ER and ERL1. These experiments were not performed in receptor pair combination. I used here only TMM or TMM with ER family proteins to check effect of ligands on SPCH protein level. The binding experiment using receptor pair combination will solve this puzzle in future, e.g. EPF1 binds with ER and ERL1 but does not physically associate with TMM. *EPF1* needs *TMM* to decrease stomatal density in *Arabidopsis*. The binding of EPF1 with, TMM and ER family receptor kinases will identify whether TMM and ER family proteins physically associate in presence of ligand or not. It might be possible EPF1 binds with TMM only in the presence of ER family. This future perspective will identify independent function of ER family.

Genetic evidence suggested that EPF1, EPF2 and stomagen compete for receptor like protein TMM. The binding experiments did not included competition assay and in future competition assay need to be tested to identify binding site/s. EPF1, EPF2 and the stomagen peptide binding with TMM and ER family receptor components pairs, including ERL2 will

help to solve, how peptides mediated cell fate is specified during stomatal development pathway. I showed clearly that ERL2 has function to modulate SPCH protein levels, so it should also have function in stomatal development pathway. To determine the specific binding preference of EPFs will allow identifying specific receptors.

Future perspectives and significance of this study

How EPF1 and EPF2 peptides degrade SPCH and which phosphorylation sites are important for SPCH degradation. To check effect of EPF1 and EPF2 in SPCH variants, in which phosphorylation sites are disrupted, will identify target sites. Identification of EPF1, EPF2 and stomagen are in same or different pathway will increase understanding of stomatal pathway. EPF1 and EPF2 activate MAP kinase (MPK6), which results in degradation of SPCH protein.

The *epf1* and *epf2* mutants increased stomatal density but the increase is less compared to *yda*, *mpk3;mpk6* double mutants. These results suggest that other negative regulators might be present in Arabidopsis.

The point of action of stomagen is not known and identification of it will help to understand this pathway. I believe stomagen acts on protodermal cell, MMC and meristemoid cell and interact with EPF1 and EPF2 pathways. The production of stomagen may be dependent on environmental factors such as light, CO_2, stress etc.

Stomatal density and placement are tightly controlled through the cell-cell signaling. It is revealed that EPF family peptides and ERs-TMM are major ligands and receptors for the signaling, and that SPCH is a master transcription factor for the entry of stomatal linage cells. This is first direct evidence that EPFs signals control the SPCH protein level through the specific receptors and a MAPK cascade. MAP kinase mediated regulation is a common mechanism for regulation of other developmental programs in plants and animals. Similar

kind of signal transduction mechanism may be involved in regulation of various biological processes in eukaryotes. Different ligands using common receptors and MAPK cascade eliciting different responses. This kind of mechanism might be conserved during evolution in plants and animals. My study suggests that similar kind of developmental regulation in plants and animals happens.

The positional information received through specific ligand–receptor and conserved downstream molecule for the orientation of asymmetric cell division is a common theme in development. In animals including invertebrates (*Caenorhabditis elegans* and *Drosophila melanogaster*) and vertebrate (including humans) well known positional cues for asymmetric cell division is Wnt-Frizzled family of signals and receptors (Goldstein et al. 2006). The downstream molecules are conserved proteins for establishment of cell polarity. The identification of EPF1 mediated regulation of cell polarity factor will be an important future perspective. My results suggest that EPF1 functions through TMM and ER family proteins and activated MAP Kinase cascade. The activated MAP Kinase (MPK6) degrades SPCH protein which might enforce pavement cell fate after asymmetric cell division occurs or decrease cell division frequency of cells that contact stomata or precursors. I believe so because SPCH is required for establishment of stomatal cell lineage pathway. This is a good example in which EPF1 controls two distinct things, including cell fate and cell polarity.

Stomatal development shares a common theme with other developmental programs in which it must communicates and coordinate to adapt different cell fates. Epidermal cells communicate to each other, through secretory peptides and environmental signals and regulate spacing and number of stomata. The spatial and temporal activities of the peptides coordinate with environmental signals and decide specific cell fate and number. The peptides are acting in competitive fashion and their binding efficiency to a particular receptor

component mediate specific response. The challenges that remain are to identify how these secretory peptides balance specify cell fates.

Materials and Methods

Plant materials and growth conditions

Nicotiana benthamiana seeds were germinated on plates with GM medium (MS salts, 1% sucrose, 1/100 volume of 2.5% MES-KOH, pH 5.7, 0.3% Phytagel) under continuous light at 22°C. Twelve-day-old plants were transferred to vermiculite supplemented with half-strength MS. Fully expanded leaves of one-month-old plants were used for infiltration.

Plasmids

Genomic sequences of genes were amplified by PCR with primers listed in Table 2. *SPCH* was cloned into the pKH1 vector, which carries *PAT* under control of the nopaline synthase (nos) promoter. All other genes were cloned in vectors carrying genes for kanamycin or hygromycin resistance. All genes of interest were under control of the duplicated CaMV 35*S* promoter.

Transient gene expression in *Nicotiana. benthamiana* leaves

Agrobacterium tumefaciens cells (strain GV3101 MP90) carrying expression constructs were grown in YEP medium with appropriate antibiotics. Bacterial cells were harvested by centrifugation at 2000 x g for 10 min at room temperature. The pellets were re-suspended in the infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, 150 µM acetosyringone). Culture densities were adjusted to an OD_{600} of 1.0, and the cells were incubated at room temperature for 4–5 h prior to infiltration. Equal volumes of cultures carrying different constructs were mixed. To enhance expression, a culture of *A. tumefaciens* cells carrying a suppressor of gene silencing p19 was added (at OD_{600} of 1.0) to the above mixture at a 1:1 ratio. The resulting mixture was infiltrated into fully expanded leaves of *N. benthamiana* (Fig. 4). Plants were kept as described above and analyzed 72 h after infiltration. The same leaves

were used for RNA purification and protein extraction.

Synthesis, Purification and refolding of synthetic Stomagen

The stomagen peptide was synthesized as described by (Kondo et al. 2010) or purchased from GenScript USA Inc (NJ, U.S.A.). It was purified and refolded according to either of the two published protocols (Katayama et al. 2001; Sugano et al. 2010). 100 nM stomagen was infiltrated into *N. benthamiana* together with *A. tumefaciens* cells.

Confocal Microscopy

Images were acquired with Zeiss LSM710 confocal microscope.

Isolation of total RNA and reverse transcription-PCR analysis

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA quality was determined spectrophotometrically ($A_{260}/A_{280} > 1.8$). RNA was treated with RNase-free DNaseI (Invitrogen) according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed in a 20 µl reaction mixture by using SuperScript II (Invitrogen). After the reaction, 10 µl of the reaction mixture was diluted with 50 µl of water. PCR primers are listed in Table 3. *PAT* was used as control.

Protein extraction and immunoblot analysis

Protein extraction was performed according to Betsuyaku et al. (2011) with some modifications. Agro-infiltrated leaves of *N. benthamiana* were ground to a fine powder in liquid nitrogen. Equal amounts of powder were homogenized in two volumes (w/v) of the extraction buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20% glycerol, 1% NP-40, 10 mM DTT, 1 mM EDTA and 1:100 of protease inhibitor (Nacalai tesque, Japan)). The

samples were centrifuged at 16000 x g for 20 min at 4°C, and supernatants used for immunoblotting. Proteins were separated by SDS-PAGE subsequently electroblotted onto a nitrocellulose membrane (GE Healthcare), which was blocked with 5% (w/v) skimmed milk in TBST buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-20) for 1 h. After a brief wash with TBST, the membrane was incubated with anti-GFP antibody overnight at 4°C.

The membrane was washed three times with TBST and further incubated with the appropriate secondary antibody for 3 h at room temperature, followed by three washes with TBST. We used primary antibodies anti-GFP (1:2000 dilution) (11814460001, Roche), anti-Myc (1:2000 dilution) (631206, Clontech), anti-HA (1:5000 dilution) (11867423001, Roche) and anti-PAT(1:10000 dilution) (P0249, Sigma), and secondary antibodies anti-rabbit (1:10000 dilution) (NA934VS, GE Healthcare), anti-mouse (1:10000 dilution) (NA931VS, GE Healthcare) and anti-rat (1:10000 dilution) (NA935V, Amersham Biosciences). Immunodetection was performed by using a chemiluminescence assay kit (299-69913, Wako).

Immunoprecipitation and MAP kinase phosphorylation assay

Total protein was extracted from the infiltrated *N. benthamiana* leaves with 2 volumes (w/v) of the IP extraction buffer containing 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1:100 protease inhibitor cocktail (Nacalai Tesque). The homogenate was centrifuged at 16000 x g for 20 min at 4°C. The supernatant 1ml was incubated with 10μL mouse anti-FLAG antibody (F3165, Sigma) at 4°C for 4 h on a rotary shaker. Then 20 μL of Protein G Dynabeads (100.04D, Invitrogen) was added, and samples were incubated on a rotary shaker at 4°C for 2 h. The beads were collected and washed five

times with the washing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl). The immunoprecipitates were eluted with a 2× SDS sample buffer by heating at 97°C for 5 min, and immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting (performed as described above) with the anti-FLAG antibody and a rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (9101, Cell Signaling Technology, Danvers, MA).

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Zhu, Y; Wang, Y; Li, R; Song, X; Wang,Q; Huang, S; et al . (2009) Analysis of interaction among the CLAVATA3 receptors reveals a direct interaction between CLAVATA2 and CORYNE in Arabidopsis. Plant J. 61: 223-233.

Table 1. Genes involved in *Arabidopsis* stomatal development in rosette leaves

Gene name	**Symbol**	**Molecular homology**	**Mutant phenotype**	**Overexpression/ Constitutive active phenotype**
EPIDERMAL PATTERNING FACTOR1	*EPF1*	Secretory peptide	Stomatal cluster, increased SD	Decrease in stomatal density
EPIDERMAL PATTERNING FACTOR2	*EPF2*	Secretory peptide	Increased SD, increased nonstomatal cells	Decrease in stomatal density
STOMAGEN	*STOMAGEN*	Secretory peptide	Mutant not reported but RNA interference (RNAi) lines decreased SD	Increase in stomatal density, and non stomatal cell density not reported
STOMATAL DENSITY AND DISTRIBUTION 1	*SDD1*	Subtilisin-like protease	Increased SI, small clusters	Repress stomatal divisions, Arrested meristemoids and GMCs
TOO MANY MOUTHS	*TMM*	Leucine-rich repeat receptor-like protein	Increased SI, stomatal cluster	ND
ERECTA-family	*ERECTA* (*ER*), *ERECTA LIKE 1*(*ERL1*), *ERECTA LIKE 2*(*ERL2*)	Leucine-rich repeat receptor-like kinases	Triple mutant show greatly increased SI, large clusters	*ER*–no phenotype, *ERL1* and *ERL2* -ND
YODA	*YDA*	Mitogen-activated protein kinase kinase	Greatly increased SI, large clusters	No stomata, pavement cells only

		kinase		
MAPKK4/ MAPKK5	*MKK4/ MKK5*	Mitogen-activated protein kinase kinase	Entire epidermis converted to stomata	No stomata, pavement cells only
MAPK3/MAPK6	*MPK3/MPK6*	Mitogen-activated protein kinase	Entire epidermis converted to stomata	No stomata, pavement cells only
SPEECHLESS	*SPCH*	Basic-helix-loop-Helix (bHLH) protein	No initiation of asymmetric cell division in the epidermis-no stomata	Excessive epidermal divisions, no extra stomata
MUTE	*MUTE*	bHLH protein	Initiation and reiteration of asymmetric cell division in the epidermis-no stomata	Entire epidermis converted to stomata
FAMA	*FAMA*	bHLH protein	Reiterative divisions of GMC-no stomata	Entire epidermis converted to single guard cell
FOUR LIPS	*FLP*	R2R3 MYB protein	Reiterative divisions of the GMC, small clusters	ND
MYB88	*MYB88*	R2R3 MYB protein	None, enhances *flp* phenotype	ND

SD, Stomatal density (number of stomata/mm^2); SI, Stomatal index (number of stomata/total cell number); ND, No data

Table 2. Primers used for cloning in this study

Target gene	Primer Name	Restriction enzyme	Sequence (5'->3')
EGFP	1091-F	Sma1	tcccccgggaaaATGGTGAGCAAGGGCGAG GAGCTGTTCAC
	5607-R	Pac1	ccttaattaaGTTACTTGTACAGCTCGTCC
EPF1	5672-F	Xho1	ccgctcgagaaaATGAAGTCTCTTCTTCTCC TTGCCT
	5673-R	Sma1	tcccccgggAGGGACAGGGTAGGACTTAT TGTTG
EPF2	5674-F	Xho1	ccgctcgagaaaATGACGAAGTTTGTACGC AAGTATATG
	5675-R	Sma1	tcccccgggAGCTCTAGATGGCACGTGAT AGTAT
STOMAGEN	5676-F	Xho1	ccgctcgagaaaATGAAGCATGAAATGATG AAC
	5677-R	Sma1	tcccccgggTCTATGACAAACACATCTATA ATGATAAG
TMM	6411-F	Asc1	ttggcgcgccATGGCACGATATGAATTCTTC CGCCA
	6483-R	Asc1	ggcgcgccacgacgacgaaaaacaaaACTAGATAT TAGCAT
ER	5682-F	Asc1	ttggcgcgccaaaATGGCTCTGTTTAGAGAT ATTGTTC
	5683-R	Sma1	tcccccgggCTCACTGTTCTGAGAAATAA CTTGTCC
ERL1	5738-F	Asc1	ttggcgcgccaaaATGAAGGAGAAGATGCA GCG
	5739-R	Sma1	tcccccgggTATGCTACTTTTGGAGATGAC
ERL2	5762-F	Xba1	tgctctagaaaaATGAGAAGGATAGAGACCA TGAAAG
	5763-R	Asc1	ggcgcgccTAAGCTACTTTTGGAGATATC TT
MPK6	5743-F	Asc1	tttggcgcgccaaaATGGACGGTGGTTCAGG TCAA
	5744-R	Asc1	tttggcgcgccTTGCTGATATTCTGGATTGA

			AAGCAAG
MPK3	5745-F	Xba1	ttttctagaaaaATGAACACCGGCGGTGGCCAATA
	5746-R	Asc1	tttggcgcgccACCGTATGTTGGATTGAGT
Td Tomato	6594	Sma1	tcccccgggATGGTGAGCAAGGGCGA
	6595	Pac1	ttaattaaCTTGTACAGCTCGTCCATGCCGTA
10X Myc	5700-F	Sma1	tcccccgggTCCTTGTACAAAGTGGTTGATAACAGC
	5701-R	Kpn1	ggggtaccATCGGGGAAATTCGAGCTCTAAGC
CLV3	9065	Xba1	gctctagaATGGATTCGAAGAGTTTTCTGCTACTACTA
	9066	Asc1	ggcgcgcccgccgctgcAGGGAGCTGAAAGTTGTTTC
WUS	7089	Xho1	ccgctcgagaaaATGGAGCCGCCACAGCAT
	7090	Asc1	ttggcgcgccGTTCAGACGTAGCTCAAGAG
SPCH	6206-F	Xba1	tgctctagaATGCAGGAGATAATACCGGATT
	6249-R	Asc1	tttggcgcgcctcctcctccGCAGAATGTTTGCTG

Table 3. The primers used for transcript amplification in RT-PCR

Gene	**Forward primer Sequence (5'->3')**	**Reverse primer Sequence (5'->3')**
SPCH	GAACACCTCAGCCAACAAGC	CGGGTCTTGTAGTTGCCGTC
PAT	ATCTACCATGAGCCCAGAACGACG	ATCAGATCTCGGTGACGGGCAGGA

