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TECHNICAL ADVANCE

Tissue-specific inactivation by cytosine deaminase/uracil phosphoribosyl transferase as a tool to study plant biology

Nathalie Leonhardt[1], Fanchon Divol[2], Serge Chiarenza[1], Sabrina Deschamps[1], Jeanne Renaud[1], Cécile Giacalone[1], Laurent Nussaume[1], Richard Berthomé[3] and Benjamin Péret[2,*]

[1]UMR7265, Laboratoire de Biologie du Développement des Plantes, Service de Biologie Végétale et de Microbiologie Environnementales, Institut de Biologie Environnementale et Biotechnologie, CNRS-CEA-Université Aix-Marseille Saint-Paul-lez-Durance, Saint Paul les Durance Cedex, France,

[2]BPMP, Univ Montpellier, CNRS, INRA, SupAgro, Montpellier, France, and

[3]LIPM, Université de Toulouse, INRA, CNRS, UPS, Castanet-Tolosan, France

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*For correspondence (e-mail benjamin.peret@supagro.fr).

SUMMARY

Recent advances in the study of plant developmental and physiological responses have benefited from tissue-specific approaches, revealing the role of some cell types in these processes. Such approaches have relied on the inactivation of target cells using either toxic compounds or deleterious genes; however, both tissue-specific and truly inducible tools are lacking in order to precisely target a developmental window or specific growth response. We engineered the yeast fluorocytosine deaminase (FCY1) gene by creating a fusion with the bacterial uracil phosphoribosyl transferase (UPP) gene. The recombinant protein converts the precursor 5-fluorocytosine (5-FC) into 5-fluorouracyl, a drug used in the treatment of a range of cancers, which triggers DNA and RNA damage. We expressed the *FCY-UPP* gene construct in specific cell types using enhancer trap lines and promoters, demonstrating that this marker acts in a cell-autonomous manner. We also showed that it can inactivate slow developmental processes like lateral root formation by targeting pericycle cells. It also revealed a role for the lateral root cap and the epidermis in controlling root growth, a faster response. The 5-FC precursor acts systemically, as demonstrated by its ability to inhibit stomatal movements when supplied to the roots in combination with a guard cell-specific promoter. Finally, we demonstrate that the tissular inactivation is reversible, and can therefore be used to synchronize plant responses or to determine cell type-specific functions during different developmental stages. This tool will greatly enhance our capacity to understand the respective role of each cell type in plant physiology and development.

Keywords: *Arabidopsis thaliana*, tissue-specific inactivation, root growth, lateral root development, stomatal regulation, technical advance.

INTRODUCTION

Plant physiological and developmental pathways rely on the coordinated action of several tissues. Whether this is associated with nutrient and water uptake by the root or light and CO_2 perception in the shoot, understanding the respective role of each cell type is of primary importance. There is now growing evidence that the ability of tissues to communicate, either adjacently or distantly, represents a key aspect of developmental processes. It is therefore becoming increasingly important to study physiological and developmental pathways from a tissular and cellular perspective. A key approach towards this goal has relied on the use of genetic ablation methodologies. Placing a toxic gene under the control of a tightly regulated promoter can selectively inactivate a cell type or group of cells and reveal their role in a complex tissue. The diphtheria toxin A chain (DTA) is a potent inhibitor of translation that is toxic for plant cells (Czako and An, 1991). Its targeted expression has been used for the past 25 years to effectively reveal the role of several cell types (Thorsness *et al.*, 1993; Tsugeki and Fedoroff, 1999; Weijers *et al.*, 2003).

Similar approaches based on genes triggering general cellular toxicity include the expression of ribonuclease (Mariani *et al.*, 1990; Koltunow *et al.*, 1990; Goldberg *et al.*, 1995); however, limitations of these tools are their constitutive activity and their irreversible effects that can trigger early developmental defects, especially if placed under a promoter in which expression is not limited in time and space. For example, such an approach induced the destruction of the root cap, resulting in plants exhibiting strong growth defects and subsequent death at an early stage (Tsugeki and Fedoroff, 1999). As a result, these strategies have been confined to the study of flower and seed development (Day *et al.*, 1995; Weijers *et al.*, 2003), as they have little effect on plant development overall.

A corrective strategy has consisted in the usage of regulatory elements from non-plant organisms. Their specificity relies on the absence of an equivalent target in plants. These heterologous elements include components regulated by the antibiotic tetracycline (Gatz *et al.*, 1992), the synthetic steroid dexamethasone (Aoyama and Chua, 1997), isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Wilde *et al.*, 1992), ethanol (Roslan *et al.*, 2001) or the copper ion (Mett *et al.*, 1993). They necessitate the combined use of two transgenes in plants, however: a transcription factor that acts as the switch and a target promoter controlling the expression of the gene of interest. The number of transgenes can go up to three if a reporter gene is included to follow the tissular specificity of the induction (Deveaux *et al.*, 2003). Although this method allows for the choice of the target gene to be induced, including a toxic gene such as *DTA* or a nuclease, it increases the complexity of the T-DNA(s) to be transferred and has not been widely used for genetic ablation. Interestingly, the recent development of estradiol-inducible transgenes with a full suite of MultiSite Gateway compatible cell type-specific systems may lead to new applications for genetic ablation studies (Brand *et al.*, 2006; Siligato *et al.*, 2016).

Instead of an inducible promoter, genetic toxicity can be itself induced by relying on an innocuous substance that is readily converted into a toxic compound by transgenic enzymatic activity. In plants, the use of the bacterial gene *indole acetamide hydrolase* (*iaaH*), originating from the tumor-inducing bacteria *Agrobacterium* sp., allows the conversion of the inactive compound auxin indole acetamide into indole acetic acid. Large auxin accumulation triggers general toxic effects, reminiscent of the use of 2,4-dichlorophenoxy acetic acid (2,4-D) as a herbicide. Similarly, the enzyme nitrate reductase (NR) can incorporate chlorate into the toxic form chlorite (Nussaume *et al.*, 1991). The major limitation of these two markers is that they are not cell autonomous because their end products can readily diffuse into the plant and have therefore been used only as negative markers at the organism level (Klee *et al.*, 1987; Nussaume *et al.*, 1991) and not for tissue-specific studies.

Here, we report the use of an inducible system for genetic toxicity at the tissular level, based on the heterologous expression of a tandem of two genes: *fluorocytosine deaminase* (*FCY*) and *uracil phosphoribosyl transferase* (*UPP*). We demonstrate that the tissue-specific expression of these genes is responsible for the incorporation of the 5-fluorocytosine (5-FC) precursor that is normally not metabolized by higher eukaryotes and we use this system to inactivate target cells or tissues. Tissular inactivation was found to be cell autonomous, acting on slow and fast developmental and growth responses alike. Moreover, its local and systemic reversible range of action offers versatile opportunities for plant physiology studies.

