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DISSERTATION

PP2C Regulates Cell Developmental Decisions

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ABSTRACT

Reversible protein phosphorylation is one of the key mechanisms to regulate a wide range of biological processes including stress and developmental signal transduction (Shpak et al., 2004; Shpak et al., 2005; Wang et al., 2007a). Protein phosphorylation via mitogen activated protein kinase (MAPK) cascades is a common module of signal transduction in eukaryotes, which in plants is characterized in respect to stress-sensing and development. Recent evidences link a MAPK signal transduction cascade with plant development where stress-activated MAP kinases control cell patterning in plant epidermis (Bergmann et al., 2004; Wang et al., 2007b). Protein phosphatases of type 2C (PP2Cs) are known to regulate MAPK pathways (Meskiene et al., 1998; Meskiene et al., 2003; Schweighofer et al., 2007). Arabidopsis PP2Cs comprise the largest plant protein phosphatase family, however only few members are characterized in detail.

This study addresses a novel stomata-specific Arabidopsis PP2C-type phosphatase STOPP, as a negative regulator of environmentally-responsive MAPKs. STOPP inactivates the MAPKs, which suppress stomata development. Thereby STOPP induces ectopic stomata formation in plant epidermis. Stomata are specialized cells controlling the carbon dioxide, oxygen and water vapor exchange with the environment, thus playing a crucial role in photosynthesis and global ecosystems (Hetherington and Woodward, 2003). The results of this research suggest that the STOPP/MAPK module regulates the development of epidermis by controlling cell cycle components. This study provides novel evidences of environmentally-responsive MAPK regulation by STOPP and suggests the link between STOPP/MAPK module and the cell cycle progression during the development of stomata.

ZUSAMMENFASSUNG

Protein-Phosphorylierung ist ein Schlüsselmechanismus in der Regulation vieler biologischer Prozesse, sowohl in der Entwicklungssteuerung als auch bei der Stressantwort. Die Signalweiterleitung durch Kaskaden aus Mitogen-aktivierte Proteinkinasen (MAPK) ist in eukaryotischen Organismen weit verbreitet. Diese Module sind in Pflanzen besonders bei Stress-Signaltransduktion und bei der pflanzlichen Entwicklung aktiv. Jüngste Forschungsergebnisse verlinken die MAPK-Signaltransduktion mit der Musterbildung der Epidermis. Protein-Phosphatasen vom Typ 2C (PP2C), welche bereits als Regulatoren von MAPK bekannt sind, bilden in der Modellpflanze Arabidopsis die größte Familie unter den Phosphatasen. Bisher wurden jedoch nur wenige PP2C charakterisiert.

Diese Studie beschreibt eine neue PP2C namens STOPP, welche spezifisch in Spaltöffnungszellen (Stomata) exprimiert wird und entwicklungsgesteuerte MAPK negativ reguliert. STOPP inaktiviert diese MAPK, blockiert die Zelldifferenzierung und induziert die Anhäufung von Stomata. Stomata kontrollieren den Austausch von Sauerstoff, Kohlendioxid und Wasserdampf mit der Atmosphäre und sind daher für das globale Ökosystem unerlässlich. Diese Studie belegt die Bedeutung des STOPP/MAPK Moduls in der Regulation der Epidermisentwicklung und –differenzierung. Die Ergebnisse lassen den Schluss zu, dass STOPP die MAPK während der Zelldifferenzierung kontrolliert und wahrscheinlich dadurch den Zellzyklus steuert. Diese Arbeit liefert einen äußerst wertvollen Beitrag zum Verständnis der Zellzyklusregulation und Zellentwicklung.

ABBREVIATIONS

ABA	abscisic acid
ABI	abscisic acid insensitive
AP2C	Arabidopsis protein phosphatase 2C
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BiFC	bimolecular fluorescence complementation
BSA	bovine serum albumine
CaMV	cauliflower mosaic virus
CDK	cyclin dependent kinase
CHX	cycloheximide
CTD	C-terminal domain
2,4-D	2,4-dichlorphenoxy acetic acid
DEPC	Diethyl Pyrocarbonate
Dex.SO_4	dextrane sulphate
DMF	dimethylformamide
DMSO	dimethylsulfoxide
dpg	days post germination
DSP	dual specificity protein tyrosine phosphatase
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis(β -aminoethylether)-tetraacetic acid
ER	estradiol
ET	ethylene
flg22	flagellin 22 amino acid peptide
GA	gibberellic acid
GC	guard cell
GC-FID	gas chromatography flame ionization detector
GFP	green fluorescent protein
GMC	guard mother cell
gof	gain-of-function
GST	glutathione S-transferase
GUS	ß-glucuronidase
h	hour
HA	hemagglutinin
HAB1	homology to *ABI1/ABI2*
His1	Histone 1
IPTG	isopropyl-β-D-thiogalactoside
JA	Jasmonic acid
KAPP	kinase associated protein phosphatase
kb	kilobasepair
kDa	kiloDalton
KIM	kinase interaction motif
LB	Luria Bertani media
lof	loss-of-function
M	meristemoid
MAPK/MPK	mitogen activated protein kinase
MAPKK/MKK/MEK	mitogen-activated protein kinase kinase

MAPKKK/MKKK/MEKK	mitogen-activated protein kinase kinase kinase
MBP	myelin basic protein
β-ME	β-mercaptoethanol
min.	minute
MKP	MAP kinase phosphatase
MMC	meristemoid mother cell
MP2C	Medicago protein phosphatase 2C
MS	Murashige Skoog media
Myc	myelocytomatosis
NC	neighbor cell
NLS	nuclear localization signal
NTD	N-terminal domain
OD	optical density
ONPG	o-nitrophenyl-β-D-galactopyranoside
PC	pavement cell
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pi	inorganic phosphate
PI	propidium iodide
PMSF	phenylmethylsulfonylfluoride
PP2A	protein phosphatase type 2A
PP2B	protein phosphatase type 2B
PP2C	protein phosphatase type 2C
PPM	protein phosphatase metalo-dependent
PPP	phosphoprotein phosphatase
ProD	Protodermal cell
RLK	receptor like kinase
RLP	receptor like protein
rpm	rounds per minute
RT PCR	reverse transcription PCR
S/Ser	serine
SA	salycilic acid
SAMK	stress-activated MAP kinase
SC	subsidiary cell
SD	synthetic dropout media
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SIMK	stress-induced MAP kinase
SLGC	stomata lineage ground cell
SM	satellite meristemoid
ssDNA	single stranded DNA
STOPP	stomata protein phosphatase
TCA	trichloroacetic acid
Thr/T	threonine
Tyr/Y	tyrosine
wt	wild type
Y2H	yeast two hybrid
YFP	yellow fluorescent protein
YPD	yeast extract/peptone/dextone

CONTENT

1. Introduction

1.1. Stomata development: signals and regulation

Plants exchange with the environment various substances, including nutrients, metabolites, gases and water. Plant epidermis is the boundary for the plant - environment interactions. Specialized epidermal cell structures, stomata, control CO_2 uptake and thus are crucial for plant photosynthesis and maintenance of the whole ecosystem (Hetherington and Woodward, 2003). Stomata consist of paired guard cells flanking a pore, which connects the intercellular airspace with the atmosphere (Essau, 1977; Nadeau and Sack, 2002). They control water loss, CO_2 fixation, gas exchange, xylem embolism, cooling and nutrient transport from roots (Raven, 2002). The evolvement of stomata enabled plants to optimize carbon fixation and limit water loss (Raven, 2002), which has been essential for the success of terrestrial plants in adaptation to different environments (Croxdale, 2000; Hetherington and Woodward, 2003; Gray and Hetherington, 2004).

Stomata development is entirely postembryonic and starts within a day after seed germination (Nadeau and Sack, 2002). In monocot plants and conifer needles stomata formation is pre-patterned by initial organization of competent cells into longitudinal arrays (Croxdale, 2000). In Arabidopsis, stomata formation in hypocotyls and cotyledons is controlled by organ-specific rules (Kono et al., 2007). For example the *tmm* mutant, that has impaired stomata development, produces stomatal clusters on leaves but lacks stomata in the hypocotyls, inflorescence stem and adaxial surface of sepals (Yang and Sack, 1995; Geisler et al., 1998). The plant hormone gibberrelic acid is essential for stomata development in hypocotyls but not in leaves (Saibo et al., 2003). Hypocotyl epidermis is patterned by the same mechanisms that control root epidermis development. In Arabidopsis hypocotyls stomata development is pre-patterned to non-protruding cell files that overlie anticline cortex cell wall. In roots the same cell files, which are the precursors for stomata, form root hairs (Berger et al., 1998). Stomata patterning in hypocotyls is controlled by the same genes, which define root epidermis patterning: WEREWOLF (WER), TRANSPARENT TESTA GLABRA (TTG) and GLABRA2 (GL2) (Berger et al., 1998; Lee and Schiefelbein, 1999). The density of stomata increases towards the apical pole of hypocotyl suggesting the conflicting influences of root- and shoot-specific rules of epidermis patterning (Berger et al., 1998).

Stomata formation in Arabidopsis leaves is not obviously pre-patterned, but some tendencies in stomata distribution have been observed. In leaves stomata tend to be excluded from

epidermis that lies over the midvein region and around the trichomes (Serna and Fenoll, 2000b; Nadeau and Sack, 2002; Martin and Glover, 2007). Often stomatal density is higher on the abaxial leaf surface than on the adaxial leaf surface, which is more exposed to the heating and water loss (Martin and Glover, 2007). The intercellular airspace cavities below the stomata pore coincides with the junctions between mesophyll cells (Nadeau and Sack, 2002; Bergmann, 2004). In one study the positioning of stomata was correlated with the junctions between mesophyll cells (Serna and Fenoll, 2000b). However, this correlation is not obligatory as other studies found that stomata may be placed independently of mesophyll cell junctions (reviewed in (Nadeau and Sack, 2002; Bergmann, 2004).

Stomata formation via oriented asymmetric and symmetric cell divisions

All stomata are formed by oriented cell divisions (Larkin et al., 1997). The epidermis of newly germinated Arabidopsis seedling consists of uniform protodermal cells. Within 24 hours some protodermal cells acquire the meristemoid mother cell (MMC) identity and commit to stomata developmental pathway (reviewed in (Nadeau and Sack, 2002; Nadeau and Sack, 2003; Bergmann, 2004; Bergmann and Sack, 2007; Nadeau, 2009).

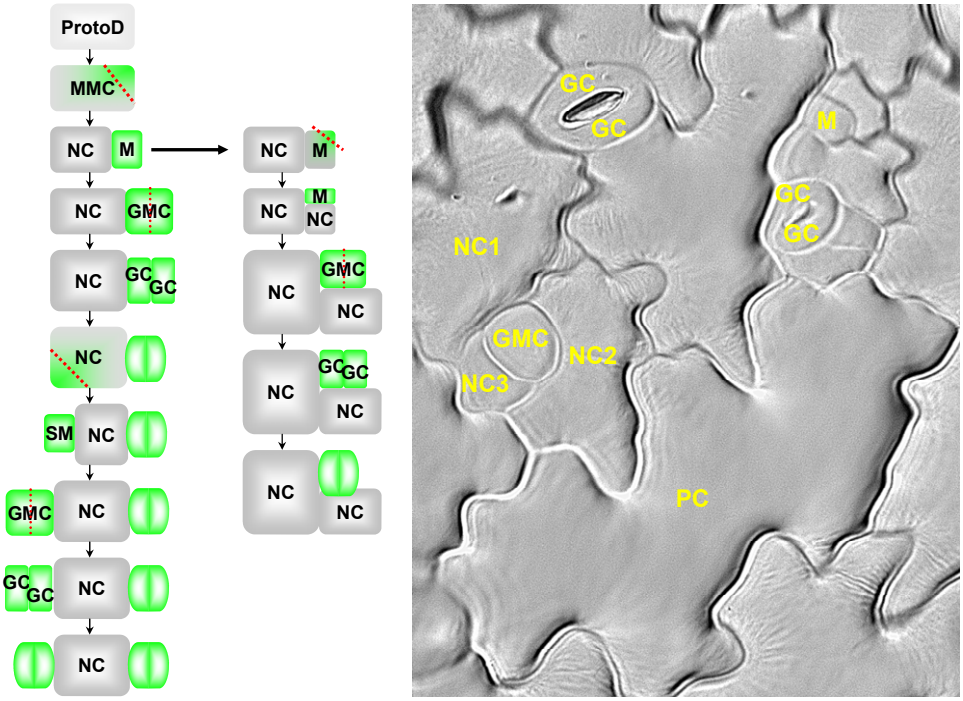

Figure 1.1. The development of stomata in Arabidopsis leaf. Left: schematic representation of asymmetric and symmetric cell divisions during stomata development. Right: wild type epidermis of Arabidopsis cotyledon contains highly crenulated pavement cells and stomata lineage cells; the anisocytic stomata complex consisting of GMC surrounded by three neighbor cells (marked in NC1, NC2 and NC3, which corresponds to the subsequent division rounds). ProtoD - protodermal cell, MMC - meristemoid mother cell, M - meristemoid, GMC - guard mother cell, GC - guard cell, NC - neighbor cell, SM - satellite meristemoid, PC – pavement cell.

The MMC divides asymmetrically to produce a smaller cell meristemoid (M) and a neighbor cell (NC) of stomatal lineage (also termed stomatal lineage ground cell – SLGC or subsidiary cell - SC) (seed Fig. 1). Eventually, the meristemoid differentiates into a guard mother cell (GMC), which divides symmetrically to produce two guard cells (GCs) that form the stomatal pore. Thus, progression through three types of stomata precursor cells (MMC, M and GMC) occurs till the terminal differentiation of stomata (Zhao and Sack, 1999).

Before the terminal differentiation, the meristemoid may divide asymmetrically two more times in an inward spiral, each time producing a meristemoid and a clonally related NC that belongs to the same stomata lineage (a monoclonal complex) (Zhao and Sack, 1999; Nadeau and Sack, 2002; Serna et al., 2002). Such stomatal complexes, containing the stomata surrounded by three NCs of unequal size, are termed anisocytic (Metcalfe and Chalk, 1950). About one third of meristemoids in a lineage do not divide or divide once and form polyclonal stomatal complexes with clonally unrelated NCs (Geisler et al., 2000; Nadeau and Sack, 2002).

NC (both of stomata and non-stomatal lineage) may differentiate into pavement cell (PC) or divide asymmetrically to produce a satellite meristemoid (SM) and larger NC (Zhao and Sack, 1999). The decision either to divide or to differentiate depends on the age of NC in the stomata complex. In young stomata complexes all NCs have equal potential to divide, whereas in older complexes, usually only the smallest and youngest NC divides (Berger and Altmann, 2000; Geisler et al., 2003). NCs that differentiate into pavement cells undergo endoreduplication and do not divide any further (Melaragno et al., 1993; Geisler et al., 2000).

The asymmetric cell division is the main mechanism to initiate and amplify stomata lineage. Stomata number is adjusted to the environmental conditions. The asymmetric division of NCs accounts for the development of 75% of stomata in leafs and cotyledons (Geisler et al., 2000). By contrast, in hypocotyls no satellite meristemoids are generated and the population of stomata lineage is amplified by the divisions of MMC precursors (Kono et al., 2007). Hypocotyl stomata typically arise after initial longitudinal and subsequent transverse divisions in non-protruding cell files (Berger et al., 1998).

In any case stomata patterning obeys to the **one-cell-spacing rule**, which ensures that stomata are not formed in direct contact to each other. The one-cell-spacing rule of stomata is maintained by oriented asymmetric cell division (Geisler et al., 2000). The plane of asymmetric cell divisions is oriented so that the new meristemoid is placed away from the pre-existing stomata or precursor cell (Zhao and Sack, 1999; Geisler et al., 2000). If two meristemoids are formed in contact, one of them either divides away placing the NC in

between of them or is arrested and does not differentiate to GMC (reviewed in (Nadeau and Sack, 2002).

Stomata initiation, entry and amplifying divisions, spacing and differentiation is tightly regulated. Generally, the gene regulators of stomata development may be considered as ***positive*** and ***negative***. Positive gene regulators are the essential factors of stomata patterning, mainly represented by bHLH proteins (overviewed in § 1.1.3). The negative regulators of stomata patterning are components of intercellular signal generation, perception and transduction cascade(s) that coordinate spacing of stomata and restrict stomata development (overviewed in § 1.1.4).

1.1.1. Signals in stomata development

Cell-to-cell signals

Intercellular signaling controls stomata patterning and distribution. Mature guard cells or their precursors, meristemoids or guard mother cells, can send positional signals to the neighbor cells and determine their fate (reviewed in (Nadeau and Sack, 2003; Bergmann, 2004; Nadeau, 2009). Positional negative signals inhibit the division of the neighbor cell adjacent to two or more stomata or control the orientation of the division to prevent stomata formation next to each other (Geisler et al., 2000).

Recently, the small secreted peptide EPIDERMAL PATTERNING FACTOR1 (EPF1) was proposed as a positional signal in stomata development in Arabidopsis (Hara et al., 2007). Meristemoid and guard mother cell-expressed EPF1 may be a ligand for putative cell surface receptors in stomata neighbor cells to suppress stomata patterning. Consistently, the ectopic over-expression of EPF1 eliminates stomata or dramatically reduces their abundance (Hara et al., 2007).

Signals derived from mesophyll cells in leaves are also important in determining the patterning of stomata (Serna and Fenoll, 2000a). Arabidopsis double mutants for the homeodomain transcription factors, such as protodermal factor2 (pdf2) and meristem layer 1 (atml1), which have no epidermis, but develop occasional patches of clustered stomata on the bare mesophyll layer (Abe et al., 2003). This suggests that either the mesophyll promotes stomata formation or the epidermis provides negative signals of stomata development (Bergmann, 2004). On the other hand, in the dominant mutant Xcl1 (extra cell layers1) of maize, which contains extra epidermal cell layers, the stomata number is decreased (Kessler et

al., 2002). Thus, stomata formation may be promoted in the proximity to the mesophyll. At the same time, epidermal cells either block the positive signals from the mesophyll layer or send the repressive signals to control stomata development (Bergmann, 2004).

Plant hormones

Plant hormones gibberellins (GA) and ethylene (ET) promote stomata formation in hypocotyls, by promoting cell division in the epidermis (Saibo et al., 2003). GA is the key hormone, which positively regulates stomata formation, while ET and auxin have an additive effect to GA action (Saibo et al., 2003; Kono et al., 2007). In cotyledons, exogenous application of these hormones has no influence on stomata development (Saibo et al., 2003). However, ET induces the differentiation of stomata in Arabidopsis leaves (Serna and Fenoll, 1996).

Auxin increases the effect of GA probably indirectly by enhancing ethylene biosynthesis (Saibo et al., 2003). The ethylene biosynthesis is regulated by mitogen-activated protein kinases (MAPKs) (Liu and Zhang, 2004). The activation of the stress MAPKs SIPK and MPK6 from tobacco and Arabidopsis, induce ethylene biosynthesis. MPK6 phosphorylates the 1-aminocyclopropane-1-carboxylic acid synthases ACS2 and ACS6 leading to their stabilization, accumulation and subsequent ethylene production (Liu and Zhang, 2004; Joo et al., 2008).

Other plant hormones, such as ABA or cytokinins can also positively regulate stomata density in various plant species (reviewed in (Martin and Glover, 2007; Casson and Gray, 2008). In hypocotyls plant hormones enhance stomata formation by promoting cell division in the non-protruding cell files but none of them induces the ectopic stomata formation in protruding cell files (Saibo et al., 2003; Casson and Gray, 2008).

Plant hormones and their precursors may act as long-distance mobile signals (Casson and Gray, 2008). Plant hormones may transduce the environmental signals, e.g. from older leaves to developing leaves, to regulate stomata initiation (Lake et al., 2001).

Environmental signals

The majority of environmental signals determine the overall stomata density (Sachs, 1991), which is calculated as stomata index (ratio of stomata to all epidermal cells). UV-B irradiation, CO_2, shading and low water content decreases stomata index, whereas increased

light intensity increases stomata index (reviewed in (Hetherington and Woodward, 2003; Casson and Gray, 2008). High humidity induces stomata clustering (Serna and Fenoll, 1997). It is suggested that CO_2 affects the pre-patterning of epidermal cells, whereas high humidity affects the patterning within the stomata lineage (Martin and Glover, 2007). CO_2 and shading can generate long-distance signals from mature to young leaves in stomata development (Lake et al., 2001).

Environmental signals are perceived at the epidermis. The cuticle, which covers and connects the epidermal cells, may be involved in the perception and transmission of signals that regulate stomatal density, distribution or function (Bird and Gray, 2003). Differences in the cuticle wax profile may alter the permeability of signaling compounds. It is also possible that products of wax biosynthesis themselves act as signaling compounds regulating epidermal cell fate (Casson and Gray, 2008). In general, a decrease in cuticle wax content increases stomata density (Gray et al., 2000). Mutations in Arabidopsis genes encoding for the long-chain fatty acids-producing enzymes *CER1* and *CER6* reduce wax accumulation on leaves and lead to highly increased stomata indices and clustered stomata (Gray et al., 2000). HIGH CARBON DIOXIDE (HIC) shares high homology to 3-ketoacyl CoA synthetase, which is involved in the synthesis of long chain fatty acids of epicuticular waxes. *HIC* is expressed only in guard cells and regulates stomata density in response to elevated CO_2. Arabidopsis *hic* mutant shows significantly increased stomata index in response to CO_2, while the wild type plants have slightly decreased stomata index in these conditions (Gray et al., 2000). This suggests that elevated CO_2-induced signaling in guard cells affects the differentiation of satellite meristemoids (Gray et al., 2000; Martin and Glover, 2007). The gain-of-function mutant of AP2/EREBP transcription factor *SHINE*, which activates wax biosynthesis, has reduced stomata index (Aharoni et al., 2004). *sdd1* plants, which have five-fold reduced level of C16 exhibit 3 – 4 - fold higher stomatal densities at ambient CO_2 demonstrating that unsaturated C16 fatty acids are important for stomata density (Fiehn et al., 2000). By contrast, mutants of WAX2, a gene required for the integrity of the internal cuticle layer have reduced stomata density (Chen et al., 2003).

1.1.2. Cell cycle control of stomata development

Cell growth and cell divisions are tightly coordinated with cell differentiation in the stomata development pathway. The cell identity in the epidermis is correlated with nuclear DNA content, as trichomes and pavement cells undergo rounds of endoreduplication, whereas guard

cells and their precursors remain diploid (Melaragno et al., 1993). The modification of endoreduplication level and cell division rate alter the cell size and number, however, multiple studies demonstrate that the ploidy level itself does not confer stomata development (De Veylder et al., 2001; Dewitte et al., 2003; Boudolf et al., 2004a; Kono et al., 2007).

A reduced level of endoreduplication and cell division rate leads to increased cell size and decreased cell number (De Veylder et al., 2001). Increased endoreduplication levels result in more but smaller cells in the epidermis (Dewitte et al., 2003). In hypocotyls, the over-expression of *E2F-DPa* or downregulation of *RETINOBLASTOMA-RELATED (RBR)* leads to a massive over-proliferation of small cells in the files that normally develop stomata, but the number of stomata is not affected (De Veylder et al., 2002; Desvoyes et al., 2006). Later in development, the cell-file restriction is lost and the *E2Fa–DPa* expression promotes divisions in all hypocotyl cell files (De Veylder et al., 2002). Therefore, Bergmann (2004) suggested that a pre-pattern in the cell files of hypocotyl influences cell ability to respond to mitogenic cues (Bergmann, 2004). Similarly, the modulation of endoreduplication levels with the cell cycle inhibitor KIP RELATED PROTEIN (KRP) or the cell cycle activator CYCD3;1 did not alter stomata index (De Veylder et al., 2001; Dewitte et al., 2003).

In contrast, the the over-expression of Arabidopsis DNA replication licensing factors *CDC6* and *CDT1* increases the density of stomata in Arabidopsis leaves (Castellano Mdel et al., 2004).

Several plant-specific cell cycle genes are involved in stomata development (as reviewed below). Although cell cycle genes and their functions are highly conserved among species, it is not surprising that some plant-specific cyclins and cyclin dependent kinases (CDKs) function in stomata development which is specific only for land plants.

The role of CDKB1;1 in stomata development

Boudolf (2004) suggested that Arabidopsis CDKB1;1 has an essential function in stomata development. The CDKB1;1 gene belongs to the unique B-type CDKs with the peak of activity at the G2/M boundary (Boudolf et al., 2004a). The transcription of CDKB1;1 is controlled by the E2F pathway and may be a part of cross talk mechanism between the G1/S and G2/M transition points (Boudolf et al., 2004b). CDKB1;1 is highly expressed in meristemoids, guard mother cells and guard cells of cotyledons (Boudolf et al., 2004a).

Reduction of CDKB1;1 activity due to a dominant negative mutation (*CDKB1;1.N161*) results in decreased stomata index in transgenic plants and aberrant unpaired guard cells that

lack stomata pores. The reason of decreased stomatal index in *CDKB1;1.N161* seedlings is the early inhibition of amplifying meristemoid divisions and satellite meristemoid formation (Boudolf et al., 2004a). The aberant GCs contain 4C nucleus whereas normal stomata contain a nucleus with 2C (Melaragno et al., 1993), indicating that aberrant GCs are blocked in the G2 phase (Boudolf et al., 2004b).

However, the activity of CDKB1;1 is not required for stomata differentiation. In *CDKB1;1.N161* seedlings divisions of stomatal precursors are inhibited but the cells still acquire stomata identity, indicating that stomata cell differentiation is uncoupled of cell division (Boudolf et al., 2004a).

The role of CYCD4 in stomata development

Other cell cycle regulators of Arabidopsis, such as cyclin D-type, CYCD4;1 and CYCD4;2, were proposed to control cell division in the stomatal lineage, specifically in the hypocotyls (Kono et al., 2007). The binding of specific cyclins to CDKs controls the CDK activity and substrate specificity (Morgan, 1997). An active cyclinD/CDK complex promotes cell cycle progression from G1 to S phase and thus promotes cell division. (de Jager et al., 2001). Both CYCD4;1 and CYCD4;2 form active kinase complexes with CDKA;1, which is the ortholog of yeast Cdc2/Cdc28p. Only CYCD4;1 can bind and activate a plant-specific CDKB2;1, which is expressed from the G2 to the M phase (Kono et al., 2003; Kono et al., 2006)., CYCD4;2 lacks exclusively the Rb binding motif (Kono et al., 2006), but is still functional as a cyclin. This suggests that CYCD4;2 may promote the divisions of stomata precursors in the hypocotyl independently of the Rb/E2F/DP pathway or CYCD4;2 may control specific transcription factors via CDK-independent mechanisms (Kono et al., 2007).

CYCD4;1 or *CYCD4;2* loss-of-function genes significantly reduce cell division in the upper region of non-protruding cell files of the hypocotyls where stomata are produced exclusively under normal conditions (Berger et al., 1998). Consistently, the number of stomata in hypocotyls is significantly reduced in *cycd4* mutants. Nevertheless, stomata differentiation was not affected either in single or in double *CYCD4* mutants (Kono et al., 2007).

Over-expression of *CYCD4* considerably increases the number of nonprotruding cells of stomatal lineage in seedlings and of stomata in the upper hypocotyl region, but it only slightly enhances the cell division in the protruding cell files. CYCD4 functions most probably early in stomatal pathway promoting the division of MMC precursors and thus amplifying the MMC population (Kono et al., 2007).

Taken together, CYCD4 plays a specific role in promoting cell divisions of MMC precursors in the nonprotruding cell files in hypocotyls. However CYCD4 seems not to be involved in the asymmetric divisions of MMCs or in GC and stomatal differentiation (Kono et al., 2007).

1.1.3. Transcription factors act as essential positive cell fate determinants of stomata development

Five basic helix–loop–helix (bHLH) proteins SPEECHLESS (SPCH), MUTE, FAMA, SCREAM/ICE1 and its paralog SCREAM2 have been identified as essential positive stomatal development regulators that successive direct cell-fate decisions (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007; Pillitteri and Torii, 2007; Kanaoka et al., 2008).
In addition, the MADS-box transcription factor AGAMOUS-like (AGL) 16 is a positive regulator of stomata development (Kutter et al., 2007). AGL16, which is regulated by microRNA-mediated decay, controls the formation of satellite meristemoids (Kutter et al., 2007).

SPCH, MUTE and FAMA direct cell fate decisions in stomata development

SPCH, MUTE and FAMA are essential positive regulators during successive steps of stomata development. (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007; Pillitteri and Torii, 2007).
SPCH is necessary and sufficient for the entry asymmetric divisions to establish the stomatal lineage (MacAlister et al., 2007; Pillitteri et al., 2007). SPCH may also promote the amplifying and spacing asymmetric divisions in stomata development (MacAlister et al., 2007).
MUTE is the key switch for meristemoid fate transition to GMCs (MacAlister et al., 2007; Pillitteri et al., 2007). The *MUTE* gene expression and protein localisation in meristemoids just before GMC differentiation suggests, that plants are able to track the rounds of asymmetric division and subsequently induce *MUTE*, which then terminates meristemoid stem-cell-like properties (Pillitteri et al., 2007).
FAMA is a key switch between cell division and terminal differentiation of stomatal lineage to GCs. FAMA restricts proliferative divisions at the end of stomatal lineage and promotes the differentiation of GCs (Ohashi-Ito and Bergmann, 2006). FAMA might be able to limit

cell division by directly regulating cell cycle genes. In *fama* mutants GMC-like cell files express *CDKB1;1,* suggesting that FAMA may negatively regulate *CDKB1;1* expression (Ohashi-Ito and Bergmann, 2006). Normally *CDKB1;1* is expressed exclusively within the stomatal lineage and is downregulated when epidermal cells cease to divide (Boudolf, 2004). In addition, both the ectopic expression of *FAMA* and the expression of dominant negative form of *CDKB1;1* cause similar unpaired GC over-production phenotypes (Boudolf et al., 2004a; Ohashi-Ito and Bergmann, 2006).

The *spch, mute* and *fama* mutants completely lack stomata. In addition no asymmetric entry division occurs in *spch* mutants and no stomata-lineage cells are produced in strong alleles (MacAlister et al., 2007; Pillitteri et al., 2007). In *mute* loss-of-function mutants the meristemoids undergo 3 – 6 rounds of excessive asymmetric divisions (normally in wild type plants meristemoids may divide 1-3 times asymmetrically) and finally are arrested as small triangular cells surrounded by excess of stomata lineage ground cells (Pillitteri et al., 2007). *fama1-1* loss-of-function mutant makes files of undifferentiated GMC-like cells(Bergmann et al., 2004). This phenotype resembles the phenotype of *flp-7* mutant of MYB transcription factor *FLP* (Lai et al., 2005). The phenotype of *fama flp* double mutant suggests that FAMA acts prior to FPL or that it is required for broader range of activities (Ohashi-Ito and Bergmann, 2006).

The over-expression of *SPCH*, *MUTE* and *FAMA* leads to diverse stomata phenotypes. Over-expression of *SPCH* induces ectopic divisions in apparently differentiated pavement cells but no extra stomata are produced (Pillitteri et al., 2007). However, the expression of *SPCH* from its native promoter results in additional asymmetric divisions and stomata clustering (MacAlister et al., 2007). Constitutive over-expression of *MUTE* leads to the conversion of all epidermal cell to stomata (MacAlister et al., 2007; Pillitteri et al., 2007). In case of a less severe phenotype, a subset of epidermal pavement cells may express characteristics of both pavement and guard cells, e.g. may adopt partial guard cell identity (containing chloroplasts, express the mature guard cell marker and produce a symmetric division plane with a “faux pore”) (MacAlister et al., 2007; Pillitteri et al., 2007). The over-expression of *FAMA* leads to the formation of unpaired GC clusters. These GCs make stomatal pores and express mature GC markers. Moreover, *FAMA* over-expression is able to transdifferentiate root epidermal cells and non-epidermal cells of mesophyll to GCs. Notably, the over-expression of *FAMA* can also force GMCs transition to GCs without undergoing normal symmetrical cell division (Ohashi-Ito and Bergmann, 2006).