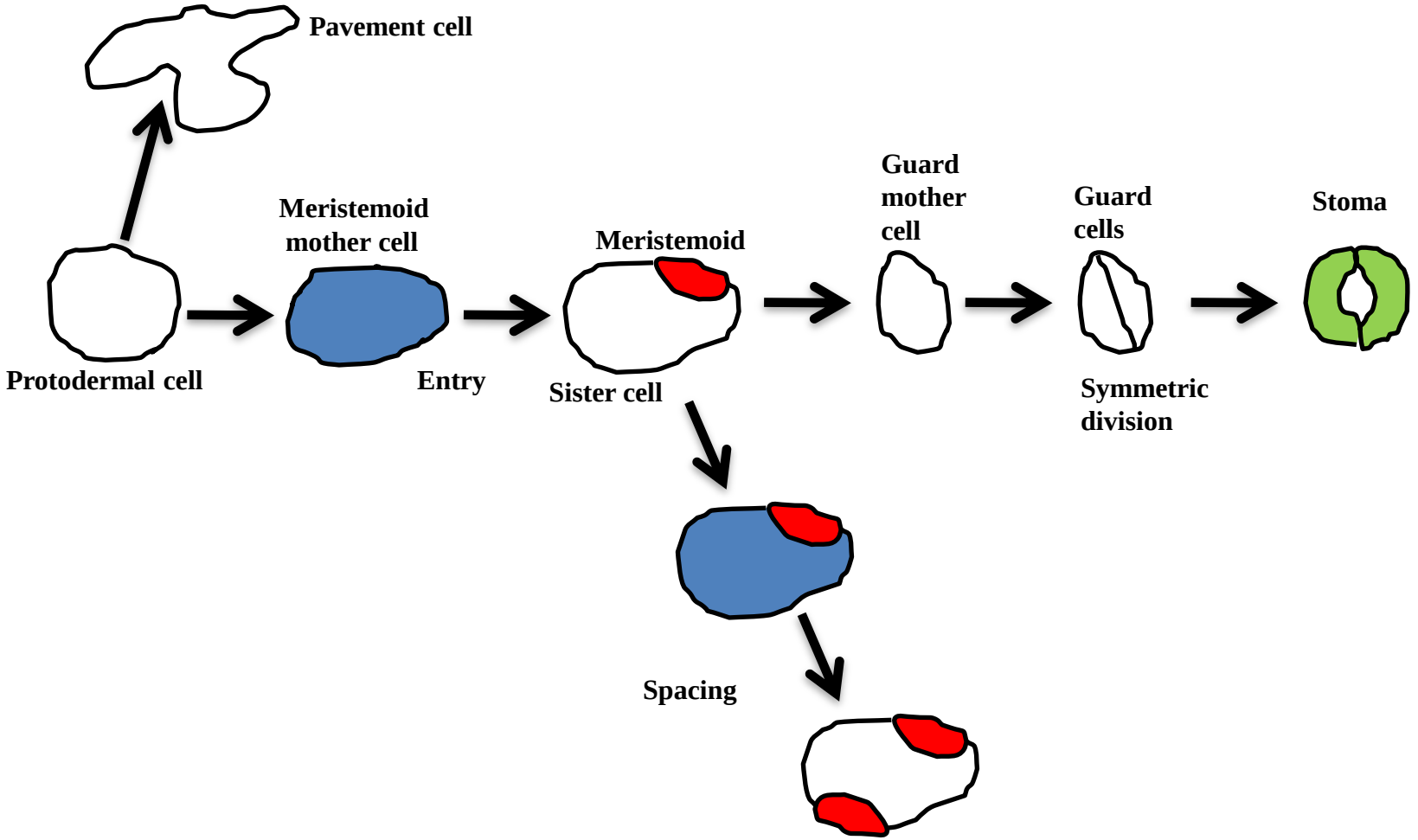

Fig. 1 Stomatal development pathway. During leaf development some of the protodermal cells convert directly in to meristemoid mother cell (MMC). MMC divides asymmetrically and produces a small triangular shaped cell, known as meristemoid and its sister cell. The meristemoid eventually becomes the oval shaped guard mother cell (GMC), which then divides symmetrically to form paired guard cells and finally converted in to a stoma.

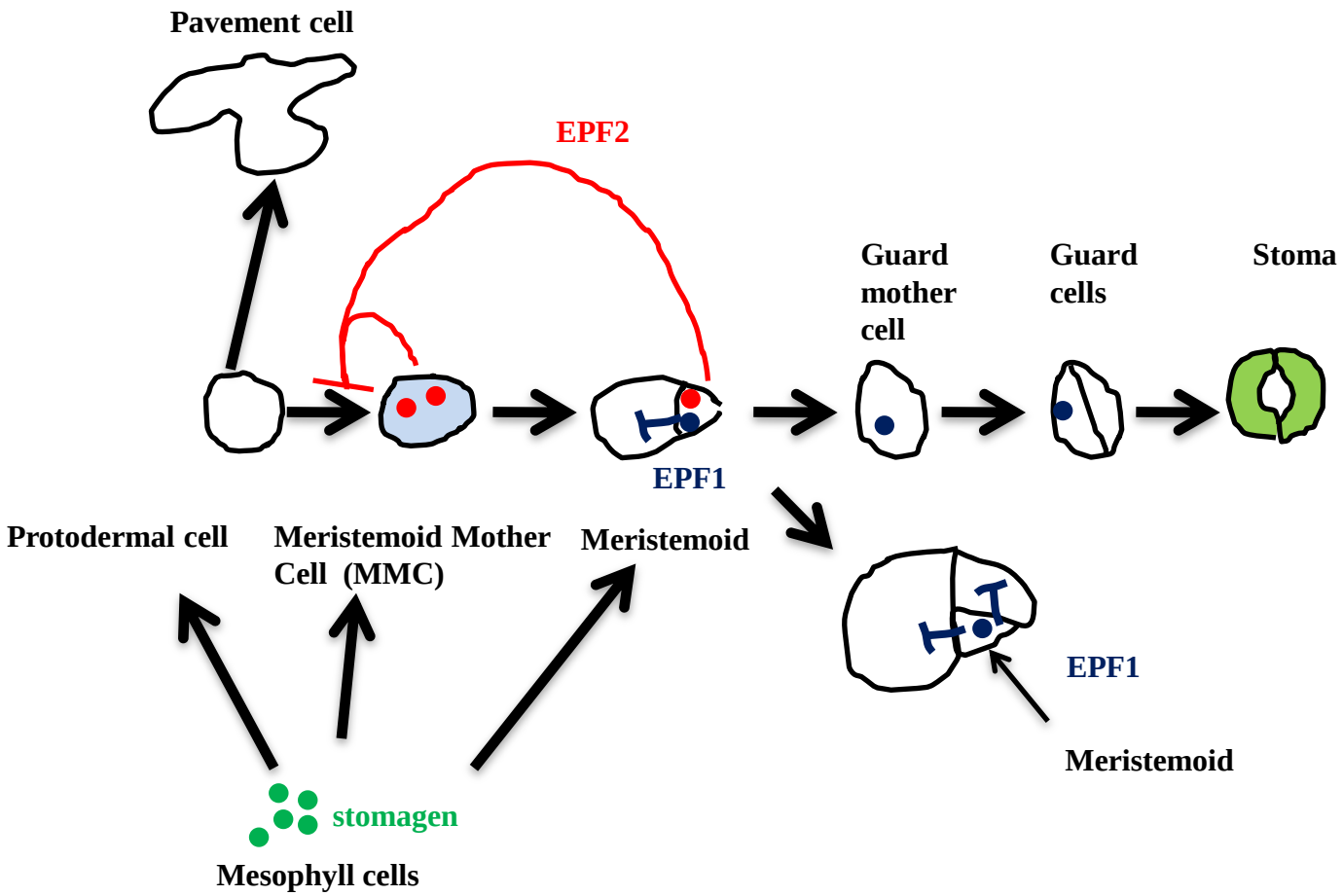

Fig. 2 *EPF1*, *EPF2* and *STOMAGEN* are expressed at different places and control stomatal development pathway. *EPF1* expressed in stomatal cells and their precursors and controls stomatal patterning through regulation of asymmetric cell division. *EPF2* expressed in MMC and protodermal cells, and inhibits additional formation of MMC. *STOMAGEN* expressed in mesophyll cells and positively controls stomatal density in *Arabidopsis*.

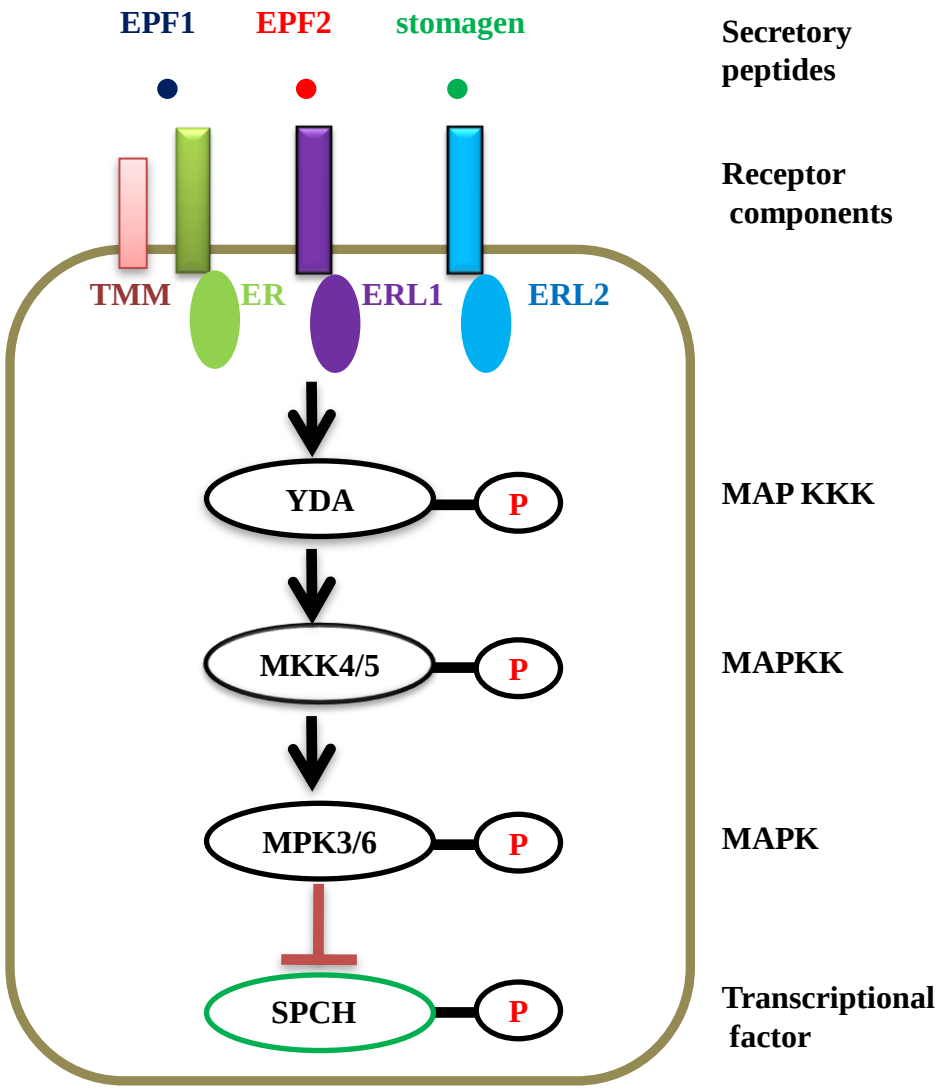

Fig. 3 A putative model for genes involved in stomatal development pathway. The extracellular signaling peptides EPF1 and EPF2 are perceived by putative receptor complexes that contain TMM and ER family protein, which in turn activates the MAPK cascade, phsphorylate SPCH, and inhibit stomatal formation. The stomagen peptide acts antagonistically to EPF1 and EPF2.

Fig. 4 Transient gene expression system in *Nicotiana benthamiana*. Leaves (length 4~5 cm) were infiltrated by 1mL needleless syringe and collected after 3days of infiltration.

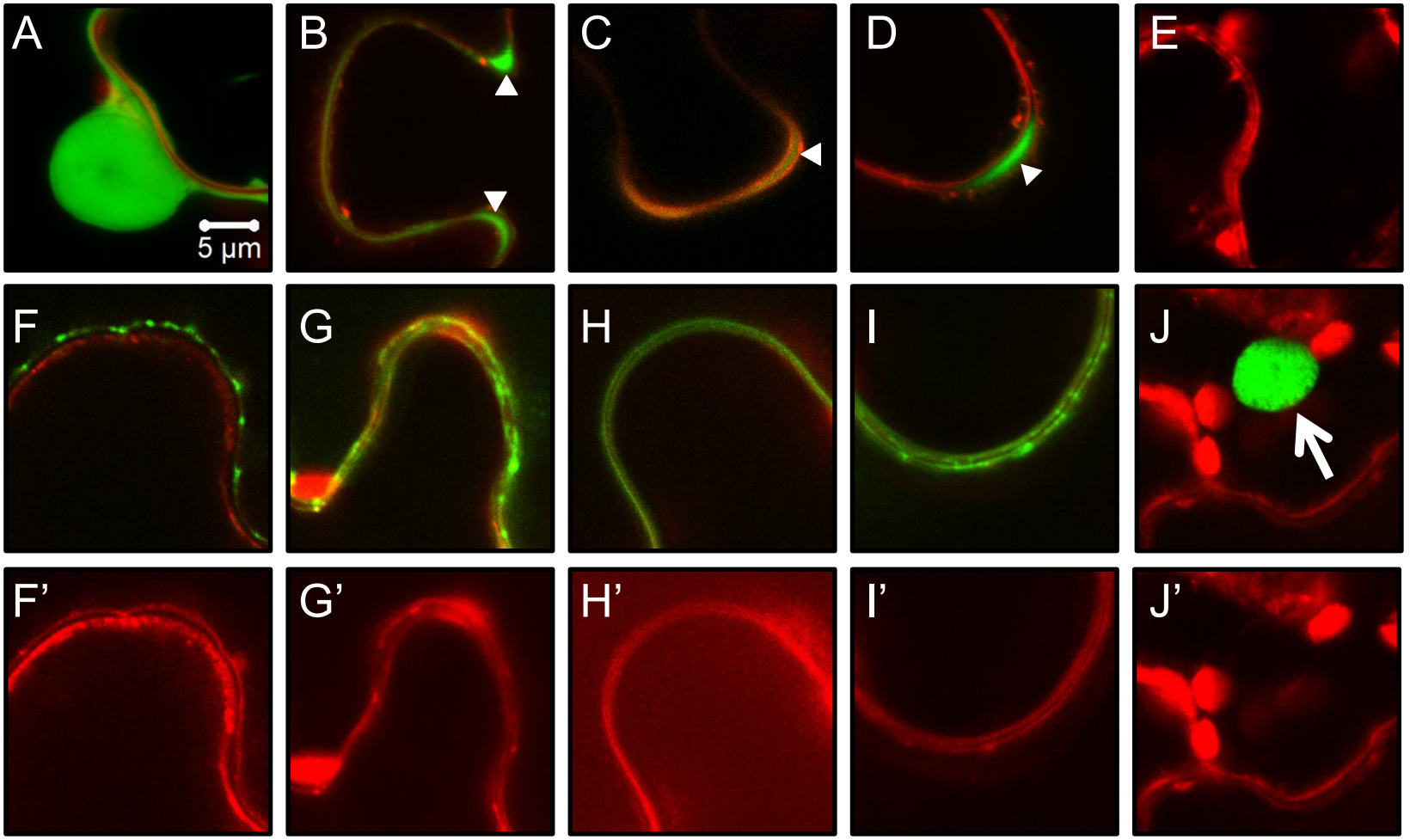

Fig. 5 Confocal microscopy of GFP fusion proteins and TMM-TdTomato expressed in *Nicotiana benthamiana* leaf epidermal cells. GFP alone (A) or fused with EPF1 (B), EPF2 (C), stomagen (D), TMM (F, F′), ER (G, G′), ERL1 (H, H′), ERL2 (I, I′) or SPCH (J, J'), and TMM-TdTomato (E). (F-J) Merged images of GFP and FM 4-64 channels. (F'-J') FM4-64 channel. GFP is present in the nucleus and cytoplasm. EPF1, EPF2 and stomagen are apoplastic (white arrowheads indicate apoplastic region). TMM, ER, ERL1 and ERL2 are localized to the cell membrane. TMM localization is shown on cell membrane of single cell. SPCH is detectable only in the nucleus (indicated by arrow). FM4-64 staining is shown in all panels except TMM-TdTomato. The red oval shaped structures are plastids. Scale bar, 5µm.

