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Running title: Tissue specific genetic ablation

(1) 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 cell autonomously. We also showed that it can inactivate slow developmental processes like lateral root formation by targeting pericycle cells. It 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 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.

(2) Significance statement

Combining tissue specific-expression of cytosine deaminase and uracil phosphoribosyl transferase and application of their precursor 5-fluorocytosine allows specific inactivation of targeted tissues. This tool reveals a role for the lateral root cap and the epidermis in controlling primary root growth and demonstrates to be acting in a tissue-specific and reversible manner therefore opening the way to a better understanding of physiological and developmental processes at the tissular and cellular level.

(3) INTRODUCTION

Plant physiological and developmental pathways rely on the coordinated action of several tissues. Whether it is 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 are now growing evidence that the ability of tissues to communicate, either adjacent or distant, 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 a general cellular toxicity include 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 whose expression is not limited in time and space. For example, such approach induced the destruction of the root cap resulting in plants exhibiting strong growth defect 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) since they have little effect on plant overall development.

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*

66 *al.*, 1992), the synthetic steroid dexamethasone (Aoyama and Chua, 1997), IPTG (Wilde *et*
67 *al.*, 1992), ethanol (Roslan *et al.*, 2001) or the copper ion (Mett *et al.*, 1993). However, they
68 necessitate the combined use of two transgenes in plants: a transcription factor that acts as the
69 switch and a target promoter controlling the expression of the gene of interest. The number of
70 transgenes can go up to 3 if a reporter gene is included to follow the tissular specificity of the
71 induction (Deveaux *et al.*, 2003). Although this method allows for the choice of the target
72 gene to be induced, including a toxic gene, such as DTA or a nuclease, it increases the
73 complexity of the T-DNA(s) to be transferred and has not been widely used for genetic
74 ablation. Interestingly, the recent development of estradiol inducible transgenes with a full
75 suite of MultiSite Gateway compatible cell-type specific systems may lead to new
76 applications for genetic ablation studies (Brand *et al.*, 2006; Siligato *et al.*, 2016) .
77 Instead of an inducible promoter, the genetic toxicity can be itself induced by relying on an
78 innocuous substance that is readily converted into a toxic compound by a transgenic
79 enzymatic activity. In plant, the use of the bacterial gene indole acetamide hydrolase (iaaH)
80 originating from the tumor inducing bacteria *Agrobacterium sp.* allows the conversion of the
81 inactive compound auxin indole acetamide into indole acetic acid. Large auxin accumulation
82 triggers general toxic effects, reminiscent of the use of 2,4 dichlorophenoxy acetic acid (2,4D)
83 as an herbicide. Similarly, the enzyme nitrate reductase (NR) can incorporate chlorate into the
84 toxic form chlorite (Nussaume et al., 1991). The major limitation of these two markers is that
85 they are not cell autonomous since their end products can readily diffuse into the plant and
86 have therefore been used only as negative markers at the organism level (Klee *et al.*, 1987;
87 Nussaume *et al.*, 1991) and not for tissue specific studies.
88 Here, we report the use of an inducible system for genetic toxicity at the tissular level, based
89 on the heterologous expression of a tandem of two genes: fluorocytosine deaminase (FCY)
90 and uracil phosphoribosyl transferase (UPP). We demonstrate that 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. Besides, its local and systemic reversible range of action offers versatile opportunities for plant physiology studies.

(4) 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 dUMP into 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-fluorocytosine (5-FC) since it is inactive in plants due to lack of cytosine deaminase (FCY) activity (Figure 1a). Early work on negative selection markers demonstrated that *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 to wild-type controls (Perera *et al.*, 1993; Kobayashi *et al.*, 1995). Interestingly, 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, germination of seedlings on plates containing 1mM 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 neighbouring transgenic plants, thereby demonstrating that the action of 5-FC incorporation is plant autonomous (Figure 1c).

The toxic effect of 5-FC was shown to be concentration dependent in the independent transgenic lines that we produced with a strong primary root growth reduction observed on 100µM 5-FC and a total arrest of seedling growth after germination at 500µM 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 250µM and a total growth arrest at 1000µM (Figure S1b and c). The sensitivity of the plants to 5-FC correlated well with the level of expression of the *FCY-UPP* transgene in these 2 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 the UAS (Upstream Activated Sequence). This construction was transformed into the xylem pole pericycle cell specific J0121 line (Laplaze *et al.*, 2005) whose expression starts in the elongation zone and is maintained in the shootward direction up to the mature tissues (Figure 3a, b and 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 1000µM and a total disappearance of LR formation at 2500µM 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 minutes for the duration of the experiment (setup shown in Figure S2a). No difference in the growth of the control (non-treated) versus 5-FC plants was observed at 1000µM upon 70 hours after transfer, therefore demonstrating the tissue autonomous nature of the inactivation system (Figure 3i). However, at higher 5-FC concentrations (2500µM and above) 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 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 to 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 blocked lateral root primordium seemed similar to those of untreated plants (Figure S3c and f).

Fast growth responses are impacted 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 and b). Treatment with 5-FC resulted in arrested primary root growth in the treated versus non-treated *J0951>>FCY-UPP* line (Figure 4c and d). This effect was found to be 5-FC concentration dependent with a minimum effective concentration tested at 100µM (Figure S4a and b). Real-time tracking of primary root growth revealed that 5-FC altered growth *ca.* 48 hours after transfer on the 5-FC medium versus

control plants transferred on a regular medium (Figure 4e). Root growth was not totally abolished but reduction was drastic. A quicker effect was obtained on the constitutive *35S:FCY-UPP* lines (Figure S4c,d), the stronger expression level (Figure S1d) correlated with a quicker response (down to 18 and 30 hours). The expression pattern driven by the J0951 line remained unaltered by 5-FC treatment (Figure S5a-d).

The FCY-UPP-GFP protein fusion 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 2 constructs (Figure 5c,e,f). Attaching the GFP protein to the FCY-UPP tandem did not prevent its enzymatic function, dose response treatment of this line with 5-FC triggered primary root growth arrest at concentrations above 1000µM (Figure 5a and S6).

