

RESEARCH PAPER

# A fluoroarotic acid-resistant mutant of *Arabidopsis* defective in the uptake of uracil

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## Abstract

A fluoroarotic acid (FOA)-resistant mutant of *Arabidopsis thaliana* was isolated by screening M2 populations of ethyl methane sulphonate (EMS)-mutagenized Columbia seed. FOA resistance was due to a nuclear recessive gene, *for1-1*, which locates to a 519 kb region in chromosome 5. Assays of key regulatory enzymes in *de novo* pyrimidine synthesis (uridine monophosphate synthase) and salvage biochemistry (thymidine kinase) confirmed that FOA resistance in *for1-1/for1-1* plants was not due to altered enzymatic activities. Uptake studies using radiolabelled purines, pyrimidines, and [<sup>14</sup>C]FOA reveal that *for1-1/for1-1* plants were specifically defective in the uptake of uracil or uracil-like bases. To confirm such specificity, genetic crosses show that *FOR1* is a distinct locus from *FUR1* which encodes a deoxyuridine nucleoside transporter. In addition, *for1-1/for1-1* plants were restored to FOA sensitivity by transformation with the *Escherichia coli* uracil transporter gene *uraA* driven by the cauliflower mosaic virus (CaMV) 35S promoter. Molecular mapping studies reveal that *FOR1* does not correspond to loci belonging to any of the six known nucleobase transporter families identified in the *Arabidopsis* genome. Moreover, *FOR1* does not appear to regulate the transcript levels of either uracil transporter-encoding loci At2g03590 or At2g03530. The above results strongly suggest that the *for1-1* mutant allele affects a transport mechanism that is specific for the uptake of uracil.

Key words: *Arabidopsis*, fluoroarotic acid resistance, 5-fluorouracil, mutant, pyrimidine, uracil transport.

## Introduction

Pyrimidines play a substantial role in numerous aspects of plant metabolism. *De novo* synthesis, modification, reutilization, uptake, salvage, and catabolism govern nucleotide synthesis, DNA and RNA processing, the biochemistry of carbohydrates, glycoproteins, and phospholipids, as well as the synthesis of many species-specific secondary compounds found in the plant kingdom (reviewed in Ross, 1991; Kafer and Thornburg, 1999; Moffatt and Ashihara, 2002; Boldt and Zrenner, 2003; Stasolla *et al.*, 2003; Kafer *et al.*, 2004). Indeed, in plant tissues, the uracil nucleotide pool is second only to the adenine nucleotide pool in abundance.

Pyrimidines are in demand during much of plant development including embryogenesis, germination, flowering, and pollen tube growth. *De novo* synthesis, salvage, and recycling biochemistry meet the plant's needs for replenishing the uracil nucleotide pool. Evidence shows a tight regulation of both *de novo* synthesis and salvage reactions. During seed germination, directly following imbibition, high levels of free bases reflect enhanced salvage pathway activities. In the following days, salvage activities wane while *de novo* synthesis activities wax and produce most of the pyrimidine pool (Stasolla *et al.*, 2003; Kafer *et al.*, 2004). Transgenic approaches decreasing enzyme levels for *de novo* pyrimidine synthesis result in

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Abbreviations: 8-AZG, 8-azaguanine; BUdR, 5-bromo-2'-deoxyuridine; CAPS, cleaved amplified polymorphic sequence; Col, Columbia wild type; EMS, ethyl methane sulphonate; ENT, equilibrative nucleoside transporter; FOA, fluoroarotic acid; 5FU, 5-fluorouracil; FUDR, 5-fluoro-2'-deoxyuridine; *Ler*, Landsberg *erecta*; MAM, minimal *Arabidopsis* medium; NAT, nucleobase-ascorbate transporter; PRT, purine-related transporter; PUP, purine permease; SSLP, simple sequence length polymorphism; TK, thymidine kinase; TS, thymidylate synthase; UMP, uridine monophosphate; UPS, uracil permease.

increases in salvage biochemistry (Geigenberger *et al.*, 2005; Schröder *et al.*, 2005). Although less well understood, the transport of pyrimidines from the cotyledons or endosperm into the developing seedling goes hand in hand with alterations in pyrimidine biochemistry.

Both intracellular and intercellular transport processes are associated with pyrimidine biochemistry. Although plastids are the main site of *de novo* pyrimidine synthesis, the conversion of dihydroorotate to orotic acid occurs in the mitochondria, necessitating the intracellular transport of pyrimidine base intermediates within the cell. Transport of pyrimidines between cells is important during germination since the initial source of pyrimidines is from liberated free bases. In *Arabidopsis*, two loci in the ureide permease (UPS) family encoding uracil transporters (AtUPS1 and 2) are differentially expressed and mirror pyrimidine salvage pathway enzyme activities or subsequent elevation of *de novo* synthesis enzyme activities during seed germination, respectively (Schmidt *et al.*, 2004).

Plants contain a plethora of loci encoding nucleobase transporters. No fewer than six distinct multigene families have been identified to date in the *Arabidopsis* genome. Nucleobase-ascorbate transporters (NATs), present in bacteria, fungi, insects, animals, and plants, transport oxidized purines, xanthine, hypoxanthine, and uric acid, the pyrimidine uracil, or ascorbate (Diallinas *et al.*, 1995; De Koning and Diallinas, 2000). The only functionally characterized plant NAT—maize leaf permease 1 (Schultes *et al.*, 1996)—is a high affinity transporter of xanthine and uric acid that competitively binds but does not transport ascorbate (Argyrou *et al.*, 2001). In plants, equilibrative nucleoside transporters (ENTs) (Möhlmann *et al.*, 2001; Li *et al.*, 2003; Wormit *et al.*, 2004; Hirose *et al.*, 2005) act primarily as nucleoside transporters, but some protozoan and mammalian ENTs also transport nucleobases (Yao *et al.*, 2002; Burchmore *et al.*, 2003; De Koning *et al.*, 2003; Henriques *et al.*, 2003).

In contrast, both purine permease (PUP) and UPS genes are found only in plants. Several *Arabidopsis* PUPs transport adenine, cytosine, or secondary compounds such as cytokinins and caffeine (Gillissen *et al.*, 2000; Bürkle *et al.*, 2003). Two transporters in the *Arabidopsis* UPS family transport uracil, allantoin, and the purines xanthine and hypoxanthine (Desimone *et al.*, 2002; Schmidt *et al.*, 2004), and a UPS from French bean transports allantoin (Pélissier *et al.*, 2004). *Arabidopsis* loci At5g50300 and At3g10960 encode proteins with significant amino acid similarity to the recently characterized AzgA adenine–guanine–hypoxanthine transporter of *Aspergillus nidulans* (Cecchetto *et al.*, 2004). *Arabidopsis* locus At5g03555 encodes a protein with significant amino acid similarity to the *FUR4* uracil transporter of *Saccharomyces cerevisiae* in the purine-related transporter (PRT) family. However, the functions of At5g50300, At3g10960, and At5g03555 remain unknown.

