DOI: 10.1007/s10535-013-0387-0 BIOLOGIA PLANTARUM 58 (2): 231-240, 2014

Expression pattern of *AtABCC13/MRP11* reveals developmental, hormonal, and nutritional regulations

T. EL GUIZANI[1,2], N. BLANC[1], S. TRIKI[2], B. ST-PIERRE[1], and E. DUCOS[1]*

EA2106, Biomolécules et Biotechnologies Végétales, Université François Rabelais de Tours, F-37200 Tours, France[1]
Laboratoire de Biochimie, Faculté des Sciences de Tunis, 2092 Tunis, Tunisia[2]

Abstract

ATP binding cassette (ABC) proteins constitute one of the largest families of transporters. In *Arabidopsis thaliana*, over 100 genes encoding ABC transporters have been identified. Here, we described the expression pattern of *AtABCC13/MRP11*, a member of the multidrug resistance associated protein MRP/ABCC subfamily, previously uncharacterized. The histochemical analysis of transgenic *Arabidopsis* harboring *AtABCC13* promoter-β-glucuronidase gene fusion shows that the *AtABCC13* expression was specifically associated with vascular bundles. Moreover, *AtABCC13* displayed a complex hormonal regulation. β-glucuronidase (GUS) fluorimetric assays revealed that the gene expression was induced by gibberellic acid and downregulated by naphthalene acetic acid, abscisic acid, and zeatin. Because *AtABCC13* is also expressed during seed development and during germination, its expression was assessed upon exposure to various nutrients: nitrate, phosphate, and sucrose stimulated the *AtABCC13* expression in seedlings, whereas their lack strongly reduced it.

Additional key words: ABC transporters, abscisic acid, gibberellic acid, β-glucuronidase, minerals, naphthalene acetic acid, plant development, saccharose, vascular bundles, zeatin.

Introduction

ABC transporters constitute a large superfamily of proteins described in all kingdoms of life. Some families have a restricted distribution, like the ABC subclass C (or multidrug resistance associated protein, MRP) which is limited to eukaryotes. In plants, ABCC/MRPs are subdivided in three phylogenetic clusters (Kolukisaoglu *et al.* 2002, Schulz and Kolukisaoglu 2006), two of which are fairly well characterized in *Arabidopsis*.

Cluster I contains four genes including *AtMRP1* and *AtMRP2* which are involved in cellular detoxification processes. These transporters display a ubiquitous expression in plants, mediate metal/metalloid tolerance and transport various compounds, such as phytochelatins (Song *et al*. 2010), glutathione conjugates (Lu *et al.* 1997), folate (AtMRP1), as well as chlorophyll catabolites (AtMRP2, Lu *et al.* 1998). The two other members of cluster I (AtMRP11 and AtMRP12) have been poorly investigated; they share transport properties of cluster I MRPs except for chlorophyll catabolites, which seems to be specific to AtMRP2 within this cluster (Frelet-Barrand *et al.* 2008).

Ten of the fifteen *Arabidopsis* ABCC/MRPs belong to cluster II. The *AtMRP3* expression is induced in leaf and root under cadmium or several other metal treatments (Bovet *et al.* 2003), and confers tolerance to cadmium in yeast (Tommasini *et al.* 1998). AtMRP3 exhibits glutathione S-conjugate transport activity, and as described for AtMRP1 and AtMRP2, chlorophyll catabolites transport activity has also been reported (Tommasini *et al.* 1998). AtMRP6 and AtMRP7 have the highest sequence similarity to AtMRP3 and are also up-regulated by Cd^{2+} treatments. In *AtMRP6* mutants, leaf development is more sensitive to Cd^{2+} treatment than in wild type. However, the expression pattern of *AtMRP6* suggests that it is not only involved in Cd^{2+} tolerance but also in developmental processes. Indeed, AtMRP6 is

Submitted 2 May 2013, *last revision* 16 July 2013, *accepted* 19 July 2013.
Abbreviations: ABA - abscisic acid; GA_3 - gibberellic acid; GUS - β-glucuronidase; NAA - naphthalene acetic acid; NO_3^- - nitrate; PO_4^- - phosphate; SO_4^- - sulfate.
Acknowledgments: This work was partly supported by grants from the Tunisian Ministry of Higher Education and Scientific Research, from the French Institute of Cooperation, and from the French Ministry of Research and Higher Education. The first two authors contributed equally to this work.
* Corresponding author; fax: (+33)247276660, e-mail: eric.ducos@univ-tours.fr

expressed during early seedling development, in root apical meristem and in lateral root initiation (Gaillard *et al.* 2008). *AtMRP7* is constitutively expressed in roots and leaves and the protein was localized to both plasmalemma and tonoplast (Wojas *et al.* 2009). Overexpression of *AtMRP7* in tobacco slightly enhances plant resistance to Cd^{2+} treatment and modifies Cd^{2+} root-to-shoot translocation. These observations suggest that AtMRP3 and its closest homologues may behave as cellular metal detoxifiers although additional functions *in planta* cannot be ruled out. *AtMRP4* and *AtMRP5*, which also belong to cluster II, have a low sequence similarity, but their stomatal-specific expression patterns suggest a role in plant adaptation to water stress rather than cell detoxification. Indeed, *AtMRP4* knock-out mutants display an increased water loss due to a larger constitutive stomata aperture. Considering its strong expression in guard cells and localization in plasmalemma, AtMRP4 is suggested to be involved in a stomata ion channel regulation (Klein *et al.* 2004). *AtMRP5* is also expressed in guard cells but localizes to tonoplast (Nagy *et al*. 2009). Interestingly, Nagy *et al.* (2009) demonstrated that AtMRP5 is a high affinity inositol hexakisphosphate (InsP6) transporter, a molecule that modulates Ca^{2+} signaling and K^{+} homeostasis in guard cells. *AtMRP5* mutants are impaired in the export of InsP6 into vacuole resulting in constitutive stomata closure and a reduced water loss (Klein *et al.* 2003, Suh *et al*. 2007).

Cluster III genes have not been functionally characterized so far. In *Arabidopsis* (AGI 2000) it was revealed a single MRP/ABCC gene belonging to cluster III, *AtABCC13/MRP11* (At2g07680). In this study, we investigated in-depth the expression pattern of *AtABCC13* in *Arabidopsis*. The β-glucuronidase (GUS) reporter gene approach revealed that the *AtABCC13* expression was related to vascular elements, seed maturation, and seedling development.