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

Since 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 catalogues 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 hours prior to stomatal aperture measurement. Both *35S:FCY-UPP* and *pMYB60:FCY-UPP* lost the ability to open their stomata upon 100µM 5-FC application. Identical results were obtained in 2 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 surface leaf temperature with a thermal camera to determine whether 5-FC treatment in *pMYB60:FCY-UPP* plants affected plant transpiration. A *ca.* 2°C increase in leaf temperature was observed in the 5-FC treated *pMYB60:FCY-UPP* plants compared to controls, untreated and 5FC-treated Col-0 plants demonstrating the absence of 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 was not different across the control and transgenic lines (Figure S7b).

5-FC incorporation toxicity is reversible

The toxicity triggered by tissue specific incorporation of 5-FC strongly impacts 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 a regular medium was performed on the J0121>>FCY-UPP line. Plants were grown for 9 days after germination on a medium containing 1000µM 5-FC and then transferred to a medium without any 5-FC. Plants from 2 independent J0121>>FCY-UPP transgenic lines were able to produce as many lateral roots 3 days after transfer back to the control medium compared to 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 hours. Plants expressing the two transgenes specifically in the guard cells (*pMYB60:FCY-UPP*) were

unable to open their stomata in response to dark/light transition. After transfer back to hydroponic solution deprived of 5-FC for 48 hours, the plants from 2 *pMYB60:FCY-UPP* lines regain the ability to respond to dark/light transition and open their stomata (Figure 7b), therefore demonstrating the reversible effect of 5-FC incorporation.

(5) DISCUSSION

Early work identified that cytosine deaminase (FCY) activity produced by prokaryotes but not higher eukaryotes could be used as a negative marker together with its substrate 5-Fluorocytosine (5-FC). The deamination 5-FC by FCY into cytotoxic 5-Fluorouracil (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 rice (Dai *et al.*, 2001) and 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 neighbouring plants due to 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 fast 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 FCY-UPP protein retains its plant autonomous

properties and that the toxicity did not spread across the culture medium (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, 1mM 5-FC application can block selectively LR formation without impacting primary root growth therefore 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) compared to a direct pWER:GFP fusion. Both expression profiles were strictly identical and unaffected by 5-FC treatment (Figure 5b-f) demonstrating that the FCY-UPP-GFP protein acts cell autonomously. Similar use of the FCY-UPP tandem proteins proved to be toxic in animal systems with a high level of specificity to fight lung cancer in mice (Christensen *et al.*, 2010) and rat (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 since they are not in direct contact with the culture medium and spraying chemicals can lead to heterogeneous applications. In the case of 5-FC applications, 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 synchronisation experiments where the experimenter can block the activity of a tissue for a certain amount 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 a 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 cell in a precise spatial and temporal manner, there are some limitations. First of all, the availability of a promoter with the desired expression pattern is a requirement and generation of transgenic plant is necessary. However, since it is an inducible system, early (embryonic) expression profiles of the promoter of choice is not problematic. Secondly, the range of active 5-FC concentrations is fairly high (from 100 to 1000µM depending on the lines tested here) this 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.

(6) EXPERIMENTAL PROCEDURES

Vector construction

FCY1 from *Saccharomyces cerevisiae* and *UPP* genes from *Escherichia coli* were PCR amplified using DNA extracts from corresponding organism (FCY1-F 5'GGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGTGACAGGGGGAATG3';

FCY-R 5'TCCACGATCTTCATCTCACCAATATCTTCAAA3'; UPP-F 5'AAGATATTGGTGAGATGAAGATCGTGGAAGTC3'; UPP-R 5'GGGGACCACTTTGTACAAGAAAGCTGGGT-CTTTCGTACCAAAGATTTT3'). 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 vector pB7m34GW and pEN-UAS (from VIB Department gateway.psb.ugent.be) to generate the *UAS:FCY-UPP* construct. *FCY-UPP* was also subsequently transferred from pEN207 FCY-UPP into Gateway compatible pMDC32 vector containing 2X35S promoter or *AtMYB60* minimal promoter, $proAtMYB60_{232}$, which was cloned using the HindIII–BamHI sites. A 2.4kb sequence upstream of ATG from the *WEREWOLF* gene (*At5g14750*) was PCR amplified using primers containing an HindIII restriction site (pWER-F: 5'TCTAAGCTTAAACCCGAATCATCATGCAAT3' and pWER-R 5'TCTAAGCTTTCTTTTTGTTTCTTTGAATGA5') and subsequently cloned into a pGWB4 plasmid containing the ER-targeted GFP as a C-terminal cassette to create pWER4. Development of Series of Gateway Binary Vectors, pGWBs, for Realizing Efficient Construction of Fusion Genes for Plant Transformation. J Biosci. Bioeng). 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 T3 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 MS plates at 23°C under long days (16h light at

150 µmol.m-2. s-1). For hydroponic culture, plants were grown in a controlled environment (8 h photoperiod at 300 mmol m_2 s_1, 21 1C, 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 mMNa_2EDTA, $2H_2O$; 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 MS plates. For hydroponic cultures, the plantlets were transferred on sand after 2 weeks on vertical plates, left there for an additional week period, and finally transferred to a home-built hydroponic culture setup. 5-FC (ref. F7129 Sigma-Aldrich) was diluted in water at the concentration of 10mM 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 minutes under continuous light with a Canon EOS 700D reflex camera controlled by the 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 reflection from the overhead lights. Primary root length was determined on the image series using RootTrace (French *et al.*, 2009) that allows rapid acquisition of growth parameter. 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 of 4- to 5-week-old Arabidopsis plants was stuck on coverslips and

peeled. Peels were submersed in petri dishes containing 10 mM 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 in the same buffer supplemented with 10 or 100 mM ABA or the equivalent dose of ethanol for 2 h under light (250 $mmol.m^{-2}.s^{-1}$). Ethanol used for ABA solutions did not exceed 0.1% (v/v) final concentration. Stomatal apertures were measured with an optical microscope (Nikon; Optiphot) fitted with a camera lucida and a digitizing table (Houston Instrument) linked to a computer as described (Leonhardt *et al.*, 1997). Each data point represented the mean of at least 80 stomatal apertures. Each experiment was repeated at least twice.