The toxic pyrimidine analogues, 5-fluorouracil (5FU) and 5-fluoroorotic acid (FOA), have been used to investigate the genetics and regulation of pyrimidine biochemistry in microbial, mammalian, and, to a more limited extent, plant cells. In plants, direct selection for resistance to fluorinated uracil analogues has been performed mostly with cell cultures, and yielded alterations in pyrimidine biochemistry rather than in pyrimidine transport (Jones and Hann, 1979; Sung and Jacques, 1980; Santoso and Thornburg, 1992, 1998, 2000). In contrast, mutations affecting loci encoding *Arabidopsis* uracil transporters AtUPS1 and AtUPS2 confer a higher level of resistance to growth of seedlings on 5FU-containing media (Schmidt *et al.*, 2004). Here, a novel *Arabidopsis* mutation, *for1-1*, that confers resistance to both FOA and 5FU in plants is reported. This mutation does not correspond to loci of known nucleobase transporters yet influences the uracil transport process.

## Materials and methods

### Genetic stocks

The following *Arabidopsis thaliana* ecotypes and genotypes were used: Columbia wild type (Col); Landsberg *erecta* (*er*) (*Ler*); W100 for mapping studies (ABRC stock #CS2224) (Koorneef *et al.*, 1983); and *fur1/fur1* (obtained from ABRC) (Wu and King, 1994). Other ABRC T-DNA insertion lines (in loci) include: SALK\_001563; SALK\_007057; SALK\_024507; SALK\_114523; and SALK\_134294 (Alonso *et al.*, 2003).

### Plant growth conditions

Minimal *Arabidopsis* medium (MAM) was prepared as described by Haughn and Somerville (1986), and filter-sterilized antibiotics, pyrimidine analogues, or radiolabelled pyrimidine supplements added at the appropriate concentrations. *Arabidopsis* seeds were grown on artificial medium for 2–3 weeks and then transferred to potting mix Redi Earth (The Scotts Company, Marysville, OH, USA) at 20 °C with 12 h d<sup>-1</sup> at 100 μE m<sup>-2</sup> s<sup>-1</sup> for crosses and seed production.

### Seed mutagenesis and mutant isolation

Seeds of *A. thaliana gl1/gl1* in an otherwise Col wild-type background were mutagenized using ethyl methane sulphonate (EMS) as in Mourad and King (1995). Resulting M<sub>2</sub> seed was propagated in subpopulations of ~10 000 seed, surface-sterilized, and plated on MAM with 25 μM FOA. One FAO-resistant mutant plant was isolated from each subpopulation and identified by the ability to grow normal shoots and roots after 2–3 weeks. The individual mutant plants were transplanted separately into potting mix and allowed to self-pollinate to produce M<sub>3</sub> seed.

### Crosses between *fur1/fur1* and *for1-1/for1-1*

Adult *for1-1/for1-1* mutant plants were used as the female recipients of pollen from *fur1/fur1* plants. Resulting F<sub>1</sub> seed was selfed and the resulting F<sub>2</sub> seed was planted on MAM plates with both 0.1 μM 5-fluoro-2'-deoxyuridine (FUDR) and 20 μM FOA. Resistance and sensitivity were scored after 21 d of growth.

### Genetic and molecular mapping of FOA resistance in GM302

Stigma of hairless (nuclear recessive marker *gl1/gl1*), FOA-resistant GM302 mutant plants in an otherwise Col background were

pollinated with pollen from the hairy *Ler* ecotype. The F<sub>1</sub> seed was planted on MAM and the successful hybrids with the hairy phenotype were then transferred to soil and allowed to self-fertilize to produce F<sub>2</sub> seed. The F<sub>2</sub> seed was then planted on MAM containing 25 μM FOA to select for the FOA-resistant segregants.

DNA was extracted from 47 FOA-resistant F<sub>2</sub> individual plants of the cross GM302×*Ler* using the method described by Konieczny and Ausubel (1993).

The following primers were used: for simple sequence length polymorphism (SSLP), markers CIW2 (5'-CCCAAAAGTTAATT-ATACTGT-3'; 5'-CCGGGTTAATAATAAATGT-3'), CIW9 (5'-CAGACGTATCAAATGACAAATG-3'; 5'-GACTACTGCTCAA-ACTATTCGG-3'), CTR1.2, NGA139, NGA76 (Bell and Ecker, 1994), and SGCSNP14411 (5'-TTGAACTTCATGTGCTTGTA-3'; 5'-TATGTTGGTGATTTGGCGATG-3'); and for cleaved amplified polymorphic sequence (CAPS), markers CUE1 (5'-TCTCGTTCTG-ATGGCTCCTGTG-3'; 5'-GTGTAACCGGTGATACTCTCGCC-3'), PHYC.2 (5'-CTACAGAATCGTCCTCAACG-3'; 5'-CCTAAT-GGAGAATCATTCGG-3'), and LMYC6 (5'-GCACGTGGCGTA-TACTG-3'; 5'-CTTGCTCATAGAGACTCGC-3'). The polymerase chain reactions (PCRs) were carried out in 20 μl as described in Konieczny and Ausubel (1993) and, where appropriate, cleaved with endonuclease restriction enzymes. The SSLP and CAPS PCR products from *Ler*, Col, and F<sub>2</sub> FOA-resistant individuals were separated and documented by gel electrophoresis.

#### Construction of transgenic 35S::uraA for1-1/for1-1 plants

PCR primers were designed to amplify the *Escherichia coli* uracil transporter gene, *uraA* (Andersen *et al.*, 1995), using *Pfu* polymerase (Promega, Madison, WI, USA) including the entire coding region (5'-GGGGATCCAATACTATGACGCGTGCTATG-3' and 5'-GCGAGCTCACCACAGAATCCCATGATGTTGA-3'). The resulting PCR product was gel purified, digested with *SacI* and *BamHI*, and ligated to *BamHI/SacI*-linearized DNA from vector pBII21.1 (Clontech, Mountain View, CA, USA). The resulting plasmid p35S-*uraA* was introduced into *E. coli* DH5α and *Agrobacterium tumefaciens* strain GV3101 according to the CaCl<sub>2</sub> protocol outlined by Sambrook *et al.* (1989). p35S-*uraA* was introduced into *for1-1/for1-1* plants according to the vacuum infiltration protocol (Bechtold *et al.*, 1993). Transgenic plants were selected on MAM supplemented with 50 μg ml<sup>-1</sup> kanamycin and 500 μg ml<sup>-1</sup> vancomycin then transplanted to potting mix and selfed. The T<sub>2</sub> seed was harvested from each T<sub>1</sub> plant separately. Transgenic plants and number of inserts were verified by Southern blot analysis (Sambrook *et al.*, 1989) using a <sup>32</sup>P-labelled PCR-amplified *uraA* fragment (Sambrook *et al.*, 1989) to probe genomic DNA extracted from the T<sub>2</sub> plants using a Qiagen DNA extraction kit (Qiagen, Valencia, CA, USA).