Materials and methods

Plasmid construction and plant transformation: The *AtABCC13* promoter was obtained by PCR on genomic DNA using the following primers: forward 5'-ataccccg CTCGAGggtttataaggtacaggttaggg-3' and reverse 5'-ata ccccgCTCGAGagtgatagccatggattctctagggtc-3' (capital letters indicate the *Xho*I site). The 2092 bp PCR product obtained corresponded to the 2075 bp upstream of the start codon of *AtABCC13* and to the 17 first nucleotides of the *AtABCC13* coding sequence. Following *Xho*I digestion, the fragment was inserted into the corresponding restriction site of a pBI102 binary vector (St-Pierre and Brisson 1995) to fuse the *AtABCC13* promoter to the *GUS* reporter gene (Fig. 1). The construction *promAtABCC13::GUS* was sequenced and introduced into *Agrobacterium tumefaciens* strain LBA4404. Wild plants of *Arabidopsis thaliana* L. ecotype Columbia (Col 0), obtained from the Nottingham Arabidopsis Stock Centre, were then transformed by the floral dipping method (Clough and Bent 1998). The transgenic seedlings were selected on a Murashige and Skoog (MS) medium (*Duchefa*, Haarlem, The Netherlands) containing agar plates supplemented with 50 mg dm^{-3} kanamycin. All kanamycin resistant seedlings were assayed histo-chemically for GUS activity. Three transformed lines were analyzed for each experiment.

Wild type and transgenic *Arabidopsis* plants containing the *promABCC13::GUS* reporter fusion construct were grown in a greenhouse at temperature of 22 °C, a 16-h photoperiod, irradiance of 100 μmol m^{-2} s^{-1}, and air humidity of 70 %. For aseptic growth of seedlings, *Arabidopsis* seeds were surface sterilized with 20 % (v/v) bleach + 0.7 % (v/v) *Triton X-100* for 15 min, followed by four rinses in sterile water and kept in 0.1 % (m/v) agar. Seed dormancy was broken by a cold treatment at 4 °C in the dark for 3 to 4 d. Then the seeds were sown on solid half-strength MS plates placed vertically in a growth chamber under the same environmental conditions.

Hormonal and nutritional treatments: Three-day-old seedlings were transferred to vertically oriented plates containing a solid half-strength MS medium supplemented with increasing concentrations (0 - 5 μM) of abscisic acid (ABA), gibberellic acid (GA), naphthalene acetic acid (NAA) (all products from *Sigma*, St. Louis, USA), or zeatin (*Duchefa*) and grown for 4 d. For treatments with nitrate, sulfate, phosphate, and sucrose, plates contain solid homemade half-strength MS media in which the tested nutrient was omitted (Dixon and Gonzales 1994). Appropriate nutrients (*i.e.* KNO_3, KH_2PO_4, $MgSO_4$, or sucrose) in increasing concentrations were added from stock solutions to complement the nutrient plates. Three-day-old seedlings grown vertically on the standard half-strength MS medium were transferred on these plates and kept for mineral treatments for five days and for sucrose one for six days. The seedlings were then subjected to the histochemical localization of GUS activity and to the fluorimetric 4-methylumbelliferyl-b-D-glucoside (MUG) assay to quantify their GUS activity.

Histochemical localization of GUS activity: Histochemical staining for GUS activity in the transgenic *Arabidopsis* plants grown in the greenhouse and *in vitro* was performed using 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as substrate (Jefferson 1989). *Arabidopsis* organs, sterile plantlets, or hand-cut cross sections were incubated at 37 °C in a GUS reaction buffer (1 mg cm^{-3} X-Gluc in 50 mM sodium phosphate, pH 7.0, with 10 mM Na_2-EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 1 %, v/v, of *Triton X-100*) for 16 h (unless otherwise indicated in figure

legends). Before observation, chlorophyllous tissues and plantlets were cleared in 96 % (v/v) ethanol for 2 h and 16 h, respectively, and gradually rehydrated with ethanol series (70, 50, and 25 % for 10 min in each solution) at room temperature, and finally conserved in glycerol: water 50:50 (v/v) with 0.02 % (m/v) NaN_3. The histochemical observations were made with an *Olympus SZ40* (Tokyo, Japan) binocular coupled to a *Leica DFC280* (Wetzlar, Germany) camera or an *Olympus BX51* microscope connected to a *Leica DP71* camera.

Fluorometric assay: The fluorometric assay of GUS activity (Jefferson 1989) was performed on the cotyledons of seedlings after four, five, or six days on different culture media with hormones, nutrients or sucrose, respectively. Because root morphology varied considerably after treatments, enzymatic assays were performed with cotyledons, which offered a homogenous material more prone for quantifications. In our experiments, a GUS activity was only observed close to cotyledon veins (data not shown). For each treatment, the cotyledons from six seedlings were placed in a safe lock microtube containing two 5 mm-diameter glass beads, frozen in liquid nitrogen and shaken in a ball mill (*MM-400*, *Retsch*, Haan, Germany) at 30 cycles s^{-1} for 2 min. The crushed cotyledons were suspended in 0.45 cm^3 of a GUS extraction buffer containing 50 mM $NaHPO_4$, pH 7.0, 10 mM 2-β-mercaptoethanol, 10 mM Na_2EDTA, 0.1 % (m/v) sodium lauryl sarcosine, and 0.1 % (v/v) *Triton X-100* and centrifuged at 10 000 *g* and 4 °C for 10 min. Protein concentration of the supernatant was determined using the Bradford protein assay reagent (*BioRad*, Hercules, USA). For GUS enzyme assays, 0.1 cm^3 of the supernatant was mixed with 0.08 cm^3 of the GUS extraction buffer and 0.02 cm^3 of MUG at a concentration of 3.5 mg cm^{-3} and incubated at 37 °C for 2 h. The reaction was stopped by adding 0.45 cm^3 of 0.2 M ice-cold Na_2CO_3. The fluorescence was measured using a fluorescence spectrophotometer (*F-4500*, *Hitachi*, Tokyo, Japan) and a calibration curve with standard solutions of 4-methyl-umbelliferone (4-MU). The GUS activity was expressed in pmol 4-MU produced per mg of protein per min. Presented data are means ± SD from six independent samples and are expressed relative to the reference conditions. Statistical analyses were performed with the Student's *t*-test.

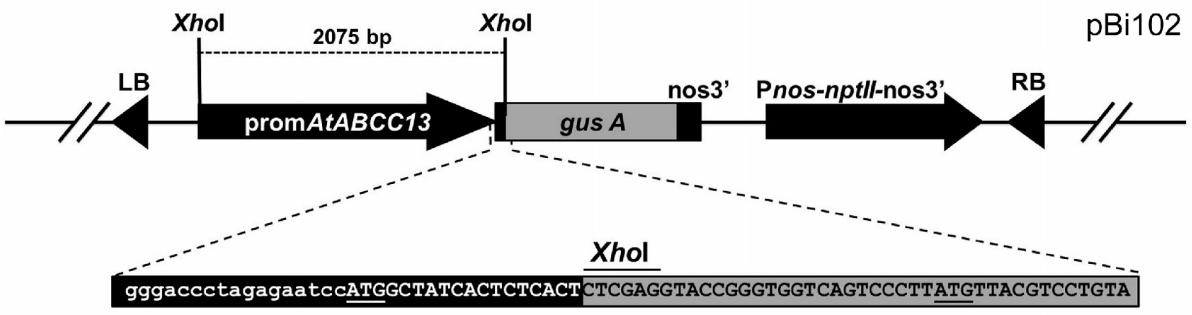

Fig. 1. The schematic representation of the *promAtABCC13::GUS* construct. LB and RB - left and right borders of T-DNA, P*nos* - nopaline synthase promoter, nos3' - nopaline synthase terminator, *nptII* - neomycin phosphotransferase. The nucleotide sequence of the translational fusion is shown. The *AtABCC13* initiation codon and *GUS* first codon (in the *frame*) are *underlined*. The *Xho*I restriction site is indicated.