Leaf temperature probing

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

Histochemical analysis and microscopy

GUS staining was done as previously described (Péret *et al.*, 2007). Plants were cleared for 24 h in 1M chloral hydrate and 33% glycerol. Seedlings were mounted in 50% glycerol and observed with an optical Leica LDM600 microscope. For confocal microscopy, plants were stained with 10 $mg.ml^{-1}$ propidium iodide for 30 s and images were captured with an inverted confocal laser-scanning microscope (Leica TCS SP2).

(7) Accession numbers;

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

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(9) Short legends for Supporting Information;

Supporting figures: Figure S1, Figure S2, Figure S3, Figure S4, Figure S5, Figure S6, Figure S7.

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(11) Tables;

N/A

(12) Figure legends;

Figure 1. 5-fluorocytosine incorporation by cytosine deaminase generates cellular toxicity. (a) 5-FC is incorporated into 2 distinct pathways leading to RNA and DNA damage. Concomitant expression of uracil phosphoribosyl transferase favors the RNA damage pathway. (b) In this study, we fused the FCY and UPP gene 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 500µM 5-fluorocytosine (n>80).

Figure 2. Dose-dependent 5-FC toxicity on primary root growth.

(a-c) Seedlings were germinated on growing concentrations of 5-fluorocytosine (0, 100, 250, 500, 1000 and 2500 µM) and primary root length was measured (data represent mean ±sem, n=20). Asterisks indicate a significant difference with corresponding non-treated control experiment by Student's t-test (* P<0.001; n=10). Bars are 1,5mm.

Figure 3. *FCY-UPP* expression in the pericycle blocks lateral root formation upon 5-FC application.

(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) 6 day-old wild-type (Col-0) seedlings germinated on control (d) or 1000µM 5-fluorocytosine medium (e) and *J0121>>FCY-UPP* seedlings germinated on control (f) or 1000µM 5-fluorocytosine 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 1000µM 5-fluorocytosine treated (5-FC) seedlings for 70 hours after transfer. Both curves overlap (data represent mean ±sem, n=10). Similar real-time tracking of non-transformed J0121 control line is shown as an insert. Asterisks indicate a significant difference with corresponding non-treated control experiment by Student's t-test (* P<0.001; n=10). Bars are 40µm (a,b), 15µm (c) and 3mm (d-g).

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 the epidermis (b). (c-d) 6 day-old *J0951>>FCY-UPP* seedlings germinated on control (c)

513 or 500µM 5-fluorocytosine medium (d). (e) Real-time primary root growth tracking of non-
514 treated (control) versus 500µM 5-fluorocytosine treated (5-FC) seedlings for 130 hours after
515 transfer (data represent mean ±sem, n=10). Similar real-time tracking of non-transformed
516 J0951 control line is shown as an insert. Bars are 40µm (a,b) and 5mm (c,d).
517
518 **Figure 5.** FCY-UPP-GFP protein is expressed in a cell autonomous manner.
519 (a) Plants containing the expression marker *pWER:GFP* or the functional *pWER:FCY-UPP-*
520 *WER* fusion after no treatment (Control) or 500µM 5-fluorocytosine treatment (5-FC) at 6
521 days after germination. (b-f) Laser-scanning confocal microscope image of epidermal
522 expression similarly driven by these 2 lines : *pWER:GFP* (b-c) and *pWER:FCY-UPP-WER*
523 (d-f) after no treatment (Control) or 500µM 5-fluorocytosine treatment (5-FC). (f) Zoom on
524 epidermal expression in the *pWER:FCY-UPP-WER* line despite 5-FC induced morphological
525 changes. Bars are 2mm (a) and 15µm (b-f).
526
527 **Figure 6.** Systemic action of 5-FC incorporation blocks stomatal aperture and leaf
528 temperature regulation
529 (a-c) *pMYB60:GUS* expression pattern in leaves and guard cells. (d) Stomatal aperture was
530 measured on wild-type (Col-0), *35S:FCY-UPP* and *pMYB60:FCY-UPP* (2 independent lines)
531 plants in the non-treated (control), DMSO treated and 100µM 5-FC treated conditions (data
532 represent mean ±sem, n=3). (e) False colour infrared image of plants treated or not with
533 100µM 5-FC during 24h indicate temperature level according to scale on the right. Asterisks
534 indicate a significant difference with corresponding control experiment by Student's t-test (*
535 $P<0.05$). Bars are 200µm (a), 60µm (b), 20µm (c) and 20 mm(e).
536
537 **Figure 7.** Tissue inactivation resulting of 5-FC incorporation 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 (2 independent lines) 6 days after germination on regular medium (Control - white bars) or 1000µM 5-FC medium (5-FC - grey bars) and after 9 days on 5-FC medium and transfer for 3 days on a regular medium (+3 Control - black bars). (b) Stomatal aperture was measured on wild-type (Col-0), *35S:FCY-UPP* and *pMYB60:FCY-UPP* (2 independent lines) plants in the non-treated (Control), 100µM 5-FC treated and 2 days after transfer back to non-treated conditions (data represent mean ±sem, n=2). Asterisks indicate a significant difference with corresponding control experiment by Student's t-test (* $P<0.05$).