#### RT-PCR analysis

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) from 11-d-old wild-type and *for1-1/for1-1* seedlings germinated on defined media as previously described (Schmidt *et al.*, 2004). Reverse transcription was performed using M-MuLV reverse transcriptase (Roche, Basel, Switzerland) at 42 °C for 2 h and then treated with DNA-free™ (Ambion, Austin, TX, USA) as prescribed. Subsequent PCR amplification with Taq DNA polymerase (Qiagen, Valencia, CA, USA) using primers specific for *AtUPS1* (5'-GCACAATAATCGGATTGGTG-3' and 5'-ATGTTAAGTATCA-GAGCAACTACAAATGC-3'), *AtUPS2* (5'-GTATCGTGCTTA-GCCTCG-3' and 5'-TGTTCTATTTCCGGTAGATGGACC-3'), and *AtACT2* (5'-TCCTCACTTTTCATCAGCCG-3' and 5'-ATTG-TTGAATATCATCAGCC-3') was performed for 21, 24, 27, 30, 33, and 36 cycles of 94 °C 30 s, 55 °C 30 s, and 70 °C 3 min in a Mastercycler gradient machine (Eppendorf, Hamburg, Germany) (Schmidt *et al.*, 2004).

#### Radiochemicals

The radiochemicals used in this study include: [7-<sup>14</sup>C]orotic acid (56 mCi mmol<sup>-1</sup>) and [2-<sup>14</sup>C]FOA (55 mCi mmol<sup>-1</sup>) (Moravec Biochemicals, Brea, CA, USA); [6-<sup>3</sup>H]thymidine (2 Ci mmol<sup>-1</sup>) (Amersham Life Science, Piscataway, NJ, USA); [8-<sup>3</sup>H]guanine hydrochloride (8.4 Ci mmol<sup>-1</sup>), [8-<sup>3</sup>H]guanosine hydrochloride (8.0 Ci mmol<sup>-1</sup>), [5-<sup>3</sup>H]cytosine (9.5 Ci mmol<sup>-1</sup>), [5-<sup>3</sup>H]cytidine (17.4 Ci mmol<sup>-1</sup>), [5,6-<sup>3</sup>H]uracil (40.7 Ci mmol<sup>-1</sup>), and [5-<sup>3</sup>H]-uridine (15.0 Ci mmol<sup>-1</sup>) (Sigma-Aldrich, St Louis, MO, USA).

#### Uridine monophosphate (UMP) synthase assay

Frozen plant tissue was homogenized in a French press in buffer containing 50 mM TRIS-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, and 5 mM dithiothreitol (DTT) at 1 ml buffer g<sup>-1</sup> fresh weight. After two rounds of centrifugation at 16 000 g at 4 °C for 5 min each, the supernatants were then stored at -70 °C until ready for use. The UMP synthase assay used 2 μM [7-<sup>14</sup>C]orotic acid as described by Santoso and Thornburg (1992). All radioactivity measurements were performed in scintillation fluid EcoLume (MP Biomedicals, Costa Mesa, CA, USA) and counted in a Tracor Analytic Delta 300 model 6891 (Tracor Analytic Inc., Elk Grove Village, IL, USA) liquid scintillation counter. The total protein content of the extracts was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) according to the procedure of Bradford (1976).

#### Thymidine kinase (TK) assay

The procedure used for assaying the activity of TK was modified from Ohyama (1974). After homogenization, ammonium sulphate was added to 80% saturation to the samples followed by centrifugation at 10 500 g at 4 °C for 20 min. The pellet was redissolved in 3 ml of 10 mM TRIS-HCl, pH 8.0, and 1.0 mM 2-mercaptoethanol, desalted using Bio-Rad Econo-Pac 10DG columns (Bio-Rad, Hercules, CA, USA), and the protein concentration determined. Each assay mixture was 220 μl total volume containing 5.0 mM ATP, 5.0 mM MgCl<sub>2</sub>, 50 mM TRIS-HCl, pH 8.0, 1 mM 2-mercaptoethanol, 50 nmol [6-<sup>3</sup>H]thymidine (2 Ci mmol<sup>-1</sup>), and an amount of plant extract containing 50 μg of total protein. After 60 min at room temperature, two 60 μl aliquots of the reaction were removed and applied to two separate 2.1 cm Whatman DEAE-cellulose filter paper discs, washed three times with 10 mM ammonium formate and twice with ethanol, dried, and the radioactivity determined using a Tracor Analytic Delta 300 model 6891 liquid scintillation counter.

#### FOA uptake analysis

Col wild-type and *for1-1/for1-1* seeds were planted on MAM and grown for 10 d. The seedlings were then transferred to MAM containing 5 μM [2-<sup>14</sup>C]FOA and grown for 2 d. After 24 h from transfer to the [2-<sup>14</sup>C]FOA-containing medium, plants were removed, washed, weighed, and then homogenized in 1.2 ml of TE. After centrifugation at 16 000 g for 5 min, the radioactivity in 400 μl of the supernatant was measured. The FOA uptake data were compiled from four independent experiments.

#### [2-<sup>14</sup>C]FOA incorporation into RNA and DNA of for1-1/for1-1 seedlings

Seeds were planted on MAM containing 20 μM [2-<sup>14</sup>C]FOA and grown for 2–3 weeks. The plants were removed, washed, and then frozen under liquid nitrogen and stored at -70 °C. Total genomic DNA was extracted from ~2 g of the frozen plants according to Mourad *et al.* (1994). Total cellular RNA was extracted from ~3 g of frozen plants according to Mourad *et al.* (1989). DNA and RNA were quantified by spectroscopy and the radioactivity in 100 μg of each nucleic acid was determined.

### Nucleoside and nucleobase uptake assay of wild type and *for1-1/for1-1*

A modification of the protocol outlined by Wu and King (1994) was followed. Eleven-day-old seedlings were transferred from MAM onto MAM supplemented with one of the following tritiated compounds; guanine (3.7 kBq ml<sup>-1</sup>), guanosine (370 Bq ml<sup>-1</sup>), cytosine (3.7 kBq ml<sup>-1</sup>), cytidine (3.7 kBq ml<sup>-1</sup>), uridine (3.7 kBq ml<sup>-1</sup>), or uracil (37

kBq ml<sup>-1</sup>). The plants were grown for 2 d in conditions of constant light at 20 °C, then removed and weighed. Three 100 mg samples of both wild-type and *for1-1/for1-1* tissue were homogenized in 0.5 ml of 20 mM TRIS-HCl, pH 8. For each sample, the radioactivity in 0.1 ml of extract was determined.