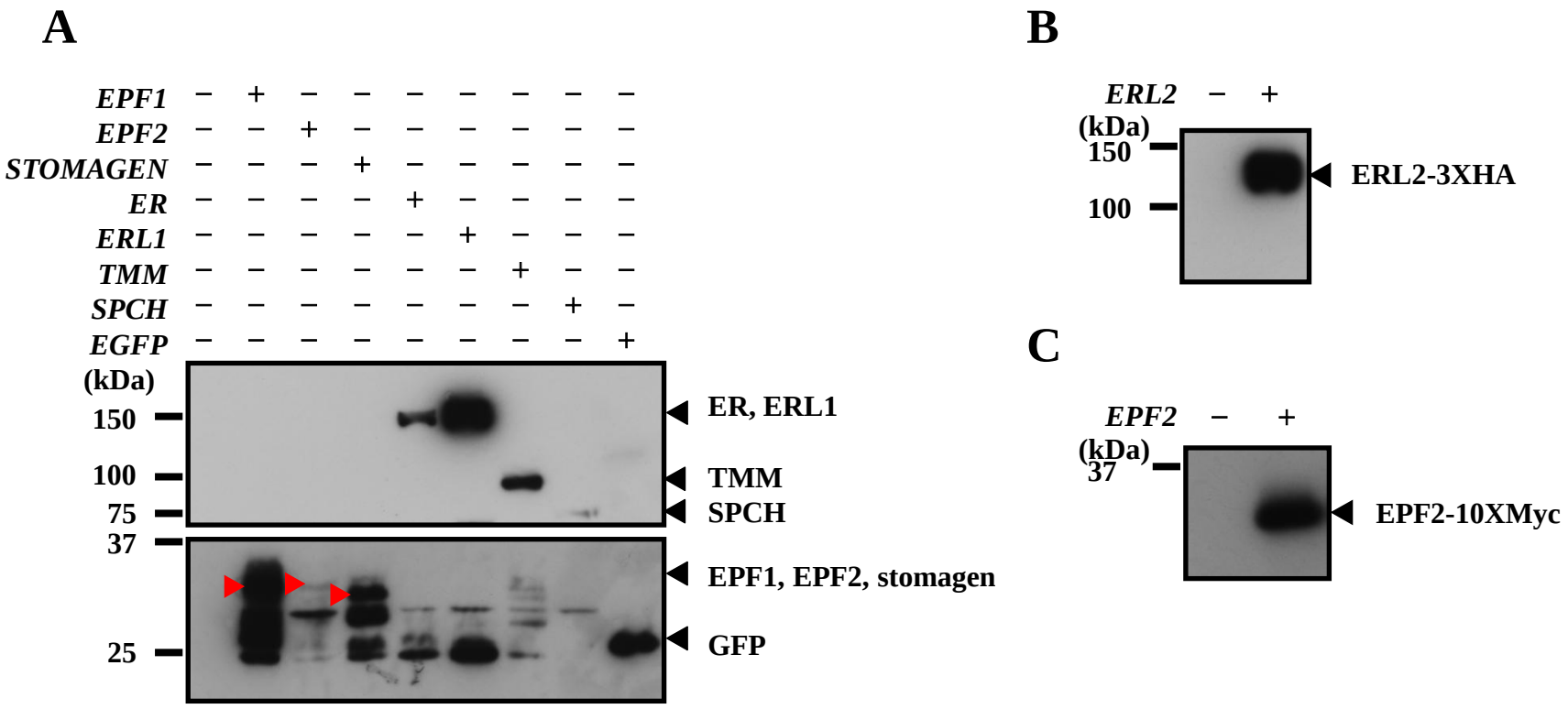

Fig. 6 Western blot analysis of GFP, GFP fusion proteins, ERL2-HA and EPF2 Myc protein. (A) Western blot analysis of EPF1, EPF2, stomagen, ER, ERL1, TMM and SPCH GFP-fusion proteins, and GFP alone, produced in *N. benthamiana* leaf cells. Proteins were detected with the anti-GFP antibody. Positions corresponding to predicted molecular weight of EPF1-GFP, EPF2-GFP and stomagen-GFP are shown with red arrowheads (description of GFP in fusion proteins is omitted in the figure). (B) Western blot analysis of ERL2-HA protein. The ERL2-3XHA protein was detected with anti-HA antibody. (C) Western blot analysis of EPF2-Myc protein. The EPF2-10XMyc protein was detected with anti-Myc antibody. Positions of MW markers are indicated on the left.

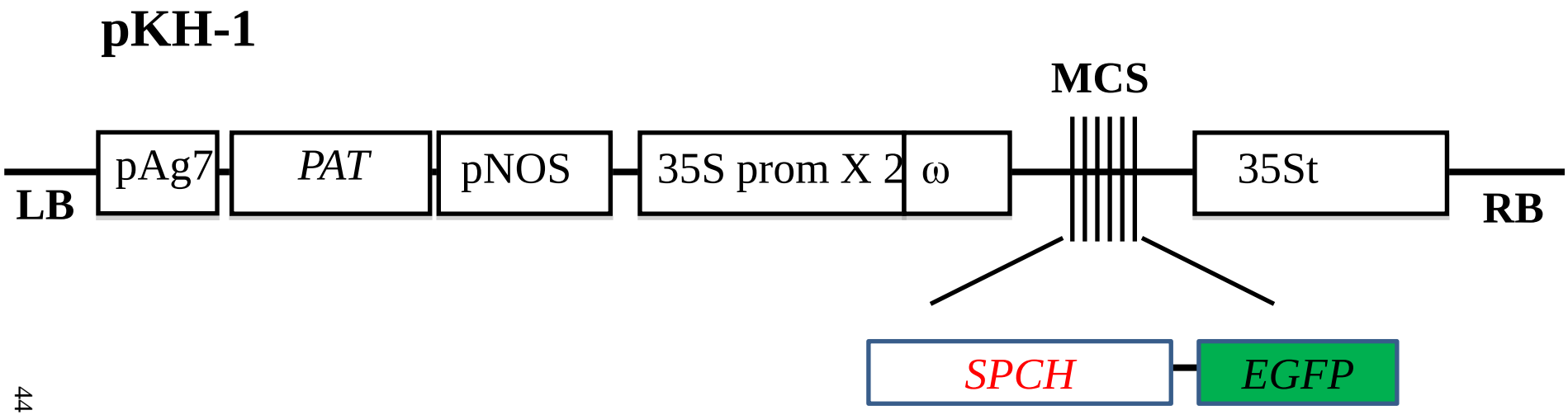

Fig. 7 *SPCH-EGFP* was cloned phosphinothricin resistant pKH1binary vector.

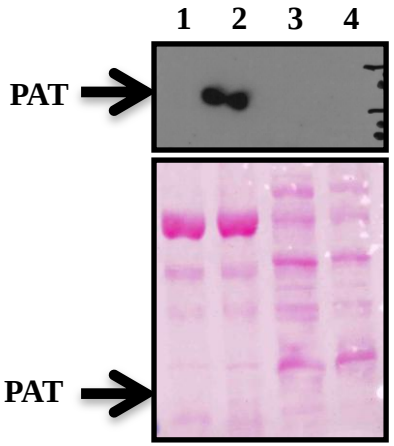

Fig. 8 NOS promoter-*PAT* is expressed in *N. benthamiana* but not in *Agrobacterium tumefaciens*. Lane 1, Extract of *N. benthamiana* leaf without transformed with pKH1-SPCH-GFP. Lane 2, Extract of *N. benthamiana* leaf transformed with pKH1-SPCH-GFP. Lane 3, Extract of *A. tumefaciens* without pKH1. Lane 4, Extract of *A. tumefaciens* carrying pKH1-SPCH-GFP. Top panel, Western blotting for PAT. Bottom, total protein detected by Ponceau S staining. Expected PAT position is marked with an arrow.

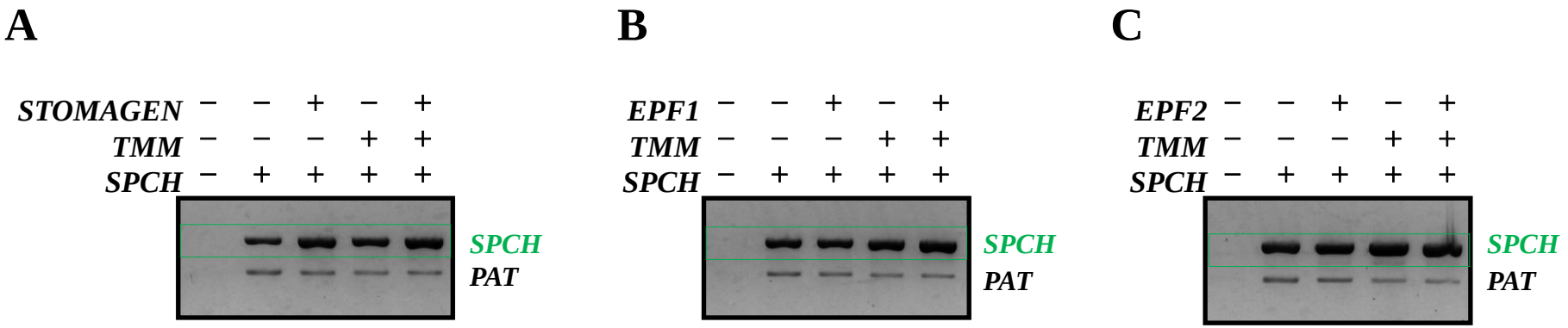

Fig. 9 *SPCH* transcript levels in leaves co-transformed with other genes. *PAT* was used as a control.

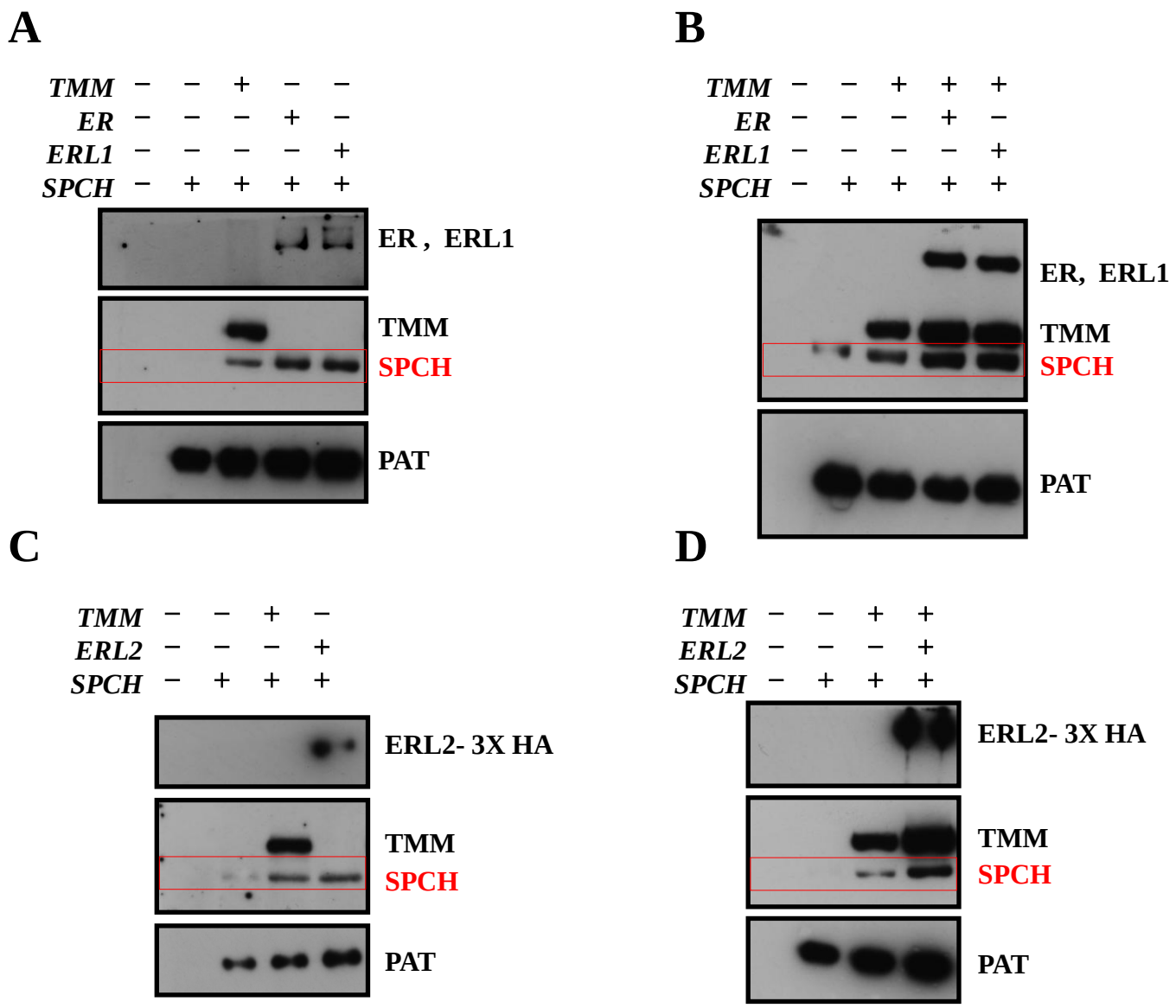

Fig. 10 Overexpression of *TMM*, *ER*, *ERL1* and *ERL2* receptor components increases recombinant SPCH protein levels. (A) Effects of *TMM*, *ER* and *ERL1*. (B) Effects of co-expression of *TMM* with *ER* or *ERL1*. (C) Effect of *TMM* and *ERL2*. (D) Effect of co-expression of *TMM* with *ERL2*. PAT was used as a loading control. Proteins were detected by Western blotting with the anti-PAT antibody or anti-HA antibody (ERL2) or anti-GFP antibody (other proteins).

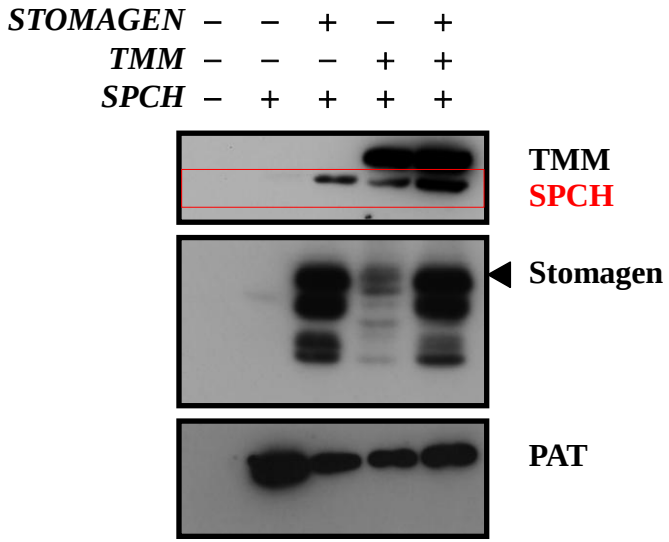

Fig. 11 Effects of *STOMAGEN* and *TMM* on the SPCH protein level. Proteins were detected by Western blotting with the anti-PAT antibody or anti-GFP antibody (other proteins). The position corresponding to the mature form of stomagen is indicated with an arrowhead. Signals in the lanes with *TMM* + *SPCH* in the STOMAGEN panel represent cleavage products of TMM-GFP fusion protein.

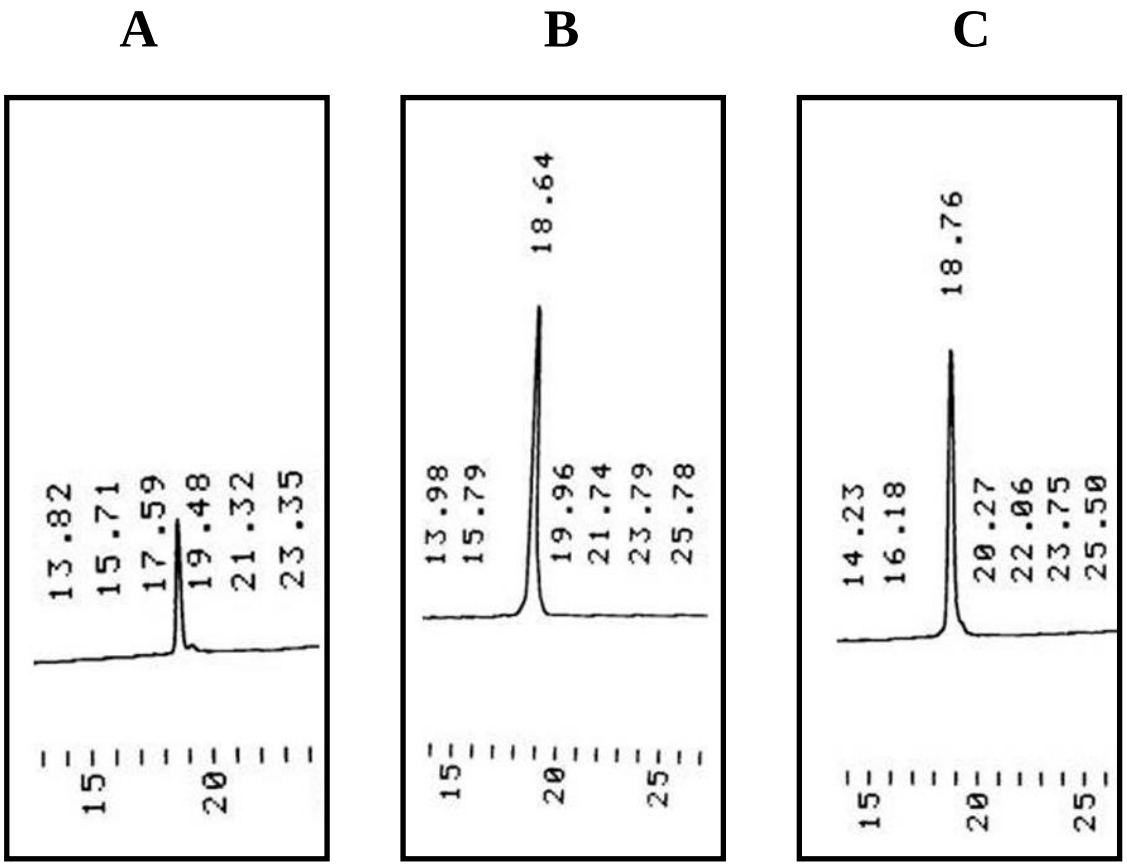

Fig. 12 Chromatogram of 45 aa the the stomagen peptide purification using reverse phase HPLC. (A) Mature 45 aa stomagen peptide obtained from Kondo et al. 2010. (B) Refolded mature stomagen peptide. (C) Co injection of refolded stomagen peptide and peptide obtained from Kondo et al. 2010.