Figure 1

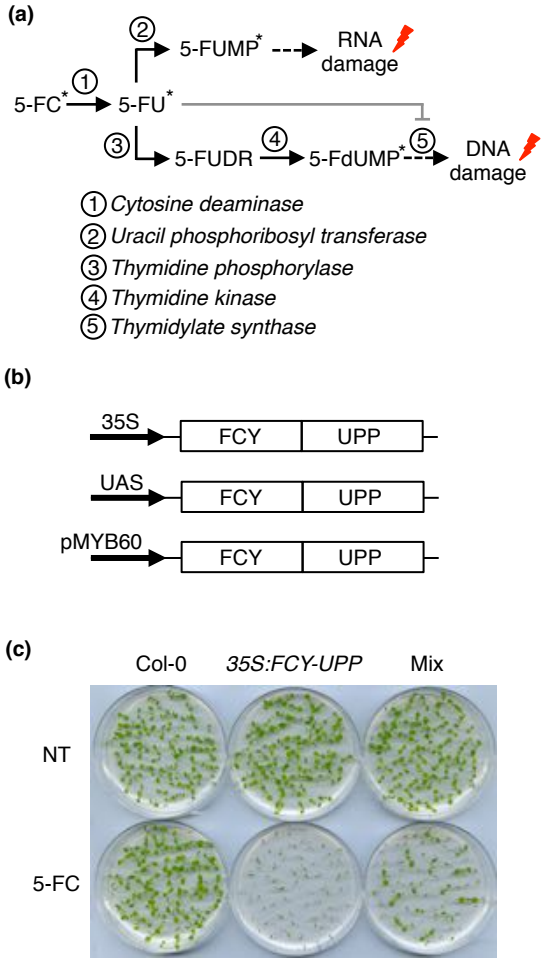
(a)
5-FC*
5-FU*
5-FUMP*
RNA damage
5-FUDR
5-FdUMP*
DNA damage
① Cytosine deaminase
② Uracil phosphoribosyl transferase
③ Thymidine phosphorylase
④ Thymidine kinase
⑤ Thymidylate synthase
(b)
35S
UAS
pMYB60
FCY
UPP
(c)
Col-0
35S:FCY-UPP
Mix
NT
5-FC

Figure 2

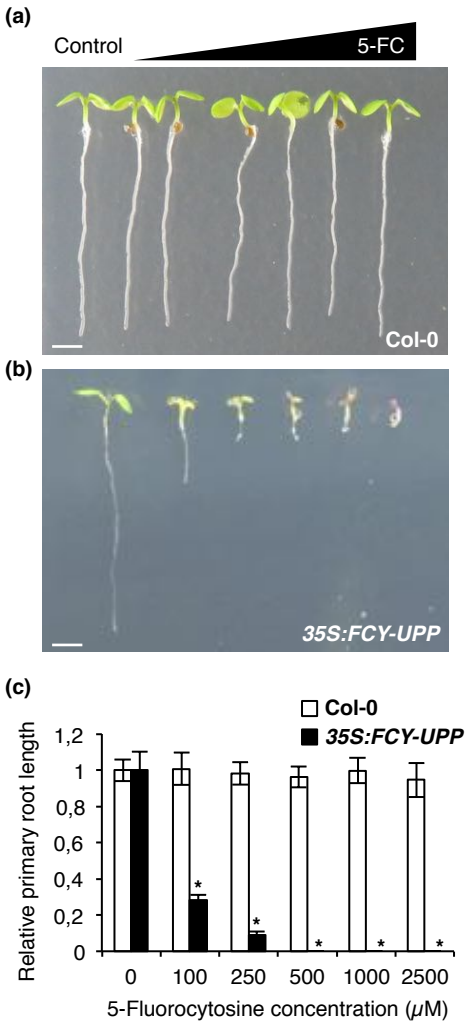
(a)
Control
5-FC
Col-0
(b)
35S:FCY-UPP
(c)
Col-0
35S:FCY-UPP
Relative primary root length
1,2
1
0,8
0,6
0,4
0,2
0
0
100
250
500
1000
2500
5-Fluorocytosine concentration (μM)

Figure 3

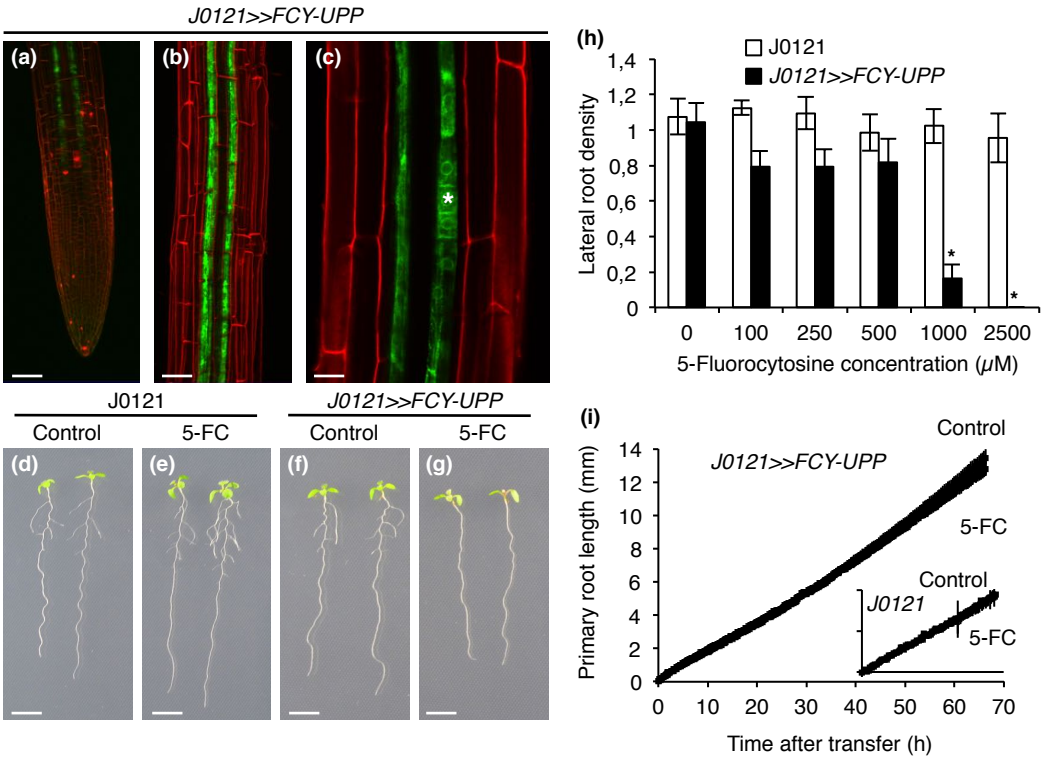
J0121>>FCY-UPP
(a)
(b)
(c)
J0121
Control
5-FC
J0121>>FCY-UPP
Control
5-FC
(d)
(e)
(f)
(g)
(h)
J0121
J0121>>FCY-UPP
Lateral root density
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0
100
250
500
1000
2500
5-Fluorocytosine concentration (μM)
(i)
J0121>>FCY-UPP
Control
5-FC
Control
J0121
5-FC
Primary root length (mm)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
60
70
Time after transfer (h)