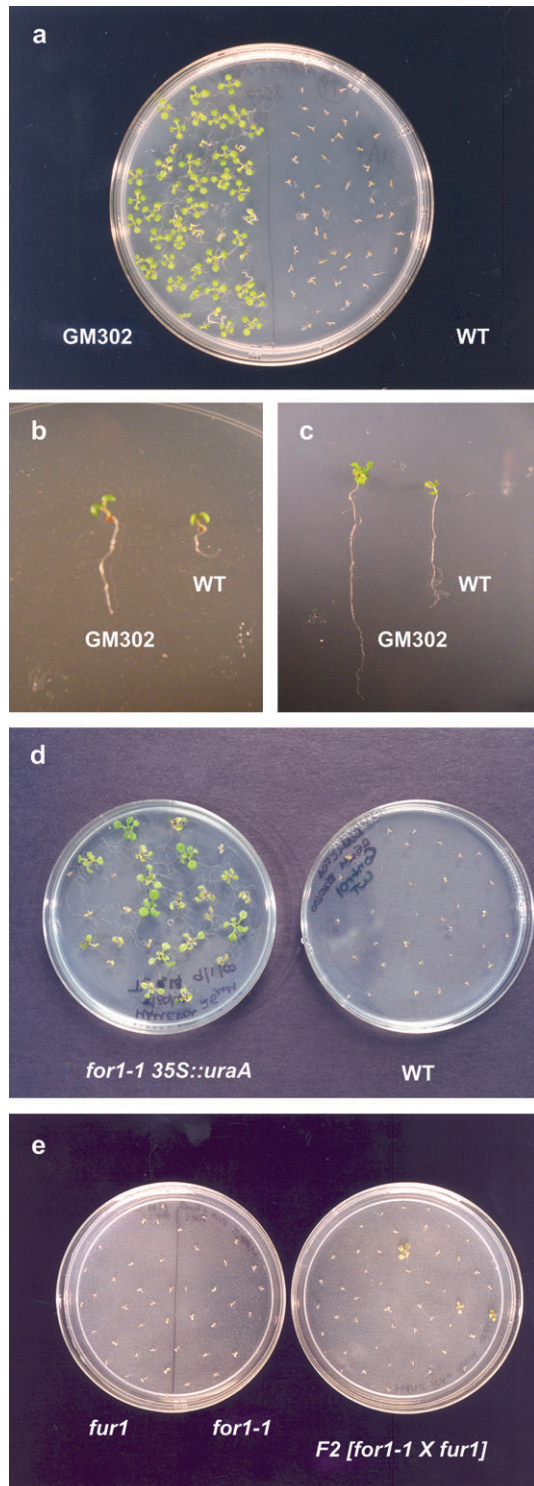
## Results

### Isolation and genetic characterization of the FOA-resistant mutant GM302

Five independently derived mutants (GM302–GM306) were selected for their ability to germinate and grow normal shoots and roots in the presence of 25 μM FOA, a concentration that completely inhibited the growth of the wild type (Fig. 1a). Mutant GM302 was 10-fold more resistant than wild type, based upon the concentration of FOA that inhibited growth by 50% (I<sub>50</sub>) (Fig. 2). The uniform FOA resistance phenotype observed in GM302 and its subsequent progeny led to it being chosen for further genetic and biochemical characterization. GM302 was crossed as both male and female to Col wild type, and FOA resistance was absent among all the F<sub>1</sub> progeny and segregated as 3 FOA-sensitive:1 FOA-resistant among the F<sub>2</sub> progeny (Table 1). In addition, GM302 plants are resistant to 200 μM of the toxic uracil analogue 5FU—a level that causes retarded growth in wild-type seedlings (Fig. 1b, c). These results suggested that FOA resistance in GM302 was inherited as a single, nuclear, recessive gene that was named *for1-1*. Thus the mutant line GM302 is *for1-1/for1-1* and will be referred to as such. The wild-type FOA-sensitive allele is *FOR1*.

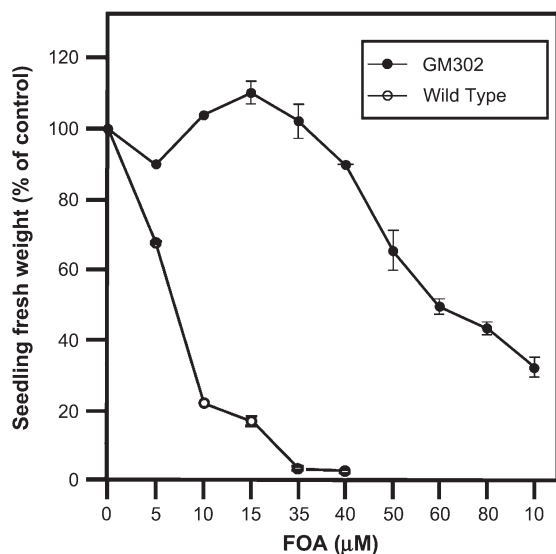
### FOA resistance in GM302 (*for1-1/for1-1*) is not due to enzyme amplification

FOA is a structural analogue of orotic acid, the precursor of UMP, the first pyrimidine nucleotide synthesized by *de novo* biosynthesis (Fig. 3). FOA is metabolized in the *de novo* pathway forming fluorinated dUMP and acts as a potent inhibitor of thymidylate synthase (TS), resulting in thymine starvation and inhibition of DNA synthesis (Fig. 3). This toxic effect of FOA is because F occupies C5 of the pyrimidine ring in F-dUMP and TS fails to replace it with a methyl group (-CH<sub>3</sub>).



**Fig. 1.** (a) Three-week-old *Arabidopsis* Col wild type (right) and GM302 (*for1-1/for1-1*) (left) grown in MAM with 25 μM FOA. (b, c) One-week-old (b) and 2-week-old (c) *Arabidopsis* Col wild type (right) and GM302 (*for1-1/for1-1*) (left) grown in MAM with 200 μM 5-FU. (d) Left: T<sub>2</sub> progeny of transgenic *for1-1/for1-1* 35S::uraA grown in MAM with 20 μM FOA and segregating in a 1 resistant:2 semi-sensitive:1 sensitive. Right: *Arabidopsis* Col wild-type grown in the same medium and displaying sensitivity to FOA. (e) Right: F<sub>2</sub> progeny of the cross *for1-1/for1-1* × *fur1/fur1* grown in MAM with 20 μM FOA plus 0.1 μM FUDR and segregating 15 sensitive:1 resistant. Left: a control plate showing *fur1/fur1* (left half of plate) and *for1-1/for1-1* plants (right half of the plate) grown in MAM with 20 μM FOA plus 0.1 μM FUDR; both mutants display sensitivity.

The recessive nature of FOA resistance in GM302 is an indication that resistance is due neither to a mutant form of UMP synthase nor to enhanced UMP synthase activity—both of which would result in dominantly inherited FOA



**Fig. 2.** Effect of varying concentrations of FOA on the growth of Col wild type and GM302 (*for1-1/for1-1*). Surface-sterilized seeds were germinated in MAM with varying concentrations of FOA. After 16 d of growth, the seedlings were gently pulled out, blotted, and weighed. Error bars represent the standard error of the mean.

**Table 1.** Genetic analysis of growth resistance to FOA

Cross	No. of plants			
	S <sup>a</sup>	R	S:R	$\chi^2$
GM302×WT F <sub>1</sub>	34.0	0.0	4:0	—
WT×GM302 F <sub>1</sub>	22.0	0.0	4:0	—
GM302×WT F <sub>2</sub>	178.0	58.0	3.06:1	0.023
W100×GM302	292.0	93.0	3.13:1	0.146

<sup>a</sup> S, sensitive; R, resistant; W100, multirecessive stock visible marker line with one marker in each chromosome arm. In a similar manner, the other mutants GM303, GM304, GM305, and GM306 segregated FOA resistance as a single recessive gene, 3 sensitive:1 resistant (data not shown).