RESULTS

Combination of exogenous 5-FC and FCY-UPP expression as a negative plant marker

The antipyrimidine effect of 5-fluorouracil (5-FU) has been largely described, mainly because of its anticancer activity on mammalian cells (reviewed in Longley *et al.*, 2003). This drug acts as an antimetabolite that inhibits synthetic processes, mainly targeting thymidylate synthase, a key enzyme for DNA synthesis converting deoxyuridine monophosphate (dUMP) into deoxythymidine monophosphate (dTMP). Concomitantly, it is incorporated into nucleic acids, resulting in an altered cellular function mainly by blocking RNA maturation (preRNA processing, tRNA post-transcriptional modifications and pre-mRNA splicing). In this study, we used the 5-FU precursor 5-FC because it is inactive in plants through a lack of cytosine deaminase (FCY) activity (Figure 1a). Early work on negative selection markers demonstrated that the *in planta* expression of the yeast (*Saccharomyces cerevisiae*) *FCY* gene under a constitutive promoter (*35S*) resulted in plants sensitive to the exogenous application of 5-FC, compared with wild-type controls (Perera *et al.*, 1993; Kobayashi *et al.*, 1995). Interestingly, the concomitant expression of the *Escherichia coli* uracil phosphoribosyl transferase (*UPP*) gene improves the cytotoxicity of the system by enhancing the RNA incorporation pathway (Tiraby *et al.*, 1998; Figure 1a). Indeed, the germination of seedlings on plates containing 1 mM 5-FC had no effect on wild-type (Col-0) plants, whereas seedlings overexpressing the two genes in tandem (*35S:FCY-UPP*; Figure 1b) died rapidly a couple of days after germination (Figure 1c). When wild-type plants were mixed with *35S:FCY-UPP* plants, the wild-type plants were not affected by the 5-FC conversion into neighboring transgenic plants, thereby demonstrating that the action of 5-FC incorporation is plant autonomous (Figure 1c).

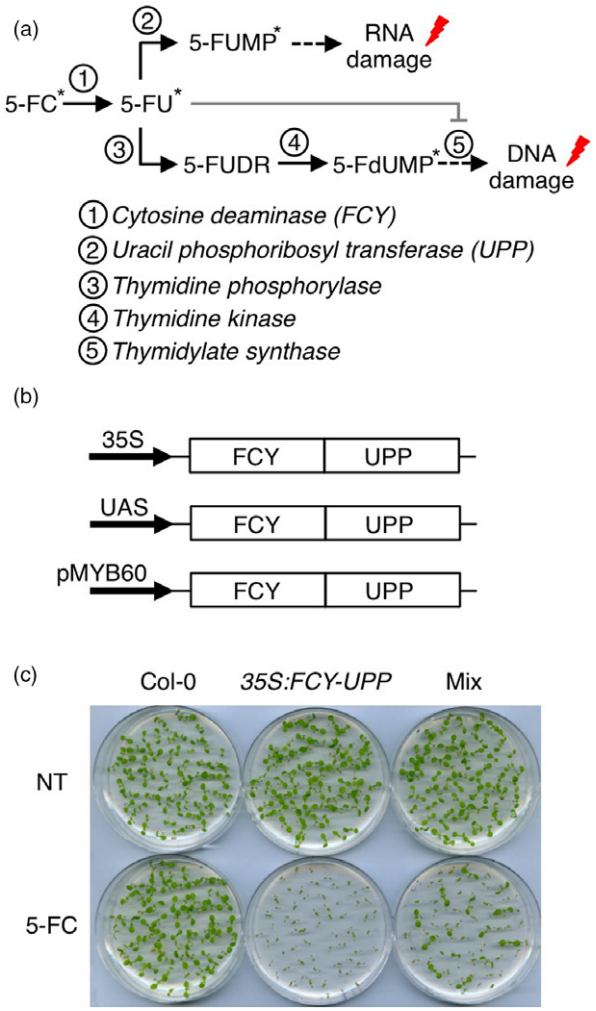

Figure 1. 5-Fluorocytosine (5-FC) incorporation by cytosine deaminase generates cellular toxicity.
(a) 5-FC is incorporated by cytosine deaminase (FCY) into two distinct pathways, leading to RNA and DNA damage. The concomitant expression of uracil phosphoribosyl transferase (UPP) favors the RNA damage pathway. (b) In this study, we fused the FCY and UPP genes to express them in tandem under the regulation of various promoters, such as *35S* for constitutive expression, the upstream-activated sequence (UAS) for transactivation by GAL4 and the guard cell-specific promoter *pMYB60*. (c) Wild-type (Col-0) and *35S:FCY-UPP* seedlings were germinated on plates in the absence (non-treated) or presence (5-FC) of 500 μM 5-FC ($n > 80$). 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracyl; 5-FUMP, 5-fuorouridine monophosphate; 5-FUDR, 5-fluorodeoxyuridine; 5-FdUMP, fluorodeoxyuridine monophosphate.