Results and discussion

The expression pattern of the *AtABCC13* promoter fused to the GUS reporter gene was examined at different developmental stages and in various organs of the plant. In vegetative organs, *AtABCC13* was expressed in the vascular tissues of cotyledons and first leaves, whereas no blue color was observed in the shoot apical meristem (Fig. 2*A*). In mature leaves, strong staining for GUS was observed in primary, secondary and tertiary veins (Fig. 2*B*). Neither trichomes nor stomata were stained (Fig. 2*C,D*). The guard cell-strong expression pattern of cluster II ABCC genes *AtMRP4* and *AtMRP5* was therefore not conserved in cluster III *AtABCC13* (Klein *et al*. 2004, Nagy *et al*. 2009). No GUS activity was detected in the cross section of a young stem (Fig. 2*E*), whereas a signal, which corresponds to the cambial zone, was observed between the primary xylem and phloem of a mature stem (Fig. 2*F*). The details of the vascular bundle area revealed that in a young stem, the *AtABCC13* expression was absent between the primary xylem and the phloem (Fig. 2*G*), but was associated with the fascicular cambium area of a mature stem (Fig. 2*H*). In *Arabidopsis*, the fascicular cambium is active and produces secondary vascular tissues before interfascicular cambium completion (Sehr *et al.* 2010). We observed that the *AtABCC13* expression was restricted to the active fascicular cambium (Fig. 2*H*, secondary xylem rays are visible) and not to the developing interfascicular cambium. These results suggest that the *AtABCC13* expression is associated with secondary vascular tissue differentiation rather than cambium establishment. *AtABCC13* was also expressed in the transition zone of a root apex, staining appeared as two spindle-shaped

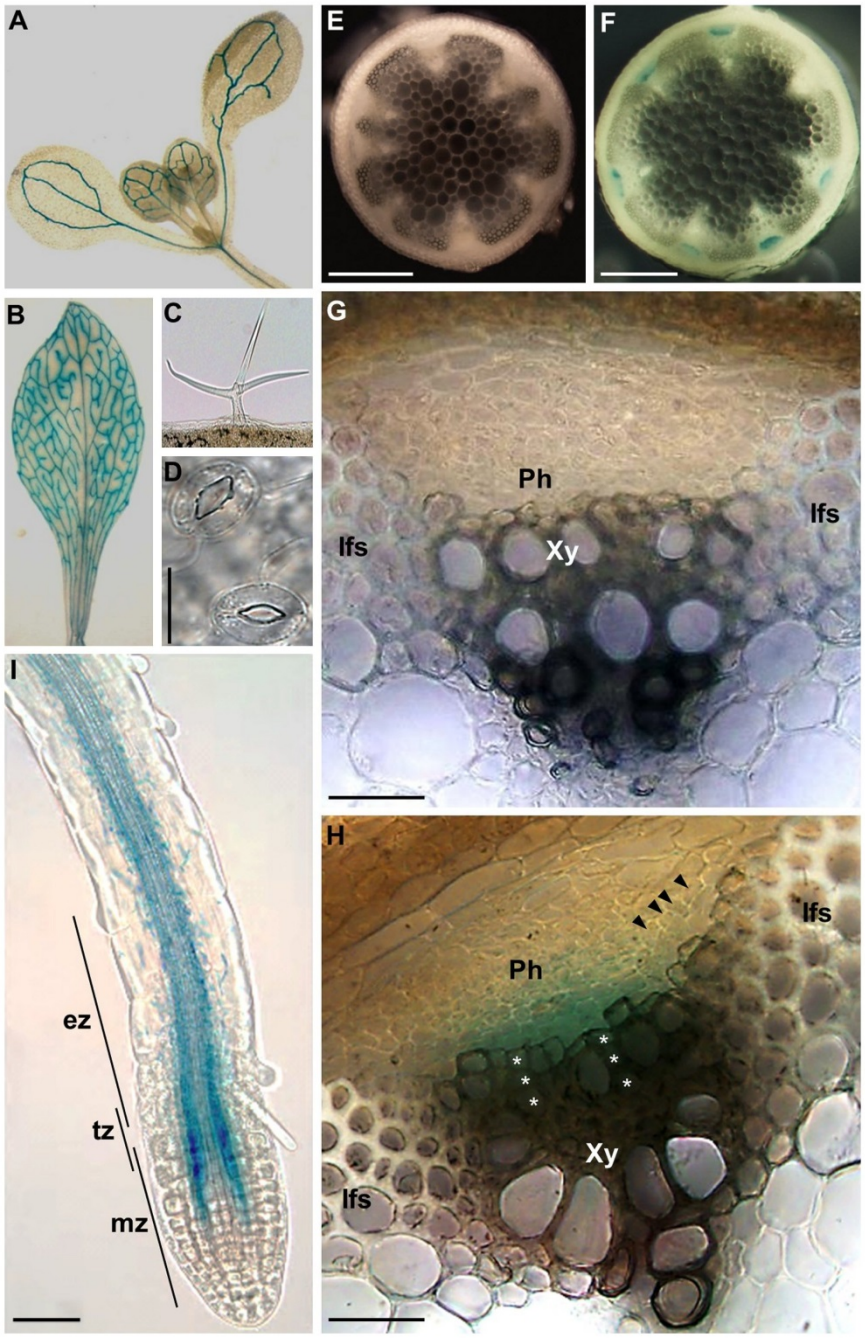

Fig. 2. *AtABCC13* is expressed in vascular tissues of vegetative organs of *A. thaliana* transformed with *promABCC13::GUS* construct. *A* - GUS staining of first leaves and cotyledons of a 10-d-old seedling; *B* - mature leaf of adult plant; *C* - trichome; *D* - stomata; *E* - cross section of a young stem; *F* - cross section of a mature stem; *G* - detail of vascular bundles of E; *H* - detail of vascular bundles of F; *I* - root apex staining with a 30 min-incubation in a GUS reaction buffer. *White stars* indicate secondary xylem cells. *Black arrows* indicate periclinal dividing cells of developing interfascicular cambium. Ph - primary phloem, Xy - primary xylem, Ifs - stem interfascicular fibers (according to Sehr *et al*. 2010), mz - meristematic zone, tz - root transition zone, ez - root elongation zone (according to Jaillais and Chory *et al*. 2010). *Scale bar E*, *F*, and *I* = 200 µm; *D*, *G,* and *H* = 20 µm.

structures confined to the pericycle layer (Fig. 2*I,* S1). The transition zone is located between the meristematic zone and elongation zone, and determines cell fate and root growth. The pericycle is composed of two different cell types, with one subset of cells being associated with xylem formation showing strong competence to initiate cell division, whereas another group of cells, associated with the phloem, appears to remain quiescent (Parizot *et al.* 2008). Symmetrical spindle-shaped GUS staining in root apex is characteristic of xylem pole pericycle cells

(De Smet *et al.* 2007, Laplaze *et al.* 2007, Parizot *et al.* 2008, Nieuwland *et al.* 2009). The pericycle cells, adjacent to protoxylem pole, acquire founder cell identity in the transition zone before tissue differentiations (Benkova and Bielach 2010) and will further give rise to lateral root initiation (Parizot *et al.* 2008). However, no GUS activity was observed at cell-cycle activation in pericycle founder cells which initiate lateral root formation (Fig. 3*A*). Figure 3*B* and 3*C* show lateral root crossing through the cortex before emergence from a main root. Later on, the *AtABCC13* promoter was activated during vascular apparatus differentiation at the pericycle forming layer of the lateral root (Fig. 3*D* and S2) as observed in the primary root (Fig. 2*I* and S1). The expression of *AtABCC13* decreased in the maturation zone and was maintained near the root apex (Fig. 3*E*).

