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A Mutant of *Arabidopsis thaliana* with a Reduced Response to Fusicoccin. I[1]

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Because fusicoccin (FC) has the the capacity to promote solute uptake, a selective procedure for isolating mutants of *Arabidopsis thaliana* with a reduced response to the toxin has been developed. The procedure is based on the incubation of *A. thaliana* seedlings in a solution containing the cation Paraquat (Pq) at a concentration that per se does not produce bleaching of the leaves upon illumination but does in the presence of FC because of the increased uptake of the toxic cation. Using this procedure, we identified, among the progenies of 2010 M_1 ethyl methanesulfonate-mutagenized plants, two mutants that stay green after exposure to FC and Pq. Some properties and inheritance of one of the two mutants (5–2) are described. Morphology of mutant plants is almost indistinguishable from that of the wild type. However, 5–2 seeds germinate and produce viable seedlings in the presence of FC plus the aminoglycoside antibiotic hygromycin B: plants of the mutant do not wilt when exposed to FC and stomata do not open or open only partially. In the presence of FC, the mutant appears less responsive than the wild type as far as the increment in fresh weight, the enlargement of leaf disc area, or the stimulation of H^+ extrusion is concerned. Inheritance of the trait is monogenic dominant or semidominant, depending on the test used.

All higher plant species investigated thus far appear responsive to the fungal toxin FC (Chain and Mantle, 1971). The responses induced by the toxin at the cellular level include, among others, hyperpolarization, proton extrusion, and increased solute uptake; at the plant level, there are promotion of germination, cell enlargement, stomatal opening, and wilting (for a review, see Marrè, 1979, 1985). Evidence from in vivo and in vitro experiments indicates that FC acts primarily by stimulating the activity of the PM H^+-ATPase (Marrè, 1979; Rasi-Caldogno and Pugliarello, 1985; Rasi-Caldogno et al., 1986); however, the receptor for the toxin is not the H^+-ATPase itself but another protein located in the PM (Stout and Cleland, 1980; Aducci et al., 1988; Feyerabend and Weiler, 1988; De Michelis et al., 1989).

To our knowledge no monogenic mutants with an altered sensitivity to FC (insensitive or hypersensitive) have been isolated so far. Such mutants may be of interest because they can help researchers understand the mechanisms regulating the activity of PM H^+-ATPase and because they can be the starting material for isolating genes involved in such regulation.

We have chosen *Arabidopsis thaliana* because it is a species ideally suited for genetic experiments. Furthermore, a PM H^+-ATPase, which is K^+ dependent, vanadate inhibited, and FC sensitive, is active in seedlings and leaves of this species (Olivari et al., 1993; M.T. Marrè, A. Talarico, C. Soave, unpublished data). The corresponding gene(s) has been cloned (Harper et al., 1989; Pardo and Serrano, 1989; Harper et al., 1990), and the presence of FC-binding sites has been demonstrated (Stout, 1988; Meyer et al., 1989).

MATERIALS AND METHODS

Seed stocks used were derived from the pure *Arabidopsis thaliana* line Landsberg *erecta*. Plants were grown in a growth chamber under a regimen of 14 h of light (50 W m^{-2}), 10 h of dark at 25/20°C, or in a greenhouse on a mixture of sterile soil and perlite (3:1, v/v).

Induction and Isolation of FC-Insensitive Mutants

The selective procedures used and the properties of mutants isolated were reported by Soave et al. (1992). Seeds were mutagenized with ethyl methanesulfonate (15 mM, 20 h, 24°C) as described by Koorneff and van der Veen (1980). The resulting M_1 plants were cultivated in soil, and M_2 seeds from pools of 10 M_1 plants were harvested.

To isolate FC-insensitive mutants, seeds from half an aliquot of each pool were grown in rotating flasks in a standard liquid mineral medium (Soave et al., 1992) for 3 d in continuous light (dormancy was broken by keeping seeds at 4°C for 4–6 d). Seedlings were washed twice (30 min each) in 0.5 mM $CaSO_4$, 1 mM Mes (pH 6.2) (BTP) and incubated in rotating flasks (about 250 seedlings in 10 mL of solution) in the same solvent plus 5×10^{-6} M FC and 5×10^{-7} M Pq for 15 h in the dark at 25°C. Light was then turned on (300 μmol m^{-2} s^{-1} of PAR) for 3 h, and the phenotype of the seedlings

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Abbreviations: BTP, BisTris propane [1,3-bis(tris(hydroxymethyl) methylamino)-propane]; FC, fusicoccin; HG, hygromycin-B; PM, plasma membrane; Pq, paraquat.