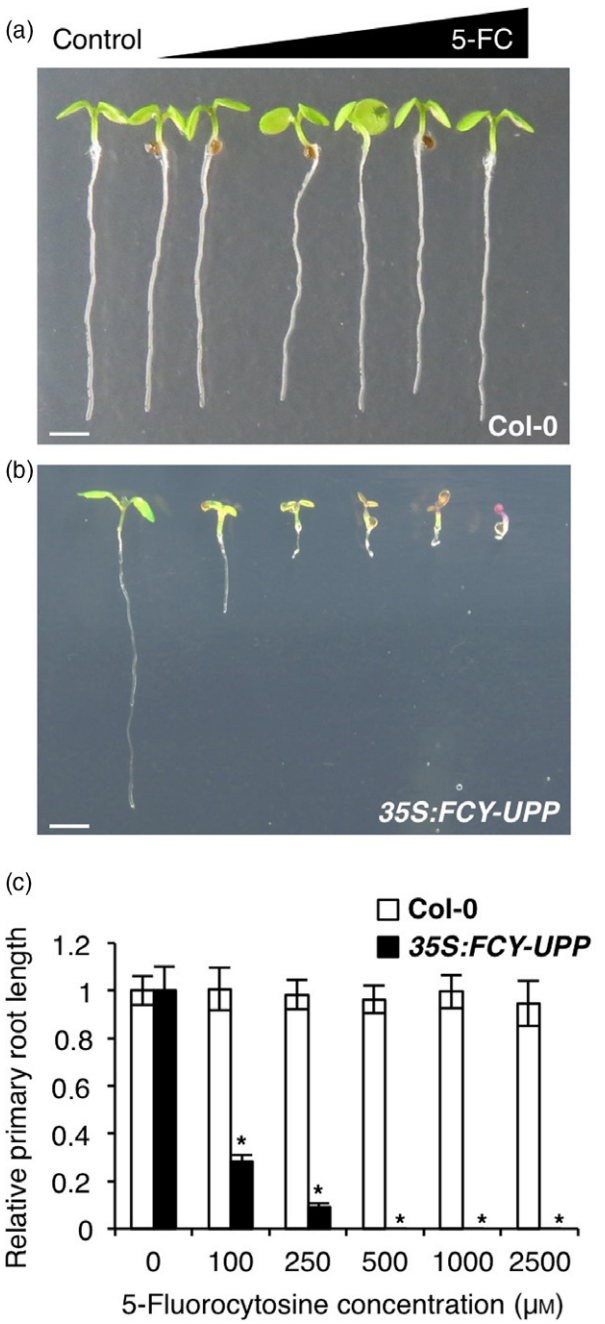

Figure 2. 5-Fluorocytosine (5-FC) toxicity on primary root growth is concentration dependent.
(a–c) Seedlings were germinated and grown with different concentrations of 5-FC (0, 100, 250, 500, 1000 and 2500 μM) and primary root length was measured (data represent means ± SEMs, $n = 20$). Asterisks indicate a significant difference with the corresponding non-treated control experiment by Student's *t*-test (*$P < 0.001$; $n = 10$). Scale bars: 1.5 mm.

The toxic effect of 5-FC was shown to be concentration dependent in the independent transgenic lines that we produced, with a pronounced reduction of primary root growth observed with 100 μM 5-FC and with a total arrest of seedling growth after germination observed with 500 μM in line 1, whereas wild-type (Col-0) plants were unaffected (Figure 2a–c). Other commonly used Arabidopsis ecotypes were also unaffected by a similar treatment (Figure S1a). A second line showed a weaker sensitivity to 5-FC with a reduction observed at 250 μM and with total growth arrest at 1000 μM (Figure S1b,c). The sensitivity of the plants to 5-FC correlated well with the level of expression of the *FCY-UPP* transgene in these two independent lines (Figure S1d).

Tissue-specific expression of *FCY-UPP* drives tissue-autonomous toxicity

In order to target 5-FC incorporation in specific tissues, we used the previously described GAL4-based transactivation system (Brand and Perrimon, 1993). The *FCY-UPP* tandem genes were fused and placed downstream of the UAS (upstream activated sequence). This construction was transformed into the xylem pole pericycle cell specific J0121 line (Laplaze *et al.*, 2005), the expression of which starts in the elongation zone and is maintained in the shootward direction up to the mature tissues (Figure 3a–c). Exogenous treatment with 5-FC had no effect on the untransformed J0121 plants whereas *J0121>>FCY-UPP* plants showed a strong reduction in lateral root (LR) number (Figure 3d,e,f and g). This toxic effect on LR formation was found to be concentration dependent, with a minimum effective concentration of 1000 μM and with a total disappearance of LR formation at 2500 μM 5-FC (Figure 3h). In order to determine whether primary root growth was also affected in this line, we followed plant growth in real time. A computer-controlled DSLR camera was used to take pictures every 10 min for the duration of the experiment (set-up shown in Figure S2a). No difference in the growth of the control (non-treated) versus 5-FC plants was observed at 1000 μM at 70 h after transfer, therefore demonstrating the tissue-autonomous nature of the inactivation system (Figure 3i). At higher 5-FC concentrations (2500 μM and above), however, primary root growth was also affected (Figure S2b), demonstrating that tissue autonomy can be overcome at higher 5-FC treatments. Primary root growth of wild-type plants remained unaffected by a similar treatment (Figure S2c). In order to determine whether lateral root initiation or subsequent primordium emergence is altered by 5-FC treatment of the *J0121>>FCY-UPP* plants, we determined the ratio of emerged and non-emerged lateral roots. The treatment did not decrease the number of initiation events but significantly reduced the emergence of lateral root primordium compared with untreated plants (Figure S2d). The expression pattern driven by the J0121 line remained unaltered by 5-FC treatment (Figure S3a,b and d,e), and the blocked lateral root primordium seemed similar to those of untreated plants (Figure S3c and f).

Fast growth responses are affected by 5-FC incorporation toxicity

The toxic effect of 5-FC incorporation into the pericycle on LR formation (a slow developmental process) prompted us to test whether the system could also affect faster growth responses. We transformed the *UAS:FCY-UPP* construct into the lateral root cap and epidermis-specific J0951 line (Figure 4a,b). Treatment with 5-FC resulted in arrested primary root growth in the treated versus non-treated *J0951>>FCY-UPP* line (Figure 4c,d). This effect was found to be 5-FC concentration dependent with a minimum effective concentration tested at 100 μM (Figure S4a,b). Real-time tracking of primary root growth revealed that 5-FC altered growth approximately 48 h after transfer to 5-FC medium versus control plants transferred to regular medium (Figure 4e). Root growth was not totally abolished but the reduction was drastic. A quicker effect was obtained on the constitutive *35S:FCY-UPP* lines (Figure S4c,d), and the higher expression level (Figure S1d) correlated with a quicker response (down to 18 and 30 h). The expression pattern driven by the J0951 line remained unaltered by 5-FC treatment (Figure S5a–d).

The FCY-UPP-GFP fusion protein is cell autonomous

In order to determine whether the FCY-UPP protein is able to move across cells, we fused it to the GFP reporter and expressed it under the control of the epidermis-specific WEREWOLF promoter region. In comparison with the direct *pWER:GFP* fusion that displays GFP fluorescence in the epidermis (Figure 5b), the expression pattern of the *pWER:FCY-UPP-GFP* construct was strictly identical (Figure 5d). Treatment with 5-FC did not alter the expression profile driven by the two constructs (Figure 5c,e and f). Attaching the GFP protein to the FCY-UPP tandem did not prevent its enzymatic function, and dose–response treatment of this line with 5-FC triggered primary root growth arrest at concentrations above 1000 μM (Figure 5a and S6).