Figure 4

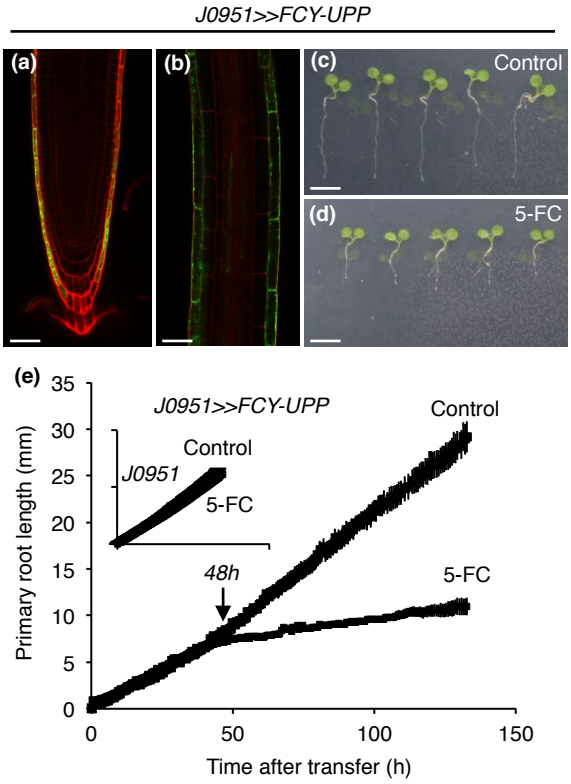
J0951>>FCY-UPP
(a)
(b)
(c)
Control
(d)
5-FC
(e)
J0951>>FCY-UPP
Control
J0951
5-FC
48h
5-FC
Primary root length (mm)
Time after transfer (h)
0
5
10
15
20
25
30
35
0
50
100
150

Figure 5

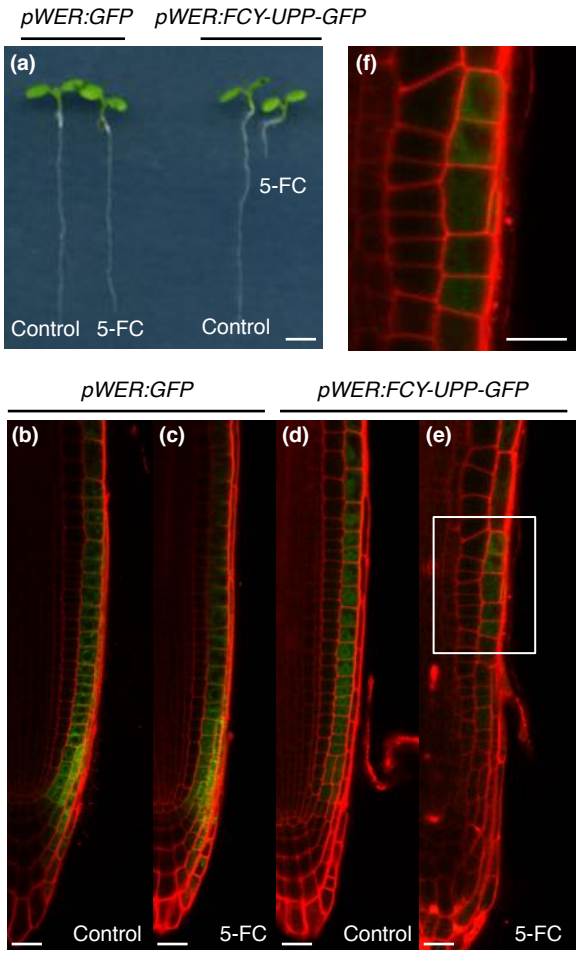
pWER:GFP
pWER:FCY-UPP-GFP
(a)
5-FC
Control
5-FC
Control
(f)
pWER:GFP
pWER:FCY-UPP-GFP
(b)
(c)
(d)
(e)
Control
5-FC
Control
5-FC

Figure 6

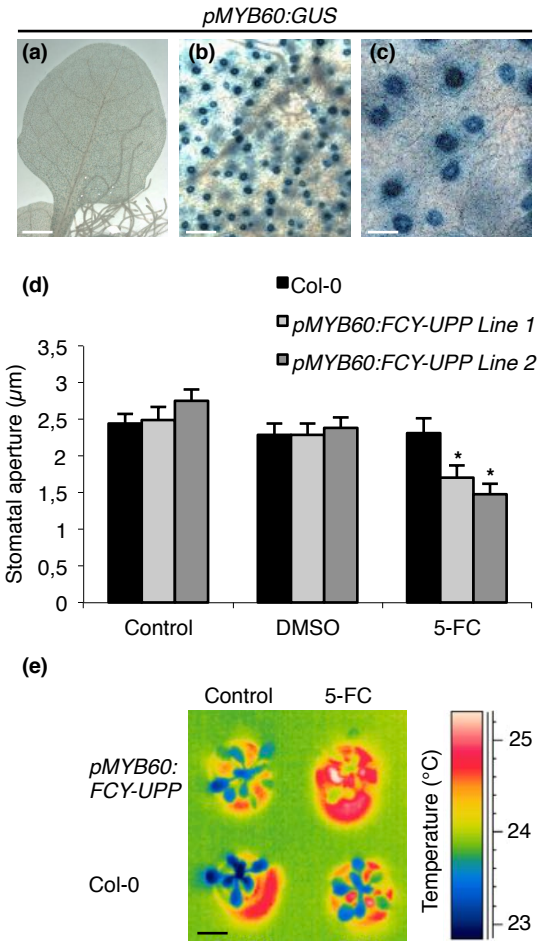
pMYB60:GUS
(a)
(b)
(c)
(d)
Col-0
pMYB60:FCY-UPP Line 1
pMYB60:FCY-UPP Line 2
Stomatal aperture (µm)
3,5
3
2,5
2
1,5
1
0,5
0
Control
DMSO
5-FC
(e)
Control
5-FC
pMYB60:
FCY-UPP
Col-0
Temperature (°C)
25
24
23