(white or green) was scored. The same test was conducted on single, detached leaves incubated in wells of tissue culture plates (11.3 mm in diameter, 1 mL of solution per well) with shaking.

Additional Tests for Assaying FC Sensitivity on an Individual Plant Basis

Wilting

Stems from flowering plants maintained for 16 h in the dark were cut near the base and inserted into tubes containing 0.5 mM $CaSO_4$, 1 mM Mes (pH 6.0) (BTP), 2.5 mM K_2SO_4, with or without 5×10^{-6} M FC. Plants were exposed to fluorescent light (15 W m^{-2}) for 3 h and then left for 15 h at 30°C in the dark in a ventilated chamber. Plants were classified as wilted or nonwilted by visual inspection of the turgor of stem, leaves, and petals.

Stomatal Aperture

Discs (about 3 mm in diameter) were punched from rosette leaves of plants at the time of initial flowering and washed for 1 h in the dark at 25°C in tissue culture wells (11.3 mm in diameter, three discs per well) containing 1 mL of 0.5 mM $CaSO_4$, 1 mM Mes (pH 6.2) (BTP) with rotatory shaking. Subsequently, discs were incubated in the same medium plus 2.5 mM K_2SO_4, 250 mM mannitol, with or without 5×10^{-6} M FC, for 2 h in the dark. Stomata were visualized under a microscope, and three randomly selected fields per disc were photographed. Apertures were measured on the pictures.

HG Resistance

Sterilized and vernalized seeds were plated in Petri dishes on an agarose-solidified medium (NO_3 medium [Soave et al., 1992]) with K_2SO_4 at a final concentration of 50 μM, 4 μg mL^{-1} of HG, and $\pm 5 \times 10^{-6}$ M FC. Germination and growth were at 25°C in light (50 W m^{-2}) for 8 d. Nongerminated seeds or seedlings with white cotyledonary leaves were classified as nonresistant, and green seedlings were considered resistant.

Fresh Weight Increment and Leaf Disc Area Enlargement

Basal leaves from plants that had just flowered were detached, cut into segments, and washed for 10 min in 0.5 M $CaSO_4$, 1 mM Mes (pH 6.0) (BTP). The segments were blotted, and aliquots of about 620 mg were weighed. The aliquots were incubated for 15 h in the dark at 25°C with shaking in 10 mL of a solution containing 2 mM $MgSO_4$, 0.85 mM $CaCl_2$, 5 mM KNO_3, 1 mM Mes, and 5.85 mM Suc (final pH 6.0) (BTP), with or without 10^{-5} M FC. Finally, leaf segments were blotted and weighed.

For leaf disc experiments, discs of about 3 mm in diameter were punched from basal leaves, washed for 1 h in 1 mM KCl, 29 mM Suc (final pH adjusted to 6.8 with KOH), and, after the medium was changed, incubated in the same solution with or without 5×10^{-6} M FC in tissue culture wells (11.3 mm in diameter) containing 1 mL of solution with three discs per well. Incubation was for 20 h in the dark at 25°C with rotary shaking. Disc diameter was evaluated with a microscope equipped with a calibrated ocular micrometer.

H^+ Extrusion

Discs (about 3 mm in diameter), punched from basal leaves of just-flowered plants, were washed for 2 h in 0.5 mM $CaSO_4$, followed by a second wash of 30 min in 0.5 mM $CaSO_4$, 15 mM Glc, and 5×10^{-6} M DCMU, with or without 10^{-5} M FC. Usually, samples of 40 mg (fresh weight) were incubated in small flasks containing 1 mL of 0.5 mM $CaSO_4$, 0.5 mM Mes (pH 6.5) (BTP), 15 mM Glc, and 5×10^{-6} M DCMU, with or without 10^{-5} M FC, and K_2SO_4 at the concentration indicated in Table III. Incubation was for 60 or 90 min in the dark with shaking. At the end, the pH of the solution was measured with a pH meter (Radiometer, Copenhagen, Denmark). Proton extrusion was measured by back-titration of the medium according to the method of Marrè et al. (1973).