AtABCC13 is therefore expressed in most plant organs according to a strict cell-specific and developmental pattern. It appears to be associated with the active cambial zones and vascular bundles differentiation processes. Because the establishment of vascular tissues in plant relies on complex interplay between several plant hormones, we investigated the effect of hormones on *AtABCC13* promoter activity.

Cambium activity and differentiation into xylem and phloem is mainly driven through the interactions of auxins, gibberellins, and cytokinins (Elo *et al.* 2009). These hormones are distributed differentially across the cambial zone of vascular bundles. Auxins are considered as key regulators of cambial activity. In stem, an auxin content peaks in the dividing cambial cells and decreases steeply towards the developing xylem and phloem (Uggla *et al.* 1996, Tuominen *et al.* 1997, Elo *et al.* 2009). Content of gibberellins and cytokinins peaks in the differentiating xylem and phloem cells, respectively (Israelsson *et al.* 2005, Bishopp *et al.* 2011). ABA has been extensively involved in water stress response and seed dormancy (Agarwal and Jha 2010), however, it is recently associated with the cambial activity cessation (Baba *et al.* 2011). In our experiments, the NAA, zeatin, and ABA treatments reduced the *AtABCC13* promoter activity in seedlings, whereas GA increased the *AtABCC13* promoter activity (Table 1). None of the hormonal treatments altered the cell-specific pattern

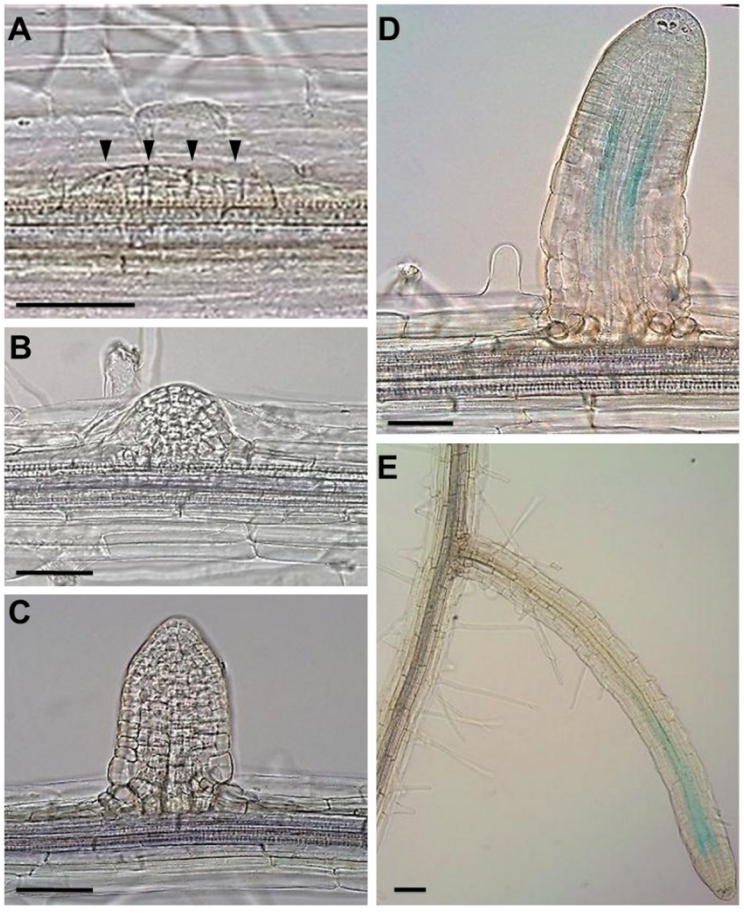

Fig. 3. *AtABCC13* is expressed during lateral root differentiation in 3-week-old seedlings. *A* - Lateral root primordium (*arrow-head*); *B* - emergence of a lateral root, still poorly differentiated tissues; *C* - development of a lateral root; *D* - GUS staining in differentiating conductive element; *E* - *AtABCC13* expression is restricted to the transition/elongation zone. *Scale bars* = 200 μm. For *D* and *E*, samples were incubated in GUS reaction buffer for 30 min instead of for 16 h.

of GUS staining in the vascular bundles (data not shown). The hormonal modulations of the *AtABCC13* promoter activity suggest that this transporter might have a role in GA-dependent developmental processes.

In the root apex, auxins, cytokinins, and gibberellins also interact to control the balance between cell division in the meristematic zone and identity acquisition in the differentiation/elongation zone (Moubayidin *et al.* 2010). The switch between auxin and cytokinin signaling that occurs in the transition/elongation zone involves gibberellins (Ubeda-Tomás *et al.* 2008). However, by developing a fluorescent-labeled GA_3, Shani *et al.* (2013) demonstrated that GA_3 accumulates in the transition/ elongating root endodermal cells in *Arabidopsis*. How endodermal GA_3 might induce pericycle-localized *AtABCC13* is not clear and may rely on other factors that monitor *AtABCC13* expression in root.