The expression and protein activity of SPCH and FAMA transcription factors may be regulated by a MAPK pathway (Bergmann et al., 2004; Lampard et al., 2008). Modifications of the potential MAPK phosphorylation target domain (MPKTD) in SPCH modulate SPCH function in stomatal development by repressing or activating SPCH in a quantitative manner (Lampard et al., 2008). *FAMA* transcript is down-regulated in Δ*N-YDA* (MAPKKK) no-stomata seedlings and up-regulated in *yda* seedlings that contain clustered stomata (Bergmann et al., 2004).

SCREAM/ICE1 and SCREAM2 specify the actions of SPCH, MUTE and FAMA

SCREAM/ICE1 (SCRM) and SCREAM2 (SCRM2) are two paralogous proteins, which specify the actions of SPCH, MUTE, and FAMA in succession from the initiation of stomatal lineage in protodermal layer to the differentiation of guard cells (Kanaoka et al., 2008). SCRM is also identified as the INDUCER OF CBF EXPRESSION1 (ICE1), an upstream key transcriptional activator of cold-induced transcriptome and freezing tolerance (Chinnusamy et al., 2003).

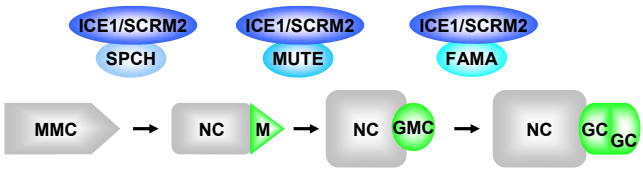

Figure 1.2. bHLH transcription factors direct the successive cell-fate decisions during stomata development (schematic representation). SPCH directs the asymmetric division into stomatal cell lineage, whereas MUTE terminates asymmetrically dividing meristemoids and triggers transition from meristemoid to GMC (Pillitteri et al., 2007). FAMA terminates symmetric divisions and promotes guard cell differentiation (Ohashi-Ito and Bergmann, 2006). SCRM/ICE1 and SCRM2 heterodimerize with SPCH, MUTE and FAMA and govern the successive steps during stomata development. The action of functionally redundant SCRM and SCRM2 is probably gene dosage dependent (Kanaoka et al., 2008). MMC – meristemoid mother cell, NC – neighbour cell, M – meristemoid, GMC – guard mother cell, GC – guard cell.

SCRM/ICE1 and *SCRM2* are broadly expressed within the stomatal cell lineage and are able to heterodimerize. They guide the sequential actions of SPCH, MUTE, and FAMA, which are expressed transiently in specific precursor cells for successive initiation, proliferation, and terminal differentiation of stomatal cell lineages. The gradual loss of *SCRM* and *SCRM2* recapitulate the phenotypes of *fama, mute,* and *spch* (Kanaoka et al., 2008).

MYB transcription factors FPL (FOUR LIPS) and MYB88 are late-acting genes in stomata development

FLP and its paralogous gene MYB88 encode two-repeat (R2R3) MYB proteins that are probable transcription activators and possess overlapping functions in stomata development (Lai et al., 2005). *FLP* is expressed in late-stage GMCs and in young, still differentiating GCs. The FPL protein accumulates just before the symmetric division (Lai et al., 2005). FLP controls the timely transition from symmetric cell division (GMC identity) to the terminal stomata differentiation (GC identity). *flp-1* mutation results in paired stomata clusters, which arise after one or more parallel divisions of GMC daughter cells. *FPL* loss-of-function causes GMC daughter cells to retain GMC identity and delays their differentiation to GCs (Lai et al., 2005).

FLP/MYB88 may function to limit division directly by regulating cell cycle genes or indirectly by promoting a transition in cell fate. Normally, GCs are probably arrested in G1 or exit the cell cycle (G0) because they do not divide and they retain a 2C level of DNA (Melaragno et al., 1993). Therefore, FLP target(s) might be a cell cycle regulator(s) expressed just before the symmetric GMC division. This target could be. CDKB1;1 (Lai et al., 2005), which is expressed in GMCs/GCs, and is required for GMC mitosis (Boudolf et al., 2004a).

1.1.4. Signal transduction components as negative regulators of stomata development

Signal production, its perception by receptor-like proteins and receptor kinases, and signal transduction via a MAPK cascade inhibits the stomata development pathway (Yang and Sack, 1995; Berger and Altmann, 2000; Bergmann et al., 2004; Shpak et al., 2005; Wang et al., 2007b).

All stomata development regulators are required for normal stomatal patterning, although genes earlier acting in the pathway, such as *SDD1 (STOMATA DENSITY DISTRIBUTION)*, *TMM (TOO MANY MOUTHS)*, *ER (ERECTA)* family and *MAPKs* are the key regulators of stomata spacing via asymmetric cell divisions. The late-acting *FLP (FOUR LIPS)/ MYB88* regulates spacing indirectly by restricting symmetric cell divisions at the end of the stomata cell lineage. Mutations in all these genes lead to increased stomata density and stomata clustering (Fig. 2)(Yang and Sack, 1995; Berger and Altmann, 2000; Bergmann et al., 2004; Lai et al., 2005; Shpak et al., 2005; Wang et al., 2007b)**.**

SDD1 and TMM are components of cell-to-cell signaling in stomata development

The *SDD1* gene encodes an extra-cellular subtilisin-like serine protease, which might cleave a yet unidentified signaling molecule precursor to produce a mature signal (Berger and Altmann, 2000) which is received by the LRR receptor-like protein TMM (Von Groll et al., 2002). SDD1, which is expressed in meristemoids and guard mother cells, might regulate the developmental fate of neighboring cells (Von Groll et al., 2002). TMM is localized to the cell membrane of stomata lineage ground cells and stomata precursors, the meristemoids. The similarity of TMM to CLAVATA2 receptor-like protein suggests that TMM dimerize with a receptor-like kinase (RLK), thereby transducing the signal leading to the restriction of stomata development (Larkin et al., 2003).

ER-family proteins were suggested as candidates for TMM co-receptor RLKs (reviewed in (Nadeau, 2009). ER and ER-like proteins, ERL1 and ERL2, negatively regulate stomatal development (Shpak et al., 2005). ER, ERL1, and ERL2 together control the initial decision of protodermal cells to enter asymmetric division to generate stomatal complexes. ERL1 and ERL2 might promote asymmetric division of the meristemoid and delay meristemoid differentiation into guard mother cells. Thus, ERL1 and ERL2 maintain the activity of stomata stem cells (Shpak et al., 2005).

However, the relationship between TMM and ER-family appears to be very complex. Shpak (2005) argues that TMM negatively regulates specific ER-family members by receptor dimerization with ER-family RLKs (most likely with ERL1). Alternatively, TMM may titrate the same ligands as does the ER family (Shpak et al., 2005). This complex interaction of TMM and ER-family genes suggests that their products are not just upstream or downstream of each other but work combinatorial to determine stomatal-lineage cell fate.

A MAP kinase cascade negatively regulates stomata development

YODA MAPKKK, MKK4 and MKK5, MPK3 and MPK6 constitute the signal transduction cascade that regulates stomata development (Bergmann et al., 2004; Wang et al., 2007b). YODA acts as a cell fate switch in stomatal development downstream of *SDD1* and *TMM* or in an independent pathway. The loss-of-function *yda* mutations lead to stomata cell clusters. Constitutive activation of YDA results in complete lack of guard cells in homozygous *ΔN-YDA* plants It suggests that YODA activity must be down-regulated to allow epidermal cells to enter the stomata lineage (Bergmann et al., 2004).

Downstream of YODA, the signal is tranduced by the MKK4/5-MPK3/6 signaling module (Wang et al., 2007b). Single *mpk3* and *mpk6* loss-of-function mutants do not have obvious developmental phenotypes. Silencing of *MPK3* by RNAi in *mpk6* mutant and embryonically-rescued *mpk3 mpk6* loss-of-function mutants show dramatic stomata development and patterning defects. Similarly, silencing of *MKK4* or *MKK5* by RNAi displays weak stomatal patterning defects (frequently 2 - 3 clustered stomata), but simultaneous silencing of both *MKK4 and MKK5* results in excessive stomata clustering. In some cases the entire epidermis of the cotyledons may consist of stomata. The seedlings with stomata defects do not survive beyond the cotyledon stage (Wang et al., 2007b). On the opposite, the activation of MKK4/5-MPK3/6 results in epidermis composed solely of pavement cells (Wang et al., 2007b).

Taken together, the main functions of MKK4/5-MPK3/6 in stomata development are to restrict asymmetric cell division frequency, to maintain the polarity of the asymmetric cell division of stomata lineage cells, to coordinate cell fate specification between daughter cells and to suppress stomata cell fate specification (Wang et al., 2007b). Moreover, the MAPKs may directly regulate positively acting transcription factors of stomata development. The stomata-initiating transcription factor SPCH contains a novel MAPK phosphorylation target domain (MPKTD), which modulates SPCH function in stomata development by repressing or activating SPCH in a quantitative manner (Lampard et al., 2008).

The functions of the MAPK signaling cascade may be linked with cell cycle progression or stress-response pathways (Bergmann, 2004; Wang et al., 2007b). The MKK4/5-MPK3/6 module is activated in both the stomata development and the stress signal transduction pathways. Therefore, this module may integrate environmental and stomata-developmental signals (Wang et al., 2007b). In addition, the mutations in *yda* and *mpk3 mpk6* confer impaired development of extra-embryonic cells and plant fertility suggesting broader range of their actions in plant development (Bergmann et al., 2004; Lukowitz et al., 2004; Wang et al., 2008).

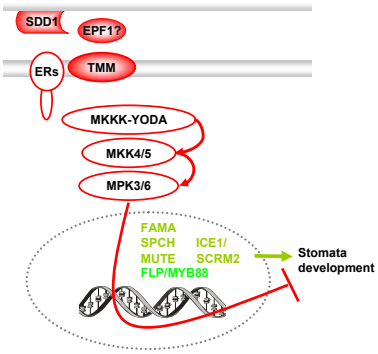

Figure 1.3. Schematic representation of cell-to-cell signaling and signal transduction in stomata development.

1.2. Protein phosphatases

Protein phosphatases are enzymes reversing the action of phosphorylation performed by protein kinases., Protein phosphatases are classified according to their specificity towards phosphorylated amino acid residues as Tyr phosphatases (PTP family) and Ser/Thr phosphatases (PPP, PPM and FCP families) (Barford et al., 1998,Cohen, 2003 #11). The catalytic domains are highly conserved within each protein phosphatase family and functional diversity is achieved through the variable N- or C-terminal extensions, regulatory domains and/or interacting subunits (Barford et al., 1998).

PTP phosphatases perform dephosphorylation reaction via the transient intermediate cysteinyl-phosphate which is further hydrolysed using an activated water molecule (Barford et al., 1994; Barford et al., 1998). Tyr phosphatases can dephosphorylate either only tyrosine or additionally also Ser/Thr amino acid residues, therefore classified into Tyr-specific and dual specificity (DSP) phosphatases.

Ser/Thr phosphatases are classified according to the structure of their catalytic domain (Table 1) (Barford et al., 1998; Cohen, 2003) and these enzymes dephosphorylate substrates in a single reaction step using a metal-activated nucleophilic water molecule (Barford et al., 1998). Many of the protein Ser/Thr phosphatases (PP1, PP2A, PP3, PP4, PP6 subfamilies) are high molecular mass heteromers consisting of a catalytic subunit and one or more regulatory subunits (Cohen, 2003).Other Ser/Thr phosphatases contain N- or C-terminal non-catalytic extensions functioning in the recognition and binding of regulatory subunit(s) or substrates (reviewed in (Barford et al., 1998; Schweighofer and Meskiene, 2008a). Ser/Thr phosphatases require the metal ion for the catalysis (Fe^{3+} and Zn^{2+} for most of PPPs, Ca^{2+} for PP2B and Mn^{2+} or Mg^{2+} for PPM, (reviewed in (Barford et al., 1998; Cohen, 2003).

Amoung the Ser/Thr phosphatases the PPM family phosphatases are acting as monomers (except mitochondrial pyruvate dehydrogenase which is a heterodimer consisting of catalytic subunit Pdpc and regulatory subunit). The PPM family contains PP2C and PP2C-like proteins (Das et al., 1996; Barford et al., 1998). PP2C type phosphatases are insensitive to okadaic acid (Barford et al., 1998), which specifically inhibits PP1 and PP2A (Lawson, 1993) and weakly inhibits PP2B. PP2C type phosphatases perform diverse functions, including regulation of cell cycle and MAP kinase pathways (see overviewed below).

Table 1.1. Nomenclature of eukaryotic protein phosphatases: structurally distinct gene families (according to (Barford et al., 1998; Cohen, 2003)

	Family	Subfamily	Type	Catalytic domain	Regulatory domains/ subunits *
Ser/Thr phosphatases	PPP	PPP1	PP1	Ppp1c	Various regulatory and targeting subunits
		PPP2/4/6 (PP2A)	PP2A	Ppp2c	A (65 kDa) and B subunit (>15)
			PP4		
			PP6		
		PPP3 (Calcineurin/ PP2B)	PP2B	Ppp3c	Calmodulin binding site and autoinhibitory sequence in C-terminal domain of A subunit; B subunit
		PPP5/7	PP5	Ppp5c	3 x TPR domain in N-terminus
			PP7	Ppp7c/ EF/ calmodulin BM	
	PPM		PP2C	PP2Cc	
			PDP	Pdpc	
	FCP		TFIIF-stimulated CTD phosphatase1		
			Small CTD phosphatases		
Tyr and Ser/Thr /Tyr	PTP	Tyr-specific	PTP1B		
			SH-PTP		SH2 domain
			RPTP		
		DSP			

* beside the non-catalytic N-and C-termini of the catalytic domain, which are responsible for recognition and binding of regulatory subunit(s)

1.2.1. Protein phosphatases type 2C

PP2Cs are monomeric enzymes, depending on divalent metal ions (Mn^{2+} or Mg^{2+}), and are insensitive to the inhibitor okadaic acid (Das et al., 1996; Barford et al., 1998). So far no specific inhibitors against PP2Cs have been identified. PP2Cs share no sequence similarity with other Ser/Thr phosphatases (PPP1, PP2A and PP2B subfamilies) but have similar protein 3D structure and catalytic mechanisms. Two Mn^{2+} ions in the catalytic site facilitate a water molecule to act as nucleophile to initiate hydrolysis of the phosphomonoester bond (Das et al., 1996). Aspartatic acid residues are crucial for binding a divalent metal ion in the catalytic center of the PP2C (Barford et al., 1998). PP2C type phosphatases consist of conserved catalytic domain and variable N-terminal and/or C-terminal domains. The non-catalytic domains may contain localization signals, interaction domains or docking sites and account for the versatility of PP2Cs (Cohen, 2003; Schweighofer et al., 2004). PP2C gene members

include 7 genes in yeast, 8 in worm, 10 in fly and 76 in Arabidopsis (Kerk et al., 2002; Schweighofer et al., 2004).

Mammalian PP2Cs include 18 members that are implicated in stress signalling cascades, PI3 kinase/Akt signalling, pre-mRNA splicing, protein ubiquitination and degradation, cell metabolism, cell death/survival signaling (Table 2, reviewed in (Lu and Wang, 2008).

PP2Cα is the best studied member of PP2C family in mammals. The numerous substrates of PP2Cα include p38 MAPK, AMPk (AMP-activated protein kinase), PI3-K, Axin, Smad2 and Smad2, Cdk2, mGluR3 (metabotropic glutamate receptor type 3), 3-hydroxy-3-methylglutaryl coenzyme A reductase, MKK3b, MKK4 and MKK7 (reviewed in (Lu and Wang, 2008). PP2Cα and PP2Cβ dephosphorylate BAD and promote apoptosis and degeneration of neurons (Klumpp et al., 2002; Klumpp et al., 2004).

Human PP2Cs are important regulators of PI3-K signaling: PP2Cα, PHLPP1 and 2 can specifically target components of PI3-K/Akt pathway and modulate cell growth, differentiation and survival. PP2Cα modulate lipid kinase activity of PI3-K by regulating the phosphorylation of the PI3-K-activating p85. PHLPP1 and PHLPP2 regulate the activity of Akt isoforms and PI3-K (reviewed in Lu and Wang, 2008).

Human PP2C-like phosphatases POPX1 and POPX2 dephosphorylate PAK (the p21(Cdc42/Rac)-activated kinase) *in vitro* and blocks *in vivo* the phenotypic effects of constitutively active PAK (which promotes loss of stress fibers and eventual contraction of the cells (Zhao, 1998)). POPX can inhibit actin stress fiber brakedown and morphological changes driven by active $Cdc42^{V12}$. POPX, PAK and PIX (PAK interacting guanidine excahnge factor) form a trimeric complex *in vitro* (Koh et al., 2002).

Table 1.2. Functional diversity of mammalian PP2C isoforms (reviewed in (Lu and Wang, 2008)

Signaling pathway/substrates*	PP2C	Process	Cell compartment
Ras/PI3-K/Akt	PP2Cα, PHLPP1, PHLPP2	Cell growth, differentiation, survival	Cell membrane
TAK1/ASK1/MKK/p38/JNK	PP2Cα/β, PP2Cε, PP2Cδ/ILKAP	Stress induced cell death/survival	Cytoplasm
ATM/ATR/Chk1&2/p53/p38	PP2Cε/ PP2Cδ (PPM1D/ Wip1)	DNA damage, cell cycle	Nucleus
H2AX/H2B	PP2Cγ	DNA damage	Nucleus
PAK	POPX1, POPX2	Actin stress fiber brakedown	
ILK1/GSK3β/ß-catenin/Tcf/Lef	PP2Cδ/ILKAP	Cell survival/ proliferation	

*ASK1 - apoptosis signal-regulating kinase 1; TAK1 - transforming growth factor-β-activated kinase 1; PAK - the p21(Cdc42/Rac)-activated kinase;

1.2.2. PP2C–type MAPK phosphatases

MAP kinases constitute signalling cascades consisting of MAP kinases and upstream activating MAPK kinase and MAPKK kinases (Seger and Krebs, 1995; Kovtun et al., 2000; Mizoguchi et al., 2000). MAPKs are activated by phosphorylation of Thr and Tyr amino acid residues in the T-loop and can be translocated upon activation into the nucleus (Su and Karin, 1996; Ahlfors et al., 2004). Dephosphorylation of one or both residues is crucial for kinase activity control and can be performed by Ser/Thr phosphatases or PTPs.

PP2Cs downregulating MAPK cascades in stress signaling were identified in budding yeast, plants and mammals (Shiozaki et al., 1994; Maeda et al., 1995; Hanada et al., 1998; Meskiene et al., 1998; Takekawa et al., 1998; Meskiene et al., 2003; Schweighofer et al., 2007). *S.cerevisiae* Ptc1p, Ptc2p and Ptc3p and their orthologues in *S. pombe* negatively regulate osmosensing MAPK cascades (Warmka et al., 2001; Young et al., 2002). *S.pombe* Ptc1 and Ptc3 dephosphorylate Thr in the activation loop of the stress-responsive Spc1 MAPK (a homolog of human p38). Ptc1 is induced by stress and its expression depends on Spc1 activation (Gaits et al., 1997). Ptc3 is expressed constitutively; it also dephosphorylates Spc1 and attenuates Spc1 activity. Ptc1 and Ptc3 act in Spc1 inactivation, when Pyp1 (the major Spc1-dephosphorylating PTP) is inhibited in heat-shocked cells (Nguyen and Shiozaki, 1999).

Mammalian PP2Cα and PP2Cβ dephosphorylate and suppress the stress-activated (such as anisomycin, UV, NaCl) and TNFα-activated p38/SAPK2A and JNK MAPK signalling (Hanada et al., 1998; Takekawa et al., 1998; Hanada et al., 2001). PP2Cα and PP2Cβ also inhibit both p38 and JNK pathways by dephosphorylation of upstream MAPK cascade components: MKK3b, MKK4, MKK6b and MKK7 (Takekawa et al., 1998; Fjeld and Denu, 1999). As PP2Cα is evenly localised to the cell nucleus and cytoplasm, it was suggested that it maintains low MAPK activities in the absence of external stimuli (Takekawa et al., 2000).

In response to IL-1 the functionally redundant PP2Cβ and PP2Cε dephosphorylate the MAPKKK TAK1 (transforming growth factor-β-activated kinase 1). TAK1 is essential for the activation of NF-κB, IKK and JNK pathways in response to pro-inflammatory cytokines and microbial pathogens during innate immune response mediated by several Toll-like receptors (TLRs). Thus, PP2Cß and PP2Cε downregulate the TAK1 signaling in innate immunity (reviewed in (Lu and Wang, 2008).

In non-stress conditions PP2Cε negatively regulates the MAPKKK ASK1 (apoptosis signal-regulating kinase 1), which is a component of the SAPK system. SAPKs play key roles in ROS-induced activation of both proliferation and apoptosis. ASK1 mediates ROS- and TNFα-

induced apoptosis. PP2Cε inactivates ASK1 by suppressing H_2O_2-enhanced phosphorylation of ASK1 (reviewed in (Lu and Wang, 2008).
In contrast to PP2Cε, PP2Cδ/ILKAP activates ASK1 by enhancing its T phosphorylation. Therefore, PP2Cδ/ILKAP might act as a positive regulator of TNFα-induced apoptosis (Trinkle-Mulcahy and Lamond, 2006).

1.2.3. Function of PP2Cs in cell cycle progression

Some of the PP2C-type MAPK phosphatases are involved in the cell cycle control. Ptc2p and Ptc3p dephosphorylate CDC28, the major budding yeast cyclin-dependent protein kinase (CDK) at Thr 169 (Cheng et al., 1999). Activating phosphorylation of CDKs is necessary for kinase activity and cell cycle progression (Morgan, 1995). The Ptc2p and Ptc3p homologues PP2Cα and PP2Cβ(?) in Xenopus (Cohen, 2003) dephosphorylate Thr-161 residue of monomeric (but not the cyclin-bound) cyclin dependent kinase Cdc2 (De Smedt et al., 2002). Thr 161 phosphorylation is required for the activation of Cdc2 (De Smedt et al., 2002). Human PP2Cα and PP2Cβ2 also dephosphorylate monomeric human Cdk2/Cdk6 *in vitro* (Cheng et al., 2000). In HeLa cell extracts PP2C-like enzymes are the predominant phosphatases toward Cdk2, suggesting a evolutionarily conserved specificity of PP2Cs towards CDKs (Cheng et al., 2000).
PP2Cδ/ILKAP inhibits integrin-linked kinase ILK1 and therefore suppresses GSK3β-mediated integrin-ILK1 signaling (Leung-Hagesteijn et al., 2001), reviewed in (Trinkle-Mulcahy and Lamond, 2006). ILK1 induces an inhibitory phosphorylation of GSK3β in epithelial cells (Tan and Kim, 1999). This action results in stabilization and nuclear translocation of β-catenin followed by subsequent activation of transcription factors Tcf/Lef supporting cell survival/proliferation (Novak et al., 1998; Dedhar et al., 1999; Novak and Dedhar, 1999). Failure to down-regulate ILK1-mediated signalling can lead to oncogenic transformation (Hannigan et al., 1996; Hannigan et al., 2005). Overexpression of PP2Cδ/ILKAP activity leads to the inhibition of cell cycle progression. On the contrary, PP2Cα stimulates β-catenin–Lef signalling and cell proliferation (Strovel et al., 2000).

Wip1 – DNA damage checkpoint phosphatase

PP2Cs have essential functions in the maintenance of genomic stability (Lu and Wang, 2008). PP2Cs in budding yeast have critical function in inactivating cell cycle arrest after DNA

damage. Mammalian PP2Cγ (along with PP2A) regulates the exchange of histones H2A and H2B. Histone H2AX is also phosphorylated in response to DNA damage and its dephosphorylation is essential for complete recovery from DNA damage (reviewed in (Lu and Wang, 2008).

Mammalian PP2Cδ isoform (PPM1D/Wip1) is involved in multiple levels of DNA damage response. Wip1 specifically dephosphorylates essential components of DNA damage response signaling including ATM, the DNA repair enzyme uracil DNA glycosylase UNG2, the MAPK p38, the tumor suppressor p53 and the DNA damage-induced kinases Chk1 and Chk2 (reviewed in (Lu and Wang, 2008).

The early response to DNA damage leads to the activation of p38 and JNK MAPK cascades (Kyriakis and Avruch, 1996b, a) and stabilization of tumor suppressor p53 (Ko and Prives, 1996).

The ATM/ATR pathway stabilizes p53 by Ser15 phosphorylation (reviewed in (Meek, 1998a, b; Prives and Hall, 1999; Caspari, 2000). p38 MAPK activated by genotoxic stress phosphorylates Ser33 and Ser46 of p53 (Bulavin et al., 1999; Huang et al., 1999; Bulavin and Fornace, 2004), both residues are essential for p53 activation (Bulavin et al., 1999). Wip1 gene expression induced by γ or UV radiation is p53-dependent (Fiscella et al., 1997). Moreover, this p53-dependent gene expression of Wip1 is E2F1-regulated (Hershko et al., 2006). Wip1 dephosphorylates both Ser33 and Ser46 of p53 and selectively dephosphorylates p38 MAPK at the conserved Thr 180 residue in the nucleus (Fiscella et al., 1997; Takekawa et al., 2000). Therefore, Wip1 promotes degradation of p53 either by direct dephosphorylation or through the inactivation of p38 kinase. Wip1 mediates a negative feedback regulation of p38/p53 signaling during the recovery phase of the damaged cells and contributes to the suppression of UV-induced apoptosis (Bulavin et al., 1999; Takekawa et al., 2000).

Wip1 attenuates DNA damage-induced base excision repair by dephosphorylation of uracil DNA glycosylase (UNG2).

Wip1 is established as an oncogene and is amplified/overexpressed in many human tumors (Bulavin et al., 2002; Li et al., 2002; Hirasawa et al., 2003; Saito-Ohara et al., 2003; Bulavin et al., 2004). The inactivation or depletion of Wip1 in *Ppm1d*-null mouse embryo fibroblasts (MEFs) results in the activation of p38 MAPK and resistance to tumor transformation by modulating the *Cdkn2a* tumor-suppressor locus and by activation of the tumor-suppressor pathways (Bulavin and Fornace, 2004).

Wip1 acts as a negative feedback regulator of E2F1-induced apoptosis (Hershko et al., 2006). E2F up-regulates the levels of the MAPKKK ASK1 (Muller et al., 2001; Stanelle et al., 2002)

which subsequently leads to the activation of p38. The transient fashion of p38 phosphorylation/activation is tightly controlled by the following induction of Wip1 expression by E2F1 (Hershko et al., 2006). E2F1 also positively regulates other Wip1 substrates. E2F1 induces stabilizing phosphorylations of p53 (Hershko et al., 2006) .22, 32, 40–43). E2F1 up-regulates the expression of uracil DNA glycosylases (Hershko et al., 2006) and the damage-induced kinase *Chk1* (Hershko et al., 2006). Thus, Wip1 may be a part of a common negative feedback regulation of E2F–induced proteins (Hershko et al., 2006).

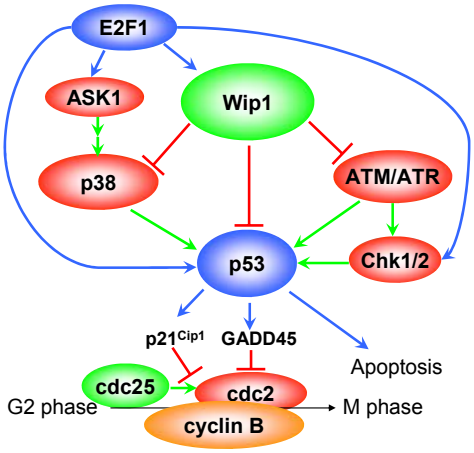

Figure 1.4. Schematic representation of WIP1 functions in cell cycle progression and apoptosis.

1.2.4. Plant PP2Cs

PP2Cs form the largest protein phosphatase family in plants. Database analysis reveals 76 PP2C gene members (Kerk et al., 2002; Schweighofer et al., 2004). In the Arabidopsis genome, 112 phosphatase catalytic subunit sequences have been identified (Kerk, 2002). Arabidopsis genome predicts 150 phosphatase candidates, among them only one PTP and 18 DSP1 (Kerk et al., 2002; Kerk, 2007). The multiplicity of PP2C enzymes in plants suggests a much broader functional diversity than in other eukaryotes (Schweighofer et al., 2004).

According to amino acid sequence alignment PP2Cs are clustered into 10 groups (A-J). The majority of genes identified in Group A are associated with ABA signaling. Group B is characterized by homology to MP2C, an alfalfa PP2C that regulates MAPK signaling. Group C includes POL-type phosphatase involved in flower development. The kinase-associated protein phosphatase (KAPP) regulates receptor-like kinases (RLKs) (Schweighofer et al., 2004).

ABA-induced PP2Cs

Abscisic acid (ABA) promotes the acquisition of desiccation tolerance and seed dormancy, and it inhibits seed germination during late embryogenesis. ABA acts as a mediator of physiological responses to low-water situations. ABA-insensitive and ABA-deficient mutants are hypersensitive to drought and salinity (Leung and Giraudat, 1998).

Arabidopsis PP2Cs (ABI1, ABI2, HAB1 and PP2CA) are negative regulators of the ABA signaling (Koornneef et al., 1984; Leung et al., 1997; Rodriguez et al., 1998; Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001).

ABI1 and ABI2 phosphatases have overlapping functions in controlling ABA signaling. Recessive loss-of-function mutants of ABI1, with phosphatase activity reduced 100-1000 fold, are hypersensitive to ABA (Gosti et al., 1999; Robert et al., 2006; Saez et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007). In contrast, dominant gain-of function mutations abi1-1 and abi2-1 reduce plant sensitivity to ABA in seed germination, seedling development, attenuation of seed dormancy and stomatal closure (Koornneef et al., 1984; Leung et al., 1997). The mutations abi1 and abi2 are single amino acid changes ABI1G180D and ABI2G168D within the catalytic domain (Koornneef et al., 1984) that impairs Mg2+ binding and reduces protein phosphatase activity (Leube et al., 1998). The catalytically diminished abi1 and abi2 might block ABA responses via a dominant negative effect (Sheen, 1998; Yang et al., 2006) by trapping positive regulators of ABA signalling in a dead complex (Saez et al., 2004). The constitutive overexpression of ABI1 inhibits ABA action in maize protoplasts (Sheen, 1998). ABA increases PP2C activity of ABI1 and ABI2 in plant extracts. These results suggest that ABI1 and ABI2 act in a negative feedback regulatory loop of the ABA signalling pathway (Gosti et al., 1999). ABI1 may be compartmentalized to the nucleus or associated with the plasma membrane (Himmelbach et al., 2002; Hoth et al., 2002; Zhang et al., 2004). Nuclear localization of abi1 is required for the insensitivity towards ABA responses (Moes et al., 2008). Non-functional mutated nuclear localization sequence (NLS) completely abolishes the negative regulation of ABA signalling by abi1 and ABI1 (Moes et al., 2008). The abi1 mutation ABI1G180D introduces a negative charge probably mimicing a phosphorylated ABI1. Therefore abi1 might escape a phosphorylation-dependent regulation of subcellular localisation (Moes et al., 2008).

The ABI1 and ABI2 homologous gene HAB1 (previously described as AtP2C-HA) is a highly ABA-induced gene and a negative regulator of ABA signaling (Rodriguez et al., 1998). *atp2c-ha-1* mutation results in increased sensitivity to ABA in ABA-induced stomatal

closing (Rodriguez et al., 1998). HAB1 transcript was identified as preferentially expressed in guard cells by microarray screening for guard cell - specific genes (Leonhardt et al., 2004). The *hab1-1* T-DNA insertion mutant shows ABA-hypersensitive inhibition of seed germination in the presence of exogenous ABA. Constitutive overexpression of HAB1 leads to the reduced ABA sensitivity both in seeds and vegetative tissues (Saez et al., 2004).
Similar to ABI1, AtPP2CA blocks ABA signal transduction when transiently expressed in protoplasts (Sheen, 1998). AtPP2CA transcription is induced by cold, drought, salt and ABA (Tahtiharju and Palva, 2001). AtPP2CA regulates AKT2 and possibly enables the control of K+ transport and membrane polarization during stress situations and phloem transport (Cherel et al., 2002).