RESULTS

Selective Procedures, Screening of the Mutagenized Population, and Mutant Isolation

Mutants with altered FC sensitivity can be isolated when a specific selective system is available. In *A. thaliana*, selection based on the promoting effect induced by FC on germination, or on wilting, or on the stimulation of acidification of the external solution cannot be used either because no clear discrimination between a control and a treated population is obtained or because the system is too laborious and expensive. Our procedure is based on the capacity of FC to promote solute uptake; therefore, it is possible to expose seedlings to a toxic cation (Pq) at a concentration that per se does not produce a phenotype (bleaching upon illumination) but does in the presence of FC, because the internal concentration of the drug is higher in FC-treated tissues (Soave et al., 1992). Such an increased uptake has been verified in *A. thaliana* seedlings (30% stimulation of the uptake in FC-treated tissue with respect to control tissue after 90 min of incubation [Soave et al., 1992]).

The synergistic action of FC on Pq toxicity formed the basis of our selective procedure. We screened with FC plus Pq the M_2 progeny of 2010 M_1 ethyl methanesulfonate-mutagenized plants. M_2 seeds from 10 M_1 plants were pooled together, and half an aliquot of each pool was germinated in liquid culture and exposed to FC plus Pq. Because the rare, green seedlings after FC plus Pq treatment do not survive transplanting in soil (even the survival of untreated seedlings from liquid culture was usually very poor in our hands), seeds of the remaining aliquot from each pool, in which at least three green seedlings were present (of about 250), were sown in soil, and the FC-Pq test was repeated on single leaves isolated from each individual plant of the pool. Plants on which the assayed leaf remained green after incubation with FC and Pq were retained and selfed. Two independent mutants were identified in this way (5–2 and 35–1). Mutant plants were selfed for three additional generations, and the transmission of the trait was tested in each progeny with the FC plus Pq test on single leaves. Starting with M_5 homogeneous proge-

Table I. *Stomatal apertures of wild type (La$^+$) and mutant (5-2) leaf discs incubated in the absence or presence of FC*

Leaf discs (about 3 mm in diameter) were incubated for 2 h in the dark in a solution containing 0.5 mM $CaSO_4$, 5 mM K^+, and 250 mM mannitol (see "Materials and Methods").

Genotype	Treatment	Stomata Counted	Closed Stomata	Percent Closed Stomata	Open Stomata	Average Aperture of Open Stomata (mean ± SE)
						μm
La$^+$		121	102	84.3	19	1.65 ± 0.15
La$^+$	FC 5 × 10^{-6} M	238	28	11.7	210	4.21 ± 0.13
5-2		140	132	94.3	8	2.32 ± 0.39
5-2	FC 5 × 10^{-6} M	216	119	55.1	97	2.50 ± 0.14

nies, we tested the mutants for resistance to Pq alone, or HG in the presence and absence of FC, wilting behavior, and stomatal aperture after exposure to FC. Results of mutant 5–2 are reported below.

Pq Resistance

Both 5–2 and wild-type seedlings bleach upon incubation with a concentration of Pq (without FC) 5 times higher than that used in the selective system (not shown).

HG Resistance

HG is an aminoglycosidic antibiotic whose uptake is energized by the electrochemical proton gradient sustained by the PM H^+-ATPase (Perlin et al., 1989; Vallejo and Serrano, 1989). Accordingly, a synergistic action of FC on HG toxicity is expected. In the presence of 100 μM K^+ in the germination medium and in the absence of FC, germination and growth of 5–2 and wild-type seeds are similar at HG concentrations of 0.1 to 16 μg mL^{-1}. The highest concentration is lethal for both genotypes. In the presence of 5 × 10^{-6} M FC instead, a concentration of HG of 4 μg mL^{-1} kills all wild-type seedlings (complete bleaching of cotyledonary leaves), whereas all of the 5–2 seedlings are still green and viable (Soave et al., 1992).

Stomatal Aperture and Wilting

Mean apertures of stomata of leaf discs of 5–2 and of wild type incubated in the dark with or without FC in a solution containing 250 mM mannitol and 5 mM K^+ are reported in Table I. In the absence of the toxin, stomata of both genotypes are for the most part closed: mean aperture of the fraction of open stomata in wild type is 1.65 μm and slightly higher in 5–2. With FC, the majority of stomata of the wild type open with a mean aperture of 4.21 μm; in the mutant, instead, less than 50% of the stomata open, and the mean aperture of this fraction is about one-half of that observed in wild type.

Further experiments (see below) were performed with the M_5 inbred population of 5–2 and, later, replicated with a 5–2 stock recovered after two backcross cycles with the Landsberg wild type.