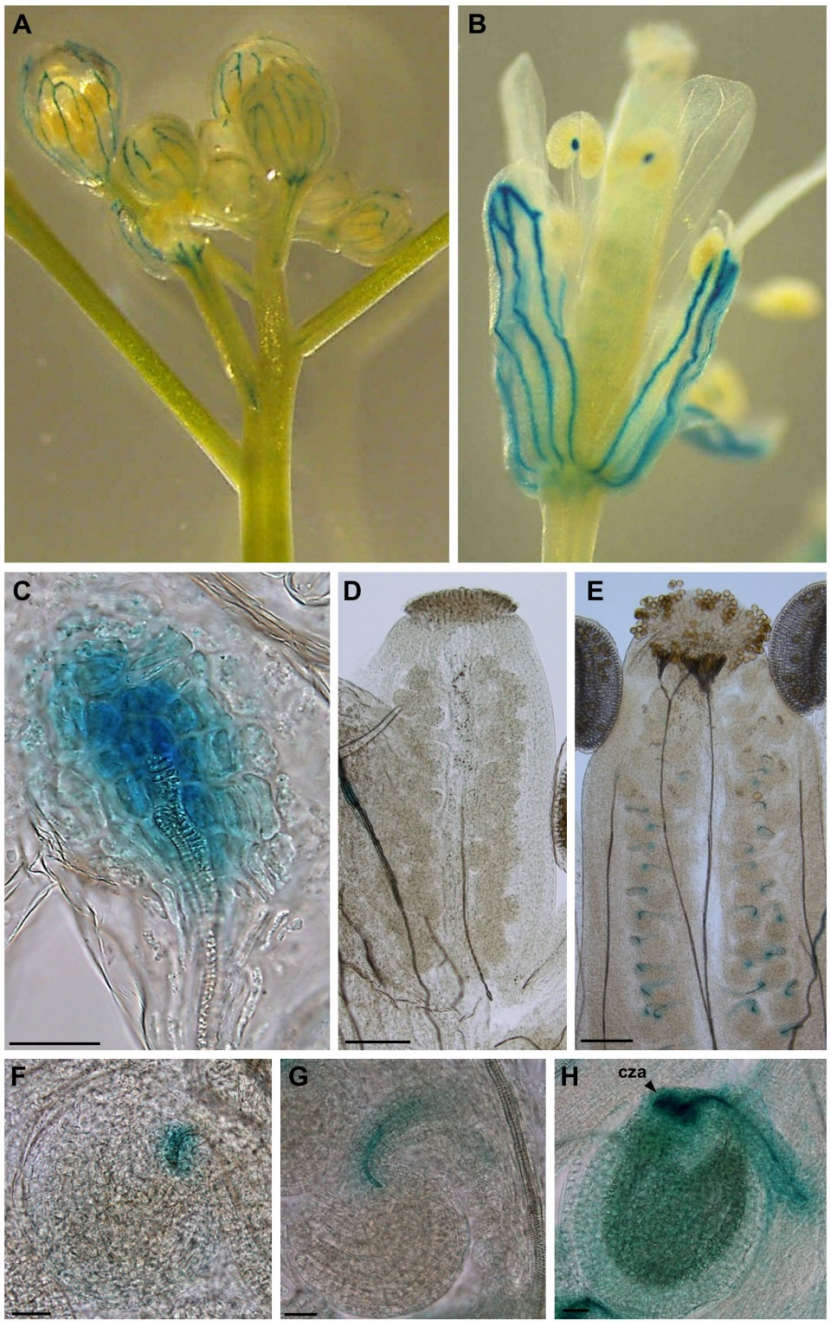

Fig. 4. *AtABCC13* is expressed in reproductive organs. *A* - GUS staining of flower buds; *B* - mature flower; *C* - magnified view of the blue spot of a stamen shown in *B*; *D* - gynoecium before pollination; *E* - gynoecium after pollination; *F, G, H* - early stages of a developing seed. *Scale bars*: *C* = 10 µm; *D*, *E* =250 µm; *F*, *G,* and *H* = 20 µm.

Table 1. The effect of increasing concentration [μM] of GA_3, zeatin, NAA, and ABA in medium for 4 d on GUS activity [μmol(MU) mg^{-1}(protein) min^{-1}] in the transgenic *Arabidopsis* seedlings with the *AtABCC13*::*GUS* construct. Means ± SD, $n = 6$. *Asterisks* indicate significant differences (* - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$) between the plants untreated and treated with the hormones; nd - not determined.

Conc.	GA	Zeatin	NAA	ABA
0	31.48±4.72	30.24±3.67	31.1±3.0	39.2±4.84
0.25	57.62±8.49*	12.44±0.81**	nd	nd
0.50	57.08±8.94*	14.68±1.05**	13.3±1.0***	14.2±0.61**
1.00	52.14±5.09*	13.87±1.78**	16.3±0.8**	10.3±2.72**
5.00	62.85±8.38*	17.87±3.31*	15.1±2.4**	11.4±1.97**

Table 2. The effect of increasing concentration of minerals in medium for 5 d or sucrose for 6 d on GUS activity [μmol(MU) mg^{-1}(protein) min^{-1}] in the transgenic *Arabidopsis* seedlings with the prom*AtABCC13*::GUS construct. Means ± SD, $n = 6$. *Asterisks* indicate significant differences (* - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$) between the untreated and treated plants; nd - not determined.

Minerals [mM]	KNO_3	KH_2PO_4	$MgSO_4$
0	21.4±4.7	36.5±3.3	34.5±5.6
0.01	nd	34.4±1.5	46.8±4.9
0.10	23.5±3.0	58.4±1.4***	44.8±5.5
1.00	46.9±3.0***	62.1±4.8***	40.1±2.0
5.00	nd	77.0±7.9***	41.9±5.0
10.00	50.5±3.0**	82.5±8.8***	27.1±3.9
35.00	36.4±3.0*	nd	nd
Sucrose [%]	**irradiance**	**dark**	
0	39.4±14.8	7.5±1.7**	
1.25	120.3±4.1***	8.9±0.8**	

In the flower bud, the *AtABCC13* expression was associated with the vascular tissues of sepals and both ends of peduncle (Fig. 4*A*). In mature flower in addition to sepal veins, a blue dot was observed in anthers (Fig. 4*B*). A detailed view shows that *AtABCC13* was expressed at the junction between filament and anther (Fig. 4*C*). The blue dot was restricted to a small group of cells surrounding the vascular element attachment to the connective tissues of anthers. No signal was observed in the gynoecium before pollination (Fig. 4*D*). The presence of pollen on the stigma was accompanied by gradually increasing staining ovules from the top to the bottom of ovary (Fig. 4*E*). Figs. 4*F* and *G* show the staining funiculus/chalaza junction in ovules (ovules from top and bottom of ovary, respectively). Four days after pollination, weak staining the endosperm central vacuole was accompanied by the strong signal of the chalazal area (Fig. 4*H*).

The *AtABCC13* expression was also investigated during silique maturation (Fig. 5*A*). The GUS activity was initially observed in the whole young developing seeds and it was later restricted to the funiculus during maturation (Fig. 5*A*,*B*). The cross section of siliques revealed that *AtABCC13* was expressed in the seed coat and embryo at the globular stage (Fig. 5*C*) and during early heart stages (Fig. 5*D*). The GUS-stained central part of globular and heart stage embryos might correspond to provascular tissues (Nawy *et al.* 2008) which would indicate that, as described for adult plants, the *AtABCC13* expression was also associated with vascular system establishment in the early step of embryo development. In freshly harvested seeds, the *AtABCC13* expression was restricted to the chalazal seed coat (Fig. 5*E*) and the signal was maintained over four months after ripening (data not shown).

No GUS staining was visible when the freshly harvested seeds were imbibed in water for 24 h, a treatment known to break seed dormancy of *Arabidopsis* Col-0 (Fig. 5*F*) (reviewed by Finkelstein *et al.* 2008). Afterwards, *AtABCC13* was expressed during all stages of seed germination (Fig. 5*F*). After 12 h on the half strength MS medium, blue staining appeared inside the seed in the radicle apex area (Fig. 5*F*). Staining covered the radicle until testa rupture and emergence (Fig. 5*F*). During germination, the *AtABCC13* expression localized to the hypocotyl and radicle vascular bundles with a strong signal observed in radicle apex (Fig. 5*G*). Cotyledon veins appear blue after they fully expand. Since ABA maintains seed dormancy, whereas GA counteracts ABA action and allows seed germination (Rodríguez-Gacio *et al.* 2009), the expression of *AtABCC13* during seed germination was thus consistent with up and down regulations observed with GA and ABA, respectively (Table 1).