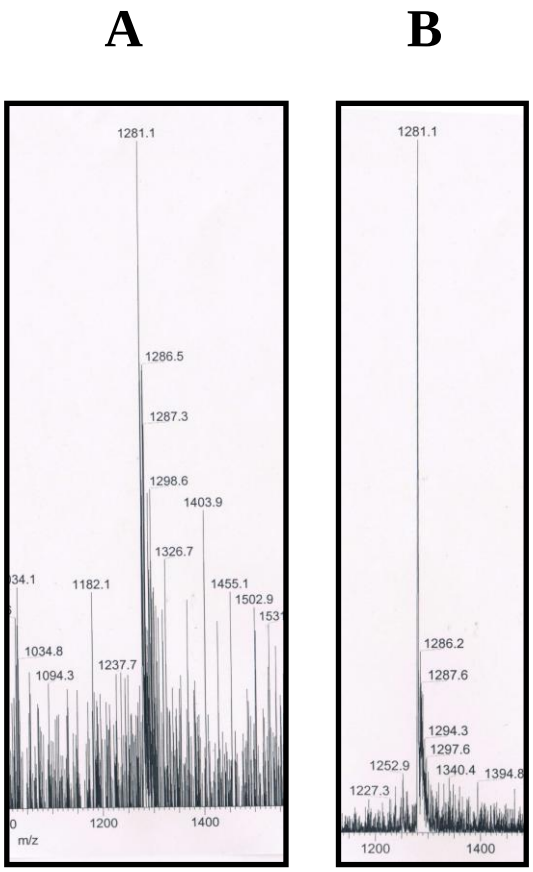

Fig. 13 ESI-MS spectrum of mature 45 aa the the stomagen peptide. (A) Mature 45 aa the stomagen peptide obtained from Kondo et al. 2010. (B) Refolded mature the stomagen peptide

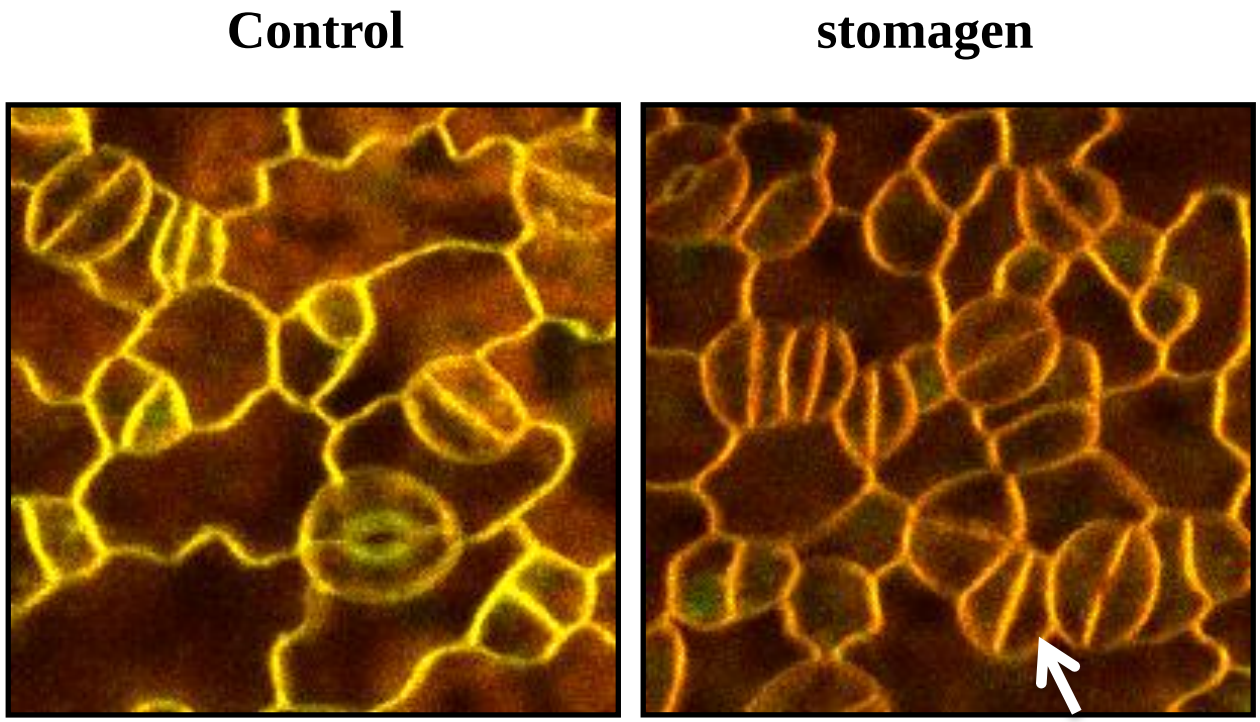

Fig. 14 Bioactivity of the stomagen peptide. The stomagen peptide treated leaf epidermis show stomatal clusters, indicated with a white arrow.

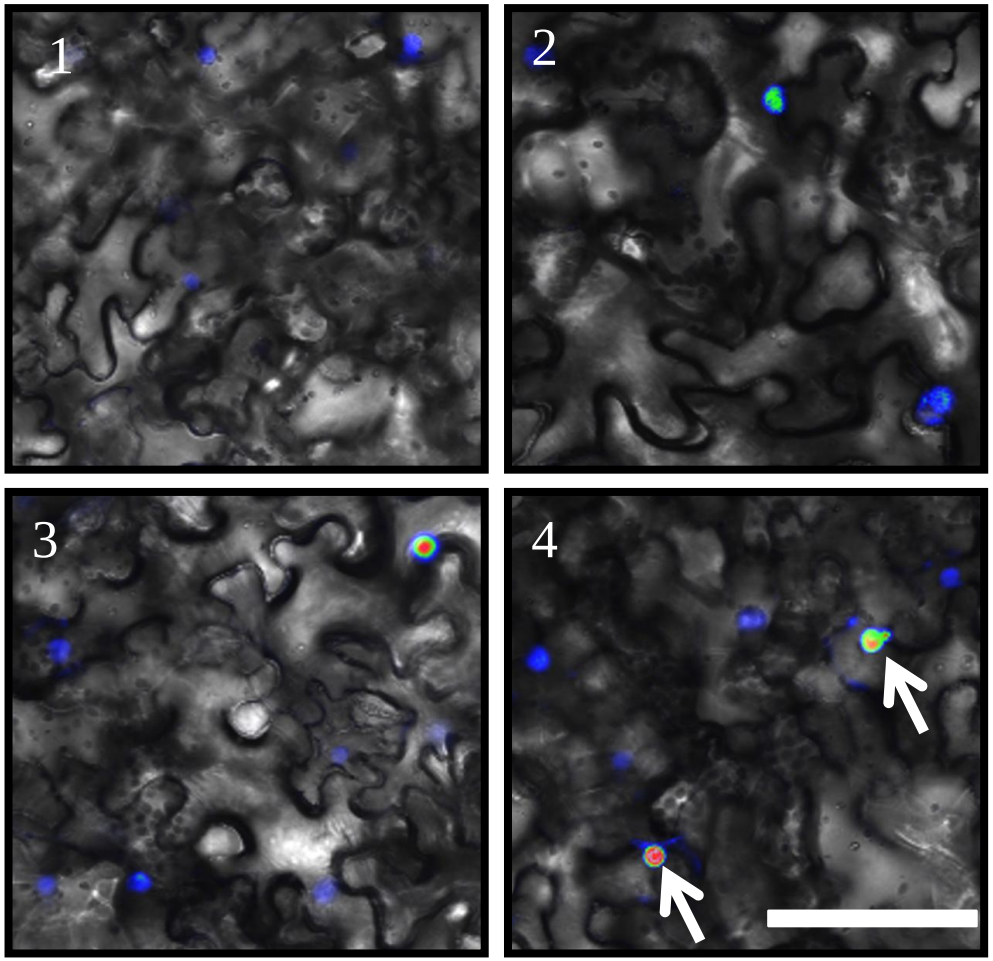

Fig. 15 The stomagen peptide increased SPCH protein levels. (A) The stomagen peptide increased *SPCH-GFP* signal intensity. (1) SPCH. (2) The stomagen peptide and *SPCH*. (3) *TMM-Td Tomato* and *SPCH*. (4) The stomagen peptide + *TMM-Td Tomato* + *SPCH*. The arrow show strong *SPCH-GFP* signal. (B) Effect of stomagen peptide on the SPCH protein level in the presence or absence of *TMM*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-GFP antibody (SPCH). Scale bar= 50μm

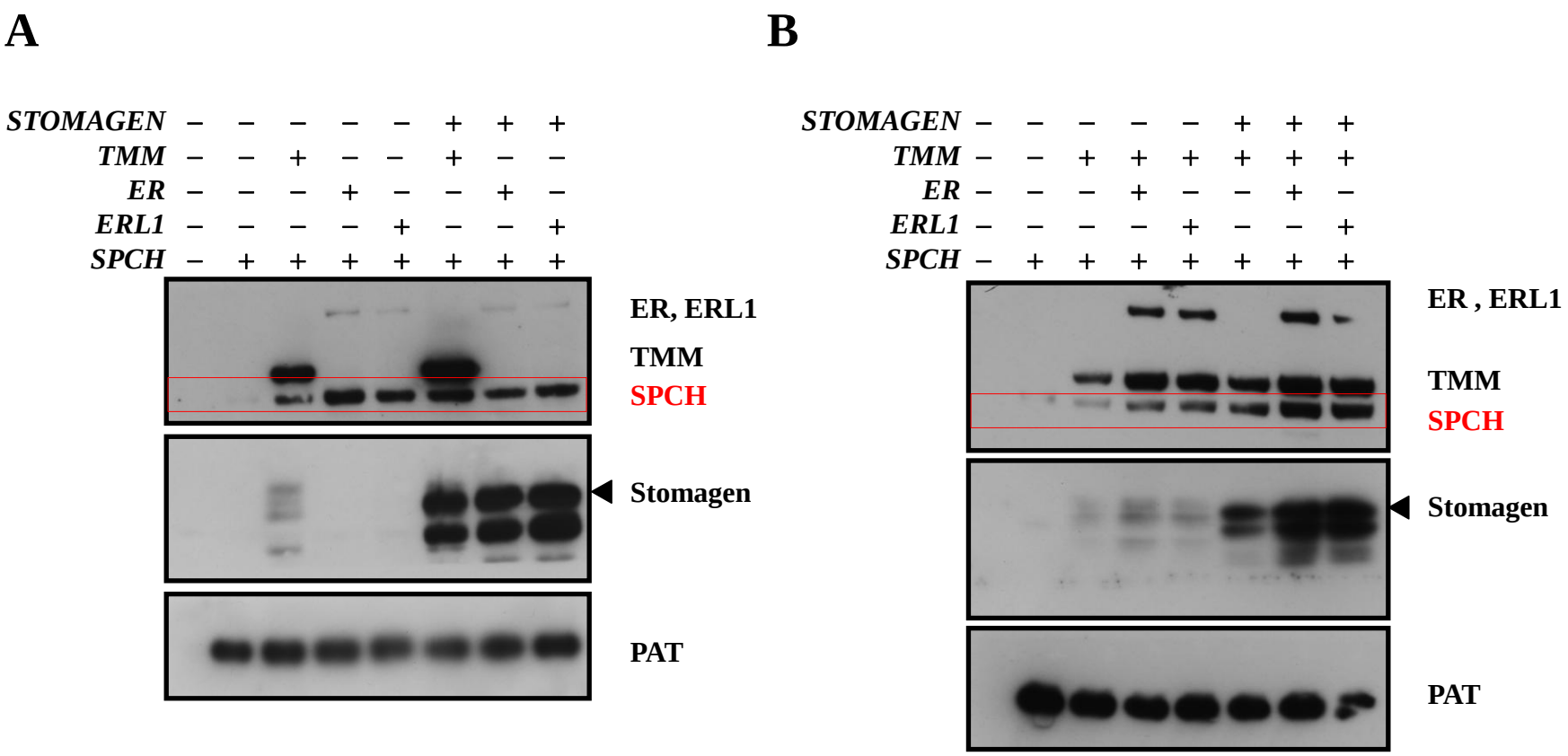

Fig. 16 Effect of *STOMAGEN* and receptor components on SPCH protein level. (A) Effect of *STOMAGEN* on the SPCH protein level, in the presence of *TMM*, *ER*, or *ERL1*. (B) Effect of *STOMAGEN* on the SPCH protein level in the presence of *TMM* + *ER* and *TMM* + *ERL1*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-GFP antibody (other proteins). The position corresponding to the mature form of stomagen is indicated with an arrowhead. Signals in the lanes with *TMM* + *SPCH* in the STOMAGEN panel represent cleavage products of TMM-GFP fusion protein.

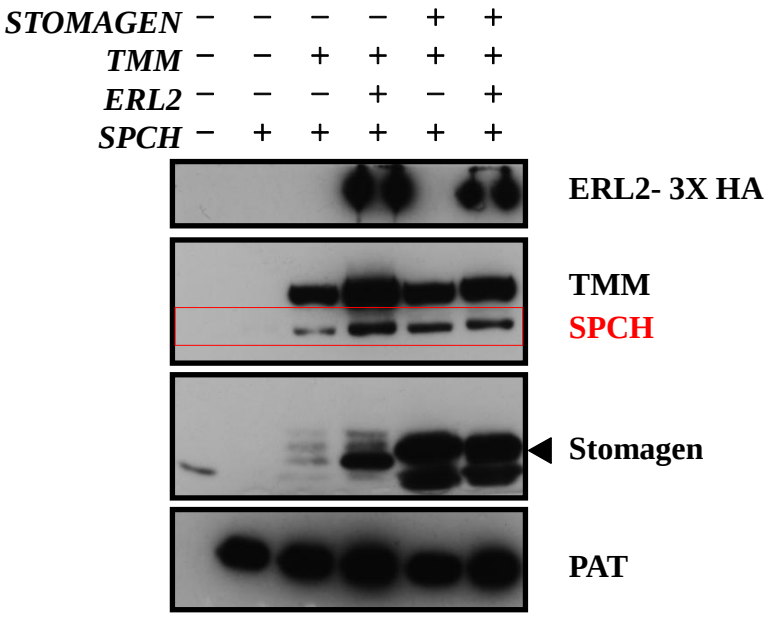

Fig. 17 Effect of *STOMAGEN* in the presence of *TMM* + *ERL2*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-HA antibody (ERL2) or anti-GFP antibody (other proteins). The position corresponding to the mature form of stomagen is indicated with an arrowhead. Signals in the lanes with *TMM* + *SPCH* in the STOMAGEN panel represent cleavage products of TMM-GFP fusion protein.

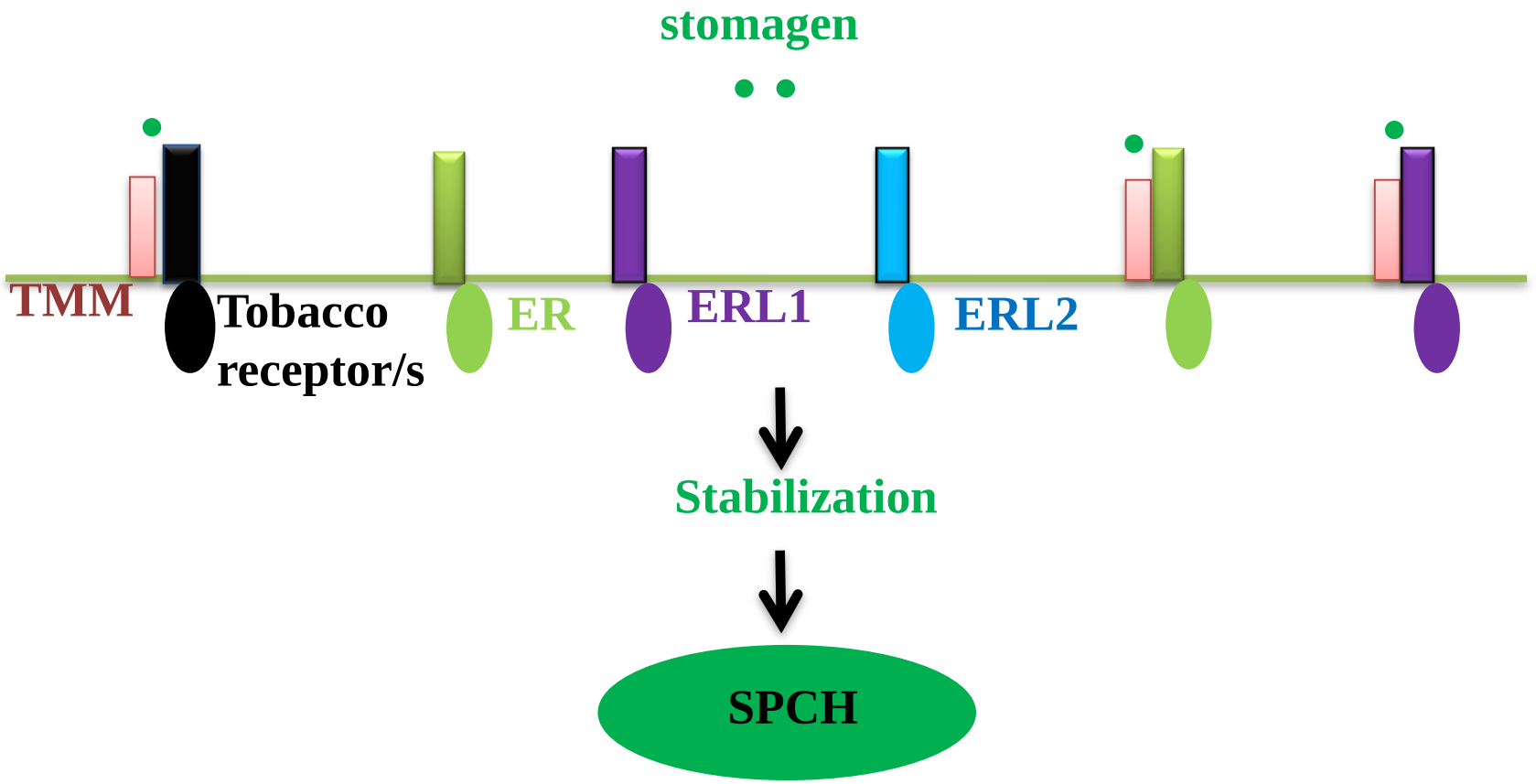

Fig. 18 Putative model of the stomagen peptide signal transduction to regulate SPCH protein level. Stomagen increased SPCH protein level with and without TMM. The effect of stomagen was higher with TMM + ER and TMM + ERL1 indicating that TMM and ER and TMM and ERL1 work together to perceive stomagen. ERL2 did not participated in stomagen mediated SPCH protein stabilization.