Fresh Weight Increments and Leaf Disc Area Enlargement Induced by FC in Wild-Type and 5–2 Leaves

Current evidence indicates that the apoplastic acidification induced by FC is accompanied by wall loosening, water uptake, and, consequently, fresh weight increment and leaf disc area enlargement (Marrè et al., 1973). The results of such experiments in the mutant and in the corresponding wild type (La$^+$) are reported in Table II. Incubation for 15 h in a

Table II. *Fresh weight increment and leaf disc area enlargement of wild type (La$^+$) and mutant (5-2) leaf discs incubated in the absence or presence of FC*

Data from two typical experiments; independent experiments gave similar results. See "Materials and Methods" for experimental details.

Geno-type	Treatment	Fresh Weight				Leaf Disc Area						
		Initial	After 15 h	Percentage over initial fresh wt	Stimu-lation by FC[a]	Initial	After 6 h	Percentage after 6 h over initial area	Stimu-lation by FC[a]	After 20 h	Percentage after 20 h over initial area	Stimu-lation by FC[a]
		mg	*mg*			*mm²*	*mm²*			*mm²*		
La$^+$		621 ± 2.0[b]	679 ± 8[b]	9.3		11.3 ± 0.1[c]	12.2 ± 0.14[c]	8.0		12.7 ± 0.10[c]	12.4	
La$^+$	FC 5 × 10^{-6} M					11.3 ± 0.1	13.0 ± 0.17	15.0	0.89	14.5 ± 0.36	28.3	1.28
La$^+$	FC 5 × 10^{-5} M	620 ± 1.0	878 ± 24	41.6	3.44							
5-2		620 ± 2.5	702 ± 5	13.0		11.3 ± 0.1	12.3 ± 0.16	8.8		12.4 ± 0.18	9.7	
5-2	FC 5 × 10^{-6} M					11.3 ± 0.1	12.4 ± 0.12	9.7	0.10	13.1 ± 0.14	16.8	0.73
5-2	FC 5 × 10^{-5} M	620 ± 0.5	763 ± 21	23.1	0.76							

[a] Calculated as fresh weight (or disc area) increment of treated minus increment of the control over increment of the control. [b] Mean ± SE, $n = 3$. [c] Mean ± SE, $n = 25$.

weakly buffered medium at pH 6.0 without the toxin promotes a similar increase in fresh weight (9.3% for the wild type and 13.0% for 5–2 over the initial fresh weight) in both genotypes; with FC, fresh weight increment of the wild type is stimulated (41.6% over the initial fresh weight), whereas the mutant responds to a lesser degree (23.1% over initial fresh weight). Consequently, the stimulation of fresh weight increment induced by FC is reduced in the mutant to about one-quarter of that observed in wild type. Results with leaf discs substantially confirm the previous one: stimulation of leaf disc area enlargement induced by FC in the mutant is lower than that observed in the wild type, especially after 6 h of incubation.

H^+ Extrusion

One of the most typical responses of plant tissue to FC is the stimulation of proton secretion leading to acidification of a weakly buffered external solution (Marrè, 1979). Accordingly, we measured the H^+ extrusion activity of wild-type and mutant plants incubated with or without FC (Table III). Leaf discs from wild-type and mutant plants incubated without the toxin induce an increase in the pH of the external solution with respect to the initial pH (6.50 in our experimental conditions). Such a net disappearance of free protons from the external solution (whose extent is variable among experiments) does not depend on the efflux from the tissue of compounds that become protonated in the medium, because the buffer capacity of the incubating solution remains unchanged before and after the experiment (not shown). It is more likely that protons disappear because they are transported together with other ions inside the mesophyll cells and/or they are trapped by unprotonated acidic groups in the cell wall. Whatever the mechanism, the alkalinization of the medium observed with wild-type and mutant leaf discs suggests that, in this tissue and in our experimental conditions, the activity of PM H^+-ATPase is low and completely subordinated to other processes subtracting protons from the bulk solution.

Treatment of wild-type tissue with FC leads to a decrease in the apparent uptake of H^+, and sometimes a net H^+ extrusion was also observed (i.e. the pH of the external solution becomes more acidic than that at time 0). This is expected because of the stimulation of proton extrusion induced by the toxin. In the mutant, however, the response to FC is much lower than that observed in the wild type. The FC effect for the mutant (calculated as ΔH^+ of the treated minus ΔH^+ of the control samples) is between 0 and 37% of the values of the corresponding wild type in the two sets of experiments.