RLK phosphatases

The CLAVATA 1 (CLV1) receptor kinase in *Arabidopsis* regulates stem cell identity and differentiation through its repression of *WUSCHEL* (*WUS*) expression. The WUSCHEL (WUS) gene, which encodes a homeodomain protein, is a key regulator of stem cell identity. In the absence of WUS, stem cells are absent, and terminated shoots are formed. *CLAVATA* genes (*CLV1*, *CLV2*, and *CLV3*) promote the progression of meristem stem cells toward differentiation through the repression of *WUS* expression. Mutations at the *POLTERGEIST* (*POL*) gene were previously described as phenotypic suppressors of mutations within the *CLV1* gene. *POL* functions in both the *CLV1*-*WUS* pathway (Yu et al., 2000) and a novel *WUS*-independent *CLV1* pathway regulating stem cell identity. *POL* encodes a protein phosphatase 2C (PP2C) with a predicted nuclear localization sequence, indicating a role in signal transduction downstream of the CLV1 receptor (Yu et al., 2003).
The PP2C KAPP contains a forkhead-associated domain (FHA) that acts to mediate binding to phosphorylated CLV1 (Li et al., 1999). KAPP is capable of dephosphorylating CLV1, and it is a negative regulator of CLV1 signaling at the plasma membrane. POL similarly contains a novel regulatory domain at the N terminus, as well as a C-terminal phosphatase domain. Thus, by analogy to KAPP, POL would bind a phosphorylated intermediate of CLV1 signaling and inactivate the component by reversing phosphorylation (Stone et al., 1998; Yu et al., 2000). KAPP binds RLKs in a phosphorylation-dependent manner and does not bind kinase-inactive mutants of RLKs (Li et al., 1999). KAPP interacts with AtSERK1, a membrane-located leucine-rich-repeat RLK expressed transiently during embryogenesis. KAPP also interacts with RLK5 in Y2H and associates *in vitro* with multiple RLKs. KAPP

may also regulate other RLK pathways but there its detailed role has to be determined (Stone et al., 1994; Braun et al., 1997; Trotochaud et al., 1999; Torii, 2000).
PLL1 functions in parallel with POL in regulation of meristem and organ development. PLL4 and PLL5 regulate leaf development, while for PLL2 and PLL3 no roles have been identified (Song and Clark, 2005).

Other PP2Cs

PP2C DIG3 may be involved in nuclear import of the VirD2 protein and, consequently, the VirD2-transferred DNA complex. *Agrobacterium tumefaciens* transfers a single-stranded DNA molecule (the T-strand) into plant cells. This DNA is covalently linked to the VirD2 protein. VirD2 contains NLS sequences that probably help directing the T-strand to the plant nucleus. Tomato DIG3 encodes an enzymatically active PP2C that interacts with the C-terminal region of VirD2. Overexpression of DIG3 in protoplasts inhibited nuclear import of a β-glucuronidase-VirD2 fusion protein (Tao et al., 2004).
A search in trapping lines for genes selectively expressed in guard cells identified amoung others a PP2C phosphatase (At1g03590). Statistical analyses of the chromosomal regions revealed an over-represented [A/T]AAAG motif, previously described as an essential cis-active element for gene expression in stomata (Galbiati et al., 2008):

PP2Cs as MAPK phosphatases

PP2Cs often act in feedback regulatory loops of stress-induced pathways. Two stress induced PP2Cs were described as MAPK phosphatases: MP2C from *Medicago sativa* (Meskiene et al., 1998; Meskiene et al., 2003) and AP2C1 from Arabidopsis (Schweighofer et al., 2007). MP2C expression is induced after wounding and MP2C regulates MAPK pathway. MP2C is a MAPK phosphatase that directly inactivates the wound-activated MAPK SIMK through Thr dephosphorylation of the pTEpY motif, which is essential for MAPK activity, but not the wound-activated MAPK SAMK (Meskiene et al., 2003).
A putative MAPK interaction motif (KIM) [$(K/R)_{3-4}X_{1-6}(L/I)X(L/I)$], similar to those found in MAPK kinases (MAPKKs), MAPK phosphatases (HOKiegerl et al., 2000; Ho et al., 2003), or transcription factors has been identified in several PP2Cs of the B-group phosphatases. In this group the KIM is localised to the N-terminal extension of the PP2C (Schweighofer et al., 2004).

AP2C1 is the closest *Arabidopsis* homolog of MP2C. AP2C belongs to a six-member subfamily of *Arabidopsis* PP2Cs, four of which contain a KIM domain in their N terminus (Meskiene et al., 2003; Schweighofer et al., 2007). Recently AP2C1 was described as a negative regulator of stress-responsive MAPKs MPK4 and MPK6 (Schweighofer et al., 2007). AP2C1 regulates MAPK signaling in defence responses to wounding and necrotrophic pathogens. Plant hormones jasmonic acid (JA) and ethylene (ET) are produced after wounding and are responsible for the induction of defence genes. Signaling by MPK4 is essential for the induction of a subset of ET-responsive genes and crucial for the antagonism between SA- and JA/Et-dependent responses (Brodersen et al., 2006). MPK6 phosphorylate and thereby stabilizes the rate-limiting enzymes in ET biosynthesis of ACC synthase family (Liu and Zhang, 2004). Thus MPK4/MPK6 positively regulate ET level or response. Jasmonic acid activates MKK3/MPK6 (but not MPK4) (Takahashi et al., 2007). ap2c1 mutant plants produce significantly higher amounts of jasmonate upon wounding and are more resistant to phytophagous mites (*Tetranychus urticae*). Plants with increased AP2C1 levels produce less ethylene and are more sensitive to the necrotrophic pathogen *Botrytis cinerea*. Pathogen and wound-induced AP2C1 regulates stress hormone levels, MAPK activities and modulates pathogen response in in Arabidopsis (Schweighofer et al., 2007).

2. Materials and Methods

2.1. Strains and plasmids

2.1.1. Strains

E.coli strains used:

DH5α, F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1

DB3.1 F– gyrA462 endA1 Δ(sr1-recA) mcrB mrr hsdS20(rB–, mB–) supE44 ara-14 galK2 lacY1 proA2 rpsL20(SmR) xyl-5 λ– leu mtl1

BL21 F – ompT hsdSB (rB– mB–) gal dcm

XL1-Blue endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)

Agrobacterium tumefaciens strain used:

GV3101::pMP90 with pSOUP helperplasmid (Konsz and Schell, 1986)

Saccaromyces cerevisiae strains used:

L40 *MATa his3Δ200 trp1-901 leu2-3,112 ade2 lys2-801am URA3::$(lexA)_8$-lacZ LYS2::$(lexA)_4$-HIS3* (Vojtek et al., 1993; Hollenberg et al., 1995)

pJ69-4A *MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal180Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ* (James et al., 1996)

2.1.2. Plasmids

2.1.2.1. Plasmids for yeast two hybrid (Y2H) assays

The 3'-end of *STOPP* (At2g40180) cDNA was amplified by PCR from cDNA clone U20838 (obtained from Arabidopsis Biological Resource Center; ABRC) with primers #237 (CGCGTGTTGTGTAACCGCTTTGATATCGA) and #182 (GCGGCCGCATGGAAGAAAGTTTTGTAGC). The 3'-end EcoRV/NotI fragment of *STOPP* cDNA and the 5'-end NcoI/EcoRV fragment without introns of *STOPP* genomic DNA (obtained from V.Kazanaviciute, unpublished) were cloned into pGAD425 vector

(Clontech; modified). Subsequently the full length *STOPP* cDNA was recloned into Y2H vectors pBTM117 (from (Vojtek et al., 1993); modified) and pBD-GAL4cam (Stratagene). MAP kinases in pGAD425 vector and MAPK kinases in pBTM117 vector (Teige et al., 2004) were used for direct Y2H assays with STOPP.
The Arabidopsis cDNA library in pACT vector (consisting of a 1:1 mix of auxin induced and uninduced seedlings roots) for Y2H screening was provided by Dr. Bert van der Zaal, Institute of Molecular Plant Sciences, Leiden University, The Netherlands.

2.1.2.2. Introduction of STOPP phosphatase mutation G163D

The point mutation G163D within the catalytic part of STOPP was introduced by site-directed mutagenesis (using Site-Directed Mutagenesis kit from Stratagene) according to manufacturer's recommendations. The PCR with gene specific primers #362 (5'-GGTTACAAAAATGCTTTCTTTGACGTCTTTGATGGTCACGGCGG-3') and #363 (5'-GCCGTGACCATCAAAGACGTCAAAGAAAGCATTTTTGTAACCTC-3') containing the mutation and an additional *AatII* restriction site was performed with Pfu Turbo polymerase from the template plasmids containing *STOPP* genomic or cDNA. The template DNA was digested by adding 1µL of *DpnI* restriction enzyme to the PCR reaction. The PCR product was transformed to XL1-Blue supercompetent E.coli cells and selected on appropriate antibiotics. Positive clones were checked by plasmid DNA restriction with *AatII* restriction enzyme which additionally cleaves in the site introduced by the mutation G163D. The correct *STOPP* genomic or cDNA fragment containing mutation G163D was cloned further to vectors for GST protein purification or for expression in plants.

2.1.2.3. Plasmids for GST protein purification

For protein GST purification the cDNA of *STOPP* G163D was cloned to pGEX-4T-1 vector (GE Healthcare). pGEX-4T-1-cSTOPPwt was obtained from C.Choopayak (unpublished).

2.1.2.4. Plasmids for transient transformation of Arabidopsis suspension protoplasts

For co-localisation of STOPP and MPK4 or MPK6 in Arabidopsis suspension protoplasts, pGreenII vectors (Hellens et al., 2000) containing double CaMV 35S promoter upstream of a) *gSTOPPwt* (obtained from V.Kazanaviciute, unpublished) tagged with sGFP(S65T) (Chiu et

al., 1996) and b) *AtMPK4wt* or *AtMPK6wt* (Teige et al., 2004) tagged with mRFP1 (Campbell et al., 2002) were used.

For bimolecular fluorescence complementation (BiFC), *gSTOPPwt* (gDNA clone obtained from V.Kazanaviciute, unpublished) was recloned into pRT100 vector (Topfer et al., 1987) downstream of CaMV 35S promoter and fused N-terminally to YFP_{NTD} (Walter et al., 2004). pRT100 vectors containing CaMV 35S promoter upstream of *AtMPK1wt, AtMPK3wt, AtMPK4wt* or *AtMPK6wt* (fused N-terminally to YFP_{CTD} (Walter et al., 2004) were used for co-expression with YFP_{NTD}-*gSTOPPwt* in protoplasts. Plasmids expressing only YFP_{CTD} or YFP_{NTD} from CaMV 35S promoter (SPYCE, SPYNE) were used as negative controls (Walter et al., 2004).

For MAP kinase activity assays from protoplasts, pGreenII vectors (Hellens et al., 2000) containing double CaMV 35S promoter upstream of *gSTOPPwt* (V.Kazanaviciute, unpublished) and *AtMPK3wt, AtMPK4wt* or *AtMPK6wt* (Teige et al., 2004) were used for co-expression in protoplasts. The phosphatase was tagged with sGFP(S65T) and MAP kinases were tagged with triple HA. HA-tagged *ΔANP1* (Asai et al., 2002) or *AtMKK5wt* and *AtMKK2EE* (Teige et al., 2004) tagged with 9-mer c-myc epitopes in pGreenII0029 or pRT100 respectively, were used for activation of downstream kinases MPK3, MPK4 and MPK6.

Table 2.1. Plasmids used for transient expression in plant protoplasts

Plasmid	References
pGreenII0029-2x35S-TL-gSTOPPwt-GFP	Vectors: pGreenII0029 (Hellens et al., 2000) pGreenII0229 modified from (Hellens et al., 2000) pRT100 (Topfer et al., 1987) pJS (Asai et al., 2002) Genes: *gSTOPPwt* (V.Kazanaviciute, unpublished) *AtMPK1wt, AtMPK3wt, AtMPK4wt, AtMPK6wt, AtMKK5wt* and *AtMKK2EE* (M. Teige, MFPL Vienna) *ΔANP1* (J.Sheen, Harvard Medical School) Epitope tags: triple HA, 9-mer c-myc and sGFP(S65T) (Chiu et al., 1996) mRFP1 (Campbell et al., 2002) YFP_{CTD} and YFP_{NTD} (Walter et al., 2004)
pGreenII0229-2x35S-TL-AtMPK4wt-mRFP1	
pGreenII0229-2x35S-TL-AtMPK6wt-mRFP1	
pGreenII0229-2x35S-TL-AtMPK3wt-HA	
pGreenII0229-2x35S-TL-AtMPK4wt-HA	
pGreenII0229-2x35S-TL-AtMPK6wt-HA	
pGreenII0029-AtMKK5wt-Myc	
pJS-ΔANP1-HA	
pRT100-AtMKK2EE-Myc	
pRT100-35S-YFP_{NTD} *-gSTOPPwt*	
pRT100-35S-YFP_{CTD} *-AtMPK1wt*	
pRT100-35S-YFP_{CTD} *-AtMPK3wt*	
pRT100-35S-YFP_{CTD} *-AtMPK4wt*	
pRT100-35S-YFP_{CTD} *-AtMPK6wt*	
pRT100-35S-YFP_{CTD}	
pRT100-35S-YFP_{NTD}	

2.1.2.5. Plasmids for stable plant transformation

For plants expressing mutant *STOPP-G163D* genomic and cDNA of *STOPP-G163D* were recloned to plant expression vector pGreenII0029 (Hellens et al., 2000) downstream of the single or double CaMV 35S promoter and tagged with sGFP(S65T) (Chiu et al., 1996).
For *STOPP* promoter activity studies, *$STOPP_{pro}$:GFP* (obtained from A.Schweighofer; unpublished) was recloned to pGreenII0029 (Hellens et al., 2000) vector.
For estradiol-inducible *STOPP* expression in plants genomic DNA of *STOPP* (obtained from V.Kazanaviciute, unpublished) was recloned to pER8 vector (Zuo et al., 2000) and tagged with YFP. *pER8-gSTOPP-Myc* was obtained from C.Choopayak (MFPL, Vienna).
pGreenII0029-$STOPP_{pro}$-gSTOPPwt-GFP and plasmids for phosphatase domain swapping *pGreenII0029-35S-TL-$AP2C1_{NTDI}$-$STOPP_{CTD}$-GFP* and *pGreenII0029-35S-TL-$STOPP_{NTDI}$-$AP2C1_{CTD}$-GFP* and were obtained from V.Kazanaviciute (unpublished; MFPL).

Table 2.2. Plasmids used for stable plant transformation

Plasmid	References
pGreenII0029-35S-TL-gSTOPP G163D-GFP	Vectors: pGreenII0029 (Hellens et al., 2000) pER8 (Zuo et al., 2000) Genes: *$STOPP_{pro}$, gSTOPPwt, $AP2C1_{NTDI}$, $AP2C1_{CTD}$, $STOPP_{CTD}$, $STOPP_{NTDI}$,* (V.Kazanaviciute, MFPL) Epitope tags: 9-mer c-myc and sGFP(S65T) (Chiu et al., 1996), YFP
pGreenII0029-35S-TL-cSTOPP G163D-GFP	
pGreenII0029-2x35S-TL-gSTOPP G163D-GFP	
pGreenII0029-$STOPP_{pro}$-GFP	
pGreenII0029-$STOPP_{pro}$-gSTOPPwt-GFP	
pGreenII0029-35S-TL-$AP2C1_{NTDI}$-$STOPP_{CTD}$-GFP	
pGreenII0029-35S-TL-$STOPP_{NTDI}$-$AP2C1_{CTD}$-GFP	
pER8-gSTOPPwt-Myc	
pER8-gSTOPPwt-YFP	

2.2. GST protein purification and phosphatase activity assay

2.2.1. GST protein purification

E.coli strain BL21 containing GST expression vector pGEX-4T-1 (GE Healthcare) with *cSTOPPwt* or *cSTOPP G163D* was cultivated in 10 mL of LB^{Amp} at 37°C overnight. The overnight culture was diluted in 100 mL of LB^{Amp} to OD_{600} 0.1 and cultivated to OD_{600} 0.8. 300 µL of the suspension was aliquoted as a negative control before the induction with

isopropyl-β-D-thiogalactopyranoside (IPTG). The expression of GST-fused STOPP proteins was induced by cultivating cells with 0.1 – 1 mM of IPTG for 2h at 28°C. 300µL of the suspension was aliquoted as a control after the induction with IPTG. The GST fused STOPP proteins were purified as follows: the suspension was placed on ice for 2 - 3 min. and centrifuged at 4000 rpm for 10 min. at 4°C. The pellet was resuspended in 20 mL of lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (Roche)) and sonicated (Bandelin HD 200 Sonoplus) twice for 10 sec. on ice. After the centrifugation at 14000 rpm for 10 min. at 4°C the supernatant was mixed with 150 – 200 µL of GSH-sepharose 4B (GE Healthcare) and rolled for 30 min. at 4°C. The sepharose was washed three times with lysis buffer containing protease inhibitors and the GST-fused STOPP was eluted five times with 100 µL each of elution buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM reduced GSH). The protein amounts in eluted fractions were checked on poly acrylamid (PAA) gel stained with Coomassie Blue (12.5% v/v glacial acetic acid, 50% v/v methanol, 0.25% w/v Coomassie Briliant Blue R-250).

2.2.2. Phosphatase activity assay on γ^{32}P-casein *in vitro*

The phosphatase activity was assayed *in vitro* on γ^{32}P-labelled casein. The casein was labelled as follows: 1 mg of casein (partially dephosphorylated casein lysate, Sigma) was phosphorylated by 50 U of bovine heart protein kinase A catalytic subunit (Sigma) with 100 µCi of γ^{32}P-ATP (Amersham Biosciences) in 100 µL of kinase A buffer (0.05 M Tris pH 7.0, 5 mM $MgCl_2$) for 30 min at 30°C. γ^{32}P-casein was precipitated with 0.5 mL of 20% w/v trichloroacetic acid (TCA) in 20 mM NaH_2PO_4 for 20 min. on ice and centrifuged at 13000 rpm for 10 min. at room temperature (RT). The γ^{32}P-casein pellet was washed with 0.7 mL of 20% w/v TCA in 20 mM NaH_2PO_4 and centrifuged at 13000 rpm for 1 min. at RT. The radioactivity of the supernatant was measured in the scintillation counter (Tri-carb 16000, Packard). The washing was repeated till the radioactivity of the supernatant was less than 200 cpm (counts per minute). The pellet was resuspended in 300µL of 0.2 M Tris pH 8.0 and stored at -20°C in aliquots. The radioactivity of 5 µl of γ^{32}P-casein had to be more than 200 000 cpm for good dephosphorylation assay.

For the phosphatase activity assay 0.5 - 1 µg of GST-purified phosphatase was incubated with 5 µL of γ^{32}P-casein in 50 µL of phosphatase buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 0.1 mM EGTA, 10 mM dithiotreitol) containing 100 nM okadaic acid (from Prorocentrum concavum, Sigma) for 1h at 30°C. The reaction was stopped by adding 750 µL NoritA

solution (0.9 M HCl, 90 mM Na-pyrophosphate, 2 mM NaH_2PO_4, 4% w/v Norit A) for 10 min. γ^{32}P-casein was adsorbed to activated carbon and pelleted by centrifugation at 13000 rpm for 5 min. The released γ^{32}P was measured in 650 µL of supernatant by scintillation counter.

For phosphatase activity assay in crude protein extracts from plants see § 2.5.7.

2.3. Yeast Two Hybrid (Y2H) assay

2.3.1. One-step yeast transformation for direct Y2H assay

The yeast strain was refreshed on YPD (2% w/v Difco peptone, 1% w/v yeast extract, 0.008% adenine hemisulfate, 2% v/v glucose) or SD (0.67% w/v yeast nitrogen base, 2% v/v glucose, 1x nutrient Dropout solution without appropriate nutrients) 2% w/v agar plates. The concentrations of nutrients in 100x Dropout solution were: 1 mg/mL adenine hemisulfate (Ade), 2 mg/mL L-Arginine, 1 mg/mL L-Histidine, 6 mg/mL L-Isoleucine, 6 mg/mL L-Leucine (L), 4 mg/mL L-Lysine, 1 mg/mL L-Methionine, 6 mg/mL L-Phenylalanine, 5g mg/mL L-Threonine, 4g L-Tryptophan (W), 5 mg/mL L-Tyrosine, 4 mg/mL uracil.

Approximately 25 µL of yeast cells were transformed with 2 µL of plasmid DNA (~ 0.5 – 1 µg) in 150 µL of transformation mixture (39% v/v PEG4000, 0.2 M lithium acetate, 0.1 M dithiotreitol and 0.357 mg/mL ssDNA) and vortexed well. Reaction was incubated at 42°C for 30 min. Subsequently cells were washed with 1 mL of SD media without glucose and amino acids, centrifuged for 2 min. at 3000 rpm, resuspended in 50 µL of SD media without glucose and amino acids and plated on SD agar plates with 2% v/v glucose without appropriate nutrients. Colonies appeared on the plates after 2-3- days at 28°C.

2.3.2. Y2H cDNA library screening

Yeast strain pJ69-4A (James et al., 1996) was used for cDNA library screening. The Arabidopsis cDNA library in pACT vector (consisting of a 1:1 mix of auxin induced and uninduced seedlings roots) for Y2H screening was provided by Dr. Bert van der Zaal, Institute of Molecular Plant Sciences, Leiden University, The Netherlands. The large-scale yeast transformation protocol was adapted from (Minet et al., 1992)

2.3.2.1. Large-scale yeast transformation

The pJ69-4A yeast were transformed with pBDcam vector containing *STOPP* cDNA as described in § 2.3.1. Transformants were selected on SD-W (with 2% v/v glucose) agar plates, inoculated 10 mL of SD-W (with 2% v/v glucose) ($OD_{600} \geq 0.1$) and cultivated at 30°C overnight. The yeast overnight cell culture was diluted to OD_{600} 0.5 (~ 5 x 10^6 cells/mL) in 80 mL of SD-W (with 2% v/v glucose) and cultivated till OD_{600} 2.0 (~ 2 x 10^7 cells/mL) at 30°C with 200 rpm agitation. Cells were harvested by centrifugation at 3000 rpm for 5 - 10 min. The pellet was resuspended in 10 mL of SD without glucose and dropout nutrients containing 70 mM lithium acetate and incubated at 30°C for 10 min. The cells were aliquoted in 1 mL aliquots and centrifuged at 3000 rpm for 5 min. One aliquot was used for one transformation with 1 µg of library cDNA (diluted to 0.1µg/µL). The yeast cell pellet was resuspended by vortexing in 360 µL of transformation mixture (33.33% v/v PEG4000, 0.1 M lithium acetate, 0.278 mg/mL ssDNA, 1 µg library cDNA). The cells were incubated at 30°C for 30 min., heat-shocked at 42°C for 30 min. and washed by resuspending in 1 mL of SD without glucose and dropout nutrients. An aliquot of 100 µL was taken from 1 mL of one sample, centrifuged at 3000 rpm for 3 min., resuspended in 50 µL of SD without glucose and dropout nutrients and plated on SD-L-W (with 2% v/v glucose) agar plate to check the efficiency. The rest of the samples were centrifuged at 3000 rpm for 3 min. and plated on SD-L-W-Ade (with 2% v/v glucose) agar large plates (one transformation per plate). All handling of yeast was done with wide open tips. Yeast colonies appeared after 2 – 3 days on SD-L-W (with 2% v/v glucose) plates and after 3 - 4 days on SD-L-W-Ade (with 2% v/v glucose) plates at 28°C. The large-scale yeast transformation was repeated until the total number of transformants on control (SD-L-W) plates was > 10^6 to cover the entire cDNA library.

2.3.2.2. Analysis of positive yeast colonies

ADE2 positive yeast colonies from SD-L-W-Ade agar plates (identified in § 2.3.2.3.) were picked and analysed as follows: 1) re-tested for expression of *ADE2* reporter gene on SD-L-W-Ade (with 2% v/v glucose) agar plates, 2) tested for expression of *HIS3* reporter gene on SD-L-W-H (with 2% v/v glucose) agar plates, 3) propagated for the analysis of reporter ß-galactosidase activity by liquid assay (see § 2.3.4.) on SD–L-W (with 2% v/v glucose) agar plates, 4) propagated for plasmid rescue from yeast (see § 2.3.2.2.) on SD–L-W (with 2% v/v glucose) agar plates. Rescued cDNA plasmids were selected and amplified in *E.coli*. The

cDNA library plasmids were transformed by one-step yeast transformation (see § 2.3.1.) to pJ69-4A strain containing *pBDcam-cSTOPP* or *pBDcam* (empty vector) or L40 strain containing *pBTM117-cSTOPP* or *pBTM117* (empty vector) for ß-galactosidase liquid assay (see § 2.3.4.).

2.3.2.3. Plasmid rescue from yeast

The yeast was cultivated in 2 mL of SD (with 2% v/v glucose, without appropriate nutrients) at 28°C overnight. The culture was centrifuged at 6000 rpm for 3 min. and resuspended in 100 µL STET solution (8% w/v saccharose, 50 mM Tris pH 8.0, 50 mM EDTA, 5% v/v Triton X-100). ~ 200 µL of glassbeads (425 – 600 nm) were added to the suspension and vortexed for 5 min. Another 100 µL of STET solution were added to the mixture and shortly vortexed. The mixture was incubated at 95°C for 3 min., shortly cooled on ice and centrifuged at 13000 rpm for 10 min. at 4°C. 100 µL of supernatant was mixed with 50 µL of 7.5 M ammonium acetate, incubated for 1h at -20°C and centrifuged at 13000 rpm for 10 min at 4°C. 100 µL of supernatant was mixed with 200 µL of 96% ethanol. The plasmid DNA was pelleted by centrifugation at 15000 rpm for 15 min at 4°C. The pellet was washed with 70% ethanol, air-dried and resuspended in 20 µL of sterile water.

2.3.2.4. Identification of putative interacting proteins

The cDNA plasmids were rescued from the positive yeast clones (see § 2.3.3.) and transformed into *E.coli* DH5α strain. The *E.coli* containing cDNA library plasmids (in pACT vector) were selected on LB^{Amp} plates (counterselection for *pDBcam-cSTOPP* plasmid). cDNA library plasmids (in pACT vector) were amplified and analysed by restriction with *BglII* restriction enzyme flanking the cDNA insert in pACT. Unique cDNA clones were sent for sequencing (VBC Biotech) and the sequences were blasted against public Arabidopsis sequence database (blastn suite, http://www.ncbi.nlm.nih.gov/). Encoding genes were identified for partial or full length cDNA clones.

2.3.4. Y2H ß-galactosidase liquid assay

The yeast containing AD (activation domain) and BD (binding domain) plasmids were grown on SD-L-W agar media. The yeast were scraped from the plate and resuspended in 1 mL of

liquid SD-L-W media without appropriate amino acids. The suspension was diluted in 3 mL of liquid SD-L-W to OD_{600} 0.3 in three replicates per sample. After reaching OD_{600} 1.3 – 1.5 yeast cells were centrifuged at 13000 rpm for 2 min. at RT. The pellet was resuspended in 200 µL of lysis buffer (25 mM Tris-HCl pH 7.5, 20m M NaCl, 8 mM $MgCl_2$, 5 mM dithiotreitol, 0.1% NP-40) and frozen in liquid nitrogen. Total protein extracts were prepared as follows: the samples were thawed on ice. ~300 µL of glass beads (425 – 600 nm) were added per sample and cells were vortexed 20 min. at 4°C. The samples were centrifuged at 13000 rpm for 10 min. at 4°C. The protein concentration in the supernatant was measured by Bradford assay (see § 2.5.3.).

The ß-galactosidase reaction was set on ice in triplicates: 50 µL of total protein extract was added to the 650 µL of Z-buffer (0.06 M $Na_2HPO_4 \cdot 2H_2O$, 0.04 M NaH_2PO_4, 0.01 M KCl, 0,001 M $MgSO_4 \cdot 7H_2O$, pH 7.0) containing 0.5% v/v ß-mercaptoethanol and afterwards mixed with 150 µL *o*-Nitrophenyl β-D-galactopyranoside (ONPG) (diluted to 4 mg/mL in Z-buffer). The reaction was incubated at 37°C till the solution developed yellow colour. The reaction was stopped by adding 400 µL of 1M Na_2CO_3. The OD of the solution was measured at 420 nm wavelength. The units of the ß-galactosidase activity were calculated according to formula: $U_{nits}=A_{420}x25x1000/(45xTxC)$ where A_{420} is absorbance at 420 nm, T is the reaction time in minutes, C is the protein concentration in mg/mL.

2.4. Plant material, genetic crosses, transformation and growth conditions

2.4.1. Plant material

Arabidopsis thaliana Col-0 wild type plants were used for transformation.

Table 2.3. Produced transgenic plant lines

Construct	Selection
pGreenII0029-35S-TL-gSTOPP-G163D-GFP	Kan^R
pGreenII0029-35S-TL-cSTOPP-G163D-GFP	Kan^R
pGreenII0029-2x35S-TL-gSTOPP-G163D-GFP	Kan^R
pGreenII0029-35S-TL-AP2C1-NTDI-STOPP-CTD-GFP	Kan^R
pGreenII0029-35S-TL-STOPP-NTDI-AP2C1-CTD-GFP	Kan^R
pGreenII0029- $STOPP_{pro}$-GFP	Kan^R
pER8-gSTOPP-Myc	Hyg^R
pER8-gSTOPP-YFP	Kan^R
pGreenII-$STOPP_{pro}$-gSTOPPwt-GFP	Kan^R

Wild type Col-0 ecotype plants were used as a control in all experiments.

T-DNA insertion line for *stopp* (At2g40180) SALK_109986 was obtained from SALK collection (http://signal.salk.edu/).

Transgenic T2 plant lines expressing *pGreenII0029-2x35S-TL-gSTOPPwt-GFP, pGreenII0029-2x35S-TL-gSTOPPwt-HA, pGreenII0029-2x35S-TL-gSTOPPwt-MYC* and *pGreenII0029-AtSTOPP$_{pro}$-GUSint* were obtained from V.Kazanaviciute (MFPL).

Table 2.4. Plant marker lines used for genetic crossing with *STOPP* gof lines

plant marker line	**Reference**
E1728 (GFP)	S.Poethig lab, http://enhancertraps.bio.upenn.edu/
MUTE$_{pro}$:GUS	(Pillitteri et al., 2007)
FAMA$_{pro}$:GFP	(Ohashi-Ito and Bergmann, 2006)
FAMA$_{pro}$:GUS	(Ohashi-Ito and Bergmann, 2006)
DR5$_{pro}$:GUS	J. Friml, VIB Ghent
DR5rev$_{pro}$:GFP	C.Luschnig, BOKU Vienna
PIN1$_{pro}$:PIN1-GFP	C.Luschnig, BOKU Vienna
PIN2$_{pro}$:PIN2-GFP	C.Luschnig, BOKU Vienna
PIN4$_{pro}$:PIN4-GFP	C.Luschnig, BOKU Vienna
PIN7$_{pro}$:PIN7-GFP	C.Luschnig, BOKU Vienna

2.4.2. Genetic crossing of Arabidopsis

For genetic crossings of Arabidopsis transgenic lines to plant marker lines, the latter were used as mother plants and pollinated with pollen of *STOPP* gain-off-function plants.

~1.5 month old plants on soil sprouting 2 - 3 shoots were used for the crossing. Only young and not yet opened flowers of the mother plant were selected for the crossing and the rest of the flowers were removed with forceps. The sepals, petals and anthers were removed from the selected flower of mother plant. Fully opened flowers of *STOPP* gain-off-function plants were used to pollinate the stigma of the acceptor plant flower.