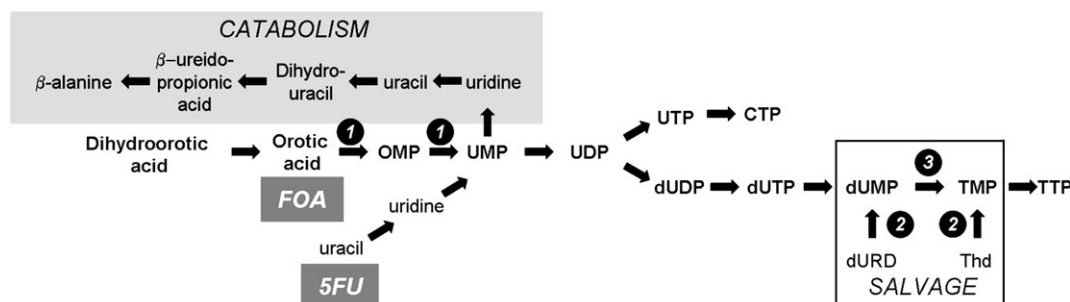
resistance. The specific activity of UMP synthase in wild-type and *for1-1/for1-1* plants is not significantly different (Table 2), confirming that UMP synthase activity is not altered. An alternative explanation for FOA resistance by enzymatic means calls for enhanced pyrimidine salvage pathway TK activity. Elevated levels of dUMP would result and thus unblock TTP starvation caused by F-dUMP inhibition of TS (Fig. 3). To test this possibility, the specific activity of TK in extracts from wild type and *for1-1/for1-1* was assayed. No significant differences were observed (Table 2), excluding amplification of TK as a cause for FOA resistance. These results support the explanation that FOA resistance in GM302 is due to a defective uptake mechanism.

#### *for1-1/for1-1* plants are defective in the uptake of [2-<sup>14</sup>C]FOA

To test the possibility that FOR1 affects a mechanism involving the uptake of FOA, the ability of wild type and *for1-1/for1-1* to take up [2-<sup>14</sup>C]FOA from the growth medium into the plant was compared. Ten-day-old seedlings of both genotypes, grown aseptically, were transferred to MAM with 5.0 µM [2-<sup>14</sup>C]FOA. The uptake of the radiolabel from the medium by both genotypes was then monitored after 24 and 48 h. At 24 h, the uptake in nmol [2-<sup>14</sup>C]FOA g<sup>-1</sup> fresh weight was 25.40±0.10 for wild type and 1.29±0.03 for *for1-1/for1-1*. The influx of [2-<sup>14</sup>C]FOA was significantly reduced in *for1-1/for1-1* seedlings when compared with the wild-type seedlings. Only 5.1% of the label that entered the wild type entered *for1-1/for1-1*.

#### The fate of [2-<sup>14</sup>C]FOA taken up by *for1-1/for1-1* plants

To investigate the fate of FOA entering GM302, incorporation of [2-<sup>14</sup>C]FOA was followed in DNA and RNA. The DNA and RNA extracted from *for1-1/for1-1* plants grown on MAM with 20 µM [2-<sup>14</sup>C]FOA revealed that the incorporation of the [<sup>14</sup>C]pyrimidine ring was 34-fold higher in RNA than DNA (Table 3).



**Fig. 3.** A flow chart showing the *de novo* biosynthesis of pyrimidine nucleotides, starting from dihydroorotic acid, and the possible salvage pathways leading to the production of pyrimidine nucleosides and their corresponding nucleotides. Solid circles with 1 indicate the position of UMP synthase, with 2 indicate TK, and with 3 indicate TS.

### *for1-1* affects an uptake mechanism with high affinity for free uracil

To test the involvement of the *FOR1* locus in the uptake of other pyrimidines and purines, an assay was performed to determine whether *for1-1/for1-1* plants were cross-resistant to the toxic pyrimidine nucleoside analogues 5-bromo-2'-deoxyuridine (BUdR) and FUdR, and the toxic purine analogue 8-azaguanine (8-AZG). *for1-1/for1-1* plants were as sensitive as the wild type for growth in the presence of BUdR, FUdR, and 8-AZG (Fig. 4a–c). This result supports a role of *FOR1* in the specific transport of free pyrimidine bases.

Since orotic acid is the precursor for all *de novo* synthesized pyrimidine nucleotides starting from UMP (Fig. 3), the question was then asked whether *for1-1* affects the transport of both types of pyrimidine bases (cytosine and uracil). To determine this, *for1-1/for1-1* and wild-type

**Table 2.** Specific activities of UMP synthase and thymidine kinase in extracts from wild type and GM302 (*for1-1/for1-1*)

Genotype	Specific activity	
	UMP synthase (nmol [ <sup>14</sup> C]CO <sub>2</sub> mg <sup>-1</sup> protein h <sup>-1</sup> )	TK (pmol [ <sup>3</sup> H]TMP mg <sup>-1</sup> protein h <sup>-1</sup> )
Wild type	0.2026±0.051	2.86±0.040
GM302 ( <i>for1-1/for1-1</i> )	0.2167±0.014	2.74±0.100

**Table 3.** Incorporation of [<sup>14</sup>C]FOA into DNA and RNA extracted from *for1-1/for1-1* plants

Nucleic acid	dpm μg <sup>-1</sup> nucleic acid
DNA	34.24
RNA	1165.8

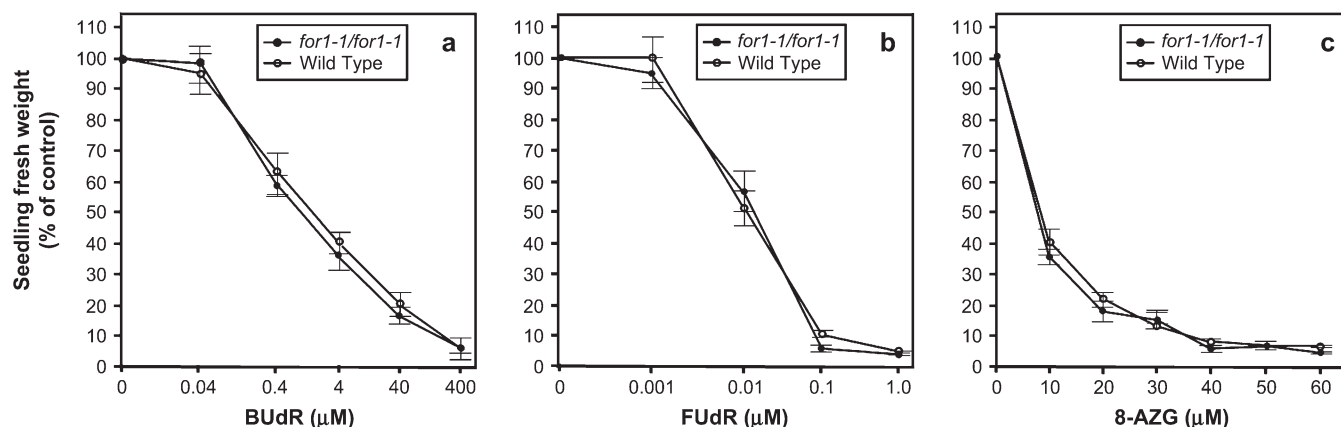
seeds were germinated in MAM supplemented with <sup>3</sup>H-labelled pyrimidine and purine free bases and nucleosides. The results showed that the wild type was 3.5-fold more efficient in the transport of uracil from the medium into the plant's tissues than the *for1-1/for1-1* mutant (Fig. 5). This difference in uracil uptake between the two genotypes was found to be statistically significant at *P*=0.05. A slight difference was observed in the uptake of cytosine between the two genotypes, but was found to be statistically insignificant. No differences in the uptake of cytidine, guanine, guanosine, or uridine were detected.