Inheritance of the Trait

5–2 plants are morphologically indistinguishable from wild-type plants at all growth stages, with the only difference being a slight reduction in size of mutant plants with respect to the wild type. Cell morphology in leaves, stems, and roots is identical in both genotypes except for a moderate reduction in cell dimensions in mutant seedlings (hypocotyls and cotyledonary leaves) cultivated in liquid medium. We, therefore, studied the inheritance of the trait by scoring the HG plus FC resistance and the wilting behavior in F_2 seeds or plants derived from a cross between the mutant and the isogenic wild-type La^+ (Table IV). With the first test, F_2 seeds can be classified into two classes: seeds producing viable seedlings with green cotyledonary leaves and seeds ungerminated or with only a protruding radicle and white cotyledonary leaves. The ratio between the two phenotypic classes indicates a monogenic type of inheritance of the factor con-

Table III. *H^+-extrusion activity of wild-type and mutant (5-2) tissues incubated in the presence or absence of FC*

Values represent the average of triplicate samples; SE never exceed ±5% of the values reported.

Experiment[a]	Genotype	FC	K^+ 200 μM		K^+ 4 mM		K^+ 10 mM	
			ΔH^+[b]	FC effect[c]	ΔH^+	FC effect[c]	ΔH^+	FC effect[c]
			μmol g^{-1} fresh wt		*μmol g^{-1} fresh wt*		*μmol g^{-1} fresh wt*	
1	La^+		−0.8		−0.85			
	La^+	10^{-5} M	+0.2	+1	+2.7	+3.55		
	5-2		−0.7		−1.1			
	5-2	10^{-5} M	−0.6	+0.1	+0.2	+1.3		
2	La^+		−2.4				−2.3	
	La^+	10^{-5} M	−1.4	+1.0			−0.8	+1.5
	5-2		−1.8				−1.6	
	5-2	10^{-5} M	−1.9	−0.1			−1.6	0

[a] Leaf discs (3 mm in diameter) from basal leaves of plants cultivated in soil and that had just flowered. Discs washed two times (1 h each) in 0.5 mM $CaSO_4$ and then for 30 min in 0.5 mM $CaSO_4$, $\pm 10^{-5}$ M FC, 5×10^{-6} M DCMU. Incubation in 0.5 mM Mes (pH 6.50) with BTP, 15 mM Glc, 5×10^{-6} M DCMU ± 10^{-5} M FC, and K^+ (as K_2SO_4) at the concentration indicated (30 mg of tissue per 0.6 mL of solution). Incubation was for 60 min in experiment 1 and 90 min in experiment 2. [b] ΔH^+, Titrated H^+ of the external solution at the end of incubation minus titrated H^+ at time 0. [c] FC effect, Calculated as titrated H^+ in FC-treated minus titrated H^+ in control samples.

Table IV. *Numbers of observed phenotypes in parental lines and in F_2 progenies from the cross (5-2 × La⁺)*

Genotype	HG-FC Test[a]											Wilting Test[e]						
	FC[b]			HG[b]			HG + FC[b]					Control (−FC)			Treated (+5 × 10^{-6} M FC)			
	N.G.[c]	G[c]	N.G.%	N.G.[c]	G[c]	N.G.%	N.G.[c]	G[c]	N.G.[d]	G	χ^2 3:1	S[f]	P.R.[f]	R[f]	S[f]	P.R.[f]	R[f]	χ^2 1:2:1
La⁺ wild type	22	199	9.9	29	85	25.4	148	0				—	—	12	12	—	—	
	31	192	13.9	26	130	16.7	170	2										
5-2	42	229	15.5	26	94	21.7	87	272	9	272		—	—	8	—	—	8	
5-2	37	241	14.9	43	148	22.5	64	210	2	210								
5-2 × *La*⁺	33	150	18.0	54	140	27.8	105	107	46	107	2.09 N.S.[g]							
(F_2 seeds)				43	120	26.4	156	184	66	184	0.26 N.S.							
5-2 (F_2 plants)												—	—	7	16	22	10	1.83 N.S.

[a] Number of seeds with the indicated phenotype present in each growing or testing condition; different lines across each genotype correspond to independent experiments. [b] Sterilized and vernalized seeds grown in Petri dishes on NO_3 medium (Soave et al., 1992), 100 μM K^+ plus 5 × 10^{-6} M FC or 3 μg mL^{-1} of HG or both. [c] N.G., Includes seeds nongerminated or with only a protruding radicle; G, germinated seeds with green cotyledonary leaves. [d] Numbers corrected by discounting from the N.G. class (HG + FC condition, N.G.[c]) the proportion of N.G. seeds when the corresponding seeds were grown in the presence of HG alone. [e] Wilting test on cut stems incubated as described in "Materials and Methods." [f] S, Sensitive, plants completely wilted; P.R., partially resistant plants, i.e. plants with initial symptoms of wilting; R, resistant plants with leaves, stem, and flowers turgid. [g] N.S., Not significant.

ferring resistance to HG plus FC with the mutant allele associated with a dominant expression of the trait.