Figure 7

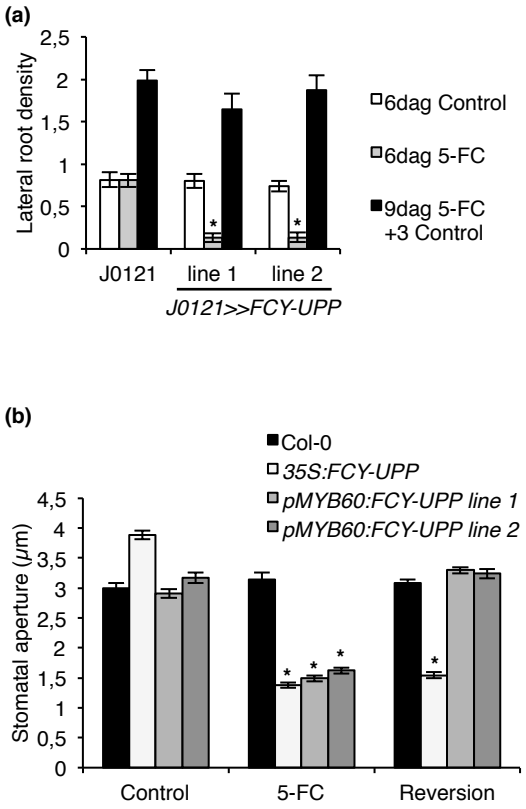
(a)
Lateral root density
2,5
2
1,5
1
0,5
0
J0121
line 1
line 2
J0121>>FCY-UPP
6dag Control
6dag 5-FC
9dag 5-FC
+3 Control
(b)
Col-0
35S:FCY-UPP
pMYB60:FCY-UPP line 1
pMYB60:FCY-UPP line 2
Stomatal aperture (µm)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
Control
5-FC
Reversion

Figure S1

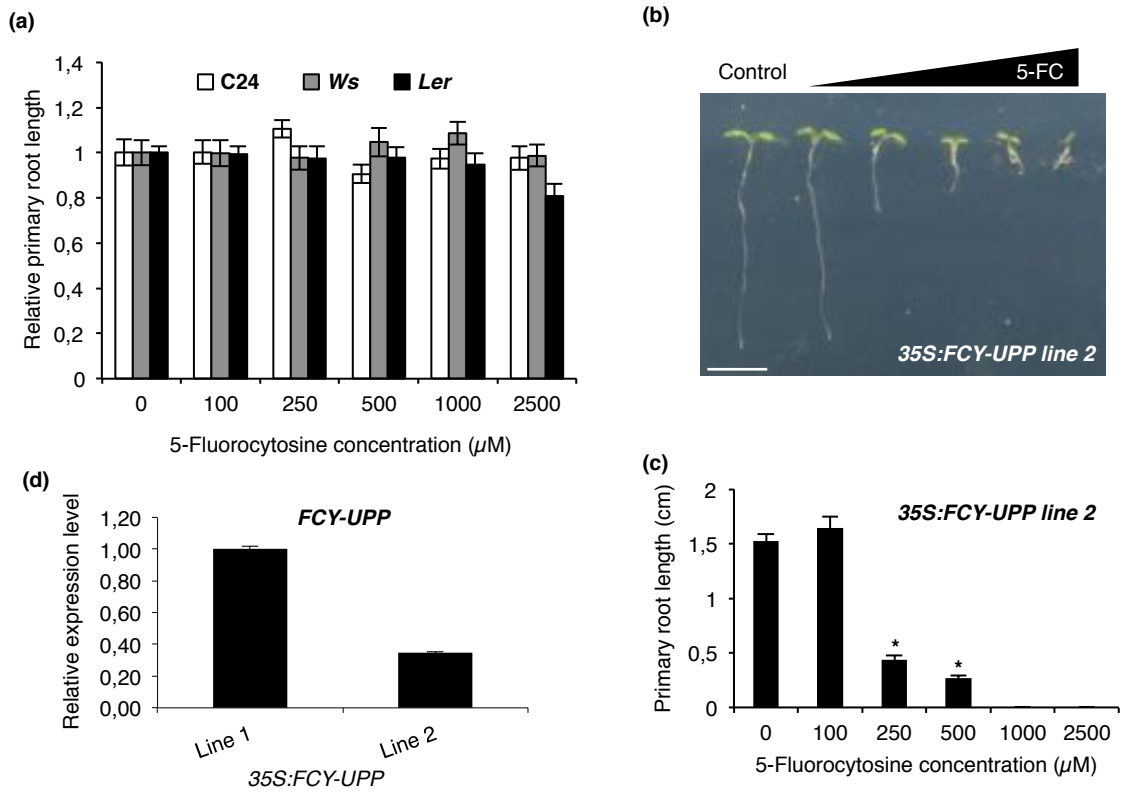

Fig. S1. Effect of 5-FC treatment on various ecotypes and independent 35S:FCY-UPP transgenic line with lower expression level of the transgene

(a-c) Seedlings were germinated on growing concentrations of 5-fluorocytosine (0, 100, 250, 500, 1000 and 2500 µM) and primary root length was measured (n=20). (d) Relative expression level of the *FCY-UPP* RNA determined by quantitative PCR. Asterisks indicate a significant difference with corresponding control experiment by Student's t-test (* P<0.001; n=10). Bar is 5mm.