### The uracil transporter of *E. coli*, *uraA*, complements *FOA* resistance in *for1-1/for1-1* plants

It became apparent that *for1-1* affects a transport mechanism with high affinity for the uptake of free uracil. To confirm such observation, *for1-1/for1-1* plants were transformed with the uracil transporter *uraA* gene of *E. coli* driven by the 35S promoter of the cauliflower mosaic virus (CaMV) and terminated with the nopaline synthase terminator sequences (*35S::uraA*). Single insert positive T<sub>1</sub> transformants were selected and selfed to produce T<sub>2</sub> seed. When planted on MAM supplemented with 20 μM FOA, the T<sub>2</sub> seedlings segregated as 1 resistant:2 semi-sensitive:1 sensitive (Fig. 1d).

### Genetic complementation confirmed specificity in transport of uracil and pyrimidine nucleosides in Arabidopsis

In *Arabidopsis*, an FUdR-resistant mutant was isolated by Wu and King (1994) and was due to a single gene designated *fur1* and mapped to chromosome 4. *fur1* was found to be specifically affecting the transport of pyrimidine nucleosides. Germinating *fur1/fur1* seeds on medium with 20 μM FOA showed that *fur1/fur1* were as sensitive to FOA as wild type. Conversely, *for1-1/for1-1* was found to

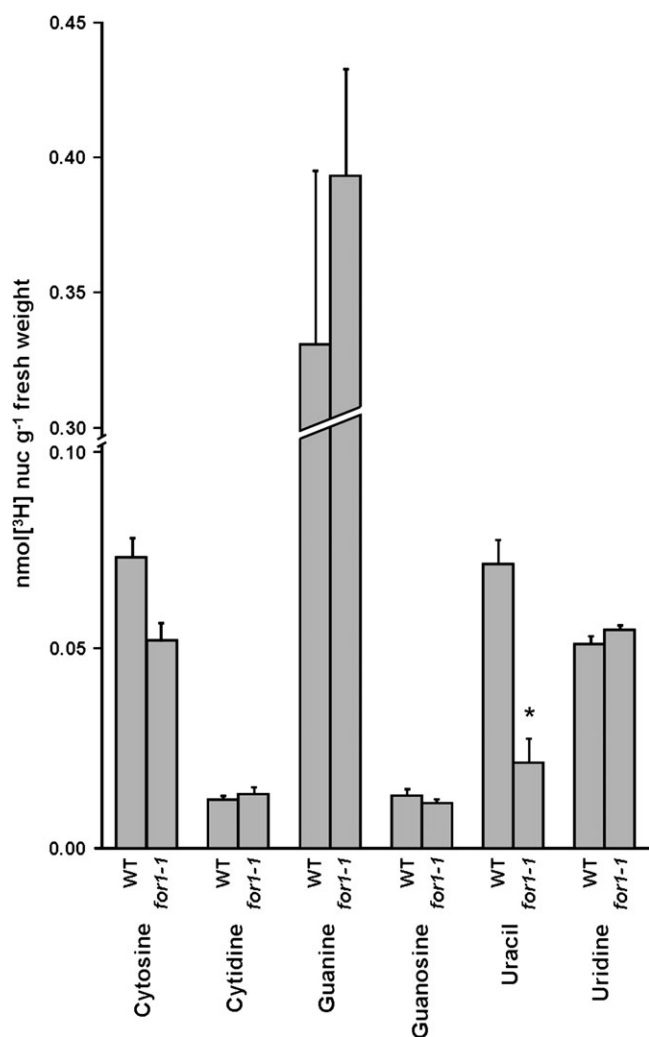


**Fig. 4.** The effects of two toxic pyrimidine nucleoside analogues (a) BUdR and (b) FUdR and a toxic purine analogue (c) 8-AZG on the growth of *Arabidopsis* Col wild type and *for1-1/for1-1*. Surface-sterilized seeds were germinated in MAM with varying concentrations of the toxic analogues. After 16 d of growth, the seedlings were gently pulled out, blotted, and weighed. Error bars represent the standard error of the mean.

be as sensitive to 0.1  $\mu\text{M}$  FUDR as the wild type. *for1-1/for1-1* to *fur1/fur1* were therefore crossed and the subsequent  $F_2$  seed were planted on MAM supplemented with 20  $\mu\text{M}$  FOA and 0.1  $\mu\text{M}$  FUDR. The pooled  $F_2$  progeny of several independent crosses segregated 363 sensitive:22 resistant to both FOA and FUDR. This ratio is statistically insignificantly different (by  $\chi^2$  at  $P=0.05$ ) from a 15 sensitive:1 resistant ratio, a perfect segregation ratio for two independently assorting genes (Fig. 1e). These results indicated that *FOR1* and *FUR1* are indeed two independent loci.

*FOR1* maps to a small interval in chromosome 5 and does not correspond to known nucleobase transporter family loci

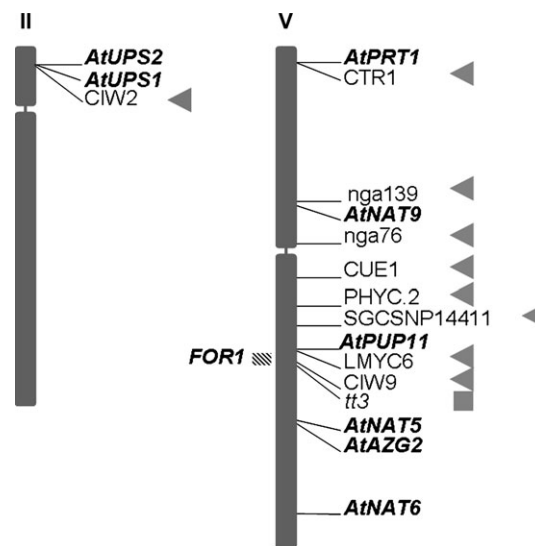
To map the new genetic locus *FOR1*, *for1-1/for1-1* was crossed as a pollen donor to the multirecessive stock visible



**Fig. 5.** Uptake assay of *Arabidopsis* Col wild type and *for1-1/for1-1* using  $^3\text{H}$ -labelled nucleobases and nucleosides. The error bar represents the standard error of the mean for three independent replicates, and nuc on the y-axis represents a nucleobase or nucleoside. \*Uptake of uracil is statistically significant as determined by a *t*-test at  $A=5\%$ .

marker line W100 and several other Michigan State University marker lines.  $F_1$  plants were allowed to self-fertilize to produce  $F_2$  seed, which was germinated on MAM with 25  $\mu\text{M}$  FOA. FOA-resistant seedlings were scored for segregation for each of the W100 or the MSU markers. The  $F_2$  data revealed a tight linkage between *for1-1* and *tt3* in chromosome 5 but with no other markers (data not shown). The  $F_2$  segregation data using visible genetic markers placed *FOR1* in chromosome 5.