(*DR5* lines were used as mother/acceptor plants for pollination with *35S$_{pro}$:gSTOPP-GFP* #2.2.7.5 or *35S$_{pro}$:gSTOPP-HA* #2.2.1 pollen). *DR5$_{pro}$:GUS* and *DR5$_{pro}$:GUS 35S$_{pro}$:gSTOPP-GFP* T2 seedlings were analysed at 3 dpg age by GUS staining.

2.4.3. Transformation of Arabidopsis by Agrobacterium by floral dip methode

Transformation of Arabidopsis by floral dip method was done according to (Clough and Bent, 1998). *Agrobacterium tumefaciens* strain GV3101-pMP90 was transformed with plasmids by electroporation as follows: competent Agrobacterium cells were thawed on ice and mixed with ~ 1 µg of plasmid DNA with a wide tip. The cells were transferred to pre-chilled electroporation cuvettes and electroporated at 2.5 kV, 400 Ω, for 1 pulse ~ 9 msec. Afterwards the cells were recovered by adding 1 mL of ice-cold LB media and shaking for 2 - 4 h at 28°C. The transformed Agrobacteria were plated on LB agar plates with appropriate antibiotics (including 50 mg/L rifampicin, 50 mg/L gentamycin and 2 mg/L tetracycline that are used to select the strain and helper plasmids). The positive clones were checked by PCR from colony with gene specific primers.

For plant transformation the Agrobacterium cell suspension containing the plasmid was grown in 50 mL of LB media with appropriate antibiotics at 28°C for 1-2 days. The suspension was diluted in 300 mL of LB with appropriate antibiotics to OD_{600} 0.3 and grown till OD_{600} 0.8. The cells were collected by centrifugation at 3500 rpm for 10 min. at room temperature and resuspended first in small volume of 5% w/v sucrose and finally in 300 mL of 5% w/v sucrose and 0,05% v/v Silvet L-177 (Vac-in-Stuff, Lehle Seeds). Wild type Col-0 plants were transformed with Agrobacteria by dipping flowers into the suspension for 15 sec. Plants were covered with plastic bags for 2 days. The bags were opened after 1 day after the transformation. The transformation was repeated again after one week.

2.4.4. Seed surface sterilisation

Seeds were sterilised before seeding with sodium hypochlorite (NaOCl) or Bayrochlor (Bayrochlormini, Bayrol France S.A). For sterilisation with NaOCl, seeds were resuspended in ~ 0.1 mL of water, mixed with 0.5 mL of 7.5% NaOCl, incubated for 5 min. and washed three times with 1 mL of sterile water. For sterilisation with Bayrochlor, seeds were mixed with 1% w/v Bayrochlor in 96% ethanol for 20 min., washed three times with 96% ethanol and air-dried for 3 - 4 h in laminar flow.

2.4.5. Arabidopsis germination, growth media and growth conditions

For the selection of transgenic Arabidopsis plants the seedlings were germinated on ½ MS (Duchefa), 1% sucrose and 0.7% plant agar (Duchefa) plates (pH5.7) with appropriate antibiotics or herbicides if not stated otherwise. For GUS staining and subsequent fluorescence microscopy seeds were germinated on ½ MS, 1% sucrose and 0.5% phytagel (Sigma) vertical plates. For ethylene measurement by gas chromatography seeds were germinated on ½ MS, 1% sucrose and 0.7% plant agar in glass vials. For various treatments such as treatment with proteasome inhibitors, induction with estradiol, hormone and stress treatment before mRNA extraction from seedlings or GUS staining, seeds were germinated in liquid ½ MS in multiwell dishes and compounds were added to the liquid media.
Before germination, seeds were stratified for 2 days at 4°C. Seedlings were germinated at 16h light photoperiod at 22°C. Plants were grown in soil at 16h or 8h light photoperiod at 22°C.

2.4.6. Selection of transgenic Arabidopsis plants

For the selection of transgenic Arabidopsis plants on plates following concentrations of antibiotics and herbicides were used: 50 mg/L kanamycin, 75 mg/L gentamicin, 20 mg/L hygromycin B (from Streptomyces hygroscopicus, Sigma), 7 mg/L Basta (glufosinate-ammonium, Pestanal, Fluka/Riedel-de Haen). For the selection with gentamicin and hygromycin B seedlings were germinated for the first 5 days in darkness.
For the selection of transgenic Arabidopsis plants on soil with Basta the seeds were germinated in soil for 7 days and sprayed four times every second day with Basta solution containing 40 mg/L Basta (ammonium glufosinate, Aventis) and 0,025% v/v Silvet L-177 (Vac-in-Stuff, Lehle Seeds).

2.4.7. Treatment with proteasome inhibitors

Seedlings were grown in multiwell dishes in liquid ½ MS. MG132 (Calbiochem) was added to the final concentration of 50 μM and MG115 was added to the final concentration of 100 μM. Solvent dimethyl sulfoxide (DMSO) was added as a control. Seedlings were incubated with inhibitors for 1h to overnight.

2.4.8. Induction of STOPP expression in estradiol-inducible STOPP lines

To induce *STOPP* over-expression in estradiol (ER)-inducible STOPP lines seedlings were germinated in multiwell dishes in ½ MS with 50 µM ER (Sigma) or the solvent dimethyl sulfoxide (DMSO) as negative control. Media was changed every day.
For ethylene measurement by GC-FID, seeds were germinated in vials on ½ MS agar (with 1% sucrose, pH5.7) and were sprayed daily with ½ MS containing 50 µM ER for 4 days and 100 µM ER for next 2 days starting from the first day after germination. In parallel another set of vials with seedlings was sprayed with ½ MS containing equal amount of solvent DMSO. After 6 days of treatment with ER, ~ 90% of *pER8-gSTOPP-Myc* seedlings obtained stomata-clustering phenotype.

2.4.9. Cultivation of Arabidopsis cell suspension and protoplast preparation

The wild type Arabidopsis cell suspension was cultivated in Arabidopsis suspension media (MS with B5 vitamins (Duchefa), 3% sucrose, 1 mg/mL 2.4-D).
Protoplasts were isolated from Arabidopsis suspension culture cells as follows: 4 - 5 days old cells were collected by centrifugation at 1500 rpm for 5 min. The cells were resuspended in 25 mL of B5 – 0.34 M media (3.164 g/L Gamborg B5 media including vitamins (Duchefa), 30.5 g/L D-glucose, 30.5 g/L D-mannitol, 1 mg/L 2.4-D, pH 5.5). The cell walls were digested by adding 25 mL of enzyme solution (1% cellulose, 0.2% macerozyme (both from Serva) in B5 – 0.34 M media) and gently shaking for ~ 1 - 2 hours in large Petri plates. The cells were collected by centrifugation at 1000 rpm for 5 min. and resuspended in 10 mL of B5 - 0.28 M S (3.164 g/L Gamborg B5 media incl. vitamins, 96 g/L sucrose, pH 5.5). The floating protoplasts were collected after centrifugation at 800 rpm for 7 min. and transferred to 13 mL tube with a wide open Pasteur pipette. Protoplasts were diluted in B5 - 0.28 M S to 4 - 6 x 10^6 cells/mL. All the centrifugation steps were done without brake in a swing-out centrifuge.

2.4.10. Transformation of Arabidopsis cell suspension culture protoplasts

50 to 70 µL of protoplast suspension was added to 7 – 15 µg of DNA (maximal amount is 15µg, maximal volume is 15 µL per transformation). The protoplasts were mixed with 150 µL of PEG solution (300 g/L PEG 6000, 82 g/L D-mannitol, 23.5 g/L $Ca(NO_3)_2$, pH 9.0) and

incubated for 10 min. The protoplasts were washed with 1 mL of 0.275 M $Ca(NO_3)_2$ and collected by centrifugation at 800 rpm (~150 *g*)for 7 min. without brake in a swing-out centrifuge. The protoplasts were resuspended in 0.25 mL of B5-0.34 M media and incubated at least for 8h in the dark. All the steps were carried out at room temperature.

2.5. Protein extraction from plant cells, protein detection and activity assays

2.5.1. Protein extraction from Arabidopsis plants

Proteins were extracted from Arabidopsis seedlings or leaves in Lacus buffer (25 mM Tris-HCl pH 7.8, 75 mM NaCl, 15 mM EGTA, 15m M $MgCl_2$, 1 mM dithiotreitol (DTT), 0.1% v/v Tween 20, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 5µg/mL leupeptin, 5µg/mL aprotinin, 15 mM 4 - nitrophenylphosphate – bis [Tris(hydroxymethyl) - aminomethan] salt, 1 mM NaF, 0.5 mM Na_3VO_4, 15 mM β-glycerophosphate). For phosphotase activity measurements proteins were extracted from seedlings in Lacus buffer containing 500 nM Okadaic Acid.

The seedlings were ground in liquid nitrogen with mortar and pestle or with a plastic grinder in the test tubes. The material was not allowed to thaw until Lacus buffer was added. For protein extraction with a homogenizer (Precellys 24, Peqlab) the seedlings were homogenized in Lacus buffer at 5000 rpm twice for 10 sec.

The leaves were ground in test tubes with a glass grinder and sand (Sigma) in Lacus buffer for 15 sec. In all cases the extracts were centrifuged at 18 000 rpm for 30 min. at 4°C. The protein concentration was measured by Bradford assay (see § 2.5.3.) and diluted to 0.5 – 2 µg/µL. For Western blot the SDS loading buffer (final concentration to 50 mM Tris pH 6.8, 100 mM DTT, 2% w/v SDS, 10% glycerol, 0.25% w/v bromophenol blue) was added to the samples. The samples were heated at 95°C for 5 min. before loading on the PAA gel.

2.5.2. Protein extraction from protoplasts

Protoplasts were centrifuged in table centrifuge at top speed for 15 sec, resuspended in 40 µL of Lacus buffer (see § 2.5.1.) and immediately frozen in liquid nitrogen. The samples were allowed to start thawing on ice and immediately vortexed for 15 sec. The extracts were centrifuged at 18000 rpm for 20 min. at 4°C. The total protein concentration was measured by

Bradford assay (see § 2.5.3.) and the concentrations were adjusted if needed. For Western blot SDS loading buffer was added to the samples (see § 2.5.1.). The samples were heated at 95°C for 5 min. before loading on the PAA gel.

2.5.3. Bradford assay

To measure protein concentration, 200 µL of dye reagent concentrate (Bio-Rad) was added to 3 µL of protein extract and 800 µL of H_2O. The solution was vortexed well and after 5 min. the absorbance at 595 nm was measured by spectrophotometer. The protein concentration (µg/µL) was calculated according to the albumin protein calibration curve.

2.5.4. Western blot

The protein samples were run in SDS running buffer (0.025 M Tris base, 0.192 M glycine, 0.1% w/v SDS) on 10% or 12.5% SDS-PAA gel at 20 mA. The PVDF (Millipore) membrane was equilibrated for 2 min. in methanol and 15 min. in transfer buffer (TB; 50 mM Tris base, 50 mM boric acid) before use. The proteins were transferred onto the PVDF membrane at 4°C at 75 V for 3h or at 15 V overnight. The membrane was stained with Ponceau S solution (0.1% Poneau S, 5% glacial acetic acid), washed in water and blocked with 5% w/v milk (dried non-fat milk powder) in TBS-T (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 2 h at room temperature with agitation. The membrane was incubated with primary antibody in 5% w/v milk in TBS-T for 1h at room temperature or overnight at 4°C with agitation.

Table 2.5. Used antibodies and sera for detection by Western blotting

Primary antibody	Produced in	Working dilution
Anti-HA (Covance)	mouse, monoclonal	1:5000
Anti-GFP (Roche)	mouse, monoclonal	1:5000
Anti-Myc (Santa Cruz)	rabbit, polyclonal	1:5000
Anti-Phospho-p44/42 (Cell Signaling)	mouse, monoclonal	1:2000
Anti-MPK3 (serum; H.Hirt MFPL)	rabbit, polyclonal	1:2500
Anti-MPK4 (L.Bögre, RHUL)	rabbit, polyclonal	1:20000
Anti-MPK6 (serum; H.Hirt MFPL)	rabbit, polyclonal	1:5000
Anti-STOPP (A.Gust, Uni Tübingen)	rabbit, polyclonal	1:5000
Anti-CDKB1;1 (L.Bögre, RHUL)	rabbit, polyclonal	1:1000
Anti-PSTAIRE (L.Bögre, RHUL)	rabbit, polyclonal	1:4000
Anti-E2Fc (L.Bögre, RHUL)	chicken, polyclonal	1:4000
Anti-α-Tubulin (Sigma)	mouse, monoclonal	1:5000

The membrane was washed 4 times for 15 min. each with 5% w/v milk in TBS-T and incubated with secondary alkaline phosphatase (AP) - conjugated antibody usually of 1:5000 dilution in 1% w/v milk in TBS-T for 1 h at room temperature with agitation.
The membrane was washed 8 times 15 min. each with TBS-T and incubated in alkaline phosphatase buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. The alkaline phosphatase reaction was developed by incubating the membrane protein-side-down into CDP-Star™ detection reagent (Amersham Biosciences) for 2 min. The membrane was wrapped into plastic sheets and exposed to Amersham Hyperfilm™ ECL film.
The Western blots with RBR, DPA, DPB, E2FB, E2FC, CDKB1;1 and PSTAIRE antibodies were done in collaboration with Dr.Z.Magyar (Royal Holloway University London, RHUL). The PVDF membranes with proteins were transferred to the RHUL where Western blot with specific antibodies was performed by Dr.Z.Magyar. These antibodies were provided by Prof.L.Bögre lab (Royal Holloway University London).

2.5.5. Dephosphorylation of crude protein extracts with λ protein phosphatase

The plant protein extracts were prepared in Lacus buffer without protein phosphatase inhibitors. 10-20 µg of crude protein extracts were incubated with 0.25µL (100 U) λ protein phosphatase (Sigma) in 50 µL of 1x λ protein phosphatase buffer (Sigma) with or without 2 mM $MnCl_2$. For control, protein extracts were incubated without λ protein phosphatase and $MnCl_2$ and with phosphatase inhibitors (20 mM EDTA, 13 mM EGTA, 40 mM ß-glycerol phosphate, 0.5 mM Na_2VO_3, 1 mM $NaVO_4$, 5 nM okadaic acid, 50 mM NaF). Another set of samples was incubated at 65°C for 10 min. before λ protein phosphatase treatment to inactivate endogenous phosphatases. The dephosphorylation reactions with λ protein phosphatase were incubated at 30°C for 30 min. and terminated by adding SDS-loading buffer (see § 2.5.1.). Samples were heated at 95°C for 5 min. before loading on SDS-PAA gel for Western blot.

2.5.6. MAP kinase activity assay on myelin basic protein (MBP)

For the immunoprecipitation of MAP kinases 35 - 100 µg of total protein extract was added to 20 µL of diluted (50%) protein A sepharose (Amersham Biosciences) with 2 µL of MAPK antibody (serum) or with 0.02 µL of purified MAPK antibody. For immunoprecipitation of

HA-tagged MAPKs from Arabidopsis protoplasts 2 µL of anti-HA serum (12CA5) was used per sample.

The protein extracts with sepharose and antibodies were rolled overnight at 4°C and washed 3 times with 1 mL of SucI buffer (50 mM TrisCl pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% v/v Tween 20, 5 mM NaF, 0.1% v/v NP-40, 0.5 mM PMSF) and 1 time with 1 mL of kinase buffer (20 mM Hepes pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). All centrifugation steps were done at 1000 rpm for 1 min. in swing-out centrifuge at 4°C. Residual liquid was removed from the sepharose by sucking off with a syringe. The sepharose was immediately mixed with 15 µL of kinase buffer containing 1 µg/µL MBP, 5 µCi/µL γ^{32}P-ATP, 0.1 mM ATP and 1 mM DTT. The reaction was incubated at room temperature for 30 minutes, terminated by adding SDS loading buffer (see § 2.5.1.), heated at 95°C for 3 minutes, centrifuged shortly (~ 10 sec) and 7 µL of supernatant per lane (~ 5 µg of MBP) were loaded on 15% SDS-PAA gels. The small gel was run at 15 mA until the runnig out of bromphenol blue dye. The gel was stained with Coomassie Blue (§ 2.2.1) for 10 min., destained with destainer solution (12.5% v/v glacial acetic acid, 10% v/v methanol) for 2 h changing the destainer every 15 min. The gel was dried on Whatman 3MM paper in a vacuum gel-dryer at 80°C for 1 h and exposed to Kodak Biomax MR film.

2.5.7. CDK activity assay on histone 1 (HisI)

For immunoprecipitation of CDKs 50µg of total protein extract was incubated with 20 µL of diluted (25%) $p13^{Suc1}$ agarose conjugate (L.Bögre RHUL or Upstate Biotechnology, USA). The beads were rolled for 1 h at 4°C and washed 3 times with 1 mL of SucI buffer (50 mM TrisCl pH 7.4, 250 mM NaCl, 5 mM EGTA, 5 mM EDTA, 0.1% v/v Tween 20, 5 mM NaF, 0.1% v/v NP-40, 0.5 mM PMSF) and 1 time with 0.5 mL of kinase buffer (50 mM Tris pH 7.4, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT). All the centrifugation steps were done at 1000 rpm for 1 min. in swing-out centrifuge at 4°C. The residual liquid was removed from the beads by sucking off with a syringe. The beads were immediately mixed with 20 µL of kinase reaction containing 1 µg/µL histone 1 (Sigma), 1 µCi/µl γ^{32}P-ATP, 10 µM ATP in kinase buffer. The reaction was incubated 30 min. at room temperature. The reaction was terminated by adding SDS loading buffer (see § 2.5.1.), heated at 95°C for 3 minutes, centrifuged shortly (~ 10 sec) and 15 µl of sample (~ 10 µg of histone) were loaded per lane on a 12.5% SDS-PAA gel. The minigel was run at 15 mA until the bromphenol blue run out. The gel was stained with Coomassie Blue (§ 2.2.1) for 10 min., destained with destainer solution (§ 2.5.6)

for 2 h changing the destainer every 15 min. The gel was dried on Whatman 3MM paper in a vacuum gel-dryer at 80°C for 1h and exposed to Kodak Biomax MR film.

2.5.8. Phosphatase activity assay on γ^{32}P-casein *in vitro* in crude protein extracts

For phosphatase activity assay in crude protein extracts from plants, total protein extracts were prepared in Lacus buffer (see § 2.5.1.) containing 500 nM okadaic acid (from Prorocentrum concavum, Sigma) as inhibitor of PP2A, PP1 (Bialojan and Takai, 1988; Holmes et al., 1990; Holmes, 1991) PP4, PP5 and possibly PP6 in 0.1 -50 nM range) and to less extent PP2B, PP7 type phosphatases (1 - 4 µM range) (reviewed in (Swingle et al., 2007). 0.5 mM sodium orthovanadate (Na_3VO_4) was used to inhibit protein tyrosine phosphatases, and 15 mM EGTA was used as a Ca^{2+} chelator and inhibitor of PP2B. For casein labelling with γ^{32}P-ATP see § 2.2.2.

5 µg of crude protein extract were taken for dephosphorylation of 1-5 µL of γ^{32}P-casein in 50 µL of phosphatase buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT) containing 100 nM okadaic acid for 30 min. at 28°C. The reaction was stopped by adding 750 µL NoritA solution (0.9 M HCl, 90 mM Na-pyrophosphate, 2 mM NaH_2PO_4, 4% v/v Norit A) for 10 min. γ^{32}P-casein adsorbed to activated carbon was pelleted by centrifugation at 13000 rpm for 5 min. The released γ^{32}P was measured in 650 µL of supernatant by scintillation counter (Tri-carb 16000, Packard) in counts per minute (cpm).

2.6. Nucleic acid extraction from plant cells, PCR and hybridization

2.6.1. Genomic DNA extraction from plants for genotyping by PCR

For plant genotyping by PCR, genomic DNA was extracted from young plant leaves by grinding with a plastic grinder in test tube in 400 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS) for 15 sec. The extract was centrifuged at 13000 rpm for 5 min. and the gDNA was precipitated by adding 300 µL of supernatant to 300 µL of isopropanol. The gDNA was pelleted by centrifugation at 13000 rpm for 5 min. and dissolved in 100 µL of sterile water or TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). All the steps were carried out at room temperature. 1 – 2.5 µL of gDNA were used per one PCR reaction in 25 µL volume.

Table 2.6. Primers used to genotype *stopp* SALK_109986 plants

ID	Sequence (5' to 3')
#237 (forward 1)	CGCGTGTTGTGTAACCGCTTTGATATCGA
#281 (forward 2)	AGCGGCCGCACCATGGGAAGACGCGGACCAAT
#365 (reverse 1)	TTGGATTCTCTACGCCTACGCAGT
#483 (reverse 2)	AGCTCTCACTTCCGATCATAATCCTT
LbaI (http://signal.salk.edu/)	TGGTTCACGTAGTGGGCCATCG

2.6.2. RNA extraction from seedlings with Trizol

All solutions were prepared in DEPC treated water. ~ 20 - 30 mg of seedlings were ground with plastic grinder in eppendorf tube in liquid nitrogen, mixed with 0.5 ml of Trizol (38% phenol in saturated buffer, 0.8 M guanidine thiocyanate, 0.4 M ammonium thyocyanate, 0.1 M sodium acetate pH5.0, 0.5% v/v glycerol, or purchased from Invitrogen), incubated at 60°C for 5 min. and centrifuged at 13000 rpm for 10 min at 4°C. 100 µL of chloroform was added to 500 µL of the supernatant, vortexed for 15 sec, incubated for 3 min. at room temperature and centrifuged at 13000 rpm for 15 min. at 4°C. The RNA was precipitated by adding 50% v/v of isopropanol and 50% v/v of 0.8 M sodium citrate/1.2 M NaCl to the aqueous phase of the supernatant. The solution was mixed and incubated for 10 min at room temperature. The RNA was pelleted by centrifugation at 13000 rpm for 10 min at 4°C. The pellet was washed with 500 µl of -20°C 75% v/v ethanol and respinning at 13000 rpm for 5 min. at 4°C. The RNA was air-dried for 3 min. at room temperature and dissolved in 40 µL of DEPC-water at 60°C for 10 min. 1 – 2 µg of RNA were used for RT-PCR.

2.6.3. RT-PCR and standard PCR

The RNA and oligo-T primers were denatured at 65°C for 10 min. prior the reaction. RT-PCR was performed with 50 U of reverse transcriptase (RT) (Roche) in 20 µL of volume containing 1x buffer for RT (Roche), 2.5% v/v RNasin, 10 mM DTT, 0.25 mM dNTPs, 30 µM oligoT and 1-2 µg RNA for 90 min. at 42°C. 1 µL of cDNA was used for standard PCR with *STOPP* gene specific primers:

Table 2.7. Gene-specific primers used for RT-PCR

STOPP	**Sequence (5' to 3')**
#237 (forward1)	CGCGTGTTGTGTAACCGCTTTGATATCGA
#365 (reverse 1)	TTGGATTCTCTACGCCTACGCAGT
#483 (reverse 2)	AGCTCTCACTTCCGATCATAATCCTT
GFP	**Sequence (5' to 3')**
#286	GCGGCCGCATGGTGAGCAAGGGCGAGGA
#287	TCTAGATTTACTTGTACAGCTCGTCCATGCCGAGAGT
ACT3	**Sequence (5' to 3')**
#260 (forward1)	ATGGTTAAGGCTGGTTTTGC
#361 (reverse 1)	AGCACAATACCGGTAGTACG

The standard PCR was performed with Taq polymerase (Fermentas) in 25 µL of reaction mixture containing 1x Taq polymerase buffer (Fermentas), 0.24 µM of each primer, 0.2 mM dNTPs, 0.1 – 10 ng of template DNA.

2.6.4. Southern blot

The probe DNA was prepared using Gene Images random prime labelling module (Amersham Biosciences) according to manufacture's regulations. For the hybridization with gDNA from SALK T-DNA insertion lines the probe was prepared by isolation of the 740 bp *AspI/ EcoRI* fragment from pBI101 vector which corresponds to the T-DNA left border sequence of pROK2 plasmid used to create SALK T-DNA insertion collection (http://signal.salk.edu). ~ 50 ng of template DNA was used for labelling with Gene Images random prime labelling module to prepare 50 µL probe. Prior to use the labelled probe was denatured as follows: 10 µL was mixed with 10 µL of sterile water, heated for 5 min. at 100°C and chilled on ice for 5 min.

Genomic DNA for Southern blot was isolated from young plant leaves by DNeasy Plant Kit (Qiagen) according manufacture's regulations. At least 1 µg of eluted gDNA was restricted with 25 - 40 U of appropriate enzyme overnight. The gDNA was precipitated with 2.5 volumes of ice cold 100% ethanol and 1/10 volume of 5.3 M sodium acetate. The gDNA was dissolved in 10 µL of TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and run on 0.8 - 1% agarose gel in TBE (0.09 M Tris base, 0.09 M boric acid, 2 mM EDTA pH 8.0) buffer at 5 V/cm^2. The image of the DNA with a reference ruler was taken prior to the other steps. Subsequently the DNA was de-purinated by incubating the gel in 0.24 N HCl for 10 min., washed for 1 min. in sterile water, denatured three times for 10 min. each in denaturation

solution (1.5 M NaCl, 0.5 M NaOH), washed for 1 min. in sterile water, neutralized 2 times 15 min. in neutralization solution (1.5 M NaCl, 1 M Tris-base, 0.001 M EDTA, pH 7.4) and washed with sterile water.

The gDNA was blotted to a nitrocellulose membrane (Hybond^{TM} -N, Amersham Biosciences) overnight in 10x SSC (1.5 M NaCl, 0.15M sodium citrate, pH 7.0), wrapped in plastic foil and cross-linked with UV for 2 min. The membrane was further incubated in 10 mL of prehybridization solution (5x SSC, 0.1% SDS, 5% w/v dextrane sulphate, 0.5 mL Liquid blocking agent (Amersham Biosciences)) rolling for 2 h at 62°C. The denatured probe was added to the prehybridization solution. The DNA was hybridized at 63°C rolling overnight . The membrane was washed with 2 - 5 mL/cm^2 of pre-warmed 1st washing solution (1x SSC, 0.1% SDS) and 2nd washing solution (0.5x SSC, 0.1% SDS) each for 15 min. rolling at 60°C.

The membrane was blocked for 1 h with 0.7- 1.0 mL/cm^2 of a 1:10 dilution of liquid blocking agent in diluent buffer (0.1 M Tris-HCl, 0.3 M NaCl, pH 9.5) with gentle agitation and rinsed in diluent buffer for 1 min. The membrane was incubated with 0.3 mL/cm^2 of 1:5000 diluted antifluorescein-AP conjugated antibody in 0.5 % (w/v) BSA in diluent buffer for 1 h with gentle agitation, washed 3 times for 10 min. each with 0.3% v/v Tween-20 in diluent buffer and rinsed in diluent buffer for 1 min. The alkaline phosphatase reaction was developed by incubating the membrane DNA-side-down into 30 - 40 µL/cm^2 of detection reagent solution CDP-Star^{TM} detection reagent (Amersham Biosciences) for 2 min. The membrane was wrapped into plastic sheets and exposed to Kodak Biomax MR film.

2.7. Histo- and immunostaining of plant material and microscopy

2.7.1. Histochemical GUS staining

Histochemical GUS staining of Arabidopsis was performed with modifications from original protocol (Malamy and Benfey, 1997). Arabidopsis seedlings or tissues were stained with GUS staining solution containing 100 mM Tris pH 7.5, 0.05 mM NaCl, 20% v/v methanol, 1 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 1 mM $K_3[Fe(CN)_6]$, 5 mg/mL 5-bromo-4-chloro-3-indoxyl-ß-d-glucoronic acid (X-GlcA) cyclohexylammonium salt (Duchefa) at 37°C for 1-3 days. Staining by this method increases specificity of the signal.

To increase the signal, seedlings were permeabilized in 90% acetone (-20°C) on ice for 2 min., washed 3 times with ice-cold 0.1 M sodium phosphate buffer pH 7.0 and stained with

GUS staining solution (0.1 M $NaPO_4$ pH 7.0, 0.5 mM $K_4[Fe(CN)_6]\cdot 3H_2O$, 0.5 mM $K_3[Fe(CN)_6]$, 1 mg/mL X-GlcA, 0.3% v/v Tween 20) at 37°C.
For the GUS staining of Arabidopsis embryos the whole ovules were stained and then opened by slightly squashing them using a coverslip. Arabidopsis leaves and flowers were stained by vacuum infiltration of GUS staining solution for 10 min.
After the staining, chlorophyll from seedlings and tissues was extracted with a series of ethanol washings (30% - 70%).

2.7.2. Propidium iodide staining of plant cell walls

Seedlings were immersed in fresh 10 µg/mL propidium iodide (PI) solution for 10 min., destained with water and observed under confocal laser scanning microscopy (CLSM).

2.7.3. Whole-mount immunolocalisation in roots of Arabidopsis

The modified protocol for whole-mount immunolocalisation in roots was used (C. Luschnig, BOKU Vienna). The seedlings were fixed in 4% w/v paraformaldehyde in MTSB buffer (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, pH 7.0) in multiwell cell culture dish for 1 hour - overnight. Seedlings were washed 5 times 10 min. in MTSB buffer with 0.1% v/v Triton X-100 and 5 times 10 min. with sterile water. Seedlings were treated with 2% w/v driselase (from Basidiomycetes ssp., Fluka) in MTSB buffer for 30 min., and then washed 5 times 10 min. in MTSB buffer with 0.1% v/v Triton X-100. Seedlings were permeabilised in 10 % w/v DMSO, 3 % v/v NP-40 for 1 hour and washed 5 times 10 min in MTSB buffer with 0.1% v/v Triton X-100. The seedlings were blocked in 2% w/v BSA in MTSB buffer for 1 hour at 30ºC and incubated with the 1:500 diluted primary anti-HA antibody (Covance) in 3% w/v BSA in MTSB buffer overnight at 30ºC. Seedlings were washed subsequently 4 times 5 min. and 4 times 10 min. in MTSB buffer with 0.1 % v/v Triton X-100 and incubated for 3 h at 30ºC with the secondary FITC conjugated antibody (Sigma) diluted to 1:40 in 3% w/v BSA in MTSB buffer. The seedlings were washed 5 times 10 min. in MTSB buffer with 0.1 % v/v Triton X-100 and 5 times 10 min. in sterile water, mounted onto slides in 77% glycerol in phosphate buffer (150 mM NaCl, 10 mM KH_2PO_4, 10 mM Na_2HPO_4 x $2H_2O$) and examined under CLSM.

2.7.4. Bright field / fluorescence microscopy

The development of epidermis and the histochemical GUS stainings of transgenic and/or wild type Arabidopsis plants were examined by Zeiss Axioplan microscope or Leica stereomicroscope. Images were taken with SPOT camera and processed with SPOT or MetaView software.
CLSM (confocal laser scanning microscopy) was used to examine protein immunolocalisation in roots of Arabidopsis seedlings. The GFP fluorescence was exited with argon/krypton laser (Leica TCS) at 488 nm line and emission was detected at 500 – 530 nm. The propidium iodide staining was detected at 600 – 650 nm. The images were obtained using Leica software.

2.7.5. Scanning electron microscopy

Seedlings were fixed in 2.5% v/v glutaraldehyde (pH 7.1) at RT overnight rolling and dehydrated through acetone series of 30%, 50%, 70%, 80%, 90% and twice with 100% each 30 min. at room temperature. Samples were immediately put for critical point drying for 1 h, mounted on stubs and coated with gold. Samples were examined by scanning electron microscope (EMBO workshop on Electron Microscopy and Stereology in Cell Biology, 2005, Ceske Budejovice).