5-FC systemically diffuses into plants from root to leaves to exert its toxic incorporation effect

As 5-FC is efficiently incorporated by FCY-UPP when the tandem genes are expressed in roots, we wanted to test whether expression in the distal parts of the plants would still be efficient. In the absence of available catalogs of enhancer trap lines in the shoot, we directly fused the *FCY-UPP* genes to the guard cell-specific promoter of the *MYB60* gene. This promoter drives expression specifically in the guard cells, as shown by the *pMYB60* promoter fusion with the glucuronidase gene (*pMYB60:GUS*; Figure 6a,b and c). We measured stomatal aperture as a response to dark–light transition in sand/hydroponic-cultivated plants. 5-FC was applied directly to the root system 24 h prior to stomatal aperture measurement. Both *35S:FCY-UPP* and *pMYB60:FCY-UPP* lost the ability to open their stomata upon treatment with 100 μM 5-FC. Identical results were obtained in two independent *pMYB60:FCY-UPP* lines with similar expression levels (Figure S7a). Stomatal aperture controls water loss from the leaves, which in turn regulates leaf surface temperature. We monitored leaf surface temperature with a thermal camera to determine whether 5-FC treatment in *pMYB60:FCY-UPP* plants affected plant transpiration. An approximately 2°C increase in leaf temperature was observed in the 5-FC-treated *pMYB60:FCY-UPP* plants compared with controls,

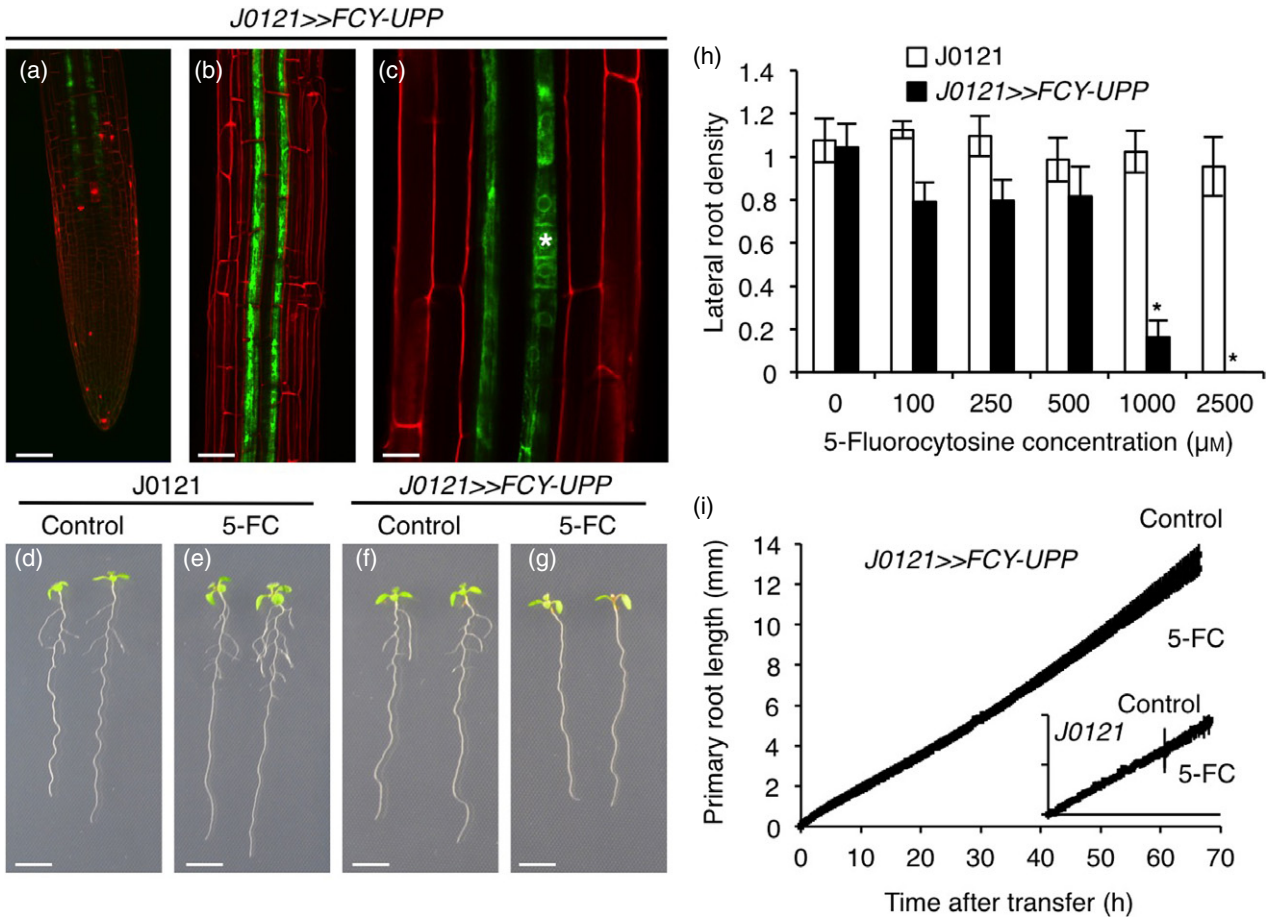

Figure 3. *FCY-UPP* expression in the pericycle blocks lateral root formation upon treatment with 5-fluorocytosine (5-FC).
(a–c) The J0121 transactivation line triggers GFP expression in the xylem pole pericycle cells, staring from the elongation zone (a), up to the mature part of the root (b) and including stage-I lateral root primordium (c). (d–g) Wild-type (Col-0) seedlings of 6 days in age germinated on control (d) or 1000 μM 5-FC medium (e) and *J0121>>FCY-UPP* seedlings germinated on control (f) or 1000 μM 5-FC medium (g). (h) Plant lateral root density (number of emerged lateral roots per cm of primary root length) was scored 6 days after germination for various 5-FC concentrations (0, 100, 250, 500, 1000 and 2500 μM). (i) Real-time primary root growth tracking of non-treated (control) versus 1000 μM 5-fluorocytosine treated (5-FC) seedlings for 70 h after transfer. Both curves overlap (data represent means ± SEMs, $n = 10$). Similar real-time tracking of the non-transformed J0121 control line is shown as an insert (units and scales are the same). Asterisks indicate a significant difference with corresponding non-treated control experiment by Student's *t*-test (*$P < 0.001$; $n = 10$). Scale bars: (a, b) 40 μm; (c) 15 μm; (d–g) 3 mm.

untreated and 5FC-treated Col-0 plants, demonstrating the absence of any effect of 5-FC on leaf temperature in the wild type and a strong reduction of leaf transpiration in the transgenic line as a result of stomatal closure (Figure 6e). Total leaf area was not affected by 5-FC treatment and did not differ across the control and transgenic lines (Figure S7b).

5-FC incorporation toxicity is reversible

The toxicity triggered by the tissue-specific incorporation of 5-FC strongly affects processes like LR formation, primary root growth and stomatal aperture. We tested whether these effects are permanent or reversible. Removal of 5-FC by transfer to regular medium was performed on the J0121>>FCY-UPP line. Plants were grown for 9 days after germination on medium containing 1000 μM 5-FC and then transferred to medium without any 5-FC. Plants from two independent J0121>>FCY-UPP transgenic lines were able to produce the same number of lateral roots 3 days after transfer back to the control medium as the wild-type Col-0 controls (Figure 7a). We next tested the reversibility of 5-FC incorporation on stomatal aperture regulation. Plants were grown for 25 days and then placed in the presence of 100 μM 5-FC for 24 h. Plants expressing the two transgenes specifically in the guard cells (*pMYB60:FCY-UPP*) were unable to open their stomata in response to the dark–light transition. After transfer back to hydroponic solution deprived of 5-FC for 48 h, the plants from two *pMYB60:FCY-UPP* lines regained the ability to respond to the dark–light transition and open their stomata (Figure 7b), thereby demonstrating the reversible effect of 5-FC incorporation.