In the wilting test, F_2 plants were classified into three phenotypic classes: plant completely wilted, plants partially wilted, and plants unwilted, in a ratio of approximately 1:2:1. Data again indicate a monogenic inheritance but with a dose-dependent phenotypic expression of the mutant allele.

DISCUSSION

In the absence of knowledge of the basic physiological role of the FC receptor (other than that of binding the toxin), a selective procedure for identifying mutants altered in the receptor function can be based either on the direct assay of FC binding to the PM receptor (practically impossible on an individual basis) or on the physiological consequences induced by the binding of the toxin. Obviously, this last approach can sort out mutants modified in several other steps in addition to those in which the formation of the complex FC receptor is altered, such as mutants in PM H^+-ATPase, in K^+ uptake, etc. Such mutants could in principle be as interesting as mutants altered in FC receptor, and a direct test of FC binding, PM H^+-ATPase activity, or K^+ uptake can be run on any independent mutant isolated.

The selective procedure adopted by us is based on one of the consequences induced by FC binding, i.e. the stimulation of cation uptake, depending, in turn, on the FC-induced activation of PM H^+-ATPase. This chain of events also holds true for the toxic cation Pq (as well as for HG) used by us as the selective agent, independent of its route of entry (carrier mediated or channel [Hart et al., 1992]), as revealed by the higher uptake of the drug in the presence of FC and by the synergistic effect of the toxin on Pq toxicity (Soave et al., 1992). Using this technique we isolated two mutants resistant to FC and Pq with a frequency (1 on about 1000 M_1 plants) approximately corresponding to that expected from random mutagenic events in *A. thaliana*.

The main feature of one of the mutants isolated (5–2) is the absence of any relevant difference in the morphology or in the physiological functions assayed in the absence of FC with respect to the isogenic wild type. Plants of both genotypes are practically indistinguishable morphologically. Sensitivity to both Pq and HG alone is similar, as is the extent of increment of fresh weight or of leaf disc area after incubation of the tissues in the absence of FC.

The two genotypes can be distinguished when tissues are treated with FC. In this condition, the mutant appears either not responsive to the toxin (for example, for the phenotypes observed with the FC plus Pq or FC plus HG treatments or in wilting behavior) or with a reduced response (stomatal aperture, increment in fresh weight or in leaf disc area, and H^+ extrusion).

The extent of the reduced response varies, depending on the type of experiment. For stomata, the fraction that opens in the mutant after FC treatment is about one-half of that observed in wild type, and the mean aperture of these stomata is 60% of that of the wild type. FC stimulation of fresh weight increment is about 20% of that of the wild type and ranges from 11% (after 6 h) to 58% (after 20 h) for leaf disc area enlargement; similar values are observed with the FC-induced stimulation of proton extrusion. These results indicate that a certain capability to respond to FC is retained in the mutant, but this residual response is not enough to produce visible toxic effects when tissues are incubated with FC and Pq or HG or to induce wilting symptoms in cut stems.

Several hypotheses explaining the reduced sensitivity of 5–2 mutant to FC, coupled with its normal morphology, can be suggested, such as a reduced accessibility of the toxin to its target. It should be noted, however, that FC stimulation of H^+-extrusion activity in wild-type *A. thaliana* seedlings saturates at concentrations of approximately 1 to 2 × 10^{-6} M (our unpublished observations), and usually our tests on the mutant have been done at concentrations of the toxin 5- to 10-fold higher. Other possibilities include alterations in the receptor or a modification in a specific H^+-ATPase isoenzyme that is activated only in the presence of FC (or, in particular, growing or stressing conditions). Until now we can only emphasize that the reduced response of the mutant to FC is

evident at external K^+ concentrations of 200 μM and 10 mM, suggesting that K^+ transport in the high- or low-affinity range is not involved in the phenotypic expression of the trait.

In conclusion, and perhaps this is the most important contribution of this work, we provide evidence that our selective procedure can pick up mutants with an altered response to FC. Therefore, it should be possible to isolate other mutants nonallelic to those described here, thus allowing genetic dissection of the steps involved in cellular responses to the toxin.

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