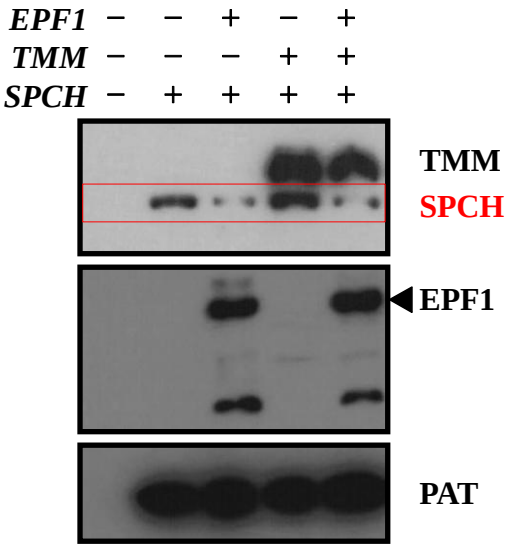

Fig. 19 Effect of *EPF1* and *TMM* on the SPCH protein levels. Proteins were detected by Western blotting with the anti-PAT antibody or anti-GFP antibody (other proteins). The position corresponding to the mature EPF1 is indicated with an arrowhead. Signals in the lanes containing *TMM* and *SPCH* in the EPF1 panel represent cleavage products of TMM-GFP fusion protein.

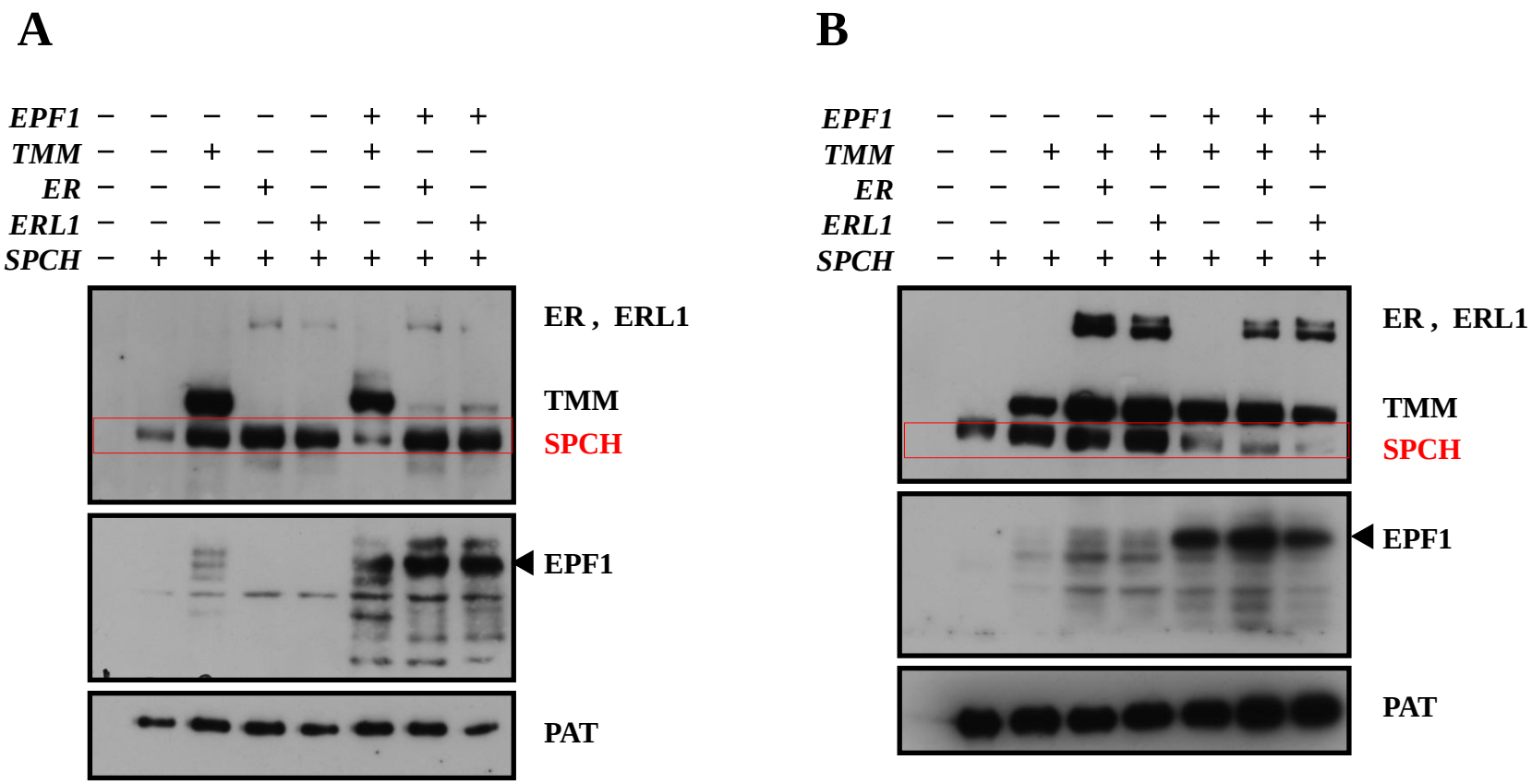

Fig. 20 Effect of EPF1 and receptor components on SPCH protein level. (A) Effect of *EPF1* on the SPCH protein level, in the presence of *TMM*, *ER*, or *ERL1*. (B) Effect of *EPF1* on the SPCH protein level in the presence of *TMM* + *ER* and *TMM* + *ERL1*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-GFP antibody (other proteins). The position corresponding to the mature EPF1 is indicated with an arrowhead. Signals in the lanes containing *TMM* and *SPCH* in the EPF1 panel represent cleavage products of TMM-GFP fusion protein.

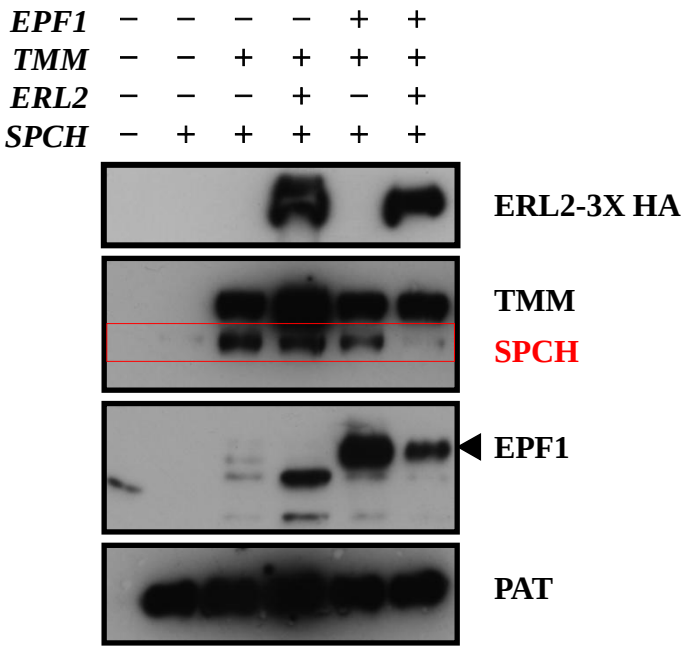

Fig. 21 Effect of EPF1 and ERL2 on SPCH protein level. Proteins were detected by Western blotting with the anti-PAT antibody or anti-HA antibody (ERL2) or anti-GFP antibody (other proteins). The position corresponding to the mature EPF1 is indicated with an arrowhead. Signals in the lanes containing *TMM* and *SPCH* in the EPF1 panel represent cleavage products of TMM-GFP fusion protein.

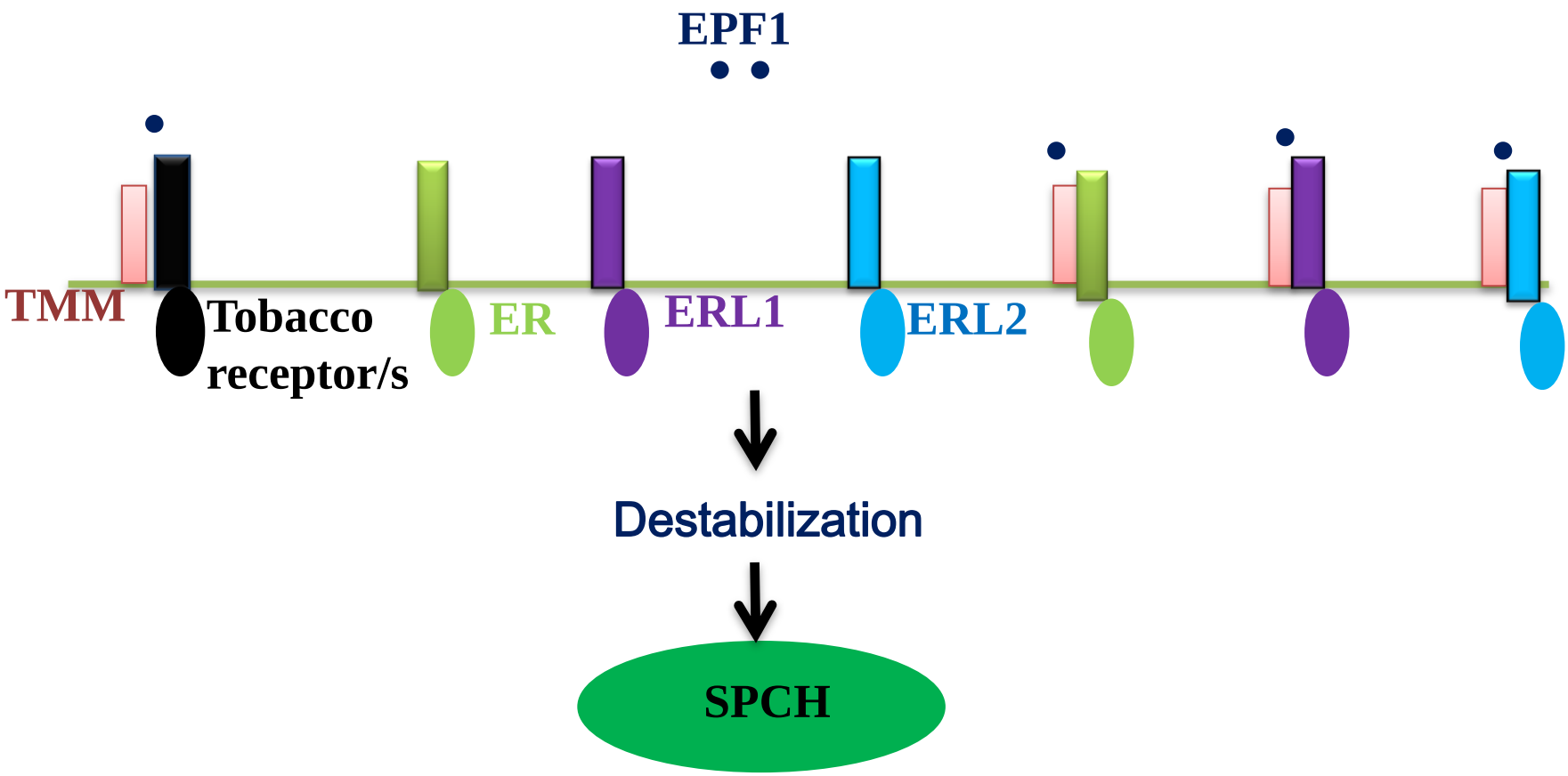

Fig. 22 Putative model of EPF1 peptide signal transduction to regulate SPCH protein level. EPF1 decreased SPCH protein level with and without TMM. *EPF1* mediated decrease in SPCH protein level was higher with *TMM + ER*, *TMM + ERL1* and *TMM + ERL2* indicating that TMM functions with ER, ERL1 and ERL2 to transduce the EPF1 signal.

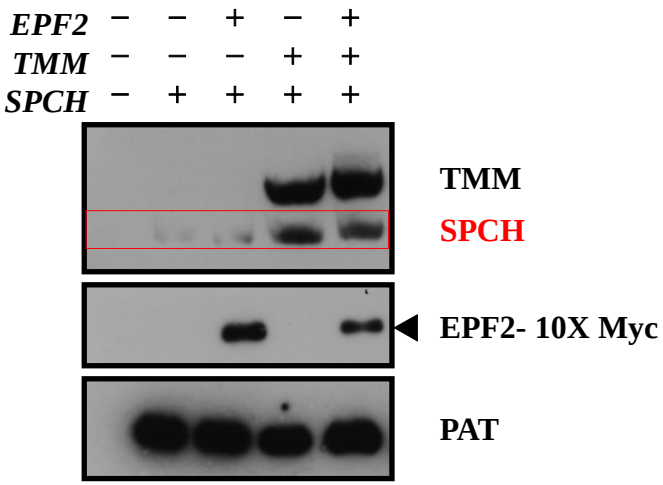

Fig. 23 Effect of *EPF2* and *TMM* on the SPCH protein levels. Proteins were detected by Western blotting with the anti-PAT antibody or anti-Myc (EPF2) or anti-GFP antibody (TMM and SPCH).

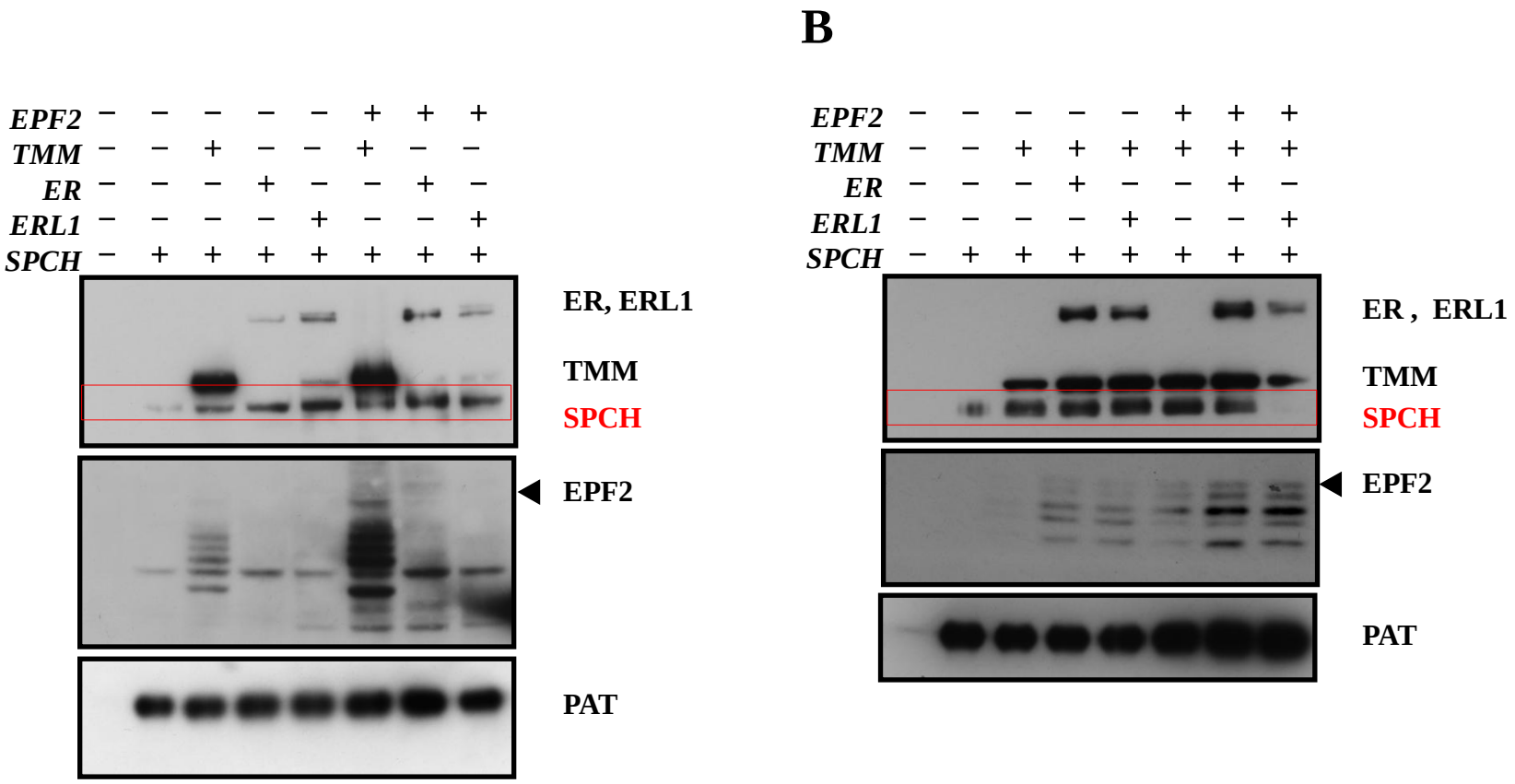

Fig. 24 *EPF2* decreased SPCH protein levels in the presence of *TMM* and *ERL1*. *(A*) Effect of *EPF2* in the presence of *TMM*, *ER*, or *ERL1*. (B) Effect of *EPF2* in the presence of *TMM* + *ER* and *TMM* + *ERL1*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-GFP antibody (other proteins).The position corresponding to the mature EPF2 is indicated with an arrowhead. EPF2-GFP signals were not clearly visible. It might be possible small undetectable amount of EPF2 was effective. Signals in the lanes without EPF2 represent cleavage products of TMM-GFP, ER-GFP, ERL1-GFP or SPCH-GFP.