Figure S2

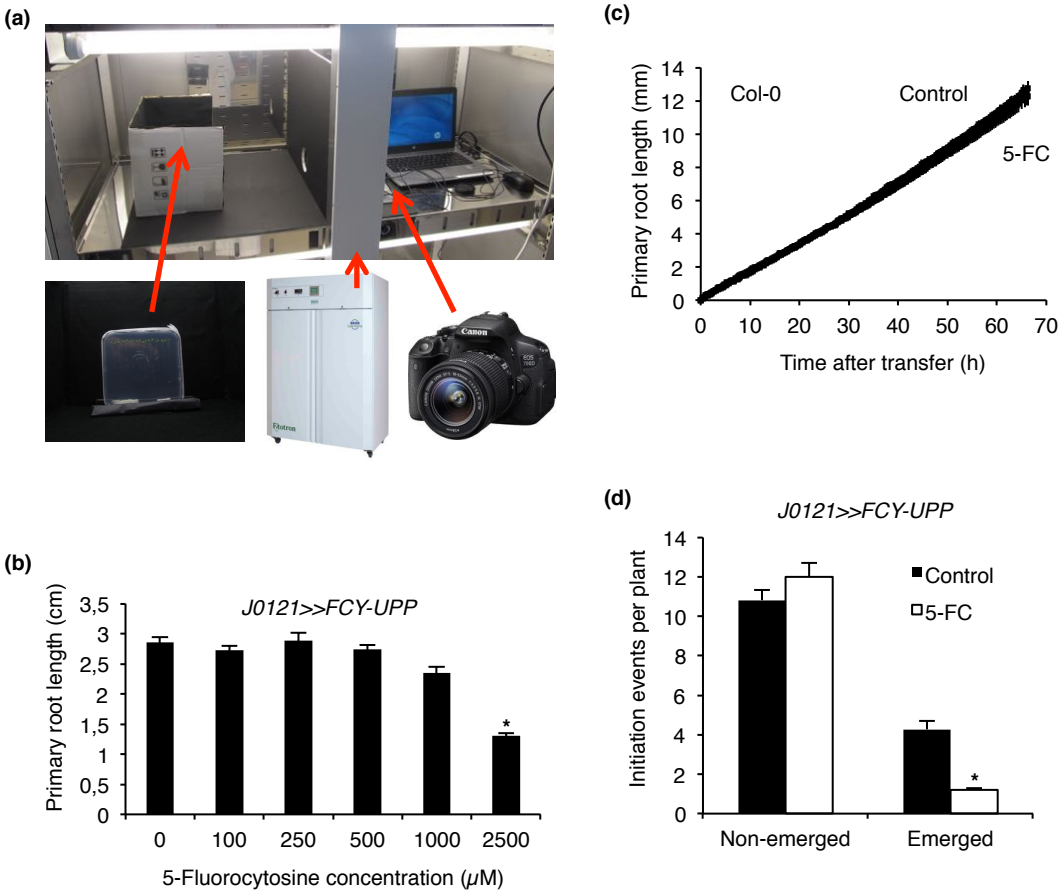

Fig. S2. Real-time root growth tracking setup for real-time tracking and single time point imaging

(a) A reflex camera was used to acquire images every 10 minutes. Setup was achieved in order to reduce light reflection and increase image quality for subsequent analysis by the RootTrace software. (b) Primary root length was measured 6 days after germination for various 5-FC concentrations (0, 100, 250, 500, 1000 and 2500 µM) in the J0121>>FCY-UPP seedlings. (c) Real-time primary root growth tracking of non-treated (control) versus 500µM 5-fluorocytosine treated (5-FC) wild-type (Col-0) seedlings for 70 hours after transfer (data represent mean ±sem,n=10). (d) Number of initiation events was counted under a microscope for control vs. 5-FC terated plants in the J0121>>FCY-UPP line (data represent mean ±sem,n=10). Asterisks indicate a significant difference with corresponding control experiment by Student's t-test (* P<0.001; n=10).

Figure S3

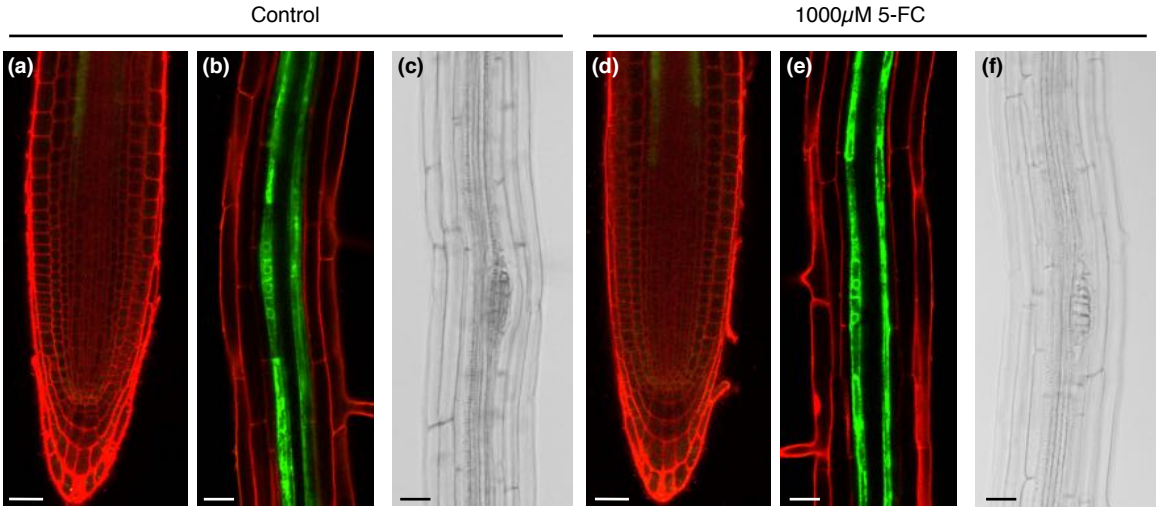

Fig. S3. 5-Fluorocytosine treatment does not alter the expression pattern driven by the J0121 line

(a,b,d,e) The *J0121* line drives expression of *GFP* and *FCY-UPP* in the pericycle cells up to the start of the elongation zone. Treatment with 5-FC blocks lateral root emergence (c *vs*. f) but does not alter the expression pattern (a,b *vs*. d,e). Bars are 25μm.