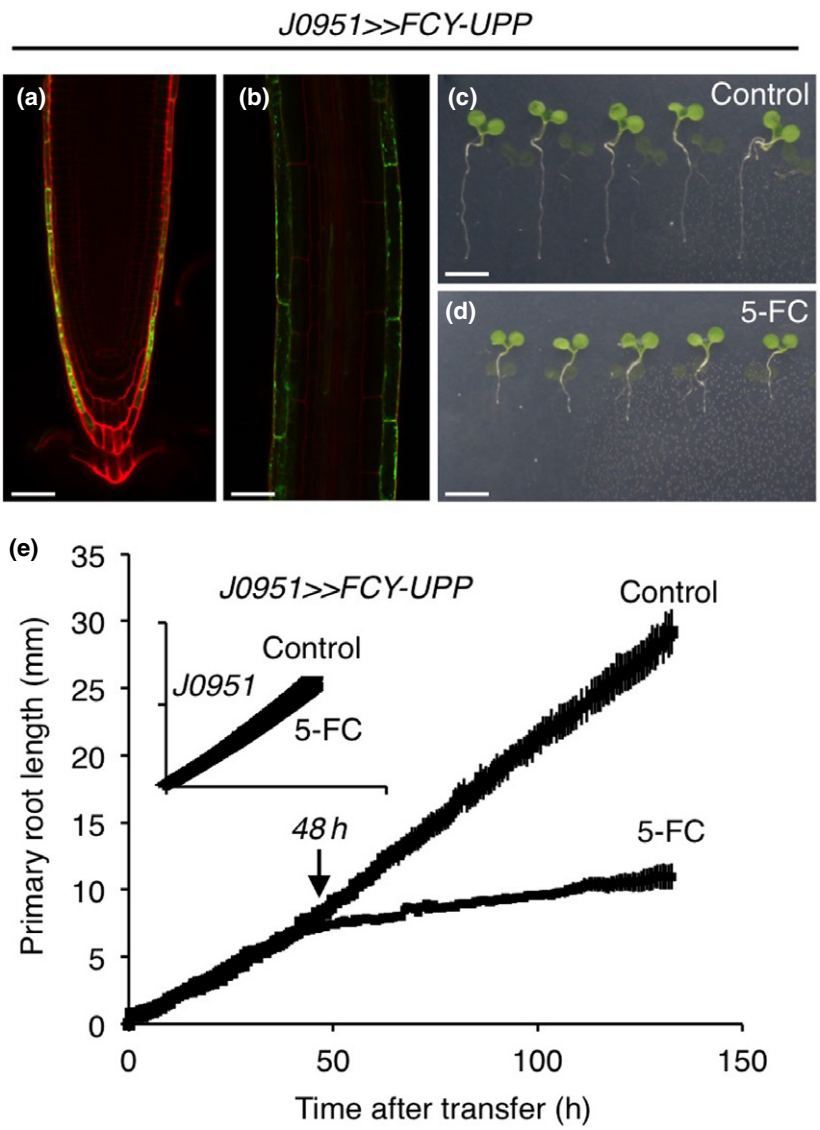

Figure 4. Tissular inactivation of the lateral root cap and epidermis blocks primary root growth.
(a–b) The J0951 transactivation line triggers GFP expression in the lateral root cap cells (a) and in the epidermis (b). (c–d) *J0951>>FCY-UPP* seedlings at 6 days of age, germinated on control (c) or 500 μM 5-fluorocytosine (5-FC) medium (d). (e) Real-time primary root growth tracking of non-treated (control) versus seedlings treated with 500 μM 5-FC for 130 h after transfer (data represent means ± SEMs, $n = 10$). Similar real-time tracking of non-transformed J0951 control line is shown as an insert (units and scales are the same). Scale bars: (a, b) 40 μm; (c, d) 5 mm.

DISCUSSION

Early work identified that cytosine deaminase (FCY) activity produced by prokaryotes but not by higher eukaryotes could be used as a negative marker together with its substrate 5-FC. The deamination of 5-FC by FCY into cytotoxic 5-FU has been used in the model plant Arabidopsis as a negative selection marker under the constitutive and strong viral *35S* promoter (Perera *et al.*, 1993; Kobayashi *et al.*, 1995). Similar results have been reported in *Oryza sativa* (rice) (Dai *et al.*, 2001) and *Solanum lycopersicum* (tomato) (Hashimoto *et al.*, 1999). These studies have shown that 5-FC incorporation triggers plant autonomous toxicity, whereas other negative markers such as iaaH (using IAM as a precursor) or NR (using chlorate as a substrate) can be toxic for neighboring plants through diffusion of their toxic product in the culture medium. Subsequently, the concomitant expression of the bacterial gene *uracil phosphoribosyltransferase* was shown to improve dramatically the sensitivity of mammalian cells to 5-FC (Tiraby *et al.*, 1998). The rapid metabolic incorporation of 5-FC into its product suggested a passive diffusion of extracellular 5-FC through the plasma membrane, resulting in increased drug uptake. In this study, we fused the yeast version of FCY and the bacterial version of UPP to create a functional protein tandem with elevated activity in plants. In order to achieve tissular specificity of 5-FC incorporation, we placed this chimeric protein under the expression of various tissue-specific promoters, including the yeast UAS activating sequence, to benefit from the available transactivation lines (Laplaze *et al.*, 2005). Our results demonstrated that the tandem expression of the FCY-UPP protein retains its plant-autonomous properties and that the toxicity did not spread across the culture medium