Six loci that map along chromosome 5 belong to one of several gene families known to encode nucleobase transporters: At5g03555 (the sole representative of the *PRT* family); At5g25420, At5g49990, and At5g62890 (members of the *NAT* family); At5g50300 (an AzgA-like transporter); and At5g41160 (a member of the *PUP* family) (Fig. 6). These loci are likely candidates for *FOR1*. None of the eight identified *ENT* loci in *Arabidopsis* maps to chromosome 5. To determine if *for1-1* is linked to any of these candidate loci, CAPS and SSLP markers along chromosome 5 and one SSLP marker of chromosome 2 closely linked to the known uracil transporters At2g03590 or At2g03530 were employed in mapping studies (Fig. 6). Wild-type *Ler* and *for1-1/for1-1* (in an otherwise Col background) were crossed and the resulting  $F_1$  progeny were selfed. Forty-seven FOA-resistant  $F_2$  progeny were selected and monitored for the genotype of each polymorphic marker. The data revealed no linkage between *for1-1* and CAP CIW2 in chromosome 2 closely linked to uracil transporter loci At2g03590 or At2g03530. Further,



**Fig. 6.** *FOR1* chromosomal mapping. Listed are graphical representations of chromosome II and V of *Arabidopsis thaliana*. Loci encoding nucleobase transporters include *AtUPS1* and 2; *AtNAT5*, 6, and 9; *AtPUP11*; *AtPRT1*, and *AtAZG2*. Triangles denote SSLP and CAPS markers used for molecular mapping of the *FOR1* locus, and a grey square identifies a genetic marker *tt3* used in genetic linkage studies. A hatched box represents the smallest interval to which the *FOR1* locus has been mapped.

recombination breakpoints place *for1-1* in a 519 kb region between markers LMYC6 and CIW9 in chromosome 5. Loci At5g03555, At5g25420, At5g49990, At5g62890, and At5g50300 map outside this region and therefore could not be *FOR1*. Although locus At5g41160 lies directly outside this region, it is very close to marker LMYC6. To ensure that *FOR1* does not correspond to this *PUP* family member, a 1640 bp genomic region encompassing the entire coding region and including 345 bp upstream of the start of translation and 218 bp downstream of the stop codon was PCR-amplified from *for1-1/for1-1* plants and the DNA sequence determined. No alteration from the Col wild-type sequence was evident (data not shown).

Within the 519 kb region in chromosome 5 are a number of loci that encode known transporters. To determine if *FOR1* was among these loci, the phenotype of available mutant alleles was investigated. Plants homozygous for a new *for1-1* allele should display FOA resistance. Seed stocks segregating exon-located T-DNA insertion alleles for loci At5g41610 (encoding a H<sup>+</sup> exchanger), At5g41800 (encoding an amino acid transporter), At5g41330 (encoding a K<sup>+</sup> channel), and At5g42420 (encoding a phosphoenol pyruvate-like transporter) were sown on FOA-containing MAM. None of the seed stocks germinated seedlings that were resistant to growth on FOA (data not shown).

#### *AtUPS1* and *AtUPS2* transcript levels are not altered in *for1-1/for1-1* plants

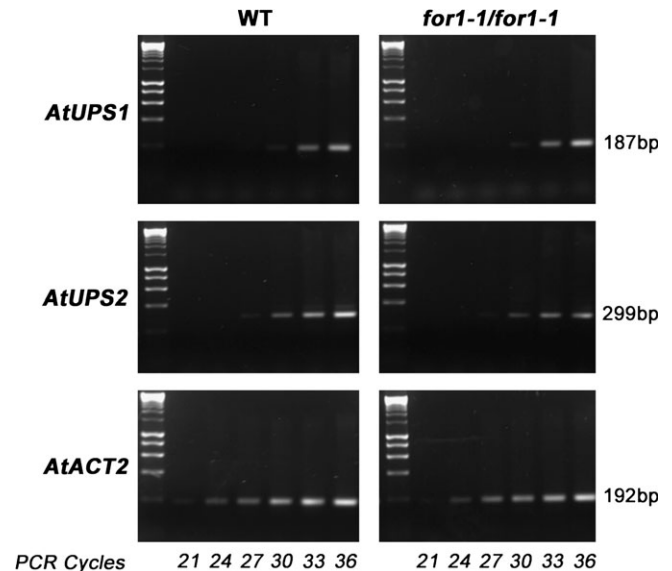
Recent work has shown that 5FU-resistant *Arabidopsis* mutant seedlings are either devoid of At2g03530 (*AtUPS2*) transcript or contain severely reduced At2g03590 (*AtUPS1*) transcripts (Schmidt *et al.* 2004). The possibility that the *for1-1/for1-1* plants gain FOA and 5FU resistance by depleting either or both *AtUPS* transcripts was investigated. *AtUPS1* and *AtUPS2* transcripts were monitored by reverse transcription–polymerase chain reaction (RT–PCR) on wild-type and *for1-1/for1-1* 11-d-old seedlings (Fig. 7). The results show no difference in the accumulation of *AtUPS* transcripts in both the wild-type and *for1-1/for1-1* seedlings.

## Discussion

This study presents a new locus, *FOR1*, in the uracil transport system of *Arabidopsis*. Mutant *for1-1/for1-1* plants have heightened resistance to toxic uracil analogues FOA and 5FU, and are deficient in the transport of uracil *in planta*. Available evidence indicates that *FOR1* does not encode a known nucleobase transporter, but is involved in the regulation of the uracil transport process.