AtABCC13 is expressed during the early stages of seed development and during germination; these processes are strongly associated with storage and mobilization of seed reserves (Penfield *et al.* 2005, Baud *et al.* 2008). Moreover, in all stages of seed development (Figs. 4*F-H*, 5*B-E*), a high GUS activity was observed in the chalazal area, a tissue believed to supply nutrients to the developing seeds across a symplasmic barrier (Hirner *et al.* 1998, Black *et al.* 2006). In order to check whether the nutrient supply may modify the *AtABCC13* expression, fluorimetric GUS assays were performed with *Arabidopsis* seedlings grown on culture media containing increasing concentrations of major mineral nutrients.

Nitrate and phosphate additions significantly upregulated the *AtABCC13* expression, whereas sulfate had no significant effect (Table 2). The chalaza-associated expression (Figs. 4*H,* 5*A-E*) suggest that *AtABCC13* might deal with nutritional resources during seed development, although an indirect role cannot be excluded. In plant, photochemical reactions produce NADPH and ATP required for the assimilation of minerals. In order to check whether sugar or irradiance may modify the *AtABCC13* expression, seedlings were grown on a sugar-free medium under irradiance or at

darkness. Sucrose strongly modified the *AtABCC13* expression in a radiation-dependent manner (Table 2). Under irradiance, the GUS activity in cotyledons was three times higher in the plants on a medium with sugar than without it, whereas under dark, the GUS activity was strongly reduced and sucrose had no effect. These results revealed that the *AtABCC13* expression in the cotyledons of the seedlings was radiation-dependent and modulated by nutrients.

Finally, *in silico* analysis of the *AtABCC13* promoter nucleotide sequence identified several putative regulatory elements including nitrate boxes, phosphate boxes, and GA responsive elements (see Fig. S3 and Table S4 for details). Interestingly, 13 putative nitrate boxes have been identified. Nitrate boxes correspond to various consensus sequences that seem to be necessary for nitrate-dependent transcription (Hwang *et al*. 1997). Moreover, six of the seven type-1 nitrate boxes are located between 0 and -650 promoter region. This might reflect a major role of nitrate in regulation of the *AtABCC13* expression.

Analyses of plant ABC expression pattern has contributed to identification of its physiological function. For instance in tobacco, *NpABC1/NpPDR1* is involved in the secretion of terpenoid compounds such as sclareol in relation to plant defense (Jasiński *et al.* 2001). The functional characterization of *NpABC1*/*NpPDR1* promoter allows the identification of sclareol-boxes that are required for the sclareol-induced expression of this transporter under pathogen attacks (Grec *et al.* 2003, Stukkens *et al.* 2005). In addition, the identification of iron deficiency *cis* elements in the promoter of the tobacco *NtPDR3* helps to highlight the involvement of this plant ABC transporter in iron homeostasis (Ducos *et al.* 2005). In maize, the promoter of the anthocyanin transporter *ZmMRP3* possesses anthocyanin responsive element (ARE). A detailed analysis of *ZmMRP3* expression pattern shows the localization of *ZmMRP3* in anthocyanin-accumulator tissues to generate a UV-B sunscreen protection (Goodman *et al.* 2004).

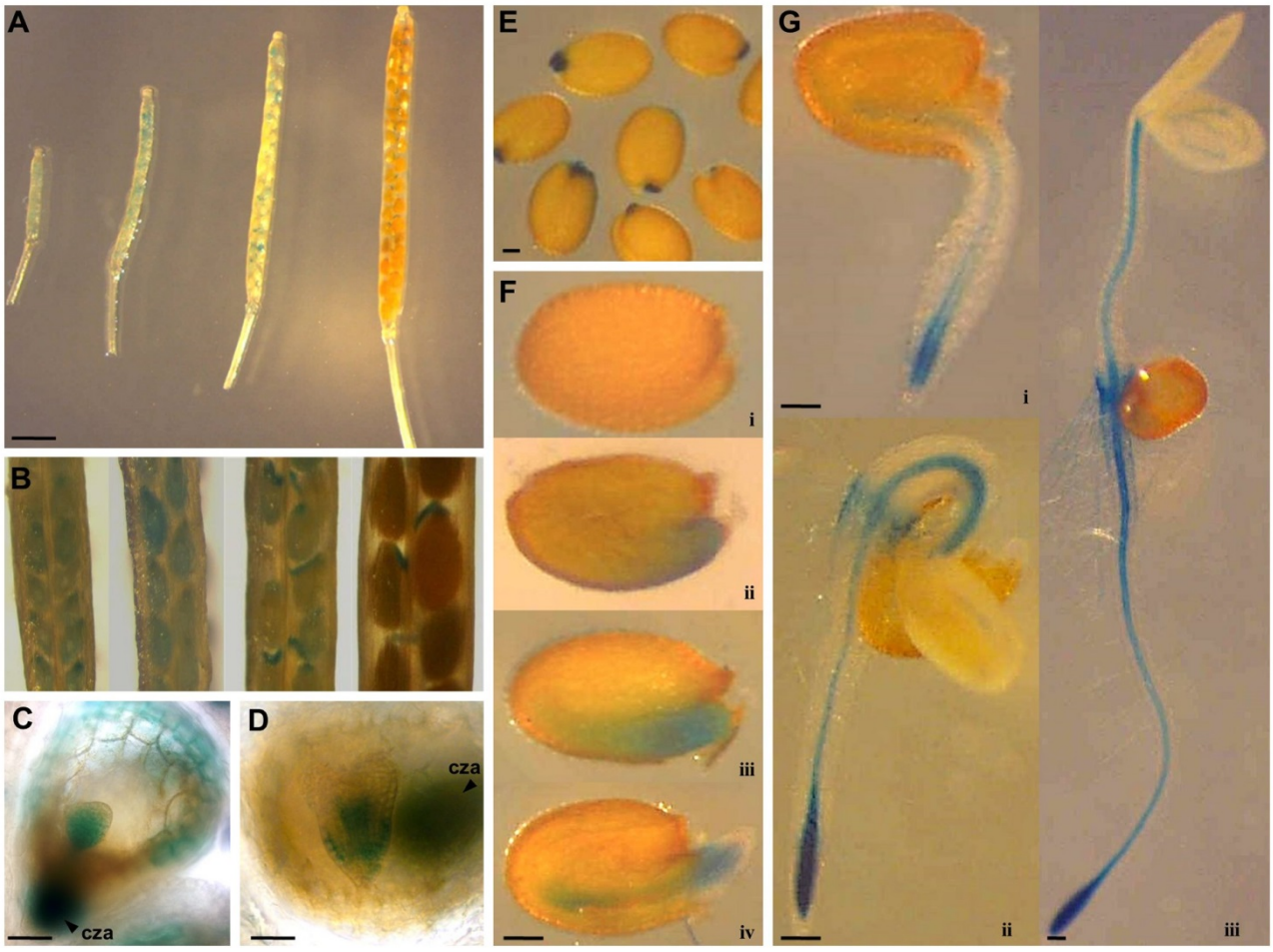

Fig. 5. *AtABCC13* is expressed during seed maturation, germination, and seedling development. *A* - Maturating siliques; *B* - detailed view of siliques shown in *A*; *C* - globular stage embryo; *D* - early heart stage embryo; *E* - freshly harvested seeds from mature siliques; *F* - germinating stratified seeds after 24 h imbibition (i), before testa rupture (ii), testa rupture (iii), emerging radicle (iv). *G* - developing seedling: radicle elongation (i), emerging cotyledons (ii), fully expended cotyledons (iii). cza - chalaza area. *Scale bars*: *A* = 1.5 mm; *C* and *D* = 50 μm; *E* and *F* = 100 μm.