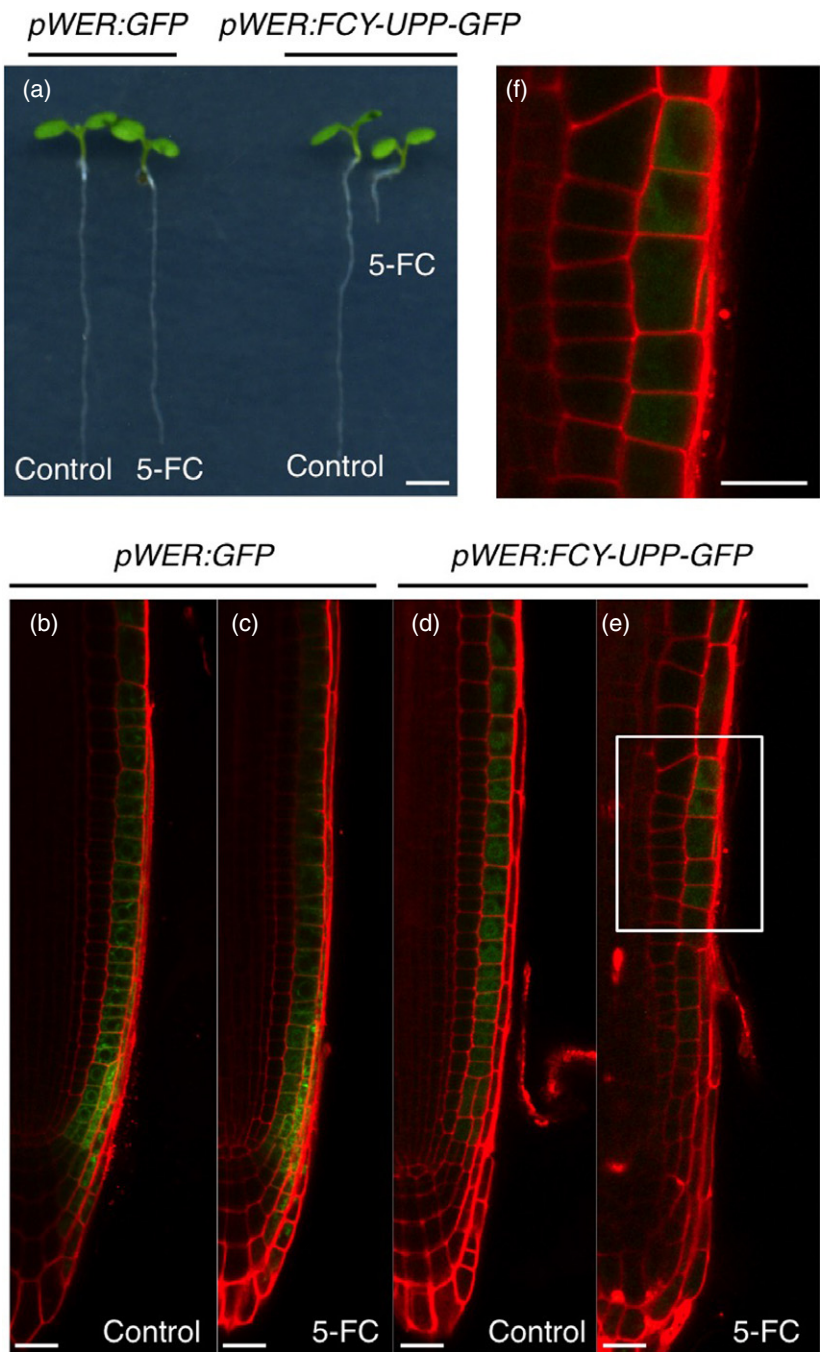

Figure 5. FCY-UPP-GFP protein is expressed in a cell-autonomous manner.
(a) Plants containing the expression marker *pWER:GFP* or the functional *pWER:FCY-UPP-WER* fusion after no treatment (Control) or after treatment with 500 μM 5-fluorocytosine (5-FC) at 6 days after germination. (b–f) Laser-scanning confocal microscope image of epidermal expression similarly driven by these two lines: *pWER:GFP* (b, c) and *pWER:FCY-UPP-WER* (d–f) after no treatment (Control) or after treatment with 500 μM 5-FC. (f) Focus on epidermal expression in the *pWER:FCY-UPP-WER* line despite 5-FC-induced morphological changes (corresponding to the white box in panel e). Scale bars: (a) 2 mm; (b–f) 15 μm.

(Figure 1c). In order to further characterize the tissue specificity of the FCY-UPP tandem, we expressed it in the xylem pole pericycle cells from which lateral roots are being formed (Dubrovsky *et al.*, 2000). In the J0121 enhancer trap line driving FCY-UPP expression, treatment with 1 mM 5-FC can block LR formation selectively without affecting primary root growth, thereby suggesting that 5-FC metabolites do not diffuse to adjacent tissues, where they would otherwise block cellular growth. Indeed, expressing FCY-UPP in the lateral root cap and epidermis using the J0951 enhancer trap line resulted in a strong reduction of primary root growth. Similarly, when FCY-UPP proteins were expressed in the guard cells using the pMYB60 promoter, no reduction in total leaf area was observed. These observations suggest that both the FCY-UPP tandem protein and the products of 5-FC incorporation remain in the cells where the construct is expressed, at least on a short timescale. We observed a tagged version of FCY-UPP fused to the GFP reporter and expressed under the epidermis-specific promoter of *WEREWOLF* (Lee and Schiefelbein, 1999),

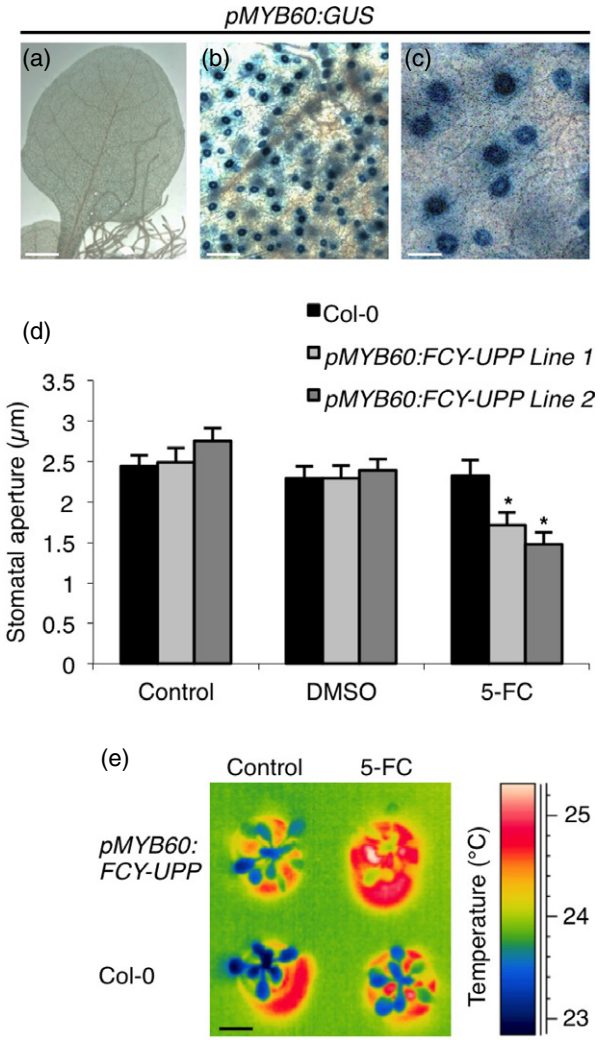

Figure 6. Systemic action of 5-fluorocytosine (5-FC) incorporation blocks stomatal aperture and leaf temperature regulation.
(a–c) *pMYB60:GUS* expression pattern in leaves and guard cells. (d) Stomatal aperture was measured from wild-type (Col-0), *35S:FCY-UPP* and *pMYB60:FCY-UPP* (two independent lines) plants in non-treated (control), dimethyl sulfoxide (DMSO) treated and 100 μM 5-FC treated plants (data represent means ± SEMs, $n = 50$). (e) False-color infrared image of plants treated or not with 100 μM 5-FC for 24 h indicate temperature levels according to the scale on the right. Asterisks indicate a significant difference from the corresponding control experiment by Mann–Whitney *U*-test ($*P < 0.05$). Scale bars: (a) 200 μm; (b) 60 μm; (c) 20 μm; (e) 20 mm.