#### FOR1 does not alter pyrimidine biochemistry

There are generally two different routes for gaining resistance to FOA—either by modifying biochemical path-



**Fig. 7.** RT–PCR analysis of *AtUPS1* and *2* expression in wild type and *for1-1/for1-1* seedlings. mRNA, isolated from *Arabidopsis* Col wild type and *for1-1/for1-1* 11-d-old seedlings, was reverse transcribed then amplified with primers specific for *AtUPS1*, *AtUPS2*, or *AtACT2* sequences for 21, 24, 27, 30, 33, and 36 cycles. DNA fragments of 187, 299, and 192 bp were resolved by agarose gel electrophoresis.

ways or through altering transport processes. Biochemical-derived resistance can be achieved by altering *de novo* pyrimidine synthesis, salvage pathways, or catabolic reactions. FOA resistance can be achieved by modifying the enzymatic activities of UMP synthase, the limiting enzyme in plant pyrimidine biosynthesis. *Nicotiana* cell cultures displaying altered levels of UMP synthase gene expression, protein level, and enzyme activity are resistant to high concentrations of FOA (Santoso and Thornburg, 1992, 1998, 2000). Alternatively, FOA resistance can be achieved by subverting FOA-induced thymine starvation via enhancing the activity of TK in the salvage pathway. Finally, altered pyrimidine catabolic activities may contribute to FOA resistance. It appears that plants have a similar pathway for pyrimidine catabolism to that found in mammalian cells (Stasolla *et al.*, 2003; Kafer *et al.*, 2004). Although FOA- or 5FU-resistant plant mutants in this pathway are unknown, 5FU-resistant mammalian tumour cells correlate with the up-regulation of genes and enzyme activities in the catabolic pathway (Kidd *et al.*, 2005). Biochemical pathways that result in direct degradation of orotic acid or directly convert orotic acid into uracil are unknown in plants, but are present in some microorganisms (Santoso and Thornburg, 2000).

The data do not support the idea that *for1-1* mutants gain FOA resistance via alterations in biochemical pathways. (i) UMP synthase and TK enzyme activities are similar in *for1-1/for1-1* and wild-type plants—no enhanced pyrimidine biosynthetic or salvage activities. (ii) The *for1-1* mutation does not map near loci encoding UMP synthase,



TK, or known catabolic enzymes dihydrouracil dehydrogenase, dihydropyrimidine dehydrogenase, or  $\beta$ -ureidopropionase. *for1-1* could not represent a mutant lesion in these loci that results in FOA resistance. (iii) FOA-resistant mutants due to enhanced enzymatic activities represent gain-of-function mutations that are semi-dominant or dominant, while *for1-1* clearly acts as recessive allele. (iv) Uptake studies reveal that *for1-1/for1-1* plants are deficient in [2-<sup>14</sup>C]FOA uptake compared to the wild type. Mutants resulting in enhanced UMP synthase or TK activities would not show any uptake deficiency. (v) The <sup>14</sup>C label ends up in RNA in *for1-1/for1-1* plants, revealing an intact biochemical pathway as would be expected in a transport mutant. (vi) An alteration in solely the *de novo* synthesis pathway or in just the salvage pathway would not allow *for1-1/for1-1* plants to be resistant to both 5FU and FOA as 5FU and FOA generate F-UMP by separate biochemical steps. (vii) The results of [<sup>3</sup>H]uracil and [<sup>3</sup>H]uridine uptake studies reveal that *for1-1/for1-1* mutant plants are deficient in uracil, but not uridine uptake. Since uridine and uracil are catabolized by the same pathway, any FOA-resistant mutant resulting from enhanced catabolic activities would be resistant to BUdR and FUdR as well. The present results do not show that (Fig. 4). Previous work by Santoso and Thornburg (2000) revealed that in plant cells, almost all of the orotic acid is converted into UMP rather than into other forms. FOA resistance through increased catabolism must proceed through a uridine step.

Could FOR1 act to up-regulate genes encoding *de novo* pyrimidine pathway enzymes? Santoso and Thornburg (1998, 2000) hypothesized that up-regulation of UMP synthase could be mediated by releasing protein transcription repressors that interact with thymine or a thymine derivative. Again, the enzymatic data and the recessive nature of *for1-1* argue against this possibility. In addition, with hyperactive UMP synthase, *Nicotiana* cell lines were found to have no defect in the uptake of [<sup>14</sup>C]FOA (Santoso and Thornburg, 2000).

#### FOR1 is involved in the uptake of uracil

The data support the hypothesis that FOR1 is specifically involved in the uracil transport process. First, *in planta* radiolabelled uptake studies show that *for1-1/for1-1* plants are deficient in the uptake of uracil or FOA compared with the wild type. No differences in the uptake of cytosine, cytidine, guanine, guanosine, or uridine were observed between *for1-1/for1-1* and wild-type plants. Secondly, *for1-1/for1-1* plants are sensitive to growth on toxic pyrimidine nucleoside analogues BUdR and FUdR and the toxic purine analogue 8-AZG, but not FOA or 5FU. Thus, FOR1 does not act in a transport process that is general for purines and pyrimidines. Thirdly, genetic and biochemical experiments show that *FOR1* and *FUR1* are distinct. Fourthly, *for1-1/for1-1* deficiency is comple-

mented by the expression of the *E. coli* uracil transporter *uraA*. Wild-type FOA sensitivity is restored in *for1-1/for1-1* plants. Fifthly, a loss of transport mutation would be inherited recessively, as in *for1-1*.

There are two ways to achieve FOA resistance via transport: either restrict entry of FOA or increase export of FOA. Mutations resulting in increased export would display a dominant not recessive mode of inheritance. FOR1 appears to be involved in the uptake and not ejection of uracil and FOA.

*FOR1* may represent a locus that encodes one of the numerous nucleobase transport proteins known to reside in the *Arabidopsis* genome, including members in the *NAT*, *PUP*, *UPS*, *PRT*, and *8-AzGA* gene families. Two top candidates are the UPS transporters, AtUPS1 and AtUPS2, that recently were shown to transport uracil and related compounds (Schmidt *et al.*, 2004). In addition, mutations in either of the loci encoding these transporters show enhanced resistance to growth on 5FU. However, genetic mapping data place *FOR1* in chromosome 5, while the *UPS* loci At2g03530 and At2g03590 map close together in chromosome 2. Further genetic mapping and DNA sequence data analysis establish that *FOR1* does not correspond to any of the five nucleobase transporter gene family members located in chromosome 5. Additional phenotypic analysis of available insertion mutants shows that *FOR1* does not correspond to four of five identified transporter-encoding loci found within the 519 kb region containing *FOR1*. Although *FOR1* does not appear to correspond to a locus encoding an identified transporter, it is still possible that *FOR1* encodes an as yet uncharacterized and unknown transporter.

An alternative possibility is that *FOR1* encodes a protein that regulates the transport mechanism in some manner. The developmental timing of *AtUPS1* and *AtUPS2* gene expression suggests that transcription factors are involved in their regulation (Schmidt *et al.*, 2004). However, evidence shows that neither *AtUPS1* nor *AtUPS2* transcripts are altered in the *for1-1/for1-1* background, suggesting that *FOR1* does not affect steady-state *AtUPS* levels.

FOR1 may have a post-transcriptional or post-translational role in uracil transport. For example, FOR1 may act as a chaperone or docking protein in the endoplasmic reticulum or vesicular system that acts on AtUPS1 and 2 proteins and influences delivery, stability, or recycling. Post-translational processing is integral to regulating the levels of uracil transporter (*FUR4*) in *Saccharomyces cerevisiae* plasma membranes (Blondel *et al.*, 2004; Bugnicourt *et al.*, 2004). Alternatively, uracil transporters may be activated by post-translational modification by FOR1 as are some human ENT proteins (Stolk *et al.*, 2005). Loss of FOR1 function would reduce uracil transport and appear as a recessive mutation. Further mapping and complementation studies are under way to reveal the molecular structure of *FOR1*.

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