2.8. Ethylene measurement

The seeds of wild type Col-0, *stopp* SALK_109986 and *pGreenII-2x35S-TL-gSTOPPwt-HA*, *pGreenII-2x35S-TL-gSTOPPwt-MYC* plant lines were surface-sterilized with Bayrochlor and 100 seeds per glas vial (45° angle) were seeded. Seedlings were germinated in 4 replicates on ½ MS media (with 1% sugar, 0.7% agar, pH 5.7) at 16 h light photoperiod, at 22°C up to 5-6 days after germination (dag). Vials were closed with air-tight caps 1 day before the measurement. Ethylene was measured by gas chromatography (Hewlett Packard 5890 SeriesII) with Al_2O_3 column (Agilent Technologies) by DI M. Schwanninger (BOKU, Vienna). The accumulated ethylene was calculated in pL (or fL) per mg of plant fresh weight.

3. Results

3.1. Analysis of *STOPP* gene expression

To investigate the expression pattern of *STOPP* gene, *STOPP* promoter and transcript levels were analyzed *in silico* and *in planta*. To see where *STOPP* gene is expressed, transgenic $STOPP_{pro}$*:GUSint* (V.Kazanaviciute, unpublished) and $STOPP_{pro}$*:GFP* plant lines were produced and analyzed by histochemical GUS staining or detection of GFP fluorescence.

3.1.1. Bioinformatic analysis suggests that *STOPP* is developmentally and stress-regulated

The expression of *STOPP* gene was studied *in silico* using the reference expression database and meta-analysis system Genevestigator V3 (https://www.genevestigator.com/gv/index.jsp) (Zimmermann et al., 2004). Changes in *STOPP* expression (transcript) were analyzed during various Arabidopsis developmental stages, in different tissues and after treatment with a range of chemical and biogenic substances. The anatomy and development meta-profiles of *STOPP* expression were created using the data from all (4075) ATH1 22K microarrays. The meta-profiling of *STOPP* expression in response to external stimuli was created using annotation tool.

STOPP transcript was the most abundant in specific cell types and generative organs, such as pollen, stamen, flowers, embryo micropylar endosperm and suspensor cell, and lateral root cap (Figure 3.1). During plant development, the *STOPP* transcript was more abundant at juvenile development stages, such as seedling, young rosette and young flower, rather than in mature plant organs (Figure 3.2).

The meta-profiling of *STOPP* expression in response to external stimuli revealed that only few substances or stress conditions could significantly alter the level of *STOPP* mRNA transcription or accumulation. *STOPP* transcript was up regulated by pathogen elicitors, including syringolin, chitin, elf18 and elf26 (18 and 26 amino acid peptides of elongation factor TU), flg22, NPP1, HrpZ (hairpin Z) and by *P.syringae* infestation (Figure 3.3). The protein synthesis inhibitor cycloheximide led to high accumulation of the *STOPP* transcript suggesting that *STOPP* may be regulated post-transcriptionally. Interestingly, closely related to *STOPP*, Arabidopsis *AP2C1* is wound-induced (Schweighofer et al., 2007). By contrast, *STOPP* transcript was not up regulated by wounding (Genevestigator V3) (Figure 3.3).

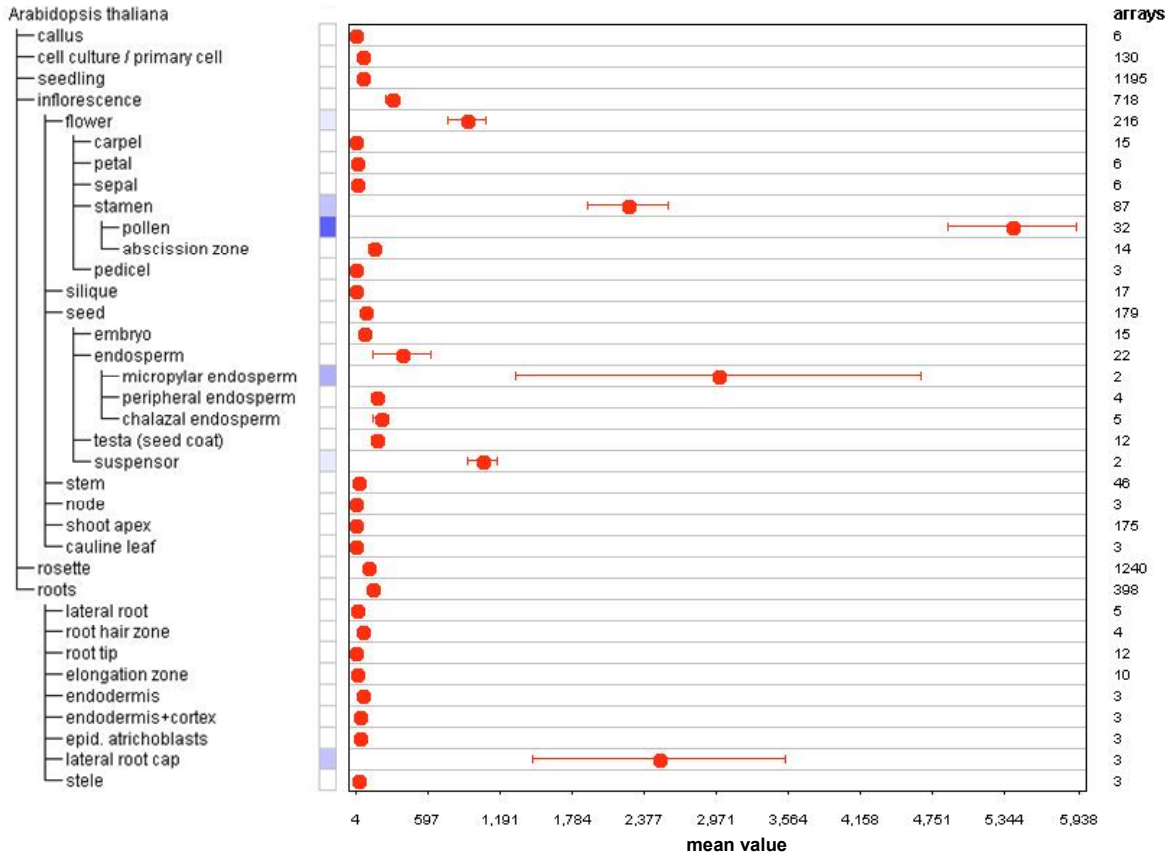

Figure 3.1. The anatomy meta-profile of *STOPP* expression in organs, tissues and cell types (Genevestigator V3). Left: heatmap of mean signal values (blue-white color coding). Right: scatterplot of mean values across the anatomy categories. Standard errors are indicated by bars. The number of arrays is shown on the very right. Array type: ATH1 22K.

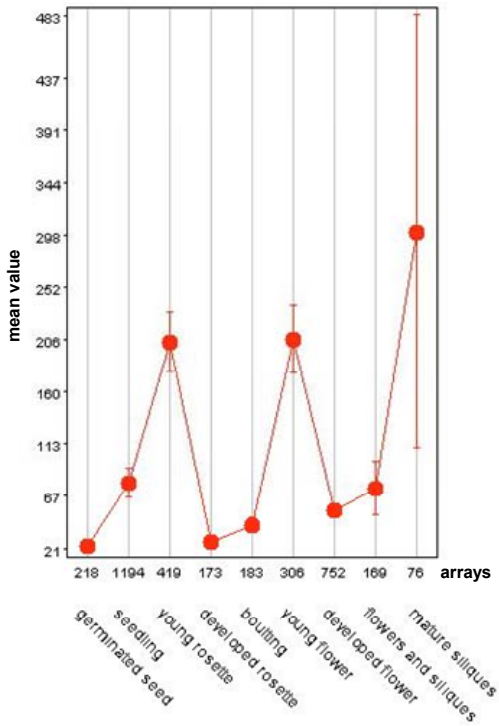

Figure 3.2. The development meta-profile of *STOPP* expression (Genevestigator V3). *STOPP* expression fluctuates during plant development. Standard errors are indicated by bars. The number of arrays is indicated for each developmental stage. Array type: ATH1 22K.

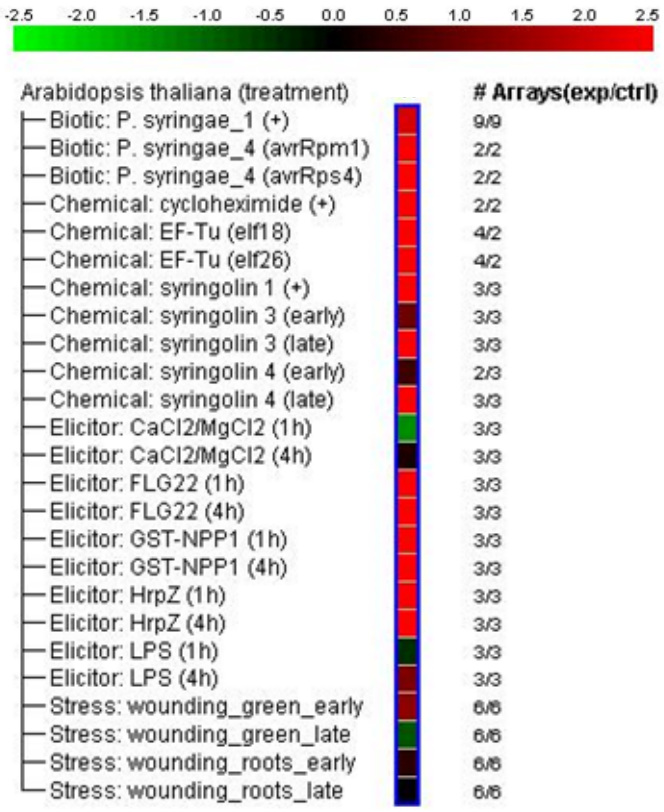

Figure 3.3. The meta-profile of *STOPP* expression in response to external stimuli (Genevestigator V3). *STOPP* is up regulated by pathogen-related stress. The red – green color coding in heatmap represents log2 signal ratio of treatment versus control values.

Taken together, the bioinformatics analysis suggests that *STOPP* expression is regulated during development and by stress application. This regulation is cell-type-specific and induced by pathogen-related stress.

The regulatory sequences of *STOPP* promoter region were searched using the Gene Regulatory Information Server (AGRIS) Arabidopsis thaliana cis-regulatory database (AtcisDB) (http://arabidopsis.med.ohio-state.edu/) (Davuluri et al., 2003; Palaniswamy et al., 2006). Such cis-regulatory binding sites were found in *STOPP* promoter as W- and G-boxes GATA and Ibox promoter motifs. RAV1-A, CCA1, MYB1 and MYB4 binding site motifs indicate that *STOPP* may be induced in response to pathogen stress or regulated by light and circadian rhythm.

3.1.2. The expression of *STOPP* during development and by stress treatment *in planta*

Developmental and tissue-specific expression of *STOPP* gene *in vivo* was studied by analyzing the *STOPP* promoter activity in plants harbouring the $STOPP_{pro}$*:GUS* or $STOPP_{pro}$*:GFP* transcriptional fusions. The activity of *STOPP* promoter was detected in a

range of cells and tissues by histochemical GUS staining. The activity of the *STOPP* promoter was detected earliest in the globular embryo and was restricted to the suspensor cell during heart and torpedo stages of embryo development (Figure 3.4). The promoter activity was also detected in the micopylar endosperm (data not shown). *STOPP* expression in 2 to 8 dpg (days post germination) seedlings was localized to the transition zone between division and cell elongation zones in the root epidermis and in stomata on hypocotyls, cotyledons and emerging true leaves. However, in later development stages the promoter activity decreased and in the adult plant was restricted to stomata on pedicel, sepals, stigma, anthers and mature pollen. Very weak activity of *STOPP* promoter was detected in stomata of rosette leaves (only after 72 h of GUS staining).

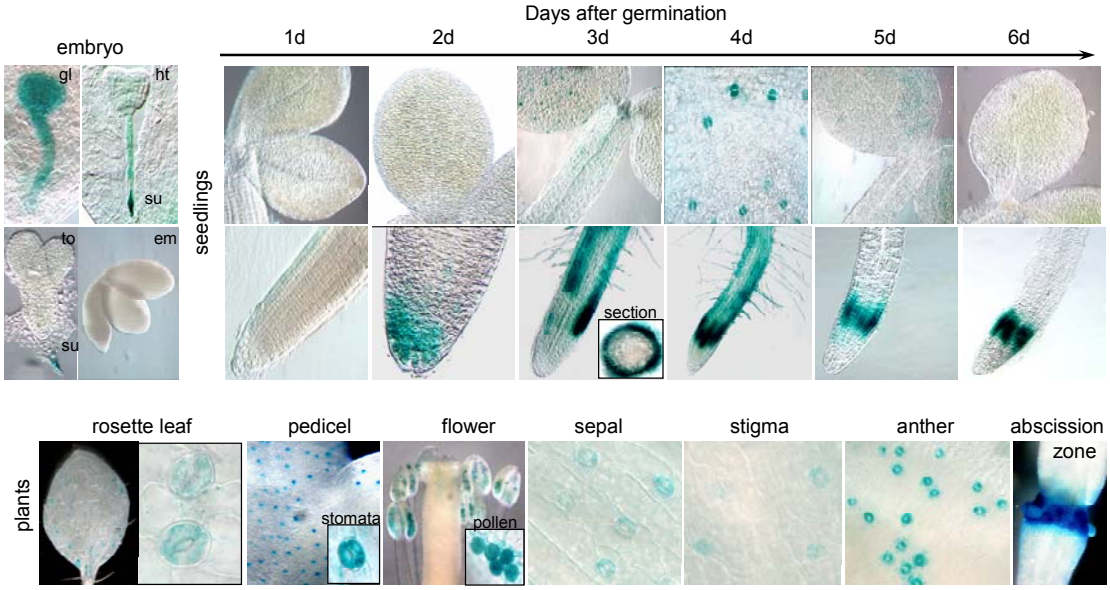

Figure 3.4. The activity of *STOPP* promoter in *STOPP$_{pro}$:GUS* plants. Histochemical staining of embryo (upper panel, left): globule (gl), heart (ht), torpedo (to) and mature embryo (em) stages. *STOPP* is expressed in globular embryo and in suspensor cell (su) during heart and torpedo stages. *STOPP* is expressed in root epidermis (starting from the second days after germination) and stomata during seedling development (upper panel, right). *STOPP* expression is localized specifically to stomata in adult plants (lower panel): very week *STOPP* promoter activity in stomata on leaves and stronger promoter activity in stomata on pedicel, sepals, stigma and anthers. *STOPP* is highly expressed in mature pollen and abscission zone of silique stem.

In agreement with the promoter - GUS activity, the GFP fluorescence in *STOPP$_{pro}$:GFP* line (#1) was detected in stomata (Supplemental figure 1). Interestingly, higher *STOPP$_{pro}$:GFP* activity was observed in young recently divided stomata than in mature stomata.

A variety of external stimuli (heavy metals, high salt, hormones, pathogen elicitors, cold and wounding) were applied to *STOPP$_{pro}$:GUS* seedlings and adult plants (summarized in Table 3.1) but all these treatments failed to induce detectable activity of the promoter (here only

wounding is shown, Figure 3.5 B). The stress application had little effect on GUS activity in stomata and had no or repressing effect on GUS expression in roots. In general, no treatment obviously induced/enhanced *STOPP$_{pro}$:GUS* activity.

Table 3.1. The activity of *STOPP$_{pro}$:GUS* after various stress application in seedlings

Treatment	Concentration	*STOPP$_{pro}$:GUS* activity*
$CuCl_2$	1 mM 10 mM	NC NC
NaCl	150 mM 250 mM	NC SU in stomata
ABA	100 µM	NC
NAA	5 µM 50 µM	NC NC
ACC	100 µM	SU in stomata
MeJA	100 µM	NC
SA	100 µM	SU in stomata, SD in roots
Xylanase	20 mM 100 mM	SU in stomata SU in stomata
flg22	10 µM 100 µM	SU in stomata SU in stomata
cold	-	NC
wounding	-	NC

* compared visually in treated and untreated *STOPP$_{pro}$:GUS* seedlings; NC – not changed, SU – slightly up regulated, SD – slightly down regulated.

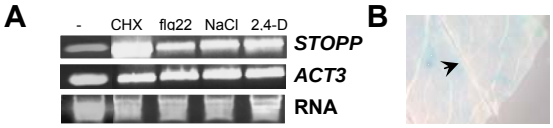

Figure 3.5. Up regulation of *STOPP* expression by stress application. *STOPP* transcript levels after the treatment with 50 µM CHX, 100 nM flg22, 100 mM NaCl or 2,4-D in seedlings by semi-quanititative RT-PCR **(A)**. The expression of *STOPP* is not induced by wounding in *STOPP$_{pro}$:GUS* plants **(B)**. Rosette leaf was wounded (cutting) and stained by GUS.

The *STOPP* transcript levels in response to biotic and abiotic stress treatment were analyzed by semi-quantitative RT-PCR. In agreement with bioinformatic data the *STOPP* expression was significantly induced by cycloheximide, however, no detectable induction with 100 nM flg22 was observed after 30 min. of treatment (Figure 3.5 A).

3.2 Analysis of *STOPP* gain-of-function and loss-of-function lines

3.2.1. *STOPP* gain-of-function lines develop stomata clusters in epidermis

Gain-of-function (gof) *STOPP* lines over-expressing epitope tagged *STOPP* gDNA were analysed morphologically and by Western blotting. In T3 generation dwarfish seedlings of *$35S_{pro}$:gSTOPP-HA* (line #2 from 2 independent lines), *$35S_{pro}$:gSTOPP-Myc* (1 independent line) and *$35S_{pro}$:gSTOPP-GFP* (lines #2, #3 and #5 from 5 independent lines) were observed (Figure 3.6 A). The *STOPP* gof 5 dpg seedlings developed ~ 60% shorter roots in comparison to the wild type seedlings when grown on vertical plates (Table 3.2 and Figure 3.6 B). The epidermal cells in basal zone of hypocotyls were ~ 66% shorter in *STOPP* gof than in wild type seedlings (Table 3.2 and Figure 3.6 C and D). The hypocotyl elongation in dark grown *STOPP* gof seedlings was ~ 23% reduced as compared to the wild type but the apical hook formation was not affected (Table 3.2 and Figure 3.6 E).

Table 3.2. Root and hypocotyl lenght of *STOPP* gof 5 dpg seedlings

Lenght	**wt**	***STOPP gof***	**P value (ttest)**
Root length (cm)	1.15 ± 0.18	0.46 ± 0.12	< 0.0001
Hypocotyl epidermal cells (µm)	131.63 ± 22.7	58.0 ± 14.76	< 0.0001
Hypocotyl elongation in dark (cm)	1.23 ± 0.24	0.95 ± 0.14	< 0.005

Seedlings with dwarf phenotype were also vitreous on the agar plates and hardly survived in soil. By contrast, almost all seedlings could survive on ½ MS plates supplemented with 1% sucrose for 1 month and longer. The strength of the phenotype varied among the survivors on the soil in the same line. In addition, the strength of the phenotype was decreasing in *$35S_{pro}$:gSTOPP-HA* ≥ *$35S_{pro}$:gSTOPP-Myc* > *$35S_{pro}$:gSTOPP-GFP* respectively. Most of the survived adult plants had a dwarf phenotype (Figure 3.6 F) and formed stunned flowers and stunned siliques (Figure 3.6 G and H). However, all the *STOPP* gof plants that had produced flowers were fertile. The fertility of pollen was normal as pollen germination was not affected. The observation of epidermis of these seedlings and plants revealed the appearance of stomata clusters or even the complete conversion of epidermal cells on the cotyledons to stomata (Figure 3.7). Stomata clusters of various sizes were observed also in the epidermis of hypocotyls, on some rosette leaves or cauline leaves, in sepals, anthers, pistils (Figure 3.7 and 3.8 A) and siliques (here is not shown).

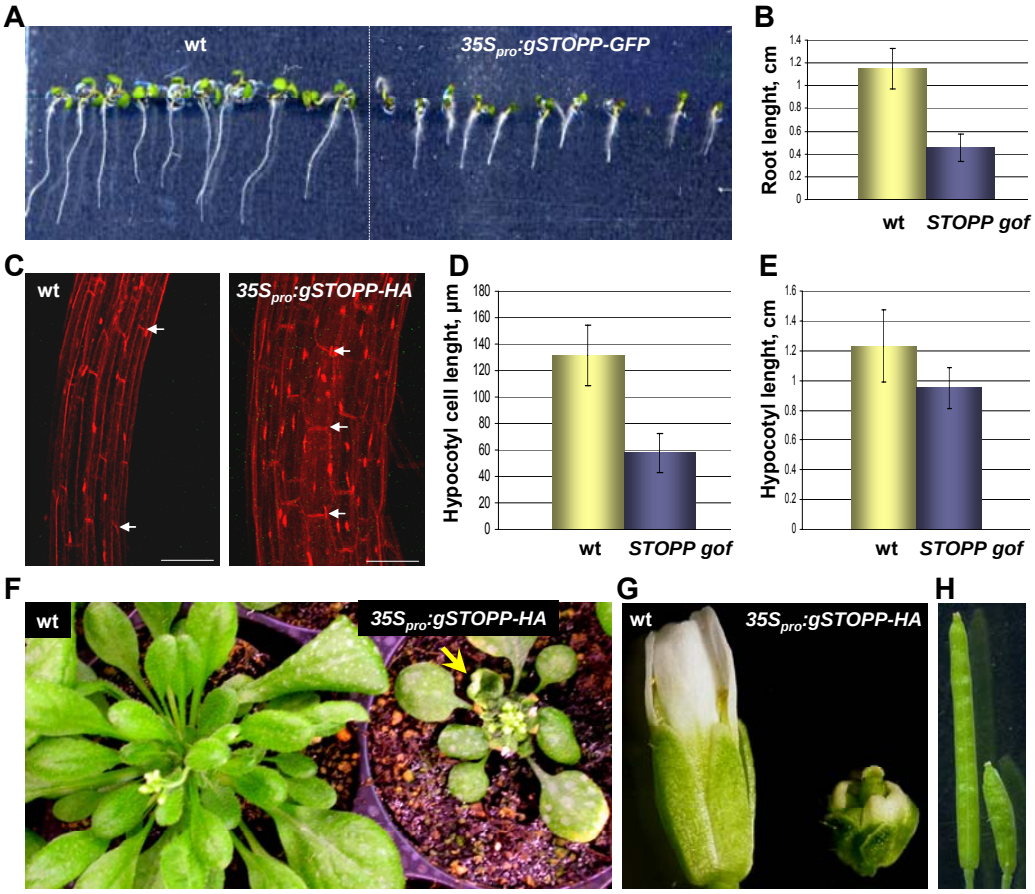

Figure 3.6. *STOPP* gof plants show dwarf phenotype. 5 dpg wild type (wt) and *STOPP gof* seedlings on vertical plates **(A)**. *STOPP* gof seedlings have ~ 60% shorter roots, ~ 66% shorter epidermal cells of basal hypocotyl and ~23% shorter hypocotyls of dark-grown seedlings than wild type **(B, D and E respectively)**. The epidermal cells of basal hypocotyl in wt and *STOPP* gof seedlings **(C)**. The junctions between epidermal cells are marked in arrows. Red – propidium iodide staining. 1-month-old wt and *STOPP* gof plants in the soil **(F).** Flowers and siliques of wt and *STOPP* gof plants **(G and H respectively)**. Wild type is on the left and *STOPP* gof is on the right in all figures. All bars 50 µm.

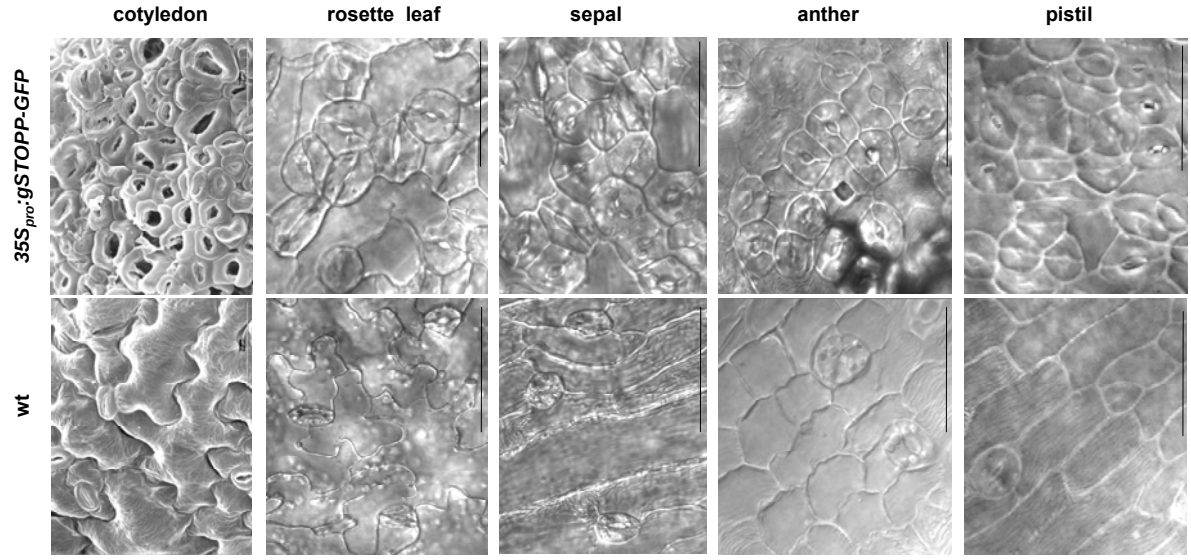

Figure 3.7. Stomata clusters on various organs of *35S$_{pro}$:gSTOPP-GFP* plants. *STOPP* gof results in the development of stomata clusters in the epidermis of cotyledons, rosette leaves, sepals, anthers and pistils. All bars 50 µm.

The stomata development was analysed in hypocotyls of *35S$_{pro}$:gSTOPP-GFP* seedlings for 5 consecutive days. The ectopic stomata developed from neighbor cells, which did not exhibit typical precursor cell morphology (Figure 3.8 A, 2 – 5 dpg). Stomata clusters were formed both in protruding and non-protruding cell files of hypocotyls (Figure 3.8 A, 12 dpg). The intensive cell divisions were observed in epidermal cells of *STOPP* gof seedlings (Figure 3.8 B). Finally, almost cells differentiated into guard cells (GCs).

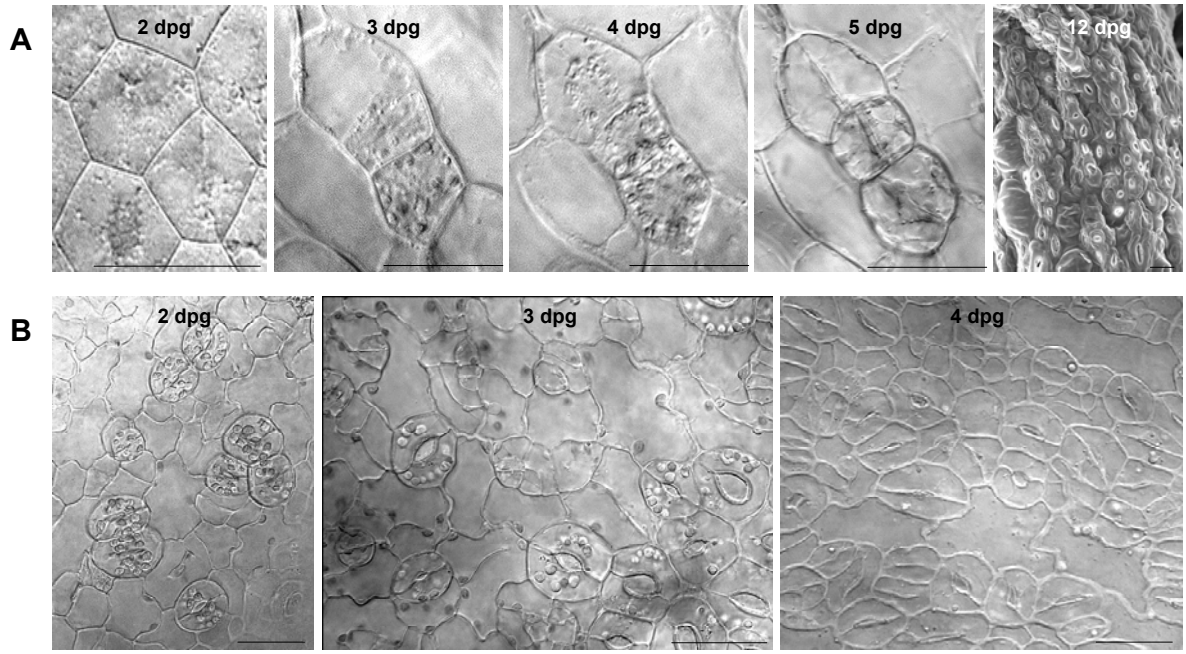

Figure 3.8. Stomata cluster formation in *STOPP* gof lines. The time-scale observation of multiple stomata formation in the hypocotyl of *35S$_{pro}$:gSTOPP-GFP* seedlings **(A)**. Almost all epidermal cells finally differentiate into guard cells. The intensive cell divisions in epidermal cells on the cotyledons of *35S$_{pro}$:gSTOPP-HA* #2.2.1.4 seedlings **(B)**. All bars 20 µm.

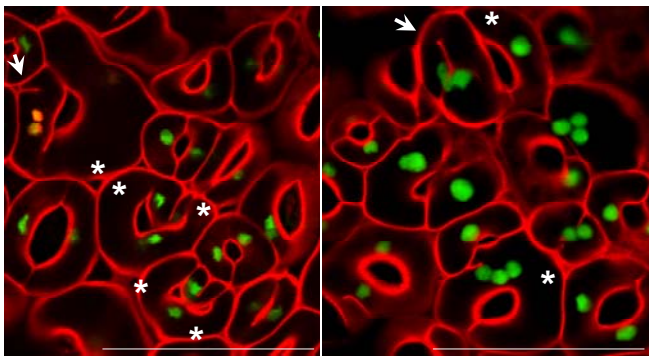

Figure 3.9. Aberrant stomata in cotyledons of *35S$_{pro}$:gSTOPP-GFP* gof 7 dpg seedlings. Aberrant stomata are indicated by asterisks; dividing GC-like cells are indicated by arrows. Green - GFP fluorescence, red – propidium iodide staining. All bars 50 µm.

In epidermis of older than 7 dpg *STOPP* gof seedlings, aberrant stomata were observed. These stomata were formed of two GC-like cells of unequal size. Such GC-like cells acquired typical guard cell characteristics but were larger than normal stomata. The aberrant stomata

contained stomata pores, which were formed often by only one guard cell in the pair (usually by the larger one). Some of the aberrant GC-like cells contained several nuclei (up to three) and could initiate asymmetric divisions (Figure 3.9).

According to the phenotype of the *STOPP* gof lines, the gene was named *STOPP* (Stomata Protein Phoshatase 2C).

3.2.2. Induction of stomata clustering by conditional *STOPP* expression

To verify if stomata phenotype is caused by *STOPP* over-expression, estradiol (ER) inducible Myc or YFP tagged *STOPP* expressing lines were produced. The seedlings were germinated in liquid ½ MS in multiwell dishes for 3 days in the presence of 50 µM ER or equal amount of solvent DMSO as a control. The seedlings were phenotypically analysed by microscopy and the transgene phosphatase protein levels were assayed by Western blot (Figure 3.10 A and C). ER-treated *XVE-gSTOPP-Myc* and *XVE-gSTOPP-YFP* seedlings developed stomata clusters in the epidermis of cotyledons (Figure 3.10 B and D) and hypocotyls (not shown). This clustering of stomata was induced in 11 out of 12 independently transformed lines.