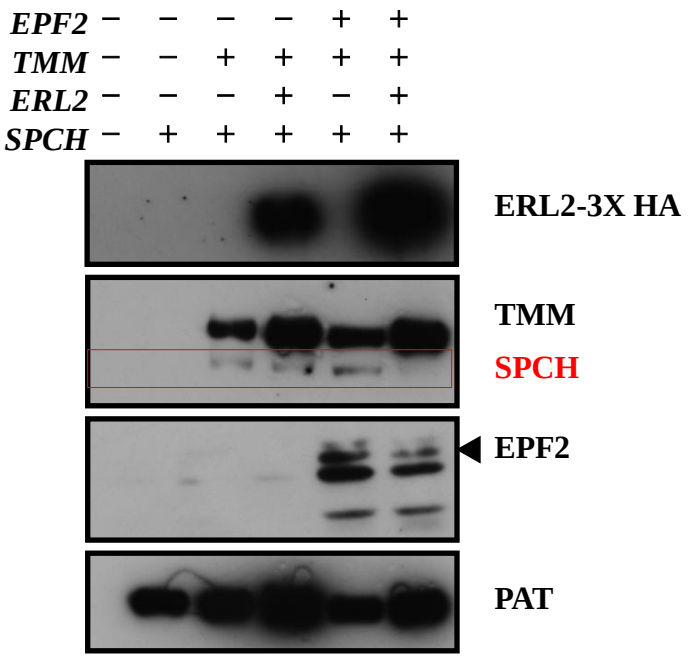

Fig. 25 Effect of *EPF2* in the presence of *TMM* + *ERL2*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-HA antibody (ERL2) or anti-GFP antibody (other proteins).The position corresponding to the mature EPF2 is indicated with an arrowhead. EPF2-GFP signals were not clearly visible. It might be possible small undetectable amount of EPF2 was effective. Signals in the lanes without EPF2 represent cleavage products of TMM-GFP, ER-GFP, ERL1-GFP or SPCH-GFP.

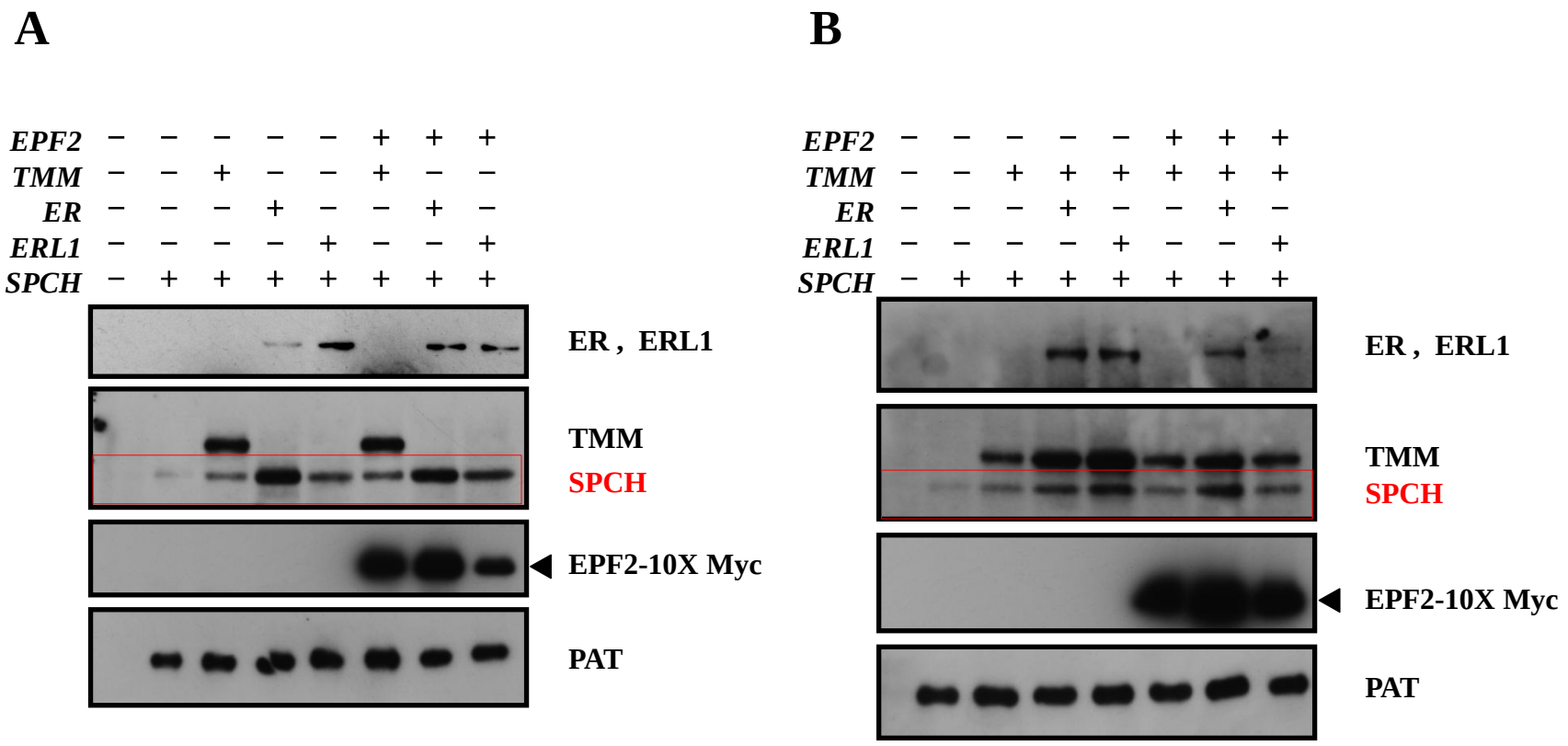

Fig. 26 *EPF2* decreased SPCH protein levels in the presence of *TMM* and *ERL1*. *(A*) Effect of *EPF2* in the presence of *TMM*, *ER*, or *ERL1*. (B) Effect of *EPF2* in the presence of *TMM* + *ER* and *TMM* + *ERL1*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-Myc antibody or anti-GFP antibody (other proteins).The position corresponding to the mature EPF2 is indicated with an arrowhead.

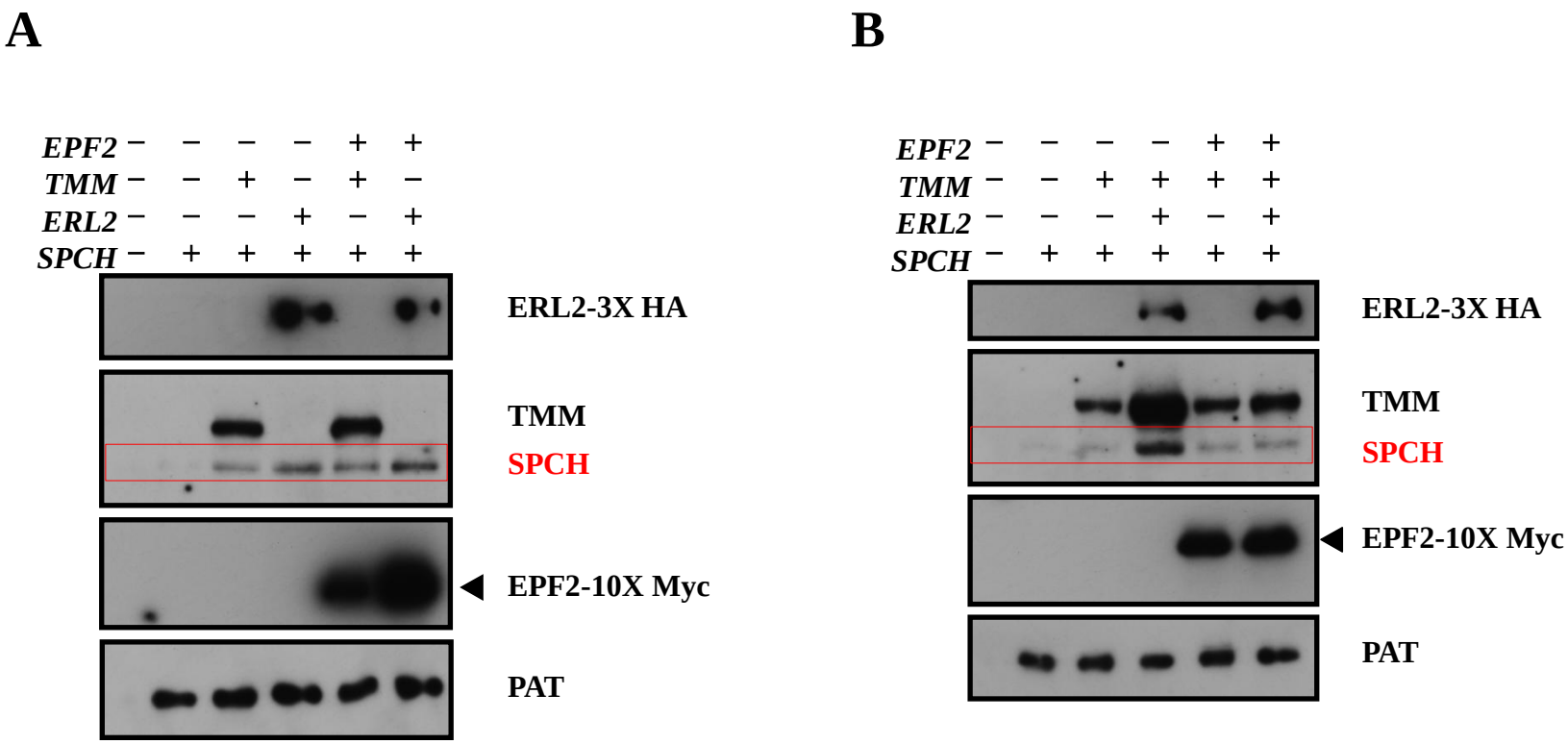

Fig. 27 Effect of *EPF2* in the presence of *ERL2*. *(A*) Effect of *EPF2* in the presence of *TMM* and *ERL2*. (B) Effect of *EPF2* in the presence of *TMM* + *ERL2*. Proteins were detected by Western blotting with the anti-PAT antibody or anti-Myc antibody or anti-GFP antibody (other proteins).The position corresponding to the mature EPF2 is indicated with an arrowhead.

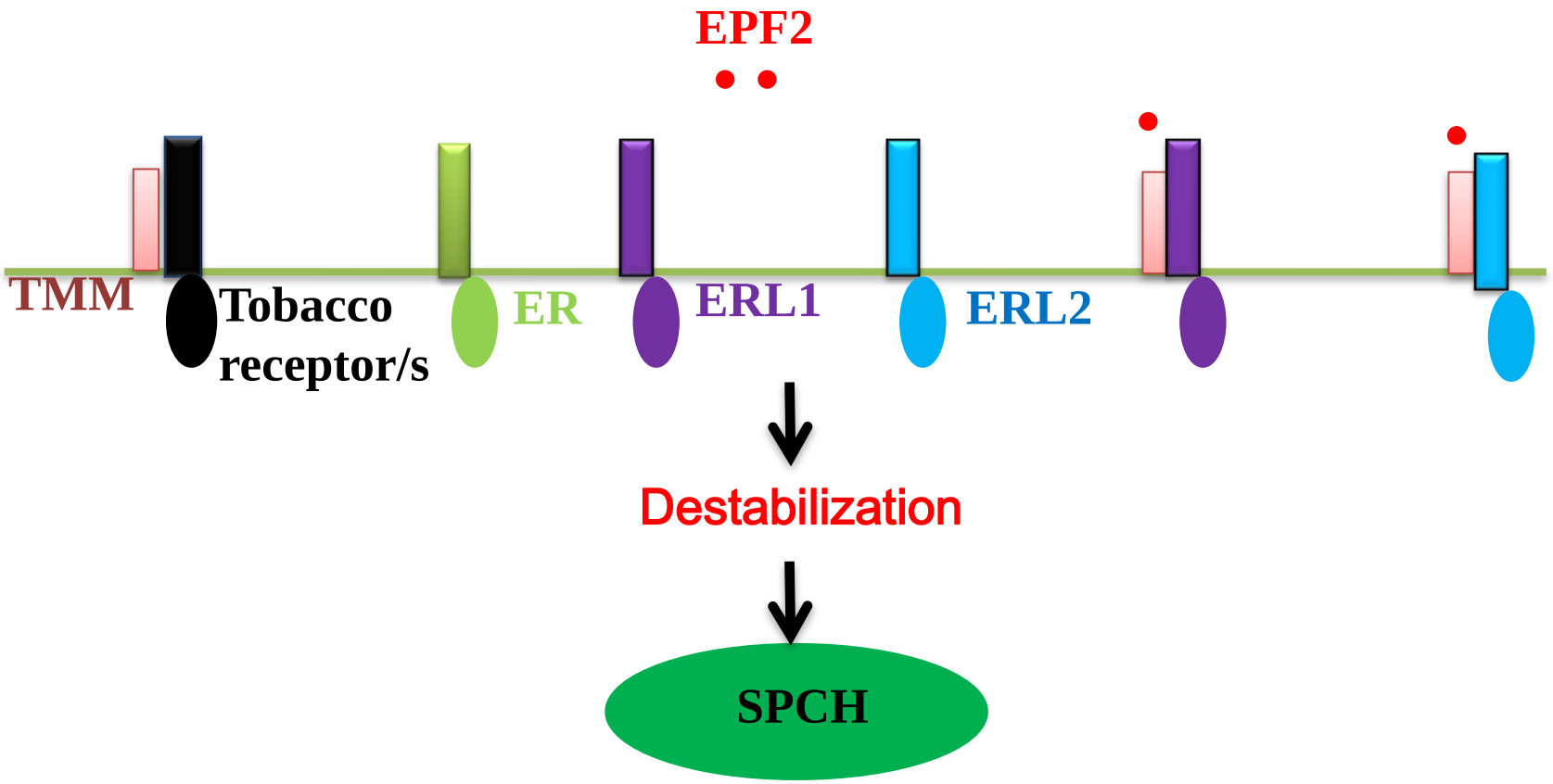

Fig. 28 Putative model of EPF2 peptide signal transduction to regulate SPCH protein level. EPF2 decreased SPCH protein level only with *TMM* + *ERL1* and *TMM* + *ERL2* suggesting that TMM functions with ERL1 and ERL2 to perceive the EPF2 signal.

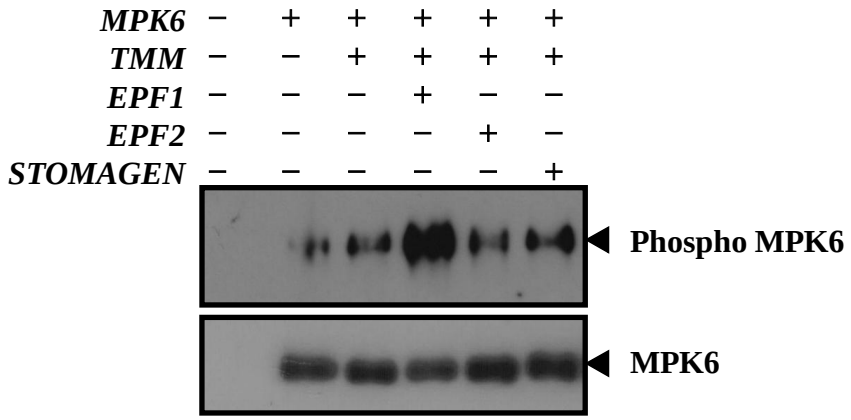

Fig. 29 Effects of *EPF1*, *EPF2* or *STOMAGEN* on MPK6 activation in the presence of *TMM*. MPK6 was immunoprecipitated with the anti-FLAG antibody, and probed with the anti-phospho-specific ERK1/2 [Thr202/Tyr204] antibody to detect the activated MAP kinase (top panel), or with the anti-FLAG antibody (bottom) to detect total amounts of immunoprecipitated MPK6.