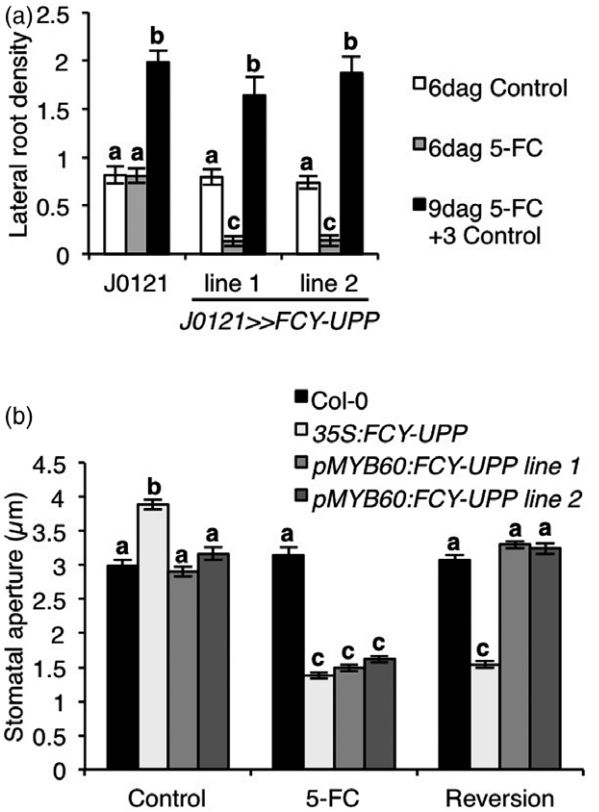

Figure 7. Tissue inactivation resulting from treatement with 5-fluorocytosine (5-FC) is reversible.
(a) Lateral root density (number of roots per cm of primary root) was determined on wild-type (Col-0) and J0121>>FCY-UPP plants (two independent lines) 6 days after germination on regular medium (Control, white bars) or 1000 μM 5-FC medium (5-FC, grey bars), and after 9 days on 5-FC medium with transfer for 3 days to regular medium (+3 Control, black bars). Data represent means ± SEMs, $n = 25$. (b) Stomatal aperture was measured on wild-type (Col-0), *35S:FCY-UPP* and *pMYB60:FCY-UPP* (two independent lines) plants in non-treated (Control), 100 μM 5-FC treated and 2 days after transfer back to non-treated conditions (data represent means ± SEMs, $n = 50$). Values were grouped into classes using Kruskal–Wallis rank sum test ($*P < 0.05$) for each condition.

compared with a direct pWER:GFP fusion. Both expression profiles were strictly identical and unaffected by treatment with 5-FC (Figure 5b–f), demonstrating that the FCY-UPP-GFP protein acts cell autonomously. A similar use of the FCY-UPP tandem proteins proved to be toxic in animal systems, with a high level of specificity for fighting lung cancer in mice (Christensen *et al.*, 2010) and rats (Johnson *et al.*, 2011); however, no report has been made on the ability of the tandem protein to be transported across animal cell membranes. Inactivating shoot tissues can be challenging as they are not in direct contact with the culture medium and spraying chemicals can lead to heterogeneous application. In the case of 5-FC application we were able to feed the plants from the root and still observe a long-distance effect, demonstrating that 5-FC is systemically transported. Indeed, by placing the FCY-UPP proteins under the control of the guard cell-specific promoter pMYB60, we were able to specifically block stomatal aperture by feeding the root with 5-FC. Reversibility is a key advantage of an inducible inactivation system because it opens the way to synchronization experiments where the experimenter can block the activity of a tissue for a certain length of time and then release it across several individuals. Surprisingly, the toxicity of the 5-FC products was not so strong that it irreversibly killed the cells. We can hypothesize that blocking RNA metabolism exerted a negative

feedback on the production of the FCY-UPP proteins themselves, therefore preventing too strong toxicity. Although this is pure speculation, further work will be needed to fully quantify this effect.

Despite the tissue-specific FCY-UPP system being a great tool to inactivate cells in a precise spatial and temporal manner, there are some limitations. First, the availability of a promoter with the desired expression pattern is a requirement and the generation of transgenic plants is necessary; however, as it is an inducible system, the use of a promoter that would display early (embryonic) expression profile is not problematic. Second, the range of active 5-FC concentrations is fairly high (from 100 to 1000 μM, depending on the lines tested here), which may be related to the necessity to reach an active dose in distant tissues. But this is not a major issue considering the relatively cheap price of 5-FC. These two limitations do not overcome the great advantages of a tool that is tissue specific, cell autonomous, reversible and acts at long distance. Future work using this tool should lead to a better understanding of the respective role of various tissues in more complex responses involving cell-to-cell communication or high levels of synchronisation across complex organs.

EXPERIMENTAL PROCEDURES

Vector construction

FCY1 from *Saccharomyces cerevisiae* and *UPP* genes from *Escherichia coli* were amplified by polymerase chain reaction (PCR) using DNA extracts from the corresponding organism (FCY1-F, 5′-GGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTGACAGGGGGAATG-3′; FCY-R, 5′-TCCACGATCTTCATCTCACCAATATCTTCAAA-3′; UPP-F, 5′-AAGATATTGGTGAGATGAAGATCGTGGAAGTC-3′; UPP-R, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-CTTTCGTACCAAAGATTTT-3′). PCR products were fused and cloned into pDONR207 by BP Gateway reaction to create pEN207 FCY-UPP. pEN207 FCY-UPP was used in a multisite Gateway LR reaction (Karimi *et al.*, 2007) with the destination vectors pB7m34GW and pEN-UAS (VIB, gateway.psb.ugent.be) to generate the *UAS:FCY-UPP* construct. *FCY-UPP* was also subsequently transferred from pEN207 FCY-UPP into a Gateway-compatible pMDC32 vector containing the *2X35S* promoter or the *AtMYB60* minimal promoter, *proAtMYB60*$_{232}$, which was cloned using the *Hind*III–*Bam*HI sites. A 2.4-kb sequence upstream of ATG from the *WEREWOLF* gene (*At5g14750*) was PCR amplified using primers containing a *Hind*III restriction site (pWER-F, 5′-TCTAAGCTTAAACCCGAATCATCATG-CAAT-3′; pWER-R, 5′-TCTAAGCTTTCTTTTTGTTTCTTTGAATGA-5′) and subsequently cloned into a pGWB4 plasmid containing the endoplasmic reticulum (ER)-targeted GFP as a C-terminal cassette to create pWER4. The pWER4 plasmid was used in a multisite Gateway LR reaction (Karimi *et al.*, 2007) as a destination vector with pEN207 FCY-UPP to create pWER:FCY-UPP-GFP. Constructs were transformed into the relevant genetic background by floral dipping and homozygous T_3 lines were generated by selfing.