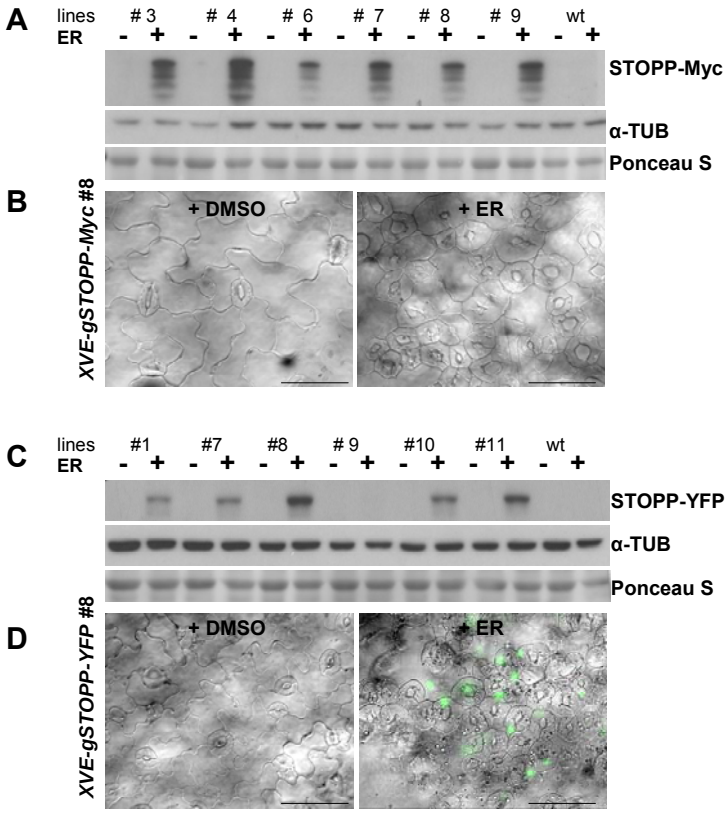

Figure 3.10. Induction of stomata cluster formation in ER-inducible *STOPP* gof lines. STOPP-Myc **(A)** and STOPP-YFP **(C)** protein accumulates after treatment of 3 dpg old seedlings with 50 M estradiol. α-tubulin (α-TUB) was used as a control for protein amounts. The accumulation of STOPP protein leads to stomata cluster formation **(B and D)**. Solvent DMSO was used as a control. Green – YFP fluorescence. All bars 50 µm.

The line which failed to develop stomata clusters (*XVE-gSTOPP-YFP #9)* also did not express STOPP-YFP protein (proven by Western blot) (Figure 3.10 C). This result provides additional evidence that over-production of STOPP protein leads to formation of multiple stomata.

3.2.3. The STOPP protein may be post-transcriptionally regulated by the proteasome

To investigate if the *STOPP* gof phenotype correlates with protein expression, STOPP protein levels were analyzed by Western blot with antibodies against HA or GFP tags.

For Western blot analysis wild type and the *35S$_{pro}$:gSTOPP-GFP* line #2.2.7 was germinated on agar plates for 19 consecutive days. STOPP-GFP protein was detected by Western blot from 0 till 11 days after germination but from 5 till 11 dpg the protein level decreased. After 11 dpg the fusion protein could not be detected (Fig. 3.11 A). The STOPP-GFP protein was further not detected in the leaves of adult *STOPP* gof plants by Western blot or by GFP fluorescence although the mRNA of endogenous *STOPP* and *STOPP-GFP* was detected by RT-PCR (Figure 3.11 B). This suggested the regulation of the STOPP protein stability. The STOPP protein stability was analyzed in *35S$_{pro}$:gSTOPP-HA* #2.2.1 seedlings. *STOPP* gof seedlings were germinated in liquid ½ MS media, treated with ubiquitin 20S and 26S proteasome pathway inhibitors (MG132 for 1 h or 12 h; MG115 for 1 h; lactacystin for 1 h and epoxomicin for 1 h) and analysed by Western blot. As a result only the reversible proteasome inhibitors MG132 and MG115 were able to slightly stabilize STOPP-HA protein (Fig. 3.11 C) whereas the more specific acting lactacystin and epoxomicin had no effect on protein stability.

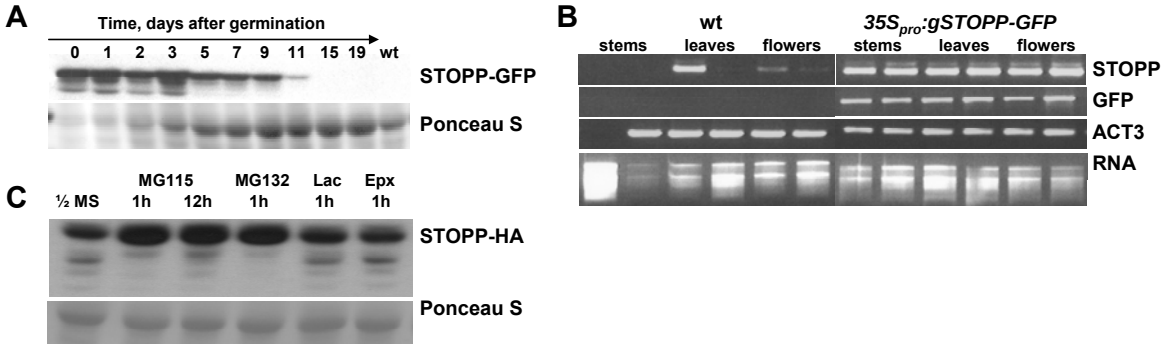

Figure 3.11. The regulation of STOPP protein levels. The timescale detection of STOPP protein levels in *35S$_{pro}$:gSTOPP-GFP* seedlings **(A)**. The *STOPP* transcript is present in stems, leaves and flowers of 1.5 month-old wild type and *35S$_{pro}$:gSTOPP-GFP* lines **(B)**. The RT-PCR was performed with primers specific for *STOPP* or *GFP*. *ACT3* was used as a control. Treatment with proteasome inhibitors of 5 dpg *35S$_{pro}$:gSTOPP-HA* seedlings **(C)**.

3.2.4. STOPP protein is localized to the cell nucleus

STOPP protein localisation was analysed by transient protein expression in Arabidopsis suspension protoplasts and in seedlings constitutively over-expressing GFP-tagged *STOPP*. STOPP-GFP was localized to cell nuclei of protoplasts transformed with *$35S_{pro}$:gSTOPP-GFP* expression vector (Figure 3.12 A) and in *$35S_{pro}$:gSTOPP-GFP* seedlings (Figure 3.12 C). These results were confirmed by whole-mount immuno-localisation in *$35S_{pro}$:gSTOPP-HA* seedlings using HA antibody in roots and cotyledons of 3 dpg seedlings (Figure 3.12 B and D respectively) indicating that GFP tag does not interfere with subcellular STOPP protein localisation.

These results also correspond to the previous observation of STOPP-YFP protein in ER-inducible *XVE*-g*STOPP-YFP* lines.

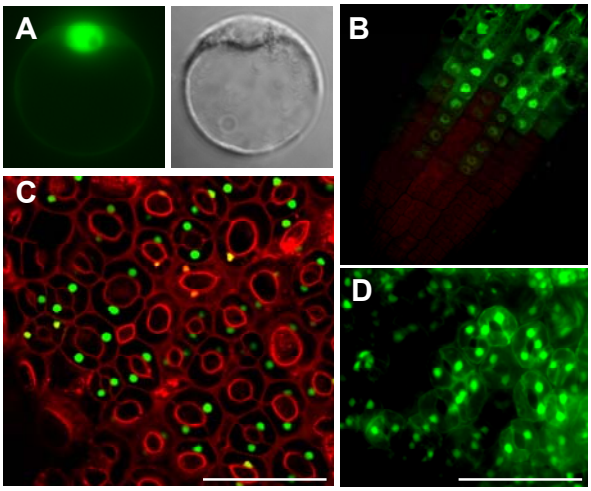

Figure 3.12. Subcellular STOPP localization. STOPP-GFP is localised to the cell nuclei in protoplasts **(A)** and seedlings **(B to D)**: stomata in the cotyledon of 3 dpg seedling expressing *$35S_{pro}$:gSTOPP-GFP* **(C)** and whole-mount immunolocalisation in root **(B)** and cotyledon **(D)** of 3 dpg seedlings expressing *$35S_{pro}$:gSTOPP-HA* with anti-HA antibody. Green – GFP fluorescence, red – propidium iodide staining. All bars 50 µm.

3.2.5. The N-terminal domain of STOPP is responsible for the nuclear localization and stomata clustering in *STOPP* gof lines

STOPP protein exhibits nuclear localization whereas another closely related phosphatase of the PP2C cluster B, AP2C1 (Schweighofer et al., 2004; Schweighofer et al., 2007), is targeted to plastids (V. Kazanaviciute unpublished). Both phosphatases share high sequence homology (especially in C-terminal catalytic domain) and substrate specificity for MAP kinases. Therefore domain-swapping of N- and C-terminal domains (NTD and CTD) between these

phosphatases was performed and fusion protein constructs were created (V.Kazanaviciute, unpublished) (Figure 3.13 A). Transgenic seedlings over-expressing (from 35S promoter) the domain-swapping proteins $AP2C1_{NTD}$-$STOPP_{CTD}$ or $STOPP_{NTD}$-$AP2C1_{CTD}$ tagged with GFP were produced and analysed by fluorescence microscopy in seedlings. As represented in Figure 3.13 B, $STOPP_{NTD}$-$AP2C1_{CTD}$ chimeric protein was located in the nucleus whereas $AP2C1_{NTD}$-$STOPP_{CTD}$ was located in plastids. Moreover, *$35S_{pro}$:$STOPP_{NTD}$-$AP2C1_{CTD}$* over-expressing seedlings developed stomata clusters in epidermis of hypocotyls, cotyledons and emerging true leaves. These results demonstrate that the NTD of STOPP determines protein localisation to the nucleus and this is sufficient for the chimeric protein to exert its function in epidermal cell conversion to stomata. The over-expression of *$35S_{pro}$:AP2C1wt-GFP* (Schweighofer et al., 2007) or *$35S_{pro}$:$AP2C1_{NTD}$-$STOPP_{CTD}$* does not lead to obvious developmental defects suggesting that CTD is interchangeable between closely-related PP2Cs.

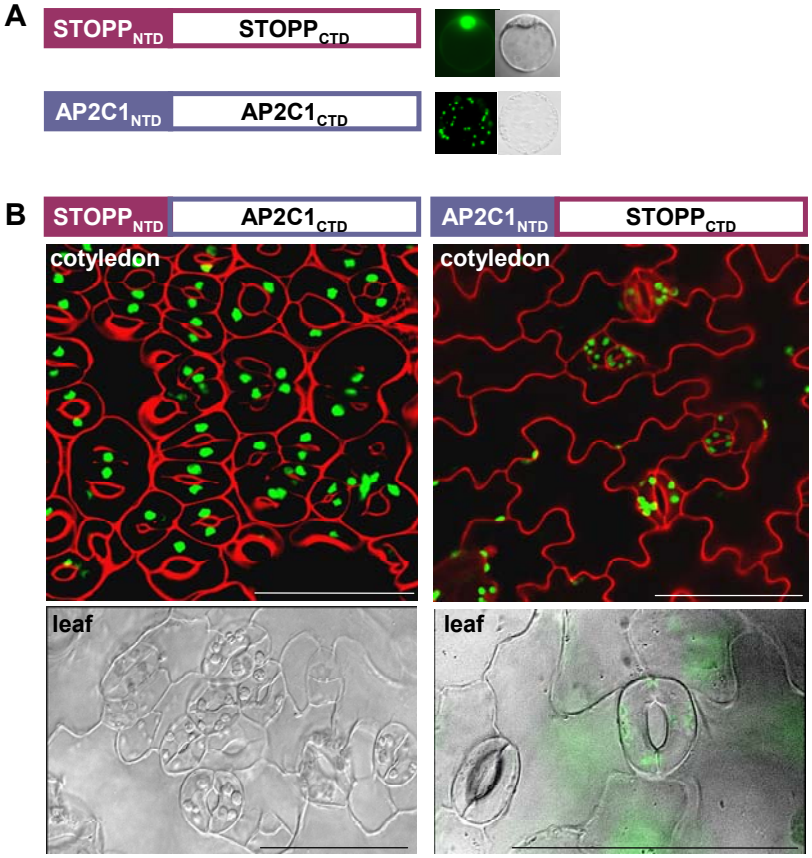

Figure 3.13. NTD of STOPP is responsible for the nuclear protein localisation and for stomata clustering. Schematic representation of domain swapping between cluster B phosphatases STOPP and AP2C1 **(A)**. Native STOPP protein is located to the nucleus and AP2C1 is targeted to the plastids as demonstrated in Arabidopsis protoplasts. After NTD/CTD domain swapping, $STOPP_{NTD}$-$AP2C1_{CTD}$ (line #1.5.) chimeric protein is located in the nucleus whereas $AP2C1_{NTD}$-$STOPP_{CTD}$ (line #1.8.) is located in the plastids (as shown in epidermis of cotyledons and leaves) in transgenic 7 dpg seedlings **(B)**. Green – GFP fluorescence, red – propidium iodide staining. All bars 50µm.

In addition the domain swapping with distant PP2C from cluster A, HAB1 does not lead to stomata clustering although the protein is also localized to the cell nucleus (Supplemental figure 2).

3.2.6. Expression of stomata development markers in *STOPP* gof plants

To identify downstream targets of STOPP, the stomata patway genes were analyzed in *STOPP* gof seedlings. Essential positive regulators of stomata development, the transcription factors FAMA and MUTE, were chosen as marker genes. The plant lines expressing FAMA and MUTE transcriptional fusions with GUS or GFP (for references see § 2.4.1.) were used as mother (acceptor) plants for genetic crosses with *STOPP* gof plants. $FAMA_{pro}$*:GFP* and $MUTE_{pro}$*:GUS* were crossed with $35S_{pro}$*:gSTOPP-HA* (line #2.2.1) whereas $FAMA_{pro}$*:GUS* and was crossed with $35S_{pro}$*:gSTOPP-GFP* (line #2.2.7.5). T1 or T2 generation seedlings of crosses were analysed by GUS staining or GFP fluorescense.

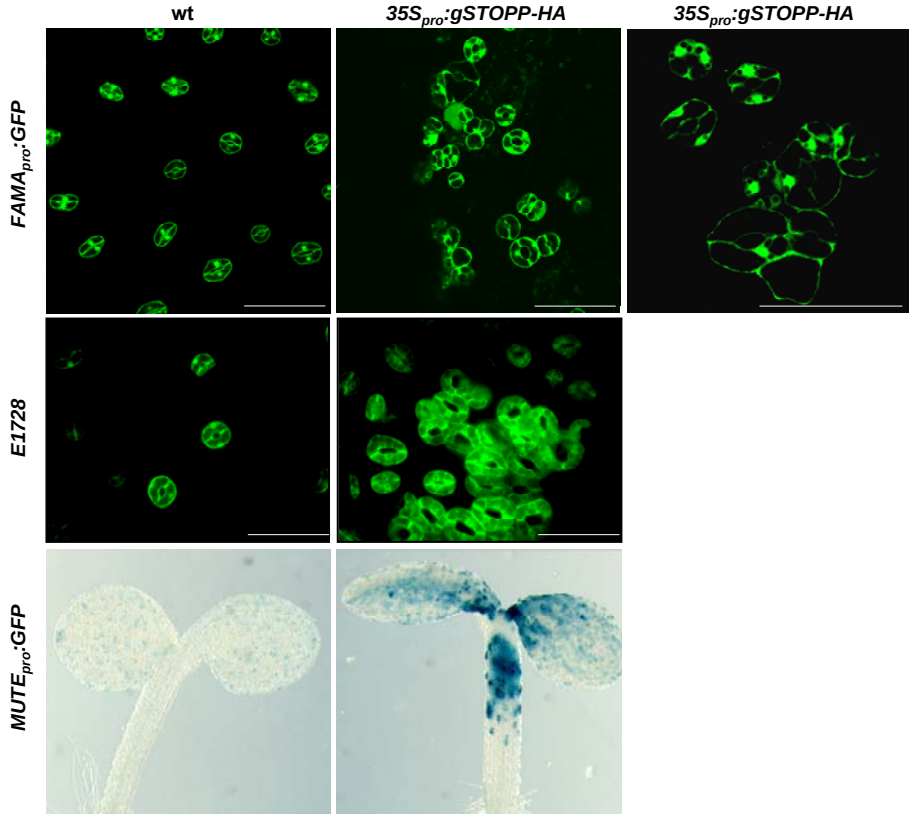

Figure 3.14. The expression of stomata development markers in *STOPP* gof genetic background. The guard cells of *STOPP* gof lines express stomata pathway markers: mature guard cell marker E1728, guard mother cell (GMC) and guard cell marker FAMA and meristemoid and GMC-specific MUTE. Promoter activities of $FAMA_{pro}$*:GFP* and $MUTE_{pro}$*:GUS* show enhanced expression of these genes in 5 dpg *STOPP* gof seedlings. Green – GFP fluorescence. All bars 50 μM.

The promoter activities of transcription factors FAMA and MUTE (Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007) were analysed in wild type and *STOPP* gof genetic background (Figure 3.14). GUS staining revealed enhanced promoter activity of the corresponding genes in *STOPP* gof lines. FAMA-GFP transcription factor is a guard cell marker (Ohashi-Ito and Bergmann, 2006). In contrast to wild type FAMA-GFP was as well expressed in stomata neighbour cells in *35S:STOPP-HA* background as shown in Figure 3.14 (close-up). Similar results were obtained with $FAMA_{pro}$*:GUS* in $35S_{pro}$*:gSTOPP-GFP* by GUS staining (not shown).

3.2.7. Identification and analysis of *stopp* T-DNA insertion lines

stopp T-DNA insertion lines SALK_139825 and SALK_009986 were obtained from SALK collection (http://signal.salk.edu) and analysed by PCR with gene specific and T-DNA primers. The T-DNA was detected in the promoter region 105 bp upstream of start codon in line SALK_139825 (V. Kazanaviciute, unpublished). However the *STOPP* transcript was still detected by RT-PCR in two homozygous lines indicating that the T-DNA insertion did not lead to the disruption of gene product. Therefore the SALK_139825 was not used in further analysis. From SALK_009986 two lines homozygous for T-DNA insertion (#2.1 and #3.2) were selected (for primers see §3.2.1). The T-DNA insertion was detected within the second exon (Figure 3.15 A). Southern blot analysis identified single T-DNA insertions in both lines of SALK_009986 (Figure 3.15 B).

RT-PCR from these lines was performed with *STOPP* specific primers (237 and 365, see §2.6.3) flanking the first and the second introns. For RT-PCR wild type, *stopp* T-DNA insertion lines #2.1 and #3.2 were germinated in liquid ½ MS and treated with 100 nM flg22 or 50 µM cycloheximide. The RT-PCR product of ~ 400 bp corresponding to mRNA of *STOPP* was detected in wild type seedlings but not in SALK_009986 knock out lines (Figure 3.15 C), ACT3 (actin) was used as a control. As a result SALK_009986 lines #2.1 and #3.2 were identified as phosphatase knock-out lines (*stopp*) lines and used for further analysis.

The analysis of epidermis using *stopp* #2.1 and #3.2 lines revealed that the absence of *STOPP* did not affect the development of stomata (epidermis of *stopp* cotyledon is shown in Fig. 3.15 D). Additionally no obvious developmental defects were observed in *stopp* seedlings and adult plants.

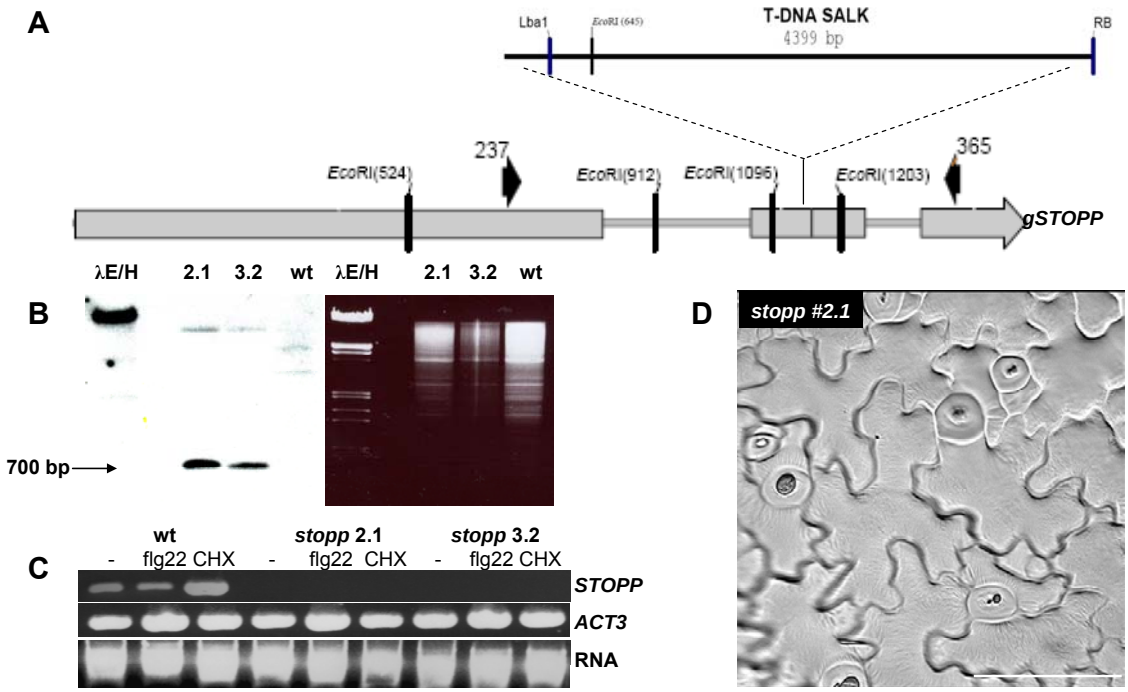

Figure 3.15. Identification of *stopp* T-DNA insertion line SALK_009986. Schematic representation of T-DNA insertion site in the 2nd exon of *STOPP*, at 1159bp from ATG **(A)**. 2 independent homozygous for T-DNA insertion lines: #2.1 and #3.2 were isolated and tested by Southern **(B)** and RT-PCR **(C)**. For Southern blotting gDNA was cut with EcoRI and hybridized with T-DNA specific fragment. Expected band size is 709 bp if the insertion is single. For RT-PCR seedlings were treated with 100 nM flg22 and 50 mM CHX. ACT3 was used as a control. The development of epidermis is not affected in stopp knock out lines as represented in 7 dpg *stopp* #2.1 seedling **(D)**. All bars 50 μm.

3.2.8. Stomata clustering phenotype induced by *STOPP* gof depends on STOPP phosphatase activity

To test if STOPP phosphatase activity is necessary to induce stomata clustering in plants, the phosphatase mutant with reduced phosphatase activity was created. The G163D mutation in the catalytic domain of STOPP was introduced according to analogous mutations in other PP2C phosphatases, ABI1-G180D and AP2C1-G178D (Bertauche et al., 1996; Schweighofer et al., 2007) (Figure 3.14 A). To test if this mutation abolishes enzymatic STOPP activity, recombinant GST-STOPP wild type (wt) and the phosphatase mutant (STOPP-G163D) proteins were produced. The phosphatase activity of recombinant STOPP proteins was assayed *in vitro* by dephosphorylation of γ^{32}P-labelled casein in the presence of 100 nM Okadaic Acid.

As a result GST-STOPP-G163D had 6-fold reduced phosphatase activity compared to GST-STOPPwt (Figure 3.14 B). Subsequently eight independent transgenic lines over-expressing *35S$_{pro}$:gSTOPP-G163D-GFP* were produced. The presence of STOPP-G163D-GFP protein in

seedlings was detected by GFP fluorescence. No stomata clusters or other developmental defects were observed in the epidermis of these seedlings as shown in Figure 3.14 D.

To assay whether the over-expression of wild type *STOPP* phosphatase results in higher overall PP2C activity *in planta*, the total PP2C activity was tested in crude protein extracts. 5 µg of crude protein extract from wild type, *stopp* knock-out (line #2.1) and *35S$_{pro}$:gSTOPP-HA* (line #2.1.6) 7 dpg seedlings was used to dephosphorylate γ^{32}P-casein in the presence of 500 nM okadaic acid. The measurement of released γ^{32}Pi was done in 9 replicates. The total PP2C activity in *35S:STOPP-HA* seedlings was ~15% higher than in wild type (t-test p = 0,006). However in the *stopp* knock-out line the total PP2C activity was only ~ 4% lower than in wild type and this difference was not statistically significant (t-test p> 0,05).

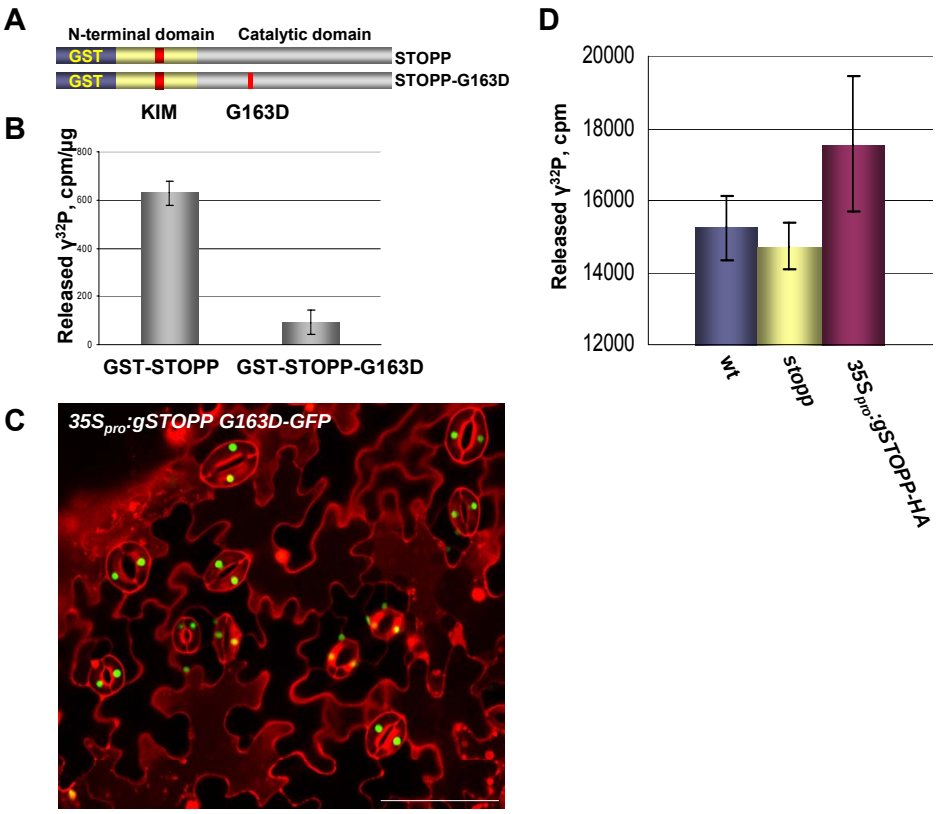

Figure 3.16. The phosphatase activity is essential for PP2C-induced stomata cluster formation. The structure of GST-STOPP and schematic representation of phosphatase mutation site **(A)**. Both STOPP versions were tagged with GST for expression in *E.coli*. Phosphatase activities of recombinant proteins were tested *in vitro* by dephosphorylation of γ^{32}P-casein in the presence of 100 nM Okadaic Acid **(B)**. *35S$_{pro}$:gSTOPP-G163D-GFP* plants have normal epidermis as represented in 7 dpg seedling of line #5 **(C)**. Green - GFP fluorescence, red – propidium iodide staining. All bars 50 µM. Total PP2C activity in crude protein extracts from wild type (wt), *stopp* knock out (line #2.1) and *STOPP* gof (line #2.1.6) 7 dpg seedlings **(D)**. 5 µg of crude protein extract were used to dephosphorylate γ^{32}P-casein in the presence of 500 nM okadaic acid.

3.3. Identification of STOPP-interacting proteins and substrates

3.3.1. MPK4 and MPK6 were identified as STOPP interacting proteins using the yeast two hybrid system

To identify molecular targets of STOPP a yeast two hybrid (Y2H) interaction screen was performed using yeast strain pJ694A transformed with BD-STOPP and an Arabidopsis cDNA library prepared from 1:1 of auxin induced and uninduced roots (provided by Dr. Bert van der Zaal, Institute of Molecular Plant Sciences, Leiden University, The Netherlands). 2.3 million clones were screened and 18 positive clones were obtained after selection. The plasmid DNA of these clones was analysed by restriction digest where 18 clones fell into 5 restriction pattern groups. The corresponding cDNAs from these 5 clones were sequenced and the encoding genes identified (Table 3) by BLAST search at NCBI (blastn; http://www.ncbi.nlm.nih.gov/).

Table 3.3. STOPP-interacting proteins identified in Y2H screening

Locus ID	Description	Clones
At4g01370	AtMPK4	1
At2g43790	AtMPK6	3
At3g61790	SINAT3, seven in absentia (SINA) family protein	1
At1g32070	GCN5 related N-acetyltransferase (GNAT) family protein / nuclear shuttle intreacting protein (NSI)	4
At1g19130	Conservative unknown protein, contains DUF985 domain that belongs to the Cupin superfamily	4

The interactions with BD-STOPP were confirmed by Y2H ß-galactosidase liquid assays and auxotrophic complementation using cDNA rescued from yeast clones (Fig. 14A and 14B).
Among other genes the MAP kinases MPK4 and MPK6 were identified as STOPP interacting partners. To verify the specificity of MAPK interaction direct Y2H interaction assays between STOPP and 18 MAPKs was performed using L40 yeast strain as a host. The ß-galactosidase liquid (Fig. 14C) and auxotrophic complementation assays confirmed that STOPP interacts specifically with MPK4 and MPK6 suggesting the biological significance of these interactions in plants.

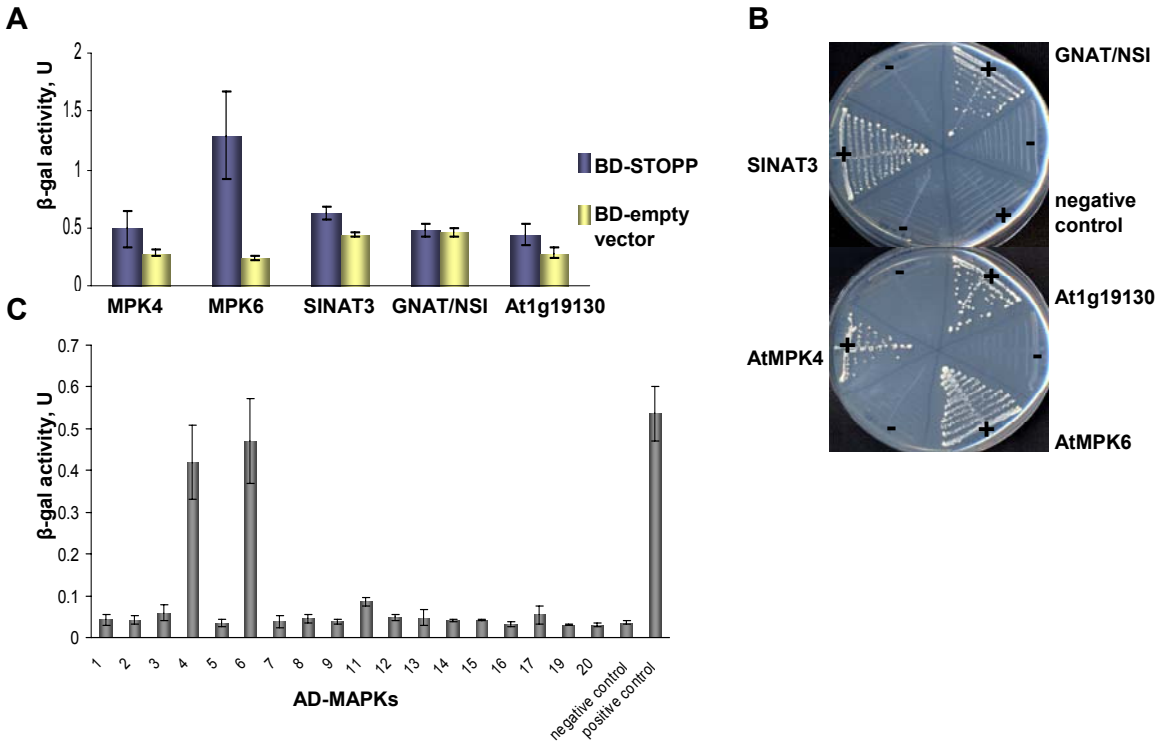

Figure 3.17. STOPP-interacting proteins in Y2H. STOPP-interacting proteins, including MPK4 and MPK4, were identified in cDNA library screening with BD-STOPP **(A)**. The interactions were confirmed by direct Y2H assays: liquid β-galactosidase assay **(A)** and auxotrophic complementation on SD-Ade media with BD-STOPP (+) or with DB-empty vector (-) as a control **(B)**. Yeast strain PJ69A was used as a host. Direct Y2H interaction assay (liquid β-galactosidase assay) between STOPP and 18 MAPKs shows the specificity of STOPP and MPK4 or MPK6 interactions **(C)**. L40 yeast strain was used as a host.