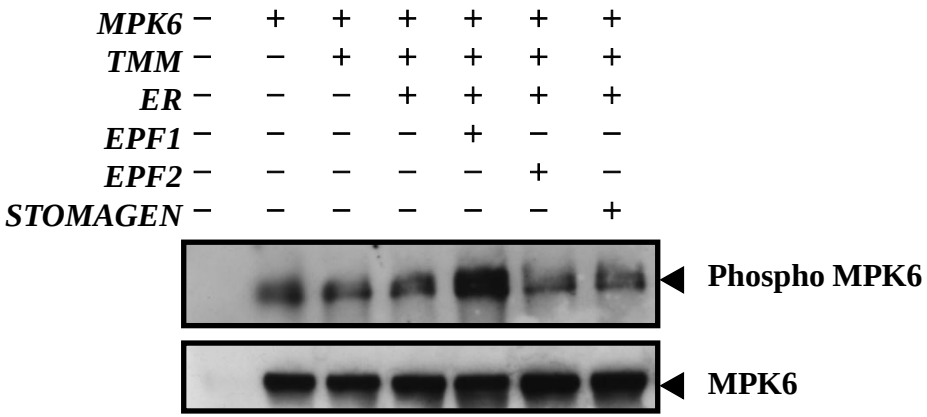

Fig. 30 Effects of *EPF1*, *EPF2* or *STOMAGEN* on MPK6 activation in the presence of *TMM.+ER*. MPK6 was immunoprecipitated with the anti-FLAG antibody, and probed with the anti-phospho-specific ERK1/2 [Thr202/Tyr204] antibody to detect the activated MAP kinase (top panel), or with the anti-FLAG antibody (bottom) to detect total amounts of immunoprecipitated MPK6.

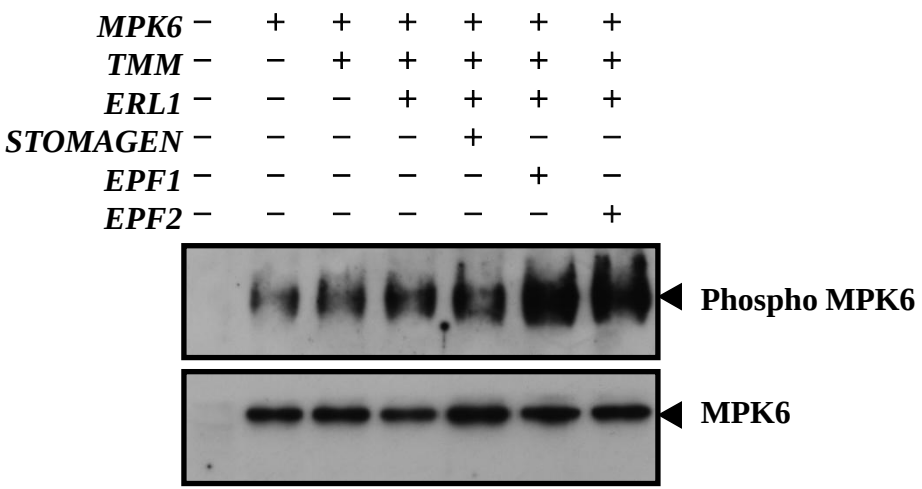

Fig. 31 Effects of *EPF1*, *EPF2* or *STOMAGEN* on MPK6 activation in the presence of *TMM.+ERL1*. MPK6 was immunoprecipitated with the anti-FLAG antibody, and probed with the anti-phospho-specific ERK1/2 [Thr202/Tyr204] antibody to detect the activated MAP kinase (top panel), or with the anti-FLAG antibody (bottom) to detect total amounts of immunoprecipitated MPK6.

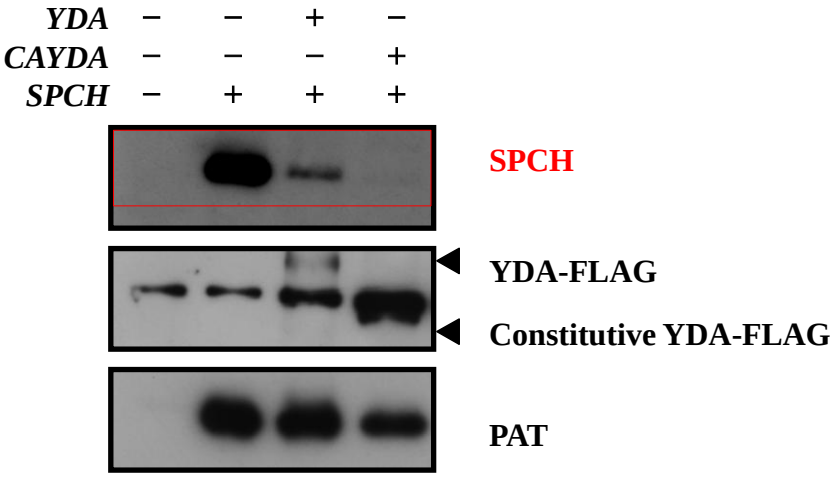

Fig. 32 *YDA* and constitutive form of *YDA* decreased SPCH-GFP protein levels. Proteins were detected by Western blotting with the anti-PAT antibody or anti-FLAG antibody (YDA and CA-YDA) or anti-GFP antibody (SPCH).The position corresponding to the YDA-5X FLAG and constitutive YDA-5X FLAG indicated with an arrowhead.

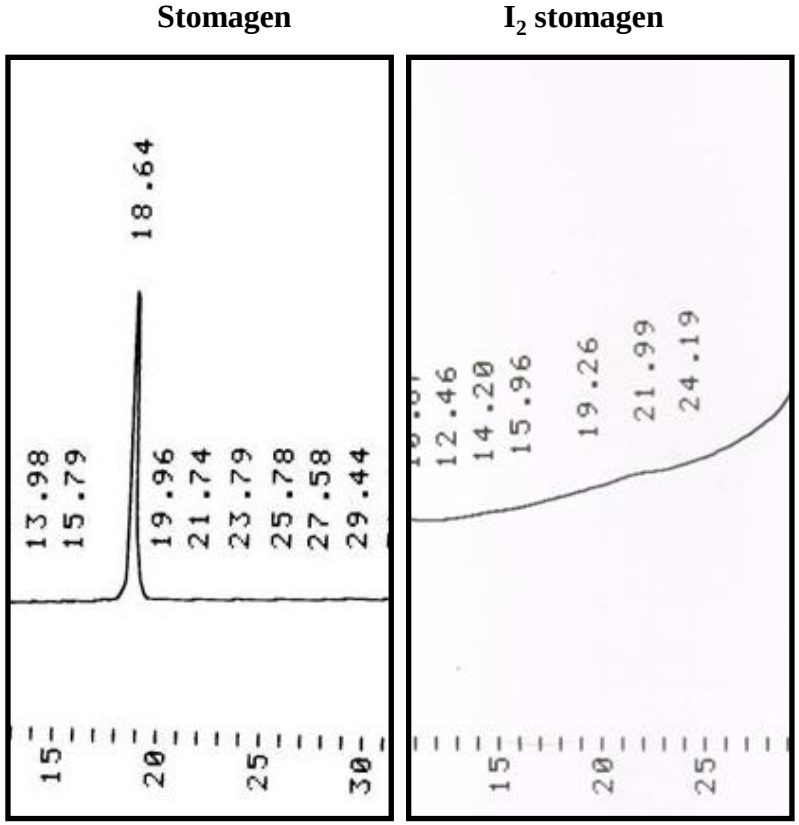

Fig. 33 I_2 labelling degrades stomagen peptide. The HPLC chromatogram show absence of stomagen peak after I_2 labelling

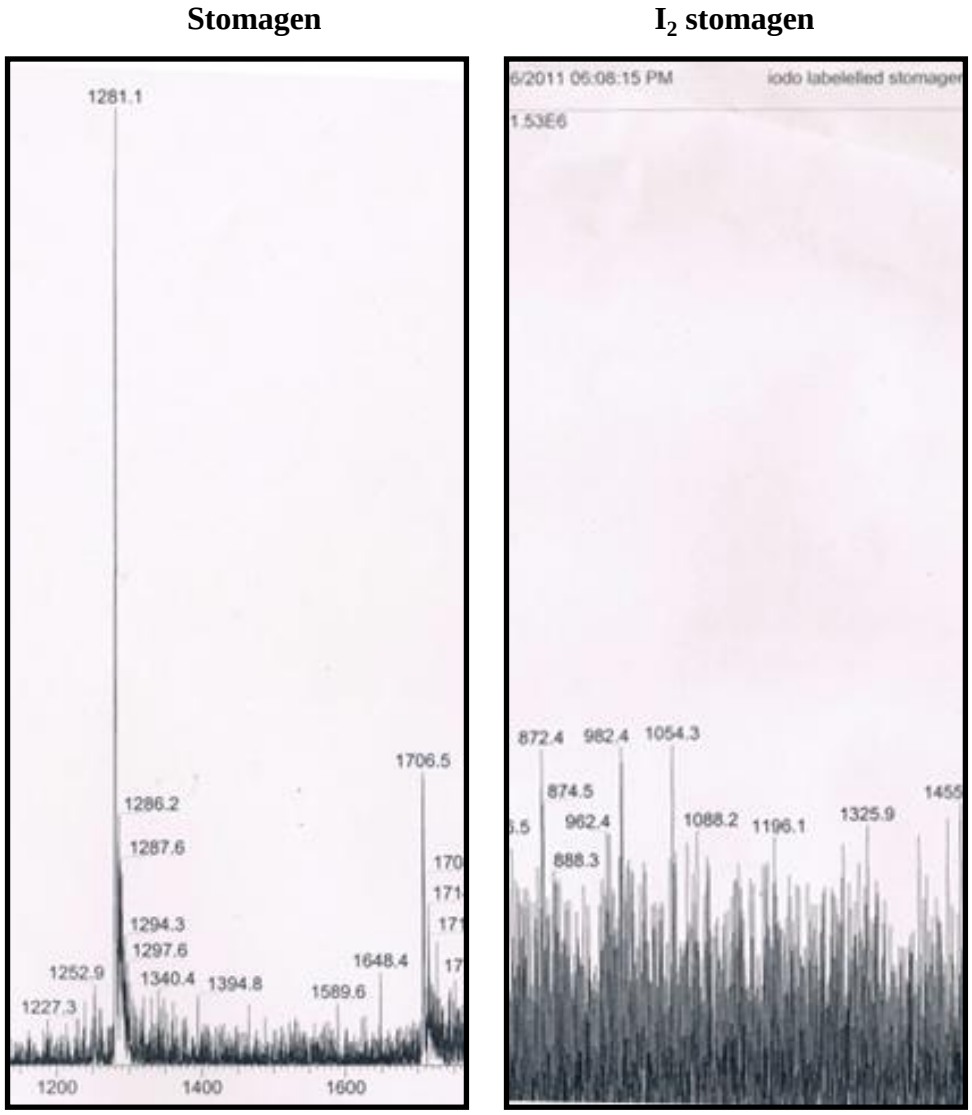

Fig. 34 I_2 labelling degrades the stomagen peptide. The ESI-MS spectrum show absence of the stomagen peptide peak after I_2 labelling

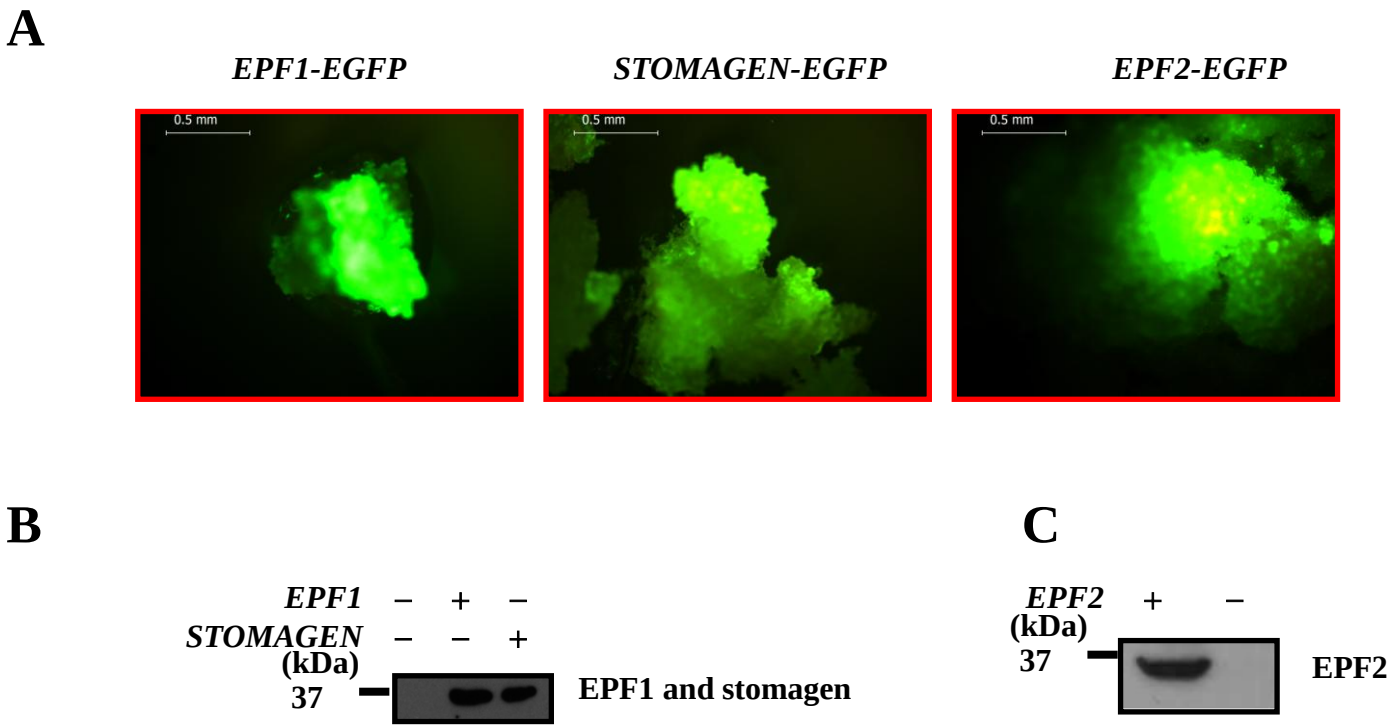

Fig. 35 EPF1, EPF2 and stomagen recombinant proteins were obtained from Tobacco BY2 cells but purified proteins were biologically inactive. Upper panel show transformed BY2 cell and lower panel indicates Western blotting with GFP antibody.

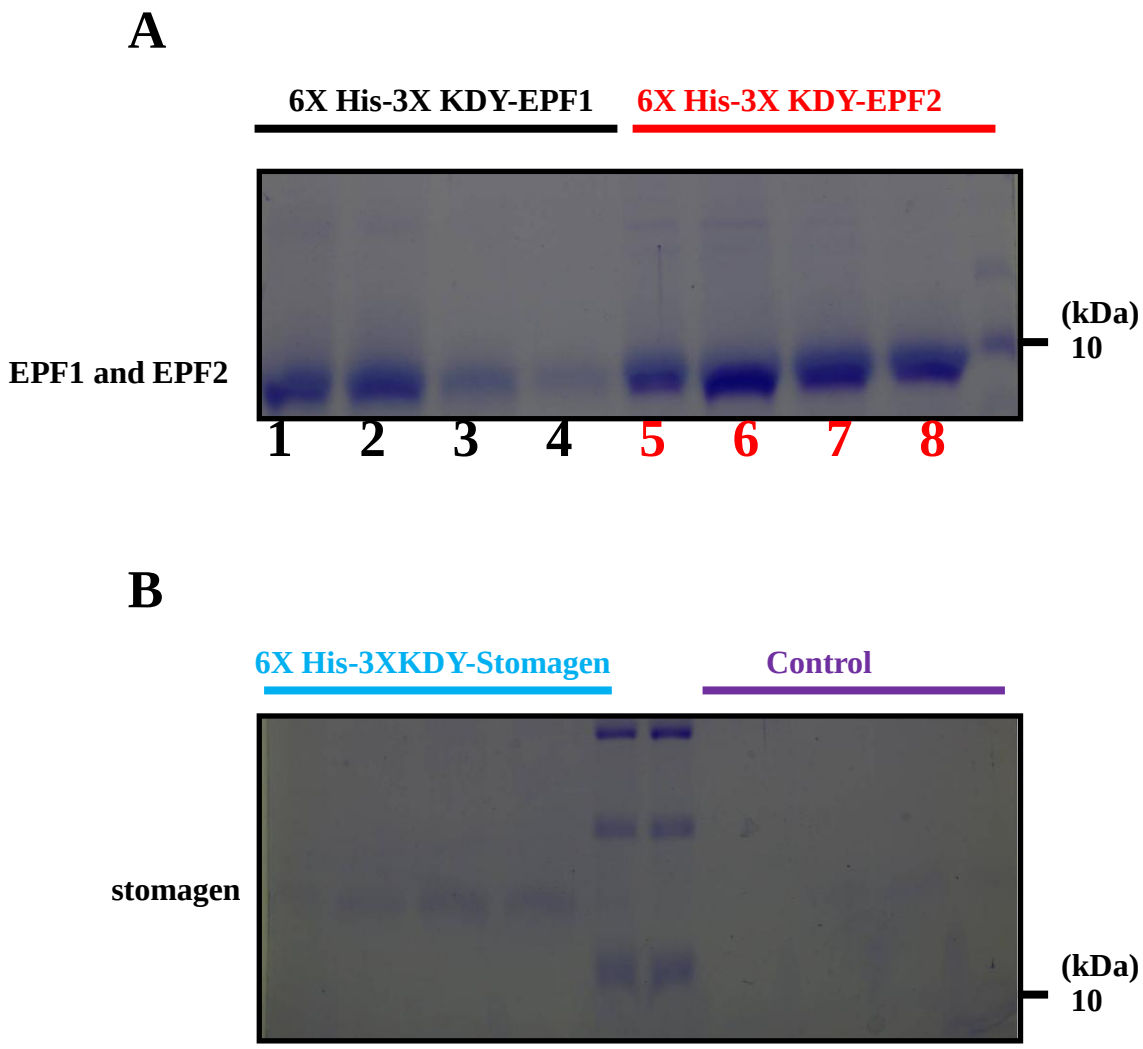

Fig. 36 EPF1, EPF2 and stomagen recombinant proteins were obtained from BL21TrexB pLysis cells but purified proteins were biologically inactive.