Plant material and growth

Seeds for the following lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC): Col-0, J0121 (Laplaze *et al.*, 2005) and J0951 (Swarup *et al.*, 2005). For *in vitro* culture, plants were grown on vertical half-strength MS plates at 23°C under long days (16 h of light at 150 μmol m^{-2} sec^{-1}). For hydroponic culture, plants were grown in a controlled environment (with an 8-h photoperiod at 300 mmol m^{-2} sec^{-1}, 21°C and 70% relative humidity) in a nutrient solution: 800 mM $Ca(NO_3)_2$, $4H_2O$; 2 mM KNO_3; 1.1 mM $MgSO_4$, $7H_2O$; 60 mM K_2HPO_4; 700 mM KH_2PO_4; 20 mM $FeSO_4$, $7H_2O$; 20 mM Na_2 EDTA, $2H_2O$; 75 nM $(NH_4)Mo_7O_{24}$, $4H_2O$; 3.5 mM $MnSO_4$, H_2O; 3 mM $ZnSO_4$, $7H_2O$; 9.25 mM H_3BO_3; 785 nM $CuSO_4$, $5H_2O$; final pH 5.8. The germination of surface-sterilized seeds was carried out in half-strength MS plates. For hydroponic cultures, the plantlets were transferred to sand after 2 weeks on vertical plates, left there for an additional period of 1 week and finally transferred to a home-built hydroponic culture set-up. 5-FC (ref. F7129, Sigma-Aldrich, https://www.sigmaaldrich.com) was diluted in water at a concentration of 10 mM and subsequently aliquoted to avoid freeze–thaw cycles that quickly denature the product (loss of activity).

Real-time root growth tracking

Photographic images were acquired every 10 min under continuous light with a Canon EOS 700D reflex camera controlled by EOS UTILITY software. The software was set such that the camera parameters, such as focus, aperture and exposure time, remained constant between exposures. Plates were arranged to minimize the reflection from the overhead lights. Primary root length was determined on the image series using RootTrace (French *et al.*, 2009), which allows for the rapid acquisition of growth parameters. Image acquisition was started upon transfer of the plants to a new medium (control or 5-FC treated). Regular primary root length measurements were performed on single images using ImageJ software equipped with the NeuronJ plugin.

Stomatal aperture scoring

The abaxial side of leaves from Arabidopsis plants of 4–5 weeks in age was stuck onto coverslips and peeled. Peels were submersed in Petri dishes containing 10 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES)/Tris, pH 6.0 and 30 mM KCl at 23°C. To test the effect of 5-FC, peels were first kept for 30 min in darkness and then transferred to the same buffer supplemented with 10 or 100 mM ABA or the equivalent dose of ethanol for 2 h under light (250 mmol m^{-2} sec^{-1}). The ethanol used for ABA solutions did not exceed 0.1% (v/v) final concentration. Stomatal apertures were measured with an optical microscope (Optiphot; Nikon, https://www.nikon.com) fitted with a camera lucida and a digitizing table (Houston Instruments) linked to a computer, as described by Leonhardt *et al.* (1997). Each data point represented the mean of at least 80 stomatal apertures, and each experiment was repeated at least twice.

Leaf temperature probing

Thermal imaging of plantlets treated with 5-FC was performed as described previously (Merlot *et al.*, 2002). In brief, plants were first grown under hydroponic conditions for 3 weeks. Then 100 μM 5-FC was added for 24 h. Thermal images were obtained using a Thermacam infrared camera (A655sc 25°, 50 Hz; FLIR, https://www.flir.com). Images were saved and analyzed on a personal computer using ResearchIR4 Max software provided by FLIR.

Histochemical analysis and microscopy

GUS staining was performed as previously described (Péret *et al.*, 2007). Plants were cleared for 24 h in 1 M chloral hydrate and 33% glycerol. Seedlings were mounted in 50% glycerol and observed

with an optical LDM600 microscope (Leica, http://www.leica.com). For confocal microscopy, plants were stained with 10 mg ml^{-1} propidium iodide for 30 sec and images were captured with an inverted confocal laser-scanning microscope (TCS SP2; Leica).

Statistical analysis

For statistical analysis, the number of samples is indicated in each figure as *n* (where *n* represents the number of plants in Figures 1c, 2c, 3h, 3i, 4e and 7a and the number of stomata in Figures 6d and 7b). Experiments were performed as at least two independent biological replicates. The normality of the parameters studied was tested under the Shapiro–Wilk test with alpha = 0.05. Primary root length and lateral root density were normally distributed, whereas stomatal aperture was not normally distributed. Pairwise comparison of normally distributed data was assessed by Student's *t*-test, pairwise comparison of non-parametric data was assessed by Mann–Whitney *U*-test and group comparison of non-parametric data was assessed by Kruskal–Wallis test with Bonferroni correction. Asterisks indicate statistically different results from these tests at a *P* value indicated in the figure legends.

ACCESSION NUMBERS

FCY1, NCBI GeneID 856175; UPP, NCBI GeneID: 946979.

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AUTHOR CONTRIBUTIONS

NL, FD, SC and SD performed root growth experiments, FD performed confocal microscopy, NL, SC and CG performed stomatal aperture experiments, SD and JR performed molecular cloning, NL, LN, RB and BP designed experiments, and BP wrote the article.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Various ecotypes are insensitive to 5-FC.

Figure S2. Real-time root growth tracking set-up.

Figure S3. J0121 expression pattern is not altered by treatment with 5-FC.

Figure S4. Primary root growth is blocked by epidermal and lateral root cap tissue-specific inactivation.

Figure S5. J0951 expression pattern is not altered by treatment with 5-FC.

Figure S6. Primary root growth response characterization of the pWER:FCY-UPP-GFP line.

Figure S7. Characterization of the expression level and leaf area in the pMYB60:FCY-UPP line.

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