3.3.2. STOPP co-localises with and inactivates MPK4, MPK6 and MPK3 in Arabidopsis protoplasts

To assay whether STOPP is capable to associate with MAP kinases in plant cells, protein co-localisation was studied. STOPP protein associations/interactions with MAP kinases in plant cells were visualised by the bimolecular fluorescence complementation (BiFC) method (Hu et al., 2002). To test whether STOPP also inactivates these MAP kinases in Arabidopsis protoplasts, MAPK activities were assayed as described (Meskiene et al., 2003).

For phosphatase and MAP kinase co-localisation studies, protoplasts were isolated from Arabidopsis cell suspension culture (Schweighofer et al., 2009) and transformed with constructs containing GFP-tagged *STOPP* and RFP-tagged *AtMPK4* or *AtMPK6* genes driven by enhanced CaMV 35S promoter. The fluorescence was monitored using filtersets for GFP and RFP where STOPP was co-localising with MPK4 and MPK6 in the nucleus (Figure 3.18).

The BiFC approach was additionally used to test whether and where these proteins associate in plant cells. Protoplasts from Arabidopsis suspension culture were co-transformed with *35S$_{pro}$:YFP$_{NTD}$-STOPP* and *35S$_{pro}$:YFP$_{CTD}$-MPKs*. Following MPKs were tested for association with STOPP in BiFC assays: MPK1, MPK3, MPK4 and MPK6 (Table 3.4). *35S$_{pro}$:YFP$_{NTD}$* (SPYNE) and *35S$_{pro}$:YFP$_{CTD}$* (SPYCE) constructs (Walter et al., 2004) were used as negative controls. As a result MPK3, MPK4 and MPK6 but not MPK1 were able to reconstitute YFP protein fluorescence with STOPP (Fig. 14B).

Table 3.4. Plasmid combinations used for BiFC assays

Split-YFP$_{NTD}$	**Split-YFP$_{CTD}$**	**Complemented YFP fluorescence***
pRT100-35S-YFP$_{NTD}$	*pRT100-35S-YFP$_{CTD}$*	nf
pRT100-35S-YFP$_{NTD}$ -gSTOPPwt	*pRT100-35S-YFP$_{CTD}$*	nf
pRT100-35S-YFP$_{NTD}$	*pRT100-35S-YFP$_{CTD}$ -AtMPK1wt*	nf
pRT100-35S-YFP$_{NTD}$ -gSTOPPwt	*pRT100-35S-YFP$_{CTD}$ -AtMPK1wt*	nf
pRT100-35S-YFP$_{NTD}$	*pRT100-35S-YFP$_{CTD}$ -AtMPK3wt*	nf
pRT100-35S-YFP$_{NTD}$ -gSTOPPwt	*pRT100-35S-YFP$_{CTD}$ -AtMPK3wt*	YF
pRT100-35S-YFP$_{NTD}$	*pRT100-35S-YFP$_{CTD}$ -AtMPK4wt*	nf
pRT100-35S-YFP$_{NTD}$ -gSTOPPwt	*pRT100-35S-YFP$_{CTD}$ -AtMPK4wt*	YF
pRT100-35S-YFP$_{NTD}$	*pRT100-35S-YFP$_{CTD}$ -AtMPK6wt*	nf
pRT100-35S-YFP$_{NTD}$ -gSTOPPwt	*pRT100-35S-YFP$_{CTD}$ -AtMPK6wt*	YF

* nf – no fluorescence, YF –YFP fluorescence

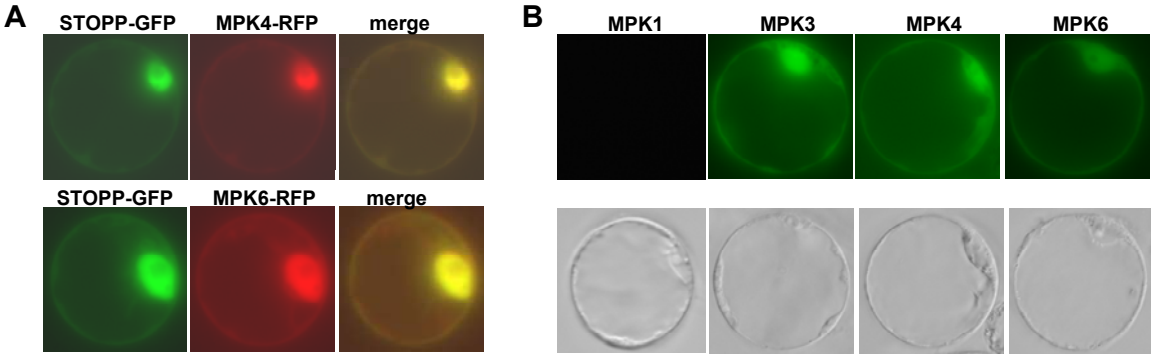

Figure 3.18. STOPP co-localization and BiFC with MAP kinases in Arabidopsis protoplasts. STOPP-GFP co-localises with MPK4-RFP and MPK6-RFP (A). Green – GFP fluorescence, red – RFP fluorescence. BiFC assay of YFP$_{NTD}$-STOPP with YFP$_{CTD}$-MPKs (1, 3, 4 and 6) **(B)**. Green – YFP fluorescence.

The failure of YFP$_{CTD}$-MPK1 and YFP$_{CTD}$-STOPP to reconstitute YFP confirms the specificity of STOPP - MAPK interactions. The reconstituted YFP fluorescence was mainly

detected in nuclei. However, low intensity YFP fluorescence was also detected in the cytoplasm, indicating that STOPP - MAPK complexes can occur also in the cytoplasm.
These results show that STOPP and the MAP kinases 3, 4 and 6 are co-localised to the nucleus. It suggests that the interaction occur in the same compartments also *in planta.*
MAP kinase activity assays were performed to test if these MAPKs are *in vivo* substrates of STOPP. Protoplasts from suspension culture cells were co-transformed with expression vectors containing HA-tagged MAPKs (*MPK3*, *MPK4* or *MPK6)* and an upstream activating MAPK(K) kinase. Here the MAPKK MKK2-EE (Teige et al., 2004), MKK5wt (L.Bögre, RHUL) or the MAPKKK ΔANP1 (Asai et al., 2002) were used (Table 5).

Table 3.5. Combinations of *STOPP* and *MAPK* expression vectors for MAPK activity assays

STOPP	***MAPK***	**Upstream *MAPKK(K)***
pGreenII0029-2x35S-TL-gSTOPPwt-Myc	*pGreenII0229-2x35S-TL-AtMPK3wt-HA*	*pJS-ΔANP1-HA*
	pGreenII0229-2x35S-TL-AtMPK4wt-HA	
	pGreenII0229-2x35S-TL-AtMPK6wt-HA	
pGreenII0029-2x35S-TL-gSTOPPwt-GFP	*pGreenII0229-2x35S-TL-AtMPK3wt-HA*	*pGreenII0029-AtMKK5wt-Myc*
	pGreenII0229-2x35S-TL-AtMPK4wt-HA	*pRT100-AtMKK2EE-Myc*
	pGreenII0229-2x35S-TL-AtMPK6wt-HA	*pGreenII0029-AtMKK5wt-Myc*
0.1 – 1 μg of each plasmid*	5 μg of each plasmid*	5 μg of each plasmid*

* per one transformation

To test if the inactivation of MAPKs depends on STOPP protein levels, increasing amounts of *$35S_{pro}$:gSTOPP-Myc* expression vector plasmid DNA were co-transformed into the protoplasts. The HA-tagged kinases were immunoprecipitated with anti-HA antibody (12CA5) or MAPK specific antibodies (anti-MPK3, anti-MPK4 and anti MPK6). MAPK activities were assayed using myelin basic protein (MBP) as substrate. As shown in Figure 14 C, STOPP could inactivate all tested MAP kinases (MPK3, MPK4 and MPK6). Even the lowest level of STOPP protein (corresponding to 0.01 μg of plasmid DNA) reduced the MAPKs activity to ~ 50% while co-transformation with the highest amount (1 μg of plasmid DNA) decreased MAPK activity to the initial background level. Similar results of MAPK inactivation by STOPP were obtained independently of the upstream activator(s) or antibodies used for immunoprecipitation (not shown). These kinase activity assays confirmed that

STOPP inactivates MPK3, MPK4 and MPK6 in plant cells and that the MAPK activity is reverse-proportional to the abundance level of STOPP protein.

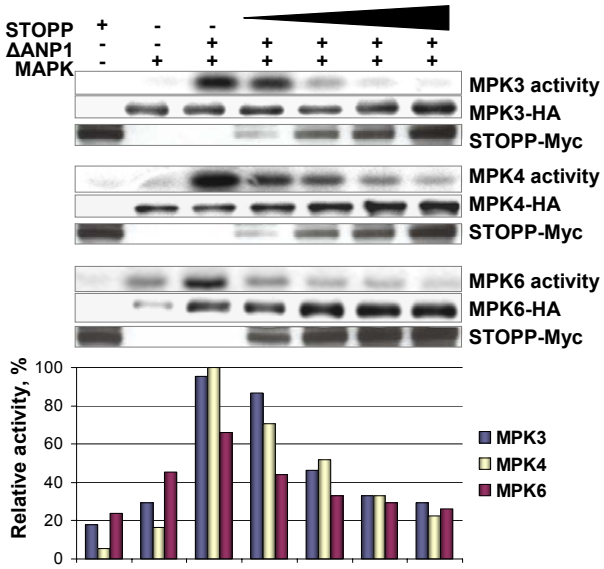

Figure 3.19. STOPP inactivates AtMPK3, AtMPK4 and AtMPK6 in Arabidopsis protoplasts. Protoplasts were co-transformed with HA-tagged *AtMAPK3, AtMAPK4* and *AtMAPK6* and 0, 0,01 0.05, 0.1, 0.5 and 1 µg of *35S$_{pro}$:gAP2C3-MYC* expression vectors. ΔANP was used as upstream activating MAPKK kinase. The kinase activities were determined by in vitro kinase assay after 8 h of co-expression. Relative kinase activity (%) was quantified by ImageQuant.

3.3.3. MAP kinase activities are affected in *STOPP*-modified lines

MAP kinase activities were assayed in wild type, *stopp* knock out and *STOPP* gof seedlings (*35S$_{pro}$:gSTOPP-HA* and *35S$_{pro}$:gSTOPP-GFP* lines). These lines were grown on vertical ½ MS agar plates and transferred to liquid ½ MS media. Seedlings were incubated in liquid ½ MS for 4 hours to achieve basal activity levels of MAP kinases and treated with 100 nM flg22 for 10 min. Seedlings were collected and immediately frozen in liquid nitrogen. Crude protein extracts were prepared for MAPK activity assay and Western blot analysis was performed with anti-phospho-p44/42 MAPK antibodies. For kinase activity assay MAPKs were immunoprecipitated from crude protein extracts with antibodies specific for MPK3, MPK4 and MPK6. Kinase activities were assayed on MBP as described (Meskiene et al., 2003).

Basal activity levels of MPK3, MPK4 and MPK6 before treatment (0 min.) were similar in all plant lines (Fig. 16). After the treatment with flg22 the MAP kinase activities increased in wild type and *stopp* lines but were suppressed in *35S$_{pro}$:gSTOPP-HA* and *35S$_{pro}$:gSTOPP-GFP* lines.

Western blotting with antibodies specific for dual-phosphorylated MAPK (recognizing phosphorylated Thr-Tyr residues within the MAPK activation loop) was performed to study

phosphorylation status of MAPKs in wild type, *stopp* and *STOPP* gof seedlings. This assay enables detection of phosphorylated active MAPK forms in flg22 treated and untreated seedlings. In untreated seedlings no dual-phosphorylated MAPKs were detected (0 min.) (Fig.16). However 10 min. after flg22 treatment dual-phosphorylated forms of MAPKs were detected in wild type and *stopp* lines but only very slight dual-phosphorylation of MAPKs appeared in *STOPP* overexpression lines. The STOPP protein was detected with anti-HA or anti-GFP antibodies. Significantly lower MAPK phosphorylation levels correlated well with lower kinase activities in *STOPP* overexpression lines. Altogether these results suggest that STOPP regulates the activities of specific MAP kinases by dephosphorylation.

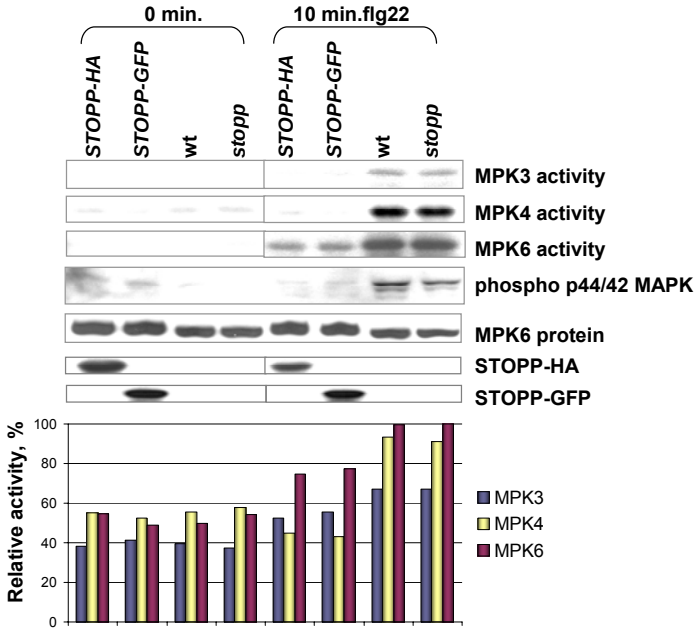

Figure 3.20. Inactivation of MAP kinases in *STOPP* gof seedlings. Seedlings were treated with 100 nM flg22 for 10 min. Cotyledons and hypocotyls were separated from roots and collected for kinase assay and Western blot. For kinase assay MAPKs were immuno-precipitated with antibodies specific for MPK3, MPK4 or MPK6. Kinase activity was assayed on MBP. Kinase activities were lower in *STOPP* over-expressing seedlings than in wild type and *stopp* knock out lines after 10 minutes activation with flg22. Relative kinase activity (%) was quantified by ImageQuant. Western blot was performed with antibodies specific for p44/42 phosphorylated MAPK.

3.4. Pathways affected by STOPP

3.4.1. Alterations of cell cycle markers in *STOPP* gof lines

The overexpression of *STOPP* causes a dramatic change in epidermal cell development, leading to the proliferation of small stomata lineage cells that all eventually differentiate into

stomata. The cyclin dependent kinase (CDK) – retinoblastoma protein (RB) - transcription factors E2F/DP pathway (CDK/RBR/E2F) is central in the switch between cell proliferation and differentiation (Dimova and Dyson, 2005; Wildwater et al., 2005; Maughan et al., 2006). Therefore cell cycle markers of Arabidopsis CDK/RBR/E2F pathway were studied in plants with modified stomata phenotype.

Cyclin-dependent kinases regulate cell cycle progression in all eukaryotes (reviewed in (Inze and De Veylder, 2006). Moreover, it is known that the plant specific B-type CDK 1;1 is essential for stomata development in Arabidopsis (Boudolf et al., 2004a). Therefore the CDK activities and protein levels of Arabidopsis CDKs were assayed in wild type, *stopp* and *STOPP* gof (*35S$_{pro}$:gSTOPP -HA* and *35S$_{pro}$:gSTOPP-GFP*) 5 and 7 dpg seedlings. p13^{Suc1} recombinant protein conjugated sepharose was used to immunoprecipitate Arabidopsis CDKs from crude protein extracts prepared from whole seedlings or cotyledons. It has been shown that the Arabidopsis CDKS CDKA1, CDKB1;1 CDKB1;2 and CDKB2;1 were interacting with the Suc1 homologue CKS1At in Arabidopsis (De Veylder et al., 1997; Boudolf et al., 2001) exhibiting their potential as p13^{Suc1}-binding CDKs. The activity of p13^{Suc1}-bound CDK was assayed on Histone 1 as a substrate (Magyar et al., 1997). Significantly higher CDK activities of p13^{Suc1}-bound CDKs were detected in *STOPP* over-expressing seedlings comparing with wild type plants. This difference was especially pronounced in cotyledons of 5 dpg seedlings (Figure 3.21 A).

To assay whether *STOPP* over-expression affects cell cycle protein levels, Western blot was performed with specific antibodies against Arabidopsis cyclin dependent kinases CDKA;1 and CDKB1;1, Adenovirus E2 promoter binding transcription factors E2FB and E2FC, E2F-dimerization partners DPA and DPB, and RETINOBLASTOMA RELATED protein (RBR). Crude protein extracts prepared from 5 and 7 dpg whole seedlings or cotyledons were used for immunodetection. As a result, significant changes in CDKB1;1 and E2FC protein levels were observed (Figure 3.21A). In Western blot with anti-CDKB1;1 antibodies two bands in 36 - 37 kDa range corresponding to CDKB1;1 were detected in seedlings. The higher molecular weight form (~37 kDa) of CDKB1;1 was predominantly present in wild type and *stopp* lines whereas the lower molecular weight form (~36 kDa) was much more abundant in *STOPP* gof seedlings. Altered electrophoretic mobility of CDKB1;1 in both *STOPP* gof lines suggested that CDKB1;1 may be posttranslationally modified in a *STOPP*-dependent manner. To verify whether the higher CDKB1;1 electrophoretic form is caused by phosphorylation, crude protein extracts from seedlings were treated with λ protein phosphatase. However no change in CDKB1;1 electrophoretic mobility was detected 30 min.

of treatment with λ protein phosphatase (not shown). In contrast to CDKB1;1, the protein levels of CDKA did not obviously differ between the plant lines.

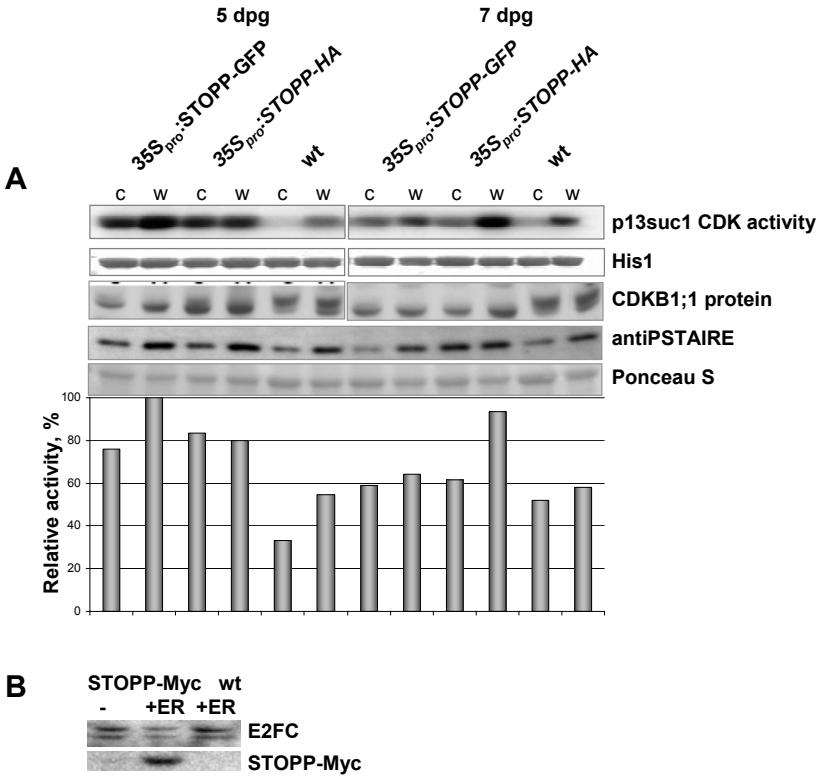

Figure 3.21. Analysis of cell cycle markers in *STOPP*-modified lines. Total $p13^{Suc1}$-precipitated CDK activity was assayed in *STOPP* gof 5 dpg and 7 dpg seedlings grown on vertical plates **(A)**. Protein extracts were prepared from 5 and 7 days old whole seedlings (w) or cotyledons (c). Total CDKs were precipitated with $p13^{Suc1}$ sepharose. Kinase activity was assayed on histone 1 substrate. Relative kinase activity (%) was quantified by ImageJ. Western blotting was performed with CDKB1;1 and PSTAIRE antibodies. E2FC protein levels were detected by specific antibody in ER-inducible *STOPP-Myc* line before and after *STOPP* induction with ER **(B)**. DMSO was used as a control.

The E2F/DP transcription factors regulate cell cycle progression and DNA replication. E2FA and E2FB act as transcriptional activators of E2F-regulated genes (Kosugi and Ohashi, 2002; Mariconti et al., 2002) promoting G1/S and S phases, while the structurally similar E2FC functions as a transcriptional repressor (de Jager et al., 2001). Probably E2FC/DPB suppresses cell division by suppressing G1/S transition and promoting endocycling G2/S in differentiating cells (del Pozo et al., 2006). Therefore, protein levels of transcription factors E2FB, E2FC, E2F-dimerization partners DPA, DPB and E2F suppressor RBR1 were analyzed.

As a result, the protein level of E2FC transcriptional repressor was dramatically reduced in *STOPP* gof lines (Supplemental figure 3). Remarkably, the protein levels of the E2FC dimerization partner DPB, the transcriptional activators E2FB, DPA and the E2F suppressor RBR1 were not affected in *STOPP* gof lines (Supplemental figure 3). A similar result was

achieved in estradiol (ER)-inducible *XVE-STOPP-Myc* 5 dpg seedlings, where E2FC protein level was significantly decreased after STOPP protein accumulation (Figure 3.21 B). However, E2FC protein abundance was not affected in *stopp* knock out seedlings (Supplemental data).

3.4.2. Ethylene and auxin levels are increased in *STOPP* gof lines

Stomata development may be modulated by ethylene and auxin, as these important hormones play essential roles in plant growth and development. Ethylene promotes stomata formation in Arabidopsis hypocotyls (Saibo et al., 2003) and leafs (Serna and Fenoll, 1996). Auxin alone has no effect on stomata density in hypocotyls but enhances the gibberellin-induced stomata development (Saibo et al., 2003). Therefore ethylene and auxin levels were assayed in *STOPP* gof seedlings.

Ethylene level was measured in wild type, *stopp* #2.1, $35S_{pro}$*:gSTOPP-HA* #2.1. and estradiol (ER)-inducible g*STOPP-Myc* (line #8) by gas chromatography. The measured ethylene levels in $35S_{pro}$*:gSTOPP-HA* seedlings were ~ 3-fold higher compared with wild type (ttest $p < 0,05$) and *stopp* lines (Figure 3.22) suggesting that *STOPP* over-expression leads to increase of developmental/non-stress ethylene. A similar tendency was observed in *XVE-gSTOPP-Myc* seedlings where the ethylene level was ~ 70% higher in ER-treated seedlings, but the difference was not statistically significant (Supplemental figure 4).

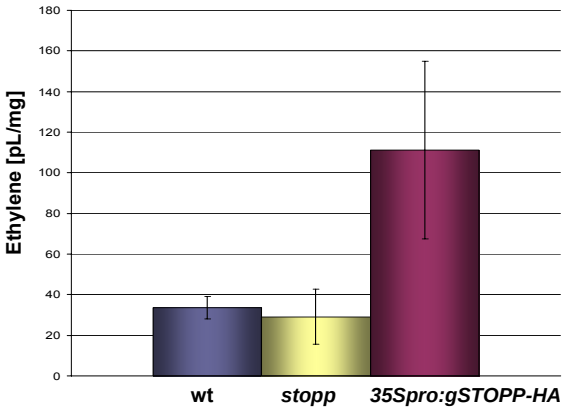

Figure 3.22. Ethylene production in *STOPP*-modified seedlings. Wild type, *stopp* and *STOPP* over-expressing lines were grown in vials in 16h light photoperiod for 7 days. Ethylene was measured after vials were closed for 20h.

To monitor auxin abundance, plants expressing $DR5_{pro}$*:GUS* or $DR5rev_{pro}$*:GFP* reporter constructs harbouring the artificial auxin-responsive promoter were used. Both lines were crossed with $35S_{pro}$*:gSTOPP-HA* #2.2.1 *or* $35S_{pro}$*:gSTOPP-GFP* 2.2.7.5 lines.

DR5rev$_{pro}$*:GFP* activity was significantly higher in *STOPP* over-expressing seedlings than in wild type (Figure 3.23 A) suggesting that these plants contain higher auxin levels. *DR5rev*$_{pro}$*:GFP* was up regulated *STOPP* gof specifically in stomata of cotyledons and hypocotyls. *DR5*$_{pro}$*:GUS* activity was higher in root of *STOPP* seedlings (Figure 3.23 B, 12h staining). In contrast to wild type plants, the *DR5*$_{pro}$*:GUS* activity in root was of dispersed pattern (Figure 3.23 B, 2 h staining).

The dispersed pattern of GUS staining in *DR5*$_{pro}$*:GUS 35S*$_{pro}$*:gSTOPP-GFP* roots suggested that the auxin transport may be affected in STOPP gof plants. Therefore *35S*$_{pro}$*:gSTOPP-HA* line was crossed with plant marker lines for protein localization of auxin transport proteins (PIN1, PIN2, PIN4 and PIN7*)* (Blilou et al., 2005); provided by C.Luschnig, BOKU. Although the localisation of PIN proteins was not affected in the roots of T1 heterozygous seedlings, the PIN1-GFP protein abundance was increased in stele cells of *35S*$_{pro}$*:gSTOPP-HA* seedlings (Supplemental figure 5).

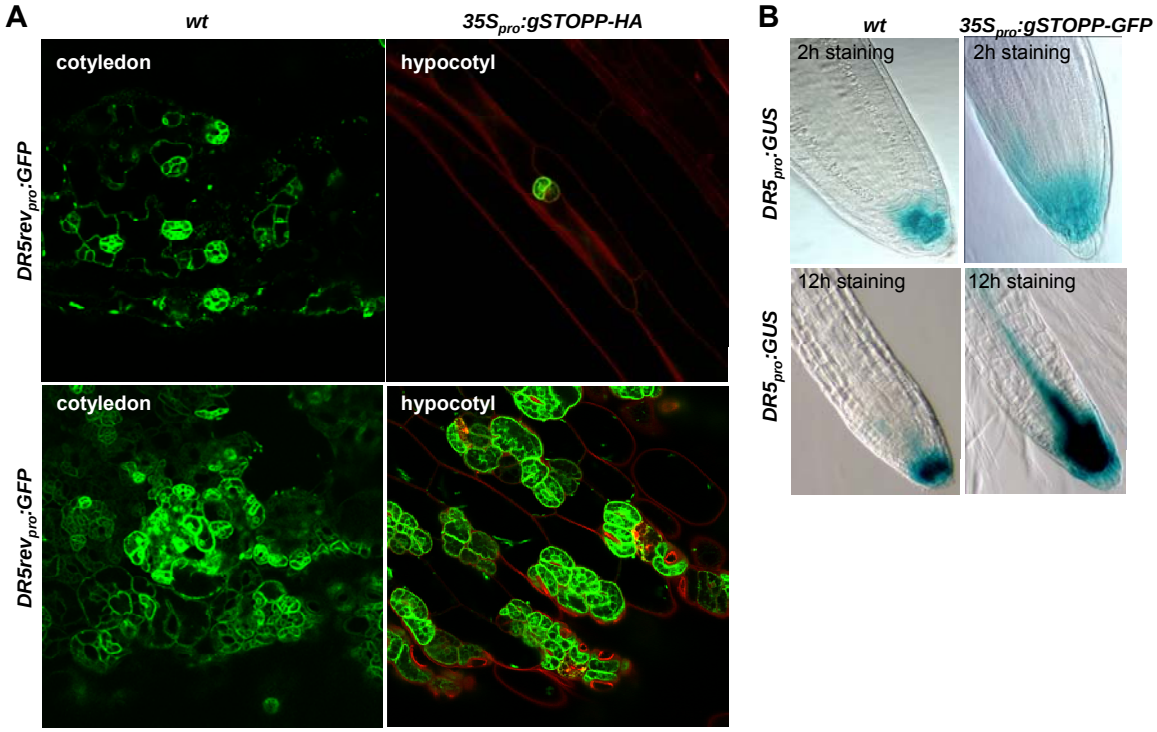

Figure 3.23. The detection of DR5 reporter gene in wild type and *STOPP* gof lines to monitoring of auxin levels. *35S*$_{pro}$*:gSTOPP-GFP DR5*$_{pro}$*:GUS* 3dpg seedlings after 2 h and 12 h of GUS staining (A). Reporter GFP fluorescence of *DR5rev*$_{pro}$*:GFP* in wild type and in *35S*$_{pro}$*:gSTOPP-HA* 3 dpg seedlings.

4. Discussion

4.1. STOPP function in epidermis development

STOPP over-expression in Arabidopsis plants leads to stomata clusters. *STOPP* over-expression induces formation of stomata clusters on cotyledons, hypocotyls, leafs and flower organs. In cotyledons and hypocotyls all epidermal cells can be converted into stomata. This phenotype is observed both in constitutive and inducible *STOPP* over-expression lines. The range of phenotype intensity suggests that there might be a threshold level for STOPP to trigger stomata development. Significantly, the *STOPP* gof plant phenotype coincides with the *STOPP* expression pattern in stomata, strongly suggesting the function of STOPP in stomata development.

4.1.1. *STOPP*-triggered stomata phenotype coincides with *STOPP* gene expression

STOPP is expressed in cells associated with asymmetric division

Asymmetric cell division generates cells of different developmental potentials (Jan and Jan, 1998). The asymmetric cell division is involved in plant developmental processes, such as division of zygote, pollen or stomata formation (reviewed in (Heidstra, 2007). The high *STOPP* expression in embryo suspensor, pollen or stomata suggests the role of STOPP in plant development associated with asymmetric cell division. In addition, *STOPP* expression correlates with the bioinformatics data on *STOPP* transcript abundance.
However, *STOPP* over-expression does not lead to the patterning defects in pollen and embryo suspensor cell. Therefore, *STOPP* gain-of-function phenotype and correlated gene expression suggest that STOPP functions primarily in stomata patterning and epidermis development.

Transient STOPP expression in development

The transient expression of *STOPP* during developmental stages suggests its time-limited function in developing tissues. The detection of strong *STOPP* expression in stomata of 2 - 6 dpg seedlings and developing leaves but very weak expression in stomata of adult leaves supports the developmental regulation of *STOPP* expression.

In adult tissues STOPP might be regulated posttranscriptionally because *STOPP* transcript is present but the protein is not detected after 11 dpg in *STOPP* gof plants. The stabilization of STOPP protein to some extent by proteasome inhibitors indicates that it might be targeted to proteasome degradation pathway. In addition, the E3 ubiquitin-protein ligase SINAT3, which has been identified as STOPP-interacting protein, may be a good candidate for STOPP targeting for ubiquitination.

4.1.2. The phosphatase activity and nuclear localization of STOPP is essential for stomata phenotype

STOPP is a nuclear-localised PP2C with phosphatase activity

STOPP is an active phosphatase according to *in vitro* assays with recombinant proteins or protein extracts from *STOPP* over-expressing seedlings. The phosphatase activity of STOPP is required for stomata cluster phenotype caused by *STOPP* gof as the over-expression of the STOPP-G163D mutant with abolished phosphatase activity does not induce stomata clusters. Therefore, only the active phosphatase is able to interfere with the stomata developmental pathway leading to stomata cluster phenotype.