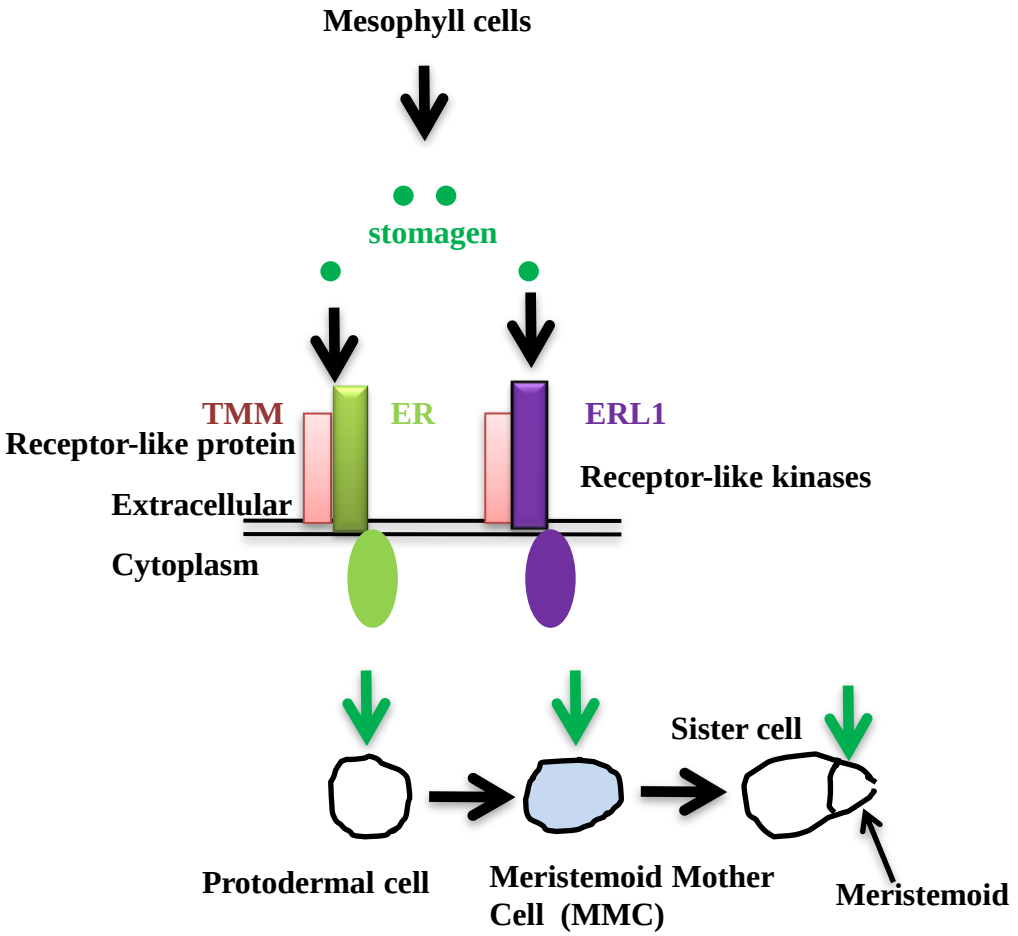

Fig. 37 Stomagen regulates stomatal development by TMM+ER or TMM+ERL1 receptor components. Stomagen acts on protodermal cell, MMC and meristemoid to increases SPCH protein level and their by progression in stomatal lineage pathway.

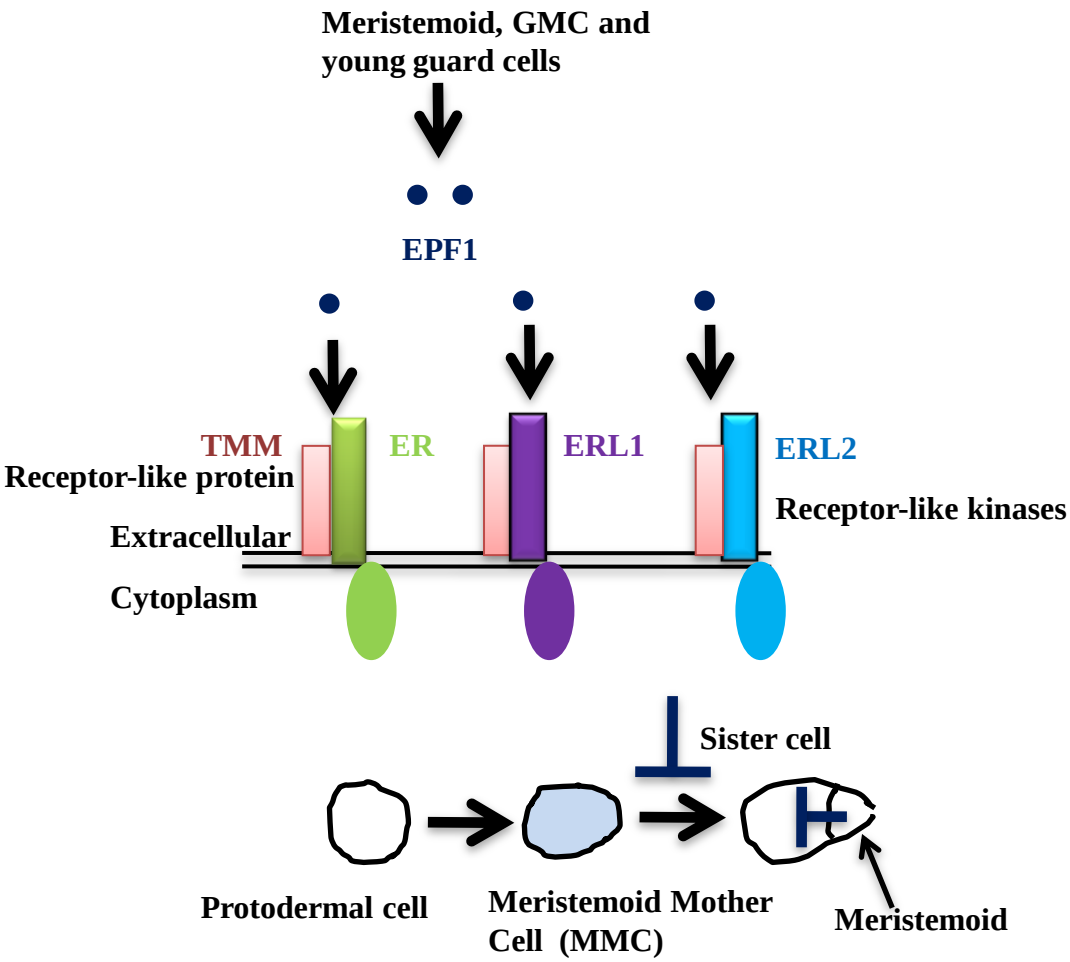

Fig. 38 EPF1 regulates stomatal development by TMM+ER, TMM+ERL1 and TMM+ ERL2 receptor components. EPF1acts on meristemoids to decrease SPCH protein level and their by stop stomatal lineage pathway.

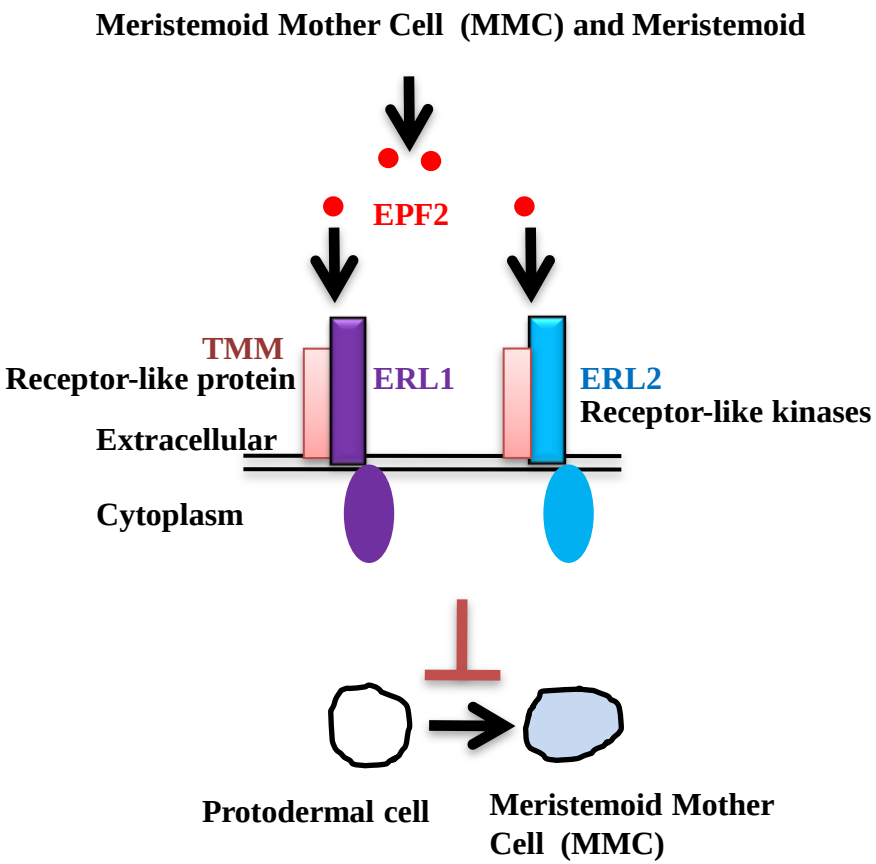

Fig. 39 EPF2 regulates stomatal development by TMM+ ERL1 or TMM+ ERL2 receptor components. EPF2 acts on protodermal cell and MMC and decrease SPCH protein level and their by stop stomatal lineage pathway.

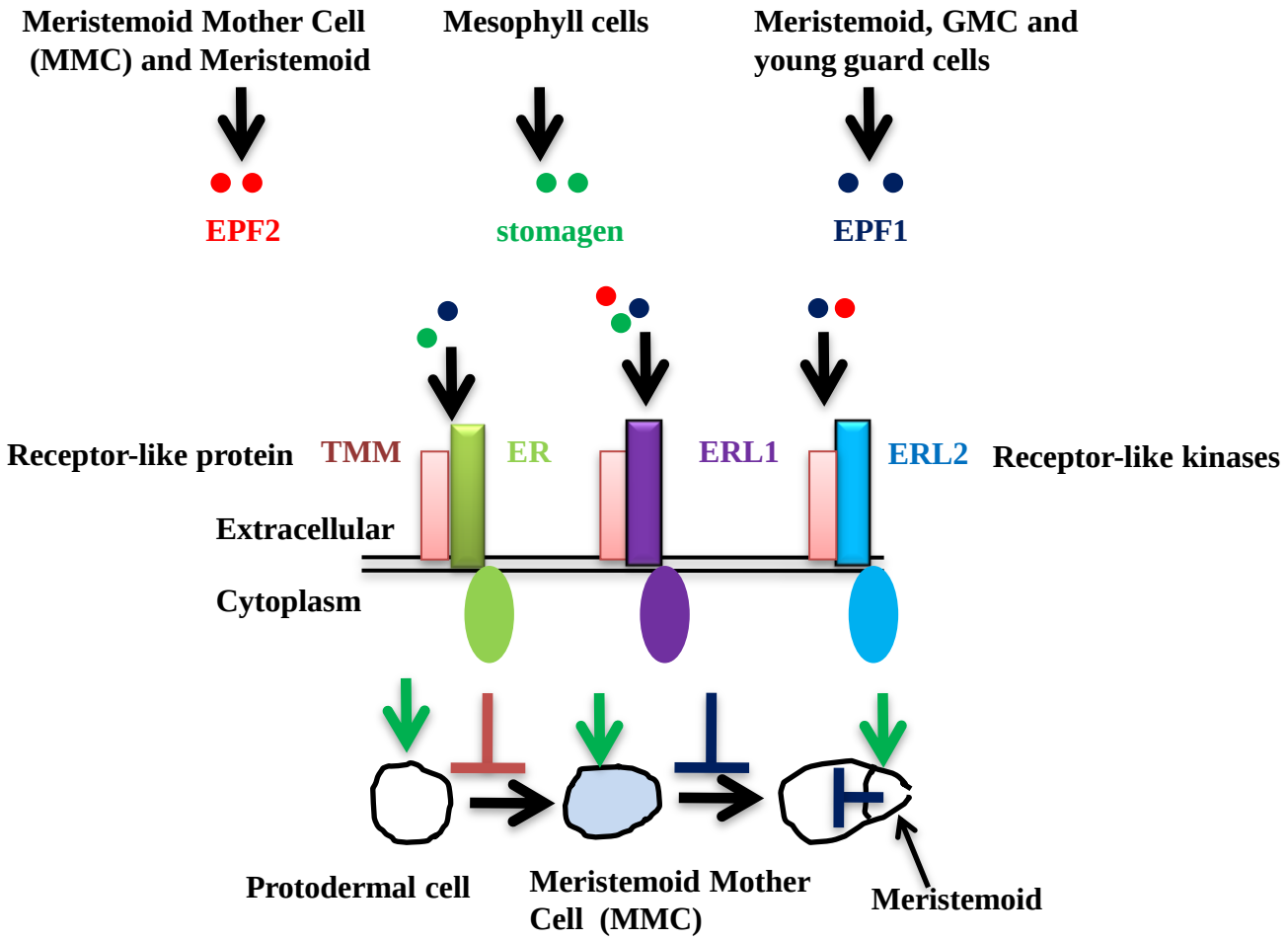

Fig. 40 EPF1, EPF2 and stomagen regulate stomatal development by different receptor components. Stomagen can acts on protodermal cells, MMCs and meristemoids whereas EPF2 and EPF1 can act on MMCs and meristemoids respectively.

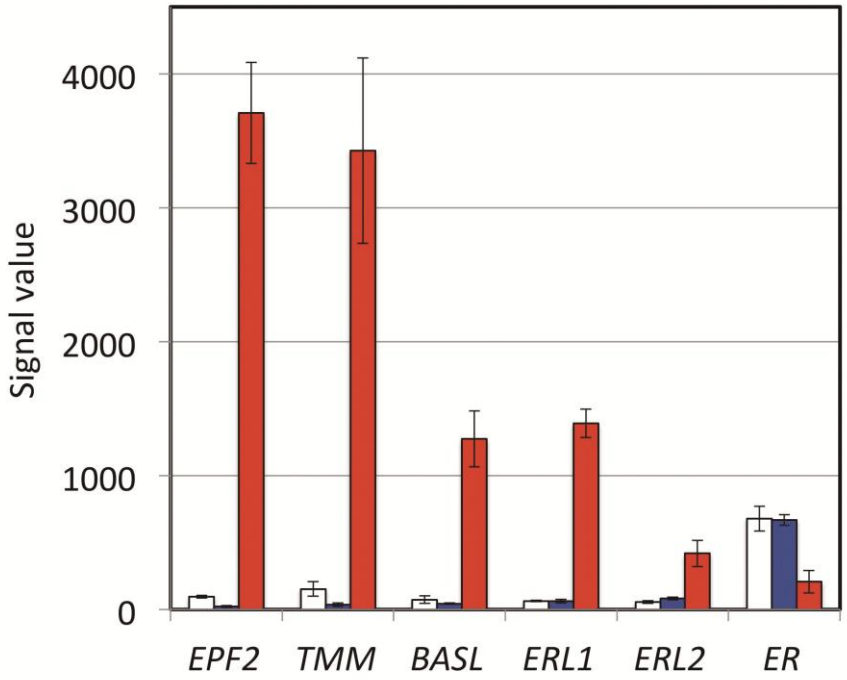

Fig. 41 Expression levels of ER-family receptor genes in wild-type (open column), *spch* (blue column), and the *scrmD mute* double mutant (red column). Epidermis of *spch* lacks stomatal lineage cells. Epidermis of *scrmD mute* consists mostly of stomatal lineage cells at early stages, consistent with the high expression of the MMC marker *EPF2*. The data were obtained from microarray data from an expression profiling experiment (GSE29814).