In conclusion, our investigation of *AtABCC13* expression pattern revealed *1*) a relation with various tissues involved in metabolic exchanges (vascular apparatus, and connective tissue of anthers and funiculus/ chalazal area of ovules and developing seeds), *2*) that phytohormones positively (GA_3) or negatively (NAA, zeatin, and ABA) modulate its expression, and *3*) a response to nutritional status and irradiance. Taken together, these results suggest that this ABC transporter may be involved in nutrient utilization in relation to vascular tissue activities and seed development.

References

Agarwal, P.K., Jha, B.: Transcription factors in plants and ABA dependent and independent abiotic stress signalling. - Biol. Plant. **54**: 201-212, 2010.

Baba, K., Karlberg, A., Schmidt, J., Schrader, J., Hvidsten, T.R., Bako L., Bhalerao R.P.: Activity-dormancy transition in the cambial meristem involves stage-specific modulation of auxin response in hybrid aspen. - PNAS **108**: 3418-3423, 2011.

Baud, S., Dubreucq, B., Miquel, M., Rochat, C., Lepiniec, L.: Storage reserve accumulation in *Arabidopsis*: metabolic and developmental control of seed filling. - Arabidopsis Book **6**: e0113, 2008.

Benková, E., Bielach, A.: Lateral root organogenesis – from cell to organ. - Curr. Opin. Plant Biol. **13**: 677-683, 2010.

Bishopp, A., Lehesranta, S., Vatén, A., Help, H., El-Showk, S., Scheres, B., Helariutta, K., Mähönen, A.P., Sakakibara, H., Helariutta, Y.: Phloem-transported cytokinin regulates polar auxin transport and maintains vascular pattern in the root meristem. - Curr Biol. **21**: 927-932, 2011.

Black, M., Bewley, J.D., Halmer, P.: The Encyclopedia of Seeds: Science, Technology and Uses. - CABI, Cambridge 2006.

Bovet, L., Eggmann, T., Meylan-Bettex, M., Polier, J., Kammer, P., Marin, E., Feller, U., Martinoia, E.: Transcript levels of MRPs: induction of AtMRP3 after cadmium treatments. - Plant Cell Environ. **26**: 371-381, 2003.

Clough, S.J., Bent, A.F.: Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. - Plant J. **16**: 735-743, 1998.

De Smet, I., Tetsumura, T., De Rybel, B., Frey, N.F., Laplaze, L., Casimiro, I., Swarup, R., Naudts, M., Vanneste, S., Audenaert, D., Inzé, D., Bennett, M.J., Beeckman, T.: Auxin-dependent regulation of lateral root positioning in the basal meristem of *Arabidopsis*. - Development **134**: 681-690, 2007.

Dixon, R.A., Gonzales, R.A.: Plant Cell Culture: A Practical Approach. - Oxford University Press, Oxford 1994.

Ducos, E., Fraysse, S., Boutry, M.: NtPDR3, an iron-deficiency inducible ABC transporter in *Nicotiana tabacum*. - FEBS Lett. **579**: 6791-6795, 2005.

Elo, A., Immanen, J., Nieminen, K., Helariutta, Y.: Stem cell function during plant vascular development. - Semin. Cell Dev. Biol. **20**: 1097-1106, 2009.

Finkelstein, R., Reeves, W., Ariizumi, T., Steber, C.: Molecular aspects of seed dormancy. - Annu. Rev. Plant Biol. **59**: 387-41, 2008.

Frelet-Barrand, A., Kolukisaoglu, H.U., Plaza, S., Rüffer, M., Azevedo, L., Hörtensteiner, S., Marinova, K., Weder, B., Schulz, B., Klein, M.: Comparative mutant analysis of *Arabidopsis* ABCC-Type ABC transporters: AtMRP2 contributes to detoxification, vacuolar organic anion transport and chlorophyll degradation. - Plant Cell Physiol. **49**: 557-569, 2008.

Gaillard, S., Jacquet, H., Vavasseur, A., Leonhardt, N., Forestier, C.: *AtMRP6*/*AtABCC6*, an ATP-binding cassette transporter gene expressed during early steps of seedling development and up-regulated by cadmium in *Arabidopsis thaliana.* - BMC Plant Biol. **28**: 8-22, 2008.

Goodman, C. D., Casati, P., Walbot, V.: A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays.* - Plant Cell **16**: 1812-1826, 2004.

Grec, S., Vanham, D., de Ribaucourt, J.C., Purnelle, B., Boutry, M.: Identification of regulatory sequence elements within the transcription promoter region of *NpABC1*, a gene encoding a plant ABC transporter induced by diterpenes. - Plant J. **35**: 237-250, 2003.

Hirner, B., Fischer, W.N., Rentsch, D., Kwart, M., Frommer, W.B.: Developmental control of H^+/amino acid permease gene expression during seed development of *Arabidopsis*. - Plant J. **14**: 535-544, 1998.

Hwang, C.F., Lin, Y., D'Souza, T., Cheng, C.L.: Sequences necessary for nitrate-dependent transcription of *Arabidopsis* nitrate reductase genes. - Plant Physiol. **113**: 853-862, 1997.

Israelsson, M., Sundberg, B., Moritz, T.: Tissue-specific localization of gibberellins and expression of gibberellin-biosynthetic and signaling genes in wood forming tissues in aspen. - Plant J. **44**: 494-504, 2005.

Jaillais, Y., Chory, J.: Unraveling the paradoxes of plant hormone signaling integration. - Natur. struct. mol. Biol. **17**: 642-645, 2010.

Jasiński, M., Stukkens, Y., Degand, H., Purnelle, B., Marchand-Brynaert, J., Boutry, M.: A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. - Plant Cell **13**: 1095-1107, 2001.

Jefferson, R.A.: The GUS reporter gene system. - Nature **342**: 837-838, 1989.

Klein, M., Perfus-Barbeoch, L., Frelet, A., Gaedeke, N., Reinhardt, D., Mueller-Roeber, B., Martinoia, E., Forestier, C.: The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signaling and water use. - Plant J. **33**: 119-129, 2003.

