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Isolation and characterization of a barley mutant with abscisic-acid-insensitive stomata

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Abstract. A barley (*Hordeum vulgare* L.) mutant ("cool") with leaf transpiration unaffected by the application of 1 mM abscisic acid (ABA) was isolated from the population of M2 seedlings using thermography (electronic visualization and quantitation of the temperature profiles on the surface of the leaves). Stomata of the mutant plants were insensitive to exogenously applied ABA, darkness, and such desiccation treatments as leaf excision and drought stress. The evaporative cooling of the leaves of the "cool" barley was always higher than that of the wild-type barley, even without ABA application, indicating that the diffusive resistance of the mutant leaves to water loss was always lower. Guard-cell morphology and stomatal density as well as ABA level and metabolism were seemingly unaltered in the mutant plants. In addition, gibberellin-induced α-amylase secretion and precocious embryo germination in the mutant barley was inhibited by ABA to the same extent as in the wild-type barley.

Key words: Abscisic acid (barley) – Abscisic acid-insensitive mutants – *Hordeum* (ABA-insensitive mutant) – Stoma (ABA-insensitivity) – Thermography.

Introduction

Plants unresponsive to certain endogenous plant growth regulators provide a valuable material for studying hormone action. Several plant mutants deficient in their ability to respond to abscisic acid (ABA) have been recently identified. For example, Koornneef et al. (1984) were able to select *Arabidopsis thaliana* plants which could grow on normally toxic levels of ABA. Robichaud et al. (1980) described viviparous mutants of corn which could germinate in the presence of ABA. Barley mutants with an increased resistance to the inhibitory effect of ABA on gibberellin (GA)-induced α-amylase secretion from aleurone tissue were discovered by Ho et al. (1980). Recently, Hickok (1985) found mutants of the fern *Ceratopteris* in which ABA did not inhibit growth and male sexual differentiation in gametophytes.

Abscisic acid is known to cause stomatal closure and is likely to function as a natural regulator of stomatal movements (see Davis et al. 1981 for a review). Therefore, identification of mutants deficient in the ability of their stomata to respond to ABA may lead to a better understanding of stomatal regulation and the nature of the ABA receptor in guard cells. Several mutants with altered stomatal behavior have been already identified. For example, wilty mutants which are unable to produce sufficient amounts of ABA have been found in potato (Quarrie 1982), tomato (Tal 1966; Imber and Tal 1970; Tal and Nevo 1973), and peas (Marx 1976; Wang et al. 1984). These plants wilt under conditions in which normal plants remain turgid. Normal turgor can be, at least partially, restored by foliar spraying with ABA.

Thermal imaging (thermography) is the electronic detection and display of long-wave or far-infrared radiation. This technique allows visualization and quantitation of temperature differences on the surface of the objects, with a 0.1° C resolution. In spite of its potential as a powerful and efficient data-collection technique, thermography has been only rarely used for the study of plants (Hashimoto et al. 1984). Since evapo-transpiration is the major component in the energy balance of

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Abbreviations: ABA = (±) cis-trans abscisic acid; GA = gibberellin

the leaf, stomatal closure results in a rapid warming of the leaf surface, making thermography a very useful tool for identifying plants deficient in the ability to close their stomata in response to ABA. In this paper we demonstrate the successful use of thermography in the identification of a barley mutant with ABA-insensitive stomata and presents data on the physiological and biochemical characterization of this mutant.

Material and methods

Chemicals. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) unless stated otherwise.

Plant material. M2 (second generation mutant) seeds (caryopses) of barley (*Hordeum vulgare* L. cv. Morex) collected from the plants grown from the seed mutagenized in 1 mM sodium azide solution (Kleinhofs et al. 1978) were a gift from Dr. R.A. Nilan (Washington State University, Pullman, USA). M2 and wild-type seeds of the same cultivar were planted 1.3 cm deep in 4.5-cm square pots filled with Pro-Mix A (A.H. Hummert Seed Co., St. Louis, Mo., USA). The pots were placed in metal trays for sub-irrigation, and the trays moved to a greenhouse equipped with supplementary sodium-halide lights providing a 16-h photoperiod with day temperature 22° C and night temperature 16° C. Seedlings were grown in the greenhouse for 7–9 d until the second leaf started to expand, and then moved to a growth chamber maintained under the following conditions: day temperature 22° C, night temperature 16° C, 16 h photoperiod with a photon fluence rate of 1200 μmol photons·m^{-2}·s^{-1} of photosynteticaly active radiation at the plant level and 50% relative humidity (RH).

Screening for mutants. On the morning following the transfer of plants to the growth chamber, seedlings were sprayed to run off with 1 mM aqueous solution of ABA containing 0.05% Triton X-100. The spraying was done 1 h after the lights went on. Two hours later the leaf temperatures of the sprayed plants were compared to these of control non-mutagenized barley plants (cv. Morex) which were sprayed only with the 0.05% Triton X-100 solution. Leaf temperature was evaluated with a Thermal Video System (Model TVS-4300; Hughes Aircraft Co., Carlsbad, Cal., USA) equipped with a Computer Interface Unit (Model 999RO40; Hughes Aircraft Co.) integrated with an IBM Personal Computer (Model 5150; IBM Corp., Armonk, N.Y., USA). The image was displayed on a color monitor (Model Color IV; Amdek Corp., Elk Grove Village, Ill., USA). Suspected mutants, with leaf temperatures close to the unsprayed control, were transplanted to 15-cm-diameter round pots and grown in the greenhouse to maturity. All the emerging spikes were bagged to prevent cross pollination. M3 caryopses of suspected mutants were germinated, grown to the second-leaf stage, and screened again for the elevation in leaf temperature in response to foliar application of ABA according to the above procedure.

Thermographic measurements of transpiration. Second (flag-2) or third (flag-3) leaves below the flag leaf were cut off from the greenhouse-grown plants at 11:00