Figure S4

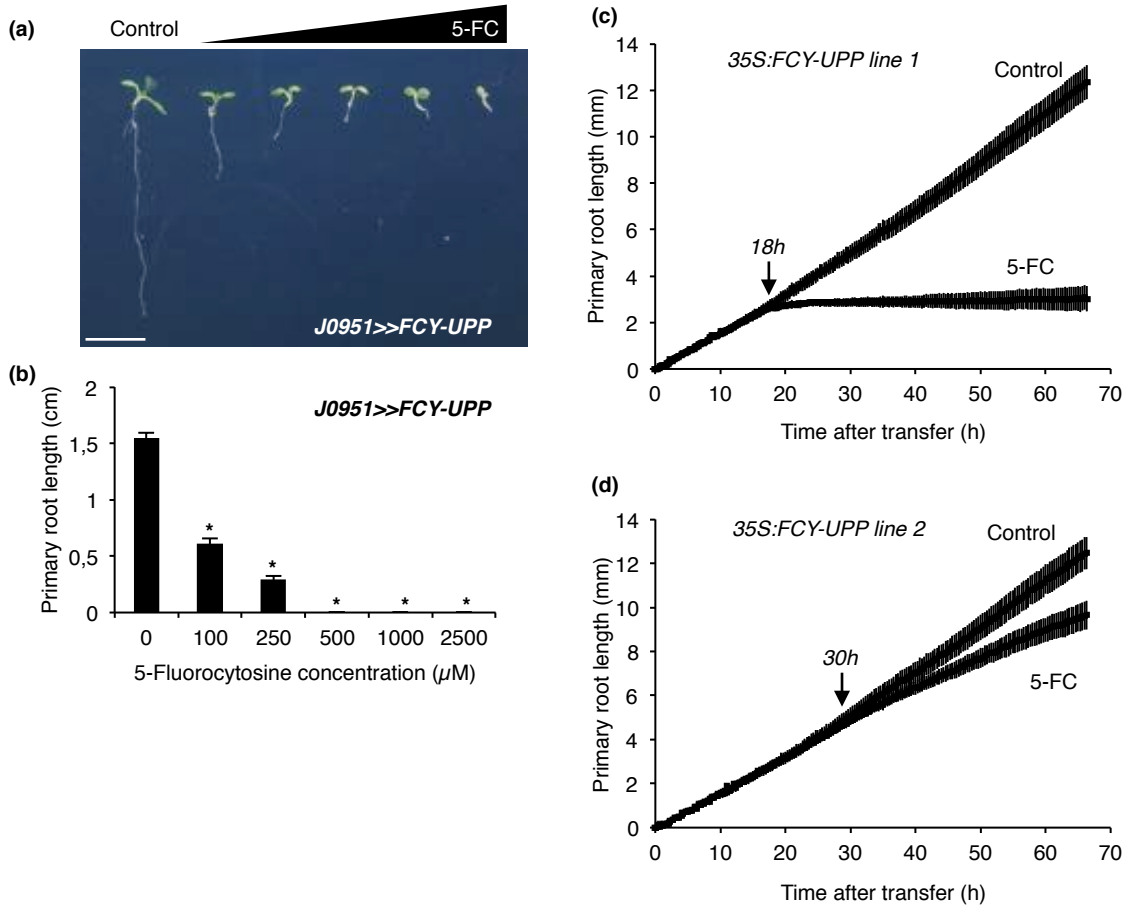

Fig. S4. Lateral root cap and epidermis expression of FCY-UPP blocks primary root growth

(a-b) 6 day-old J0951>>FCY-UPP seedlings germinated on various 5-FC concentrations (0, 100, 250, 500, 1000 and 2500 µM). (c-d) Real-time tracking of primary root length in 2 independent *35S:FCY-UPP* lines in non-treated condition (Control) or placed on medium containing 1000µM 5-FC. Bar is 5mm. Asterisks indicate a significant difference with corresponding control experiment by Student's t-test (* P<0.001; n=10).

Figure S5

Fig. S5. 5-Fluorocytosine treatment does not alter the expression pattern driven by the J0951 line

(a-d) The *J0951* line drives expression of *GFP* and *FCY-UPP* in the lateral root cap and epidermal cells. Treatment with 5-FC (c,d) blocks primary root growth compared to non-treated plants (a,b) but does not alter the marker line expression pattern. Bars are 25μm.

Figure S6

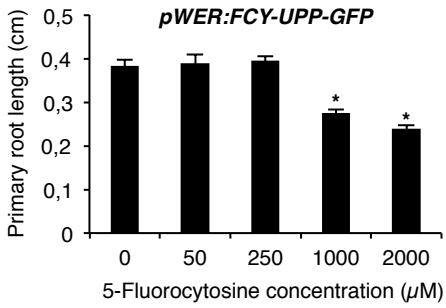

Fig. S6. Dose response of the pWER:FCY-UPP-GFP line to 5-Fluorocytosine

Seedlings were germinated on growing concentrations of 5-fluorocytosine (0, 50, 250,1000 and 2000 µM) and primary root length was measured (n=20). Asterisks indicate a significant difference with corresponding control experiment by Student's t-test (* P<0.001).

Figure S7

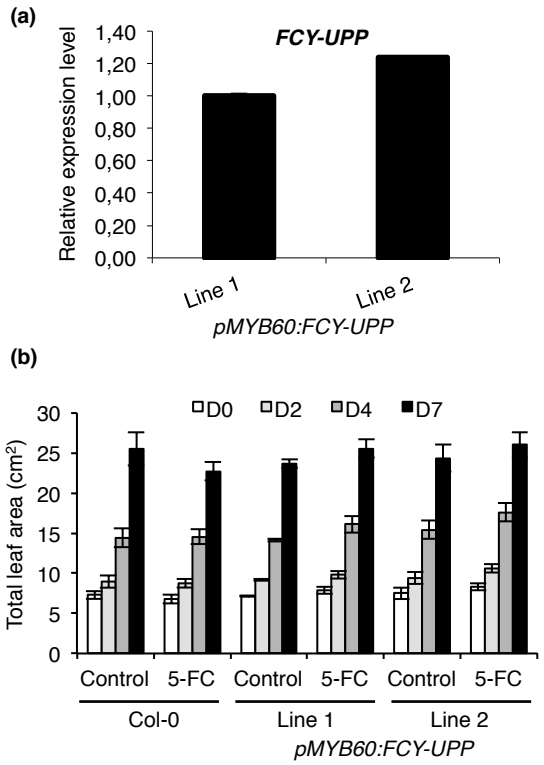

Fig. S7. Expression level of FCY-UPP RNA under the control of the *pMYB60* promoter and leaf area mesaurement

(a) Relative expression level of the FCY-UPP tandem RNA under the control of the pMYB60 promoter in 2 independent transgenic lines. (b) Total leaf area was measured in the wild-type (Col-0) and pMYB60:FCY-UPP lines in non-treated (Control) and upon 5-FC treatment (5-FC) at day 0 (D0), day 2 (D2) day 4 (D4) and day 7 (D7).