It was suggested that N-terminal domain of PP2Cs contains sequences responsible for regulation and specificity of PP2C phosphatases (Schweighofer et al., 2004). STOPP is a nuclear protein and its nuclear localisation is essential for *STOPP* stomata cluster phenotype. Domain swapping experiment with the closely related plastid-targeted PP2C AP2C1 (Schweighofer et al., 2007) suggested that the nuclear localisation of STOPP is determined by the N-terminal domain (NTD) (V. Kazanaviciute, unpublished). The over-expression of nucleus-localised $STOPP_{NTD}$-$AP2C1_{CTD}$-*GFP* but not plastid targeted $AP2C1_{NTD}$-$STOPP_{CTD}$-*GFP* induces stomata clustering phenotype. It suggests that STOPP N-terminal domain is required for nuclear localisation and induction of stomata clusters whereas C-terminal domain can be interchangeable between closely related MKPs. In addition, N-terminal domain of STOPP is necessary but not sufficient for stomata cluster phenotype as N-terminal domain swapping with distant HAB1 PP2C does not lead to stomata clustering.

The absence of stomata-related phenotype in stopp lines suggests functional redundancy between protein phosphatase members

STOPP knock out lines (*stopp*) homozygous for T-DNA insertion show no obvious developmental phenotype, suggesting a functional redundancy with other PP2Cs. There are three closely-related to STOPP Arabidopsis PP2Cs from the cluster B and nine PP2Cs from other clusters harbouring the KIM domain, providing the possibility that these PP2Cs might complement *stopp* loss-of-function (Schweighofer et al., 2004). Moreover, some of the MAPK-interacting dual specificity phosphatases (Ulm et al., 2001; Ulm et al., 2002; Lee and Ellis, 2007; Lee et al., 2008) may compete with STOPP functions in signal transduction pathways.

4.2. STOPP regulates MAPK cascade involved in stomata development

4.2.1. STOPP is a MAP kinase phosphatase that inactivates MPK3, MPK4 and MPK6

STOPP associates specifically with the MAP kinases MPK4 and MPK6 in yeast two hybrid assays. STOPP interaction with MAPKs *in vivo* localizes to the nucleus as shown by BiFC and co-localisation studies. It is known that MPK3 and MPK6 are translocated to the nucleus upon activation (Ahlfors et al., 2004). Although interaction STOPP with MPK3 was not detected in yeast, the phosphatase might form a complex with this kinase in plant cells via a scaffold protein or other regulatory proteins.

STOPP negatively regulates MAPK activity *in planta* as demonstrated by kinase assays. STOPP inactivates MPK3, MPK4 and MPK6 in plants as the phosphorylation status of MAPK correlates with the suppression of MPK3, 4 and 6 activities in *STOPP* gof seedlings.

It is unlikely but cannot be excluded that STOPP targets upstream MAPK(K) kinases. However, STOPP did not interact with any of the ten Arabidopsis MAPKKs in yeast. Analogy with other PP2Cs that inactivate similar or homologous MAPKs suggests that STOPP acts exclusively on the MAPK level comparable with previously described MP2C and AP2C1 (Meskiene et al., 2003; Schweighofer et al., 2007). Similarly, in other systems such as yeast, the PP2C Ptc1 inactivates the HOG1 MAPK pathway by dephosphorylating Hog1 but has little effect on the MAPKK Pbs2 (Warmka et al., 2001). The mammalian PP2C Wip1 inhibits the stress-activated p38 MAPK but displays little activity toward MAPKKs (Takekawa et al., 2000). STOPP structure (the KIM domain) and sequence homology with

MAPK-interacting plant PP2Cs (Meskiene et al., 2003; Schweighofer et al., 2007) additionally supports STOPP function as MAPK-phosphatase.

4.2.2. *STOPP* gof plants and *yda/mpk* mutants have similar phenotypes

STOPP gof induces stomata clusters in epidermis by promoting 1) massive ectopic asymmetric divisions in neighbouring cells (NCs) and subsidiary cells (SCs), 2) uncoordinated orientation of asymmetric division which results in incorrect spacing of smaller cells (meristemoids - Ms), 3) guard cell (GC) differentiation, as all epidermal cells including smaller cells ("Ms"), larger cells ("SCs") and undifferentiated cells of nonstomatal lineage ("NCs"), can acquire GC fate, 4) production of aberrant stomata in older than 7 dpg seedlings (as depicted in Fig. 1), 5) upregulation of stomata positive regulator genes such as *FAMA* and *MUTE*.

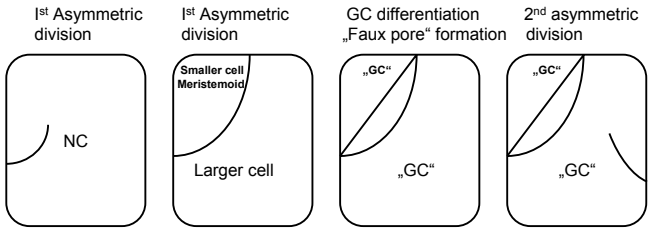

Figure 4.1. Schematic representation of aberrant stomata formation and "GC" asymmetric division. STOPP promotes GC differentiation even without GMC (guard mother cell) to GC transition. Occasionally aberrant stomata are formed of two cells of unequal size, which probably arise after asymmetric division of NC/SC. Such aberrant GCs form stomatal pores (often "faux pore" is formed only by the larger GC in the pair) and acquire typical GC characteristics. Aberrant GCs may divide asymmetrically as they retain both epidermal cell and GC identity, but unlike FLP or FAMA mutants, do not produce cell files.

The similarity between *STOPP* gof and *yda/mpk* mutant phenotypes suggests that STOPP acts in the same pathway as YDA-MAPKK4/5-MAPK3/6 module (Bergmann et al., 2004; Wang et al., 2007b). This kinase cascade restricts stomata development by maintaining cell polarity during asymmetric divisions. It restricts asymmetric divisions of meristemoids, GMC to GC transition and GC differentiation and controls probably the expression and activity of positive stomata gene regulators, such as *FAMA* and *SPCH* (Bergmann et al., 2004; Wang et al., 2007b; Lampard et al., 2008). It is suggested, that MAPK cascade transduces signal downstream of TMM/ER-family receptors and is constitutively active in the cells that are not appropriate to develop into stomata (Yang and Sack, 1995; Von Groll et al., 2002; Bergmann et al., 2004; Shpak et al., 2005; Wang et al., 2007b). This work suggests that STOPP "switches off" the MAPK cascade by negatively regulating MPK3 and MPK6 and thereby releases the suppression of development into stomata.

The YDA-MAPKK4/5-MAPK3/6 cascade is also involved in other developmental processes, such as embryo, ovule and anther development (Lukowitz et al., 2004; Wang et al., 2007b; Hord, 2008; Wang et al., 2008). *yda* mutation and *mpk3 mpk6* loss-of-function affect embryo development and plant fertility. Differently to *yda/mpk*, the phenotype caused by *STOPP* gof was not as stable and broad in plant development, being observed primarily in stomata patterning. This is underlined by the condition-dependent ability of *STOPP* gof plants to overcome the growth defect and occasionally even produce normal leaves and flowers, while *mpk3 mpk6* mutants are embryo lethal (Wang et al., 2007b).

The YDA-MKK4/5-MPK3/6 module was suggested to function downstream of TMM/ER-family receptor-like receptors, which perceive extracellular signal in stomata lineage neighbour cells and stomata precursor cells (Von Groll et al., 2002; Bergmann et al., 2004; Shpak et al., 2005; Wang et al., 2007b). However, this model may not fully explain the wild type state as the MAPKKK *YODA* is highest expressed in guard cells and much weaker in stomata precursor cells (Bergmann et al., 2004). Unfortunately, no data is available on *MPK3/6* expression pattern to correlate it with *YODA* and *STOPP* expressions. On the other hand the presumed upstream receptor of the pathway, *TMM* is expressed in meristemoids and stomata lineage ground cells, the suggested co-receptor kinase *ER* is expressed in protodermal cells early in development, and *ERL1* and *ERL2* are expressed in stomata precursor cells but not in GCs (Von Groll et al., 2002; Shpak et al., 2005). Bergmann (2004) suggested that YDA could act also in a TMM-independent pathway in the same uncommitted cells (Bergmann et al., 2004). Therefore YODA function in GCs is not fully understood.

There are several possible explanations about the difference between expression and action sites of STOPP and YDA proteins:

First, the coinciding *STOPP* and *YODA* expression in GCs suggests that in wild type plants STOPP/YDA module may act independently of TMM/ER as none of the known receptors is expressed in GCs. It would suggest that STOPP functions as a negative regulator of MAPK cascade in the last step of stomata terminal differentiation (during the GMC to GC transition and terminal GC differentiation). This regulation is similar with FAMA, which is possibly controlled by YODA pathway. At the same time, the YDA/MAPK cascade may function also in other stomata lineage cells and act both in TMM/ER-dependent (SLGCs, stomata precursors) and –independent (GCs) pathways (Bergmann et al., 2004).

Second, STOPP may act in TMM/ER-family dependent pathway in SLGCs and stomata precursors even though *STOPP* expression was detected in GCs and not in other epidermal cells. It is not excluded that the GUS detection sensitivity is not sufficient to detect very low

expression levels. It is known that very low protein levels of the phosphatase are enough to completely inactivate MAPKs (Meskiene et al., 2003) and there are indications that STOPP is required only in the very narrow time-gap of cell development. STOPP-GFP fusion protein expressed from its own promoter is not detectable by GFP fluorescence or Western blot. In addition, the endogenous STOPP protein cannot be detected by STOPP-specific antibodies in wild type seedlings.

And third, STOPP may also have functions in stomata development that are associated with other proteins such as cell cycle regulators in guard cells.

4.2.3. How does *STOPP* over-expression affect stomata development?

One possible explanation is that miss-expression of *STOPP* in epidermal cells other than GCs, counteracts the YDA/MAPK cascade in these cells thereby causing their conversion into stomata in *STOPP* gof plants (Fig.2). Altenatively, the over-expression of *STOPP* in these cells may cause a dominant negative (DN) effect, which depends on the catalytically active STOPP as over-expression of the phosphatase inactive form results in normal epidermis development. Normal development of epidermis in *stopp* mutant suggests either protein redundancy (among PP2Cs or with other phosphatases) or supports the hypothesis of the DN effect. Ectopic *STOPP* expression from GC specific promoters would be an option to explore the role of STOPP in these cells.

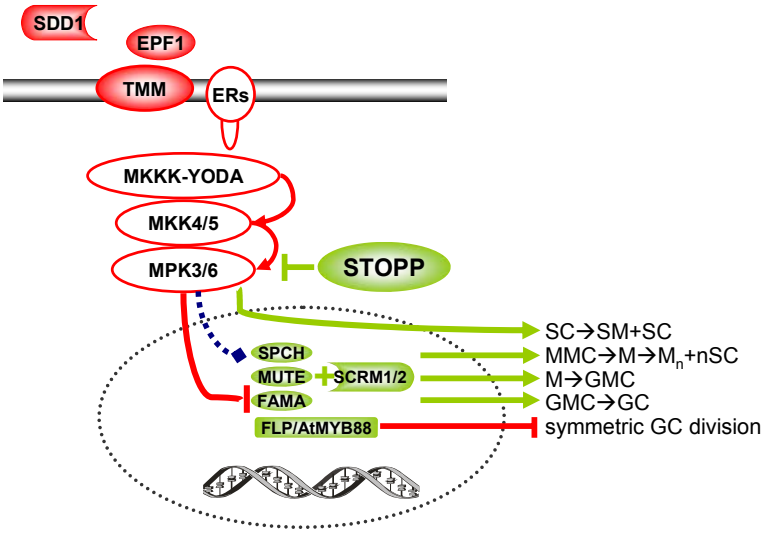

Figure 4.2. The model of STOPP action in stomata developmental pathway. STOPP switches-off the negative signal, which restricts stomata development. Thereby STOPP promotes stomata fate (green arrows). The components of signal generation, reception and transduction pathway are depicted in red; the protein kinases are depicted as empty circles; the transcription factors are depicted in green. SC – subsidiary cell, SM – satellite meristemoid, MMC – meristemoid mother cell, M – meristemoid, n – the order of meristemoid and subsidiary cell in stomata complex, GMC – guard mother cell, GC – guard cell.

4.3. Possible functions of *STOPP* in stress response

STOPP may be involved in PAMP-triggered innate immunity responses as the *STOPP* transcript level is upregulated by treatment with the pathogen elicitor peptide flg22. *STOPP* is characterized as pathogen-associated molecular pattern (PAMP)-induced, Flagellin-Rapidly-Elicited (FLARE) and described as putative ortholog of the tobacco Avr9/Cf-9 rapidly elicited (ACRE) gene (Navarro et al., 2004; Gust et al., 2007). However, no up regulation of *STOPP* transcript was detected by semi-quantitative RT-PCR suggesting that more sensitive method is required (e.g. the real-time PCR). RT-PCR with gene specific primers demonstrated the significant upregulation by cycloheximide (CHX), which is supported by *in silico* analysis of public available expression data (Zimmermann et al., 2004). Remarkably, even ~ 80% of flg22-induced genes are upregulated in Arabidopsis seedlings treated with cycloheximide suggesting that many FLARE genes are negatively regulated by rapidly turned-over repressor proteins (Navarro et al., 2004). CHX inhibits protein translation, thereby may affect the levels of many proteins, including ones involved in transcriptional regulation, transcript degradation, translational and posttranslational regulation. It is unlikely that *STOPP* expression is itself regulated by hormones as promoter-reporter studies did not detect *STOPP* induction after different hormone treatments.

STOPP may act as a converging point between developmental and stress response

As STOPP can be upregulated by pathogens or their elicitors, it may negatively regulate stress-activated MAPK cascades, which respond to pathogen attack in guard cells or regulate transpiration (reviewed in Schulze-Lefert, 2006 #3711}. Beside MPK3 and MPK6, STOPP also inactivates MPK4 that is required to repress systemic acquired response (SAR) for jasmonic acid–responsive gene expression. However, MPK4 was not shown to be involved in stomata development although it is expressed in stomata (Petersen et al., 2000; Galbiati et al., 2008). All STOPP-downregulated MAPKs are active in stress signal transductions (Asai et al., 2002; Wang et al., 2007a; Schweighofer and Meskiene, 2008b) and it is not excluded that STOPP may regulate MPK3, MPK4, MPK6 or other MPKs in response to pathogen stress. STOPP may regulate MAPK cascade in both the development and stress response, and might act as a converging point between developmental and response to environmental signals programmes.

4.4. The effect of *STOPP* gof on cell cycle regulators and plant hormones

STOPP over-expression leads to increased cell division rate

Stomata development requires both the development "competence" factors and the activity of cell cycle regulators (Bergmann, 2004; Boudolf et al., 2004a). Stomata develop through a series of coordinated cell divisions, involving the cell cycle genes and regulators. However, just the over-expression of cell cycle regulators is not sufficient to trigger cell differentiation to stomata pathway (De Veylder et al., 2001; Dewitte et al., 2003; Boudolf et al., 2004a). By contrast, stomata development factors may directly or indirectly control the cell cycle genes (Ohashi-Ito and Bergmann, 2006; Wang et al., 2007b).

STOPP over-expression leads to increased cell division ratio, as massive divisions and aberrant stomata that are also capable of initiating division, are observed in the epidermis of *STOPP* gof seedlings.

Ectopic cell divisions in *STOPP* gof may be induced by significantly increased $p13^{Suc1}$- CDK activity, increased levels of CDKB1;1 (the anticipated active form of the kinase) and decreased level of the E2FC transcriptional repressor in plant tissues. Possibly, the increase in total $p13^{Suc1}$-precipitated CDK activity is mainly associated with stomata-specific activity of CDKB1;1, which is required to amplify asymmetric divisions of meristemoids and satellite meristemoid production (Boudolf et al., 2004a). CDKB1;1 promotes G2 to M transition and (Boudolf et al., 2004a) and in contrast to other CDKs, is transcriptionally regulated by E2F transcription factors (Boudolf et al., 2004b).

At the same time, the levels of A-type CDKs (detected with anti-PSTAIRE antibodies) did not differ in *STOPP* gof and wild type lines, suggesting that STOPP may regulate the CDKB1;1 but not the CDKA;1. However, it is unlikely that STOPP regulates CDKB1;1 directly by dephosphorylation as the difference between protein electrophoretic mobility of CDKB1;1 from *STOPP* gof and from the wild type tissues is not caused by phosphorylation. The treatment with λ phosphatase did not change the mobility of CDKB1;1 nor in wild type neither in *STOPP* gof lines. Here detailed analysis of CDKB1;1 protein bands by proteomic tools is required to identify the corresponding protein modifications.

STOPP over-expression may lead to increase in cell proliferation by downregulation of E2FC, a transcriptional repressor of E2F-regulated genes. Under normal conditions these genes promote the G1 to S transition (del Pozo et al., 2002). Consistently, the expression of *CDC6*, the DNA replication licensing factor, that is known to be negatively regulated by E2FC (del Pozo et al., 2002; Castellano Mdel et al., 2004), is slightly upregulated in *STOPP* gof lines.

E2FC, phosphorylated by CDK, is degraded by the ubiquitin-SCF^{AtSKP2} pathway in response to light (del Pozo et al., 2002; del Pozo et al., 2006). SKP2A, the component of E2FC-degrading SCF complex, is itself degraded through the ubiquitin/26S proteasome pathway in auxin-dependent manner (Jurado et al., 2008). Thus it is suggesting that STOPP over-expression negatively regulates E2Fc protein stability by enhancing light-triggered E2FC degradation or auxin-dependent stabilization of SKP2A.

The balance between cell division and endoreduplication may be shifted in *STOPP* gof lines. Increased CDKB1;1 activity and the lack of E2FC protein may lead to the suppressed endoreduplication in *STOPP* gof lines. It is known that CDKB1;1 activity is required to inhibit the endoreduplication and E2FC promote endoreduplication (del Pozo et al., 2002; Boudolf et al., 2004b). Thus, STOPP may directly or indirectly regulate the cell cycle genes, protein levels and activities during stomata development.

STOPP over-expression leads to increased ethylene and auxin levels

STOPP over-expression leads to significantly increased ethylene and auxin levels in seedlings. The short root phenotype of *STOPP* gof seedlings may be conferred by increased auxin and ethylene levels. It is suggested that ethylene inhibits root cell elongation by stimulating auxin biosynthesis and basipetal auxin transport towards root elongation zone (Ruzicka et al., 2007; Swarup et al., 2007). Therefore, auxin levels in *STOPP* gof roots may be elevated either due to local auxin biosynthesis or due to PIN1-dependent transport from shoots, where auxin levels are elevated as shown by the high activity of *DR5* promoter in stomata.

As ethylene and partially auxin promote stomata development in Arabidopsis hypocotyls and leaves (Serna and Fenoll, 1996; Saibo et al., 2003) the increase in their levels may enhance stomata differentiation in *STOPP* gof lines. It is possible, that ethylene promotes stomata formation by regulating cell division and cell fate in epidermis (Serna and Fenoll, 1996; Kazama et al., 2004). However, it can not be excluded that higher ethylene level in *STOPP* gof line may merely reflect the stress conditions of severely dwarfed seedlings. The complexity of ethylene production and response in *STOPP* gof lines is suggested by the observation that hypocotyl elongation is suppressed in *STOPP* gof lines although ethylene usually promotes hypocotyl elongation (Smalle et al., 1997). In addition, the activity of MPK6, which is known to promote ethylene production (Liu and Zhang, 2004), is decreased *STOPP* gof lines. More detailed genetic and biochemical approaches are needed to investigate the role of these hormones in *STOPP*-modified plants.

Conclusions and perspectives

Conclusions

The conclusions of this work are following:

1. STOPP is a MAP kinase phosphatase
2. *STOPP* is developmentally-regulated PP2C-type phosphatase
3. STOPP inactivates MAPK cascade controlling stomata development
4. *STOPP* gain-of-function triggers stomata clustering
5. STOPP phosphatase activity and nuclear localization are essential for stomata phenotype
6. *STOPP* gain-of-function affects cell cycle regulators and plant hormone levels

Perspectives

This work suggested that STOPP may regulate cell cycle proteins and hormone production. More detailed analysis of these processes is further required. Identified STOPP-interacting proteins may be good candidates to study plant development regulation via proteasome degradation as well as STOPP function to pathogen response. As mentioned above, STOPP may have redundant functions with other MAPK Phosphatases (MKPs). Therefore genetical crosses with some candidate genes were performed and will be analized in continuation of this study.

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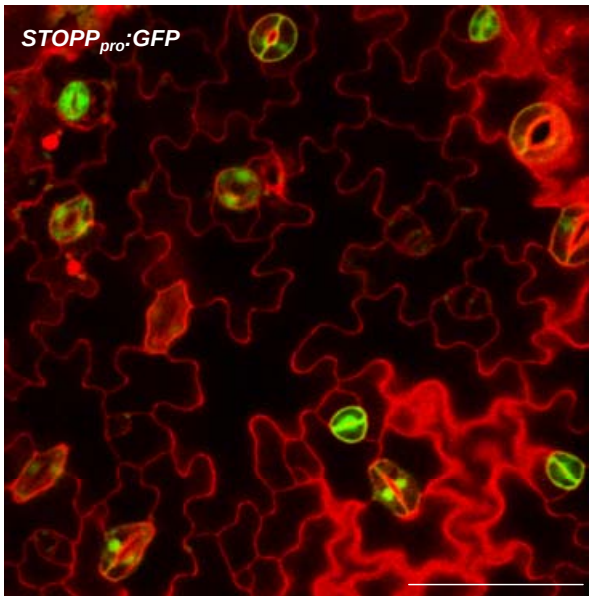
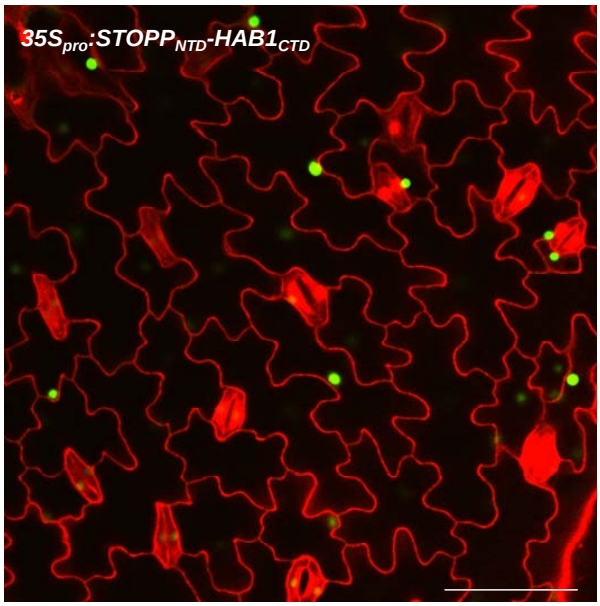
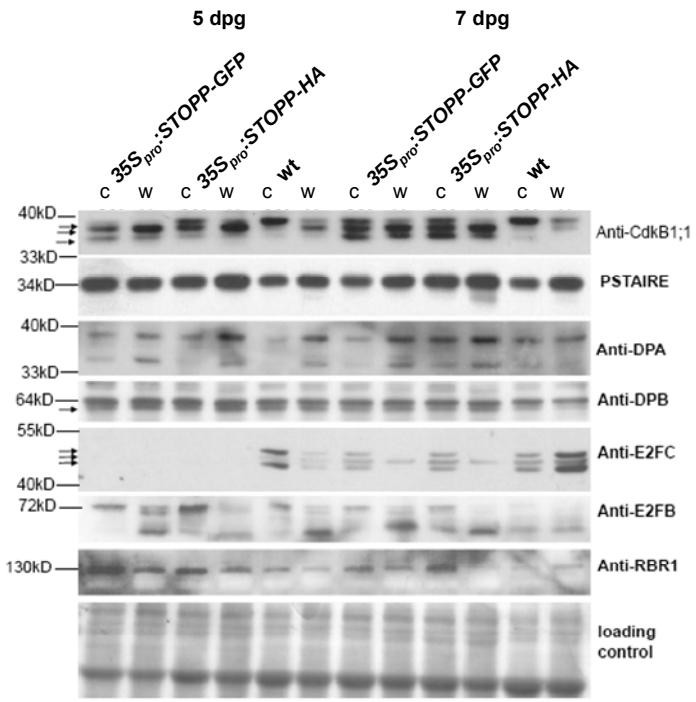
SUPPLEMENTAL DATA



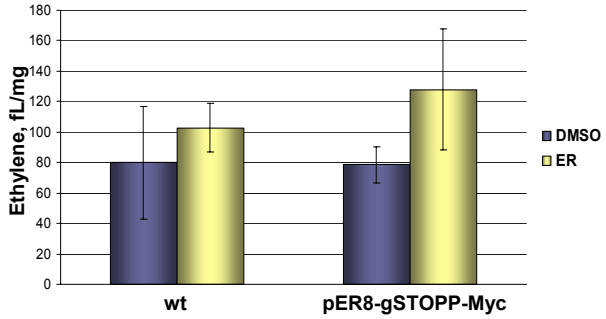
Supplemental figure 1. *STOPP* expression in stomata. The *STOPP* promoter activity in stomata of emerging true leaf of *STOPP$_{pro}$:GFP* seedlings (line #1). Bar 50 µm.



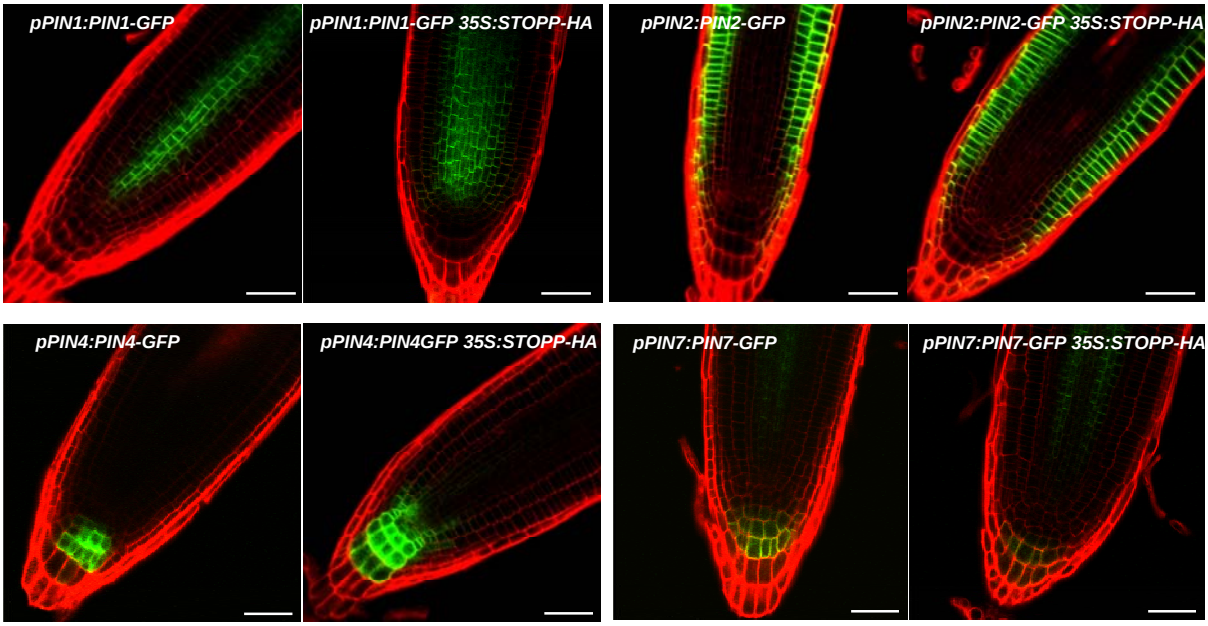
Supplemental figure 2. The localization of STOPP$_{NTD}$-HAB$_{CTD}$-GFP chimera protein. The STOPP$_{NTD}$-HAB$_{CTD}$-GFP is localized to the cell nucleus in *35S$_{pro}$:STOPP$_{NTD}$-HAB$_{CTD}$-GFP* over-expressing seedlings (line #2). Bar 50 µm.



Supplemental figure 3. Cell cycle protein levels in *STOPP*-modified seedlings. The proteins were immunodetected with specific antibodies in 5 dpg and 7 dpg seedlings. c – cotyledons, w – whole seedling.



Supplemental figure 4. Ethylene production in estradiol (ER)-inducible STOPP-Myc line. The *XVE-STOPP-Myc* (line #8) seedlings were grown in vials in 16h light photoperiod for 7 days. Ethylene was measured after vials were closed for 20h. For ER induction seedlings were sprayed once per day with 50 μM ER for 4 days and then with 100 μM ER for 2 next days. ~90% of seedlings developed stomata cluster phenotype. DMSO was used as a solvent control.



Supplemental figure 5. PIN localisation in the root of *STOPP* gof seedlings. Crosses with STOPP gof line were analyzed in T1 (heterozygous) generation in seedlings with stomata cluster phenotype. All bars 50 µM.

Curriculum vitae

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Education and professional experience

2004-2008	PhD studies at the University of Vienna, Austria, Max F. Perutz Laboratories, Vienna Biocenter, Group of Dr. Irute Meskiene. Project: "Signaling Regulation by Protein Phosphatases in Plants"
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2004-2007	Tutor at the Practical Courses of Molecular Biology, University of Vienna, Austria
2005	The EMBO Practical Course on Electron Microscopy and Stereology in Cell Biology, Ceske Budejovice, Czech Republic
2003	Diploma in Biology (Genetics), University of Vilnius, Lithuania Master Thesis: „Genotoxicity Studies of Allergens in Drugs", Supervisor prof. J. Lazutka
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Invited and Poster presentations in conferences and seminars

FEBS Workshop "Adaptation Potential in Plants", GMI, Vienna, Austria, 19 - 21 March 2009. Poster: Stress-hormone control by protein phosphatases

International Conference "Plant Abiotic Stress Tolerance", Vienna, Austria, 8 - 11 February 2009. Poster: Wound jasmonate and ethylene are controlled by PP2C phosphatases

FASEB conference on Protein Phosphatases, Snowmass Village CO, USA, 13 - 18 July 2008. Poster: Identification of protein phosphatase 2C targets by proteomics in Arabidopsis plants

Keystone Symposia on Plant Hormones and Signaling, Keystone CO, USA, 10 – 15 February 2008. Poster: An Arabidopsis PP2C as a negative regulator of specific MAP kinases.

Seminar at University of Vilnius, Lithuania, 12 December 2007. Presentation: Signal transduction in cell division/differentiation programmes in plants.

4th Tri-National Arabidopsis Meeting, Vienna, Austria, 12 - 15 September 2007. Poster: Developmental Expression Patterns of Novel Arabidopsis PP2C-type Phosphatases.

XVII International Botanical Congress, Vienna, Austria, 17 - 23 July 2005. Poster: Gene Expression Patterns of PP2C Phosphatase.

Seminar at the Royal Holloway University of London, UK, 11 April 2006. Presentation: The Role of Arabidopsis PP2C.

EMBO Practical Course on Electron Microscopy and Stereology, Ceske Budejovice, Czech Republic, 19 – 29 June 2005. Presentation: Localisation of Arabidopsis PP2Cs.

Publications

J. Umbrasaite, A. Schweighofer, V. Kazanaviciute, C. Choopayak, Z. Magyar, J.A. H. Murray, L. Bögre and I. Meskiene. Control of stomata cell development by a PP2C-type phosphatase. Manuscript in preparation: invited for submission as Letters to „Nature"

J. Umbrasaite, A. Schweighofer and I. Meskiene. Substrate Analysis of Arabidopsis PP2C-type Protein Phosphatase. 2009 In: "Plant kinases" ed. By A. Schnittger "Methods in Molecular Biology" series of Humana Press

Other activities

2007-2008 The member of Austrian Society of Biochemistry and Molecular Biology

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