Klein, M., Geisler, M., Suh, S.J., Kolukisaoglu, H.U., Azevedo, L., Plaza, S., Curtis, M.D., Richter, A., Weder, B., Schulz, B., Martinoia, E.: Disruption of AtMRP4, a guard cell plasma membrane ABCC-type ABC transporter, leads to deregulation of stomatal opening and increased drought susceptibility. - Plant J. **39**: 219-236, 2004.

Kolukisaoglu, H.U., Bovet, L., Klein, M., Eggmann, T., Geisler, M., Wanke, D., Martinoia, E., Schulz, B.: Family business: the multidrug-resistance related protein (MRP) ABC transporter genes in *Arabidopsis thaliana*. - Planta **216**: 107-119, 2002.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., Offringa, R., Graham, N., Doumas, P., Friml, J., Bogusz, D., Beeckman, T., Bennett, M.:

Cytokinins act directly on lateral root founder cells to inhibit root initiation. - Plant Cell **19**: 3889-3900, 2007.

Lu, Y.P., Li, Z.S., Rea, P.A.: *AtMRP1* gene of *Arabidopsis* encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ATP-binding cassette transporter gene. - Proc. nat. Acad. Sci. USA **94**: 8243-8248, 1997.

Lu, Y.P., Li, Z.S., Drozdowicz, Y.M., Hortensteiner, S., Martinoia, E., Rea, P.A.: AtMRP2, an *Arabidopsis* ATP binding cassette transporter able to transport glutathione *S*-conjugates and chlorophyll catabolites: functional comparisons with AtMRP1. - Plant Cell **10**: 267-282, 1998.

Moubayidin, L., Perilli, S., Dello Ioio, R., Di Mambro, R., Costantino, P., Sabatini, S.: The rate of cell differentiation controls the *Arabidopsis* root meristem growth phase. - Curr. Biol. **20**: 1138-1143, 2010.

Nagy, R., Grob, H., Weder, B., Green, P., Klein, M., Frelet-Barrand, A., Schjoerring, J.K., Brearley, C., Martinoia, E.: The *Arabidopsis* ATP-binding cassette protein AtMRP5/ AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. - J. biol. Chem. **284**: 33614-33622, 2009.

Nawy, T., Lukowitz, W., Bayer, M.: Talk global, act local-patterning the Arabidopsis embryo. - Curr. Opin. Plant Biol. **11**: 28-33, 2008.

Nieuwland, J., Maughan, S., Dewitte, W., Scofield, S., Sanz, L., Murray, J.A.H.: The D- type cyclin CYCD4:1 modulates lateral root density in *Arabidopsis* by affecting the basal meristem region. - Proc. nat. Acad. Sci. USA **106**: 22528-22533, 2009.

Parizot, B., Laplaze, L., Ricaud, L., Boucheron-Dubuisson, E., Bayle, V., Bonke, M., De Smet, I., Poethig, S.R., Helariutta, Y., Haseloff, J., Chriqui, D., Beeckman, T., Nussaume, L.: Diarch symmetry of the vascular bundle in Arabidopsis root encompasses the pericycle and is reflected in distich lateral root initiation. - Plant Physiol. **146**: 140-148, 2008.

Penfield, S., Graham, S., Graham, I.A.: Storage reserve mobilization in germinating oilseeds: *Arabidopsis* as a model system. - Biochem. Soc. Trans. **33**: 380-383, 2005.

Rodríguez-Garcio, M del C., Matilla-Vázquez, M.A., Matilla, A.J.: Seed dormancy and ABA signaling: the breakthrough goes on. - Plant Signal Behav. **4**: 1035-1049, 2009.

Schulz, B., Kolukisaoglu, H.U.: Genomics of plant ABC transporters: the alphabet of photosynthetic life forms or just holes in membranes? - FEBS Lett. **580**: 1010-1016, 2006.

Sehr, E.M., Agusti, J., Lehner, R., Farmer, E.E., Schwarz, M., Greb, T.: Analysis of secondary growth in the *Arabidopsis* shoot reveals a positive role of jasmonate signaling in cambium formation. - Plant J. **63**: 811-822, 2010.

Shani, E., Weinstain, R., Zhang, Y., Castillejo, C., Kaiserli, E., Chory, J., Tsien, R.Y., Estelle, M.: Gibberellins accumulate in the elongating endodermal cells of *Arabidopsis* root. - PNAS **110**: 4834-4839, 2013.

Song, W.Y., Park, J., Mendoza-Cózatl, D.G., Suter-Grotemeyer, M., Shim, D., Hörtensteiner, S., Geisler, M., Weder, B., Rea, P.A., Rentsch, D., Schroeder, J.I., Lee, Y., Martinoia, E.: Arsenic tolerance in *Arabidopsis* is mediated by two ABCC type phytochelatin transporters. - Proc. nat. Acad. Sci. USA **107**: 21187-21192, 2010.

St-Pierre, B., Brisson, N.: 5' deletion analysis of the potato starch phosphorylase gene: an upstream sequence defines distal regulatory elements and a proximal organ-dependent promoter. - Plant Sci. **110**: 193-203, 1995.

Stukkens, Y., Bultreys, A., Grec, S., Trombik, T., Vanham, D., Boutry, M.: NpPDR1, a pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana plumbaginifolia*, plays a major role in plant pathogen defense. - Plant Physiol. **139**: 341-352, 2005.

Suh, S.J., Wang, Y.F., Frelet, A., Leonhardt, N., Klein, M., Forestier, C., Mueller-Roeber, B., Cho, M.H., Martinoia, E., Schroeder, J.I.: The ATP binding cassette transporter AtMRP5 modulates anion and calcium channel activities in *Arabidopsis* guard cells. - J. biol. Chem. **282**: 1916-1924, 2007.

Tommasini, R., Vogt, E., Fromenteau, M., Hörtensteiner, S., Matile, P., Amrhein, N., Martinoia, E.: An ABC-transporter of *Arabidopsis thaliana* has both glutathione-conjugate and chlorophyll catabolite transport activity. - Plant J. **13**: 773-780, 1998.

Tuominen, H., Puech, L., Fink, S., Sundberg, B.: A radial concentration gradient of indole-3-acetic acid is related to secondary xylem development in hybrid aspen. - Plant Physiol. **115**: 577-585, 1997.

Ubeda-Tomás, S., Swarup, R., Coates, J., Swarup, K., Laplaze, L., Beemster, G.T., Hedden P., Bhalerao, R., Bennett, M.J.: Root growth in *Arabidopsis* requires gibberellin/DELLA signalling in the endodermis. - Nat. Cell Biol. **10**: 625-628, 2008.

Uggla, C., Moritz, T., Sandberg, G., Sundberg, B.: Auxin as a positional signal in pattern formation in plants. - Proc. nat. Acad. Sci. USA **93**: 9282-9286, 1996.

Wojas, S., Hennig, J., Plaza, S., Geisler, M., Siemianowski, O., Skłodowska, A,. Ruszczyńska, A., Bulska, E., Antosiewicz, D.M.: Ectopic expression of *Arabidopsis* ABC transporter MRP7 modifies cadmium root-to-shoot transport and accumulation. - Environ. Pollut. **157**: 2781-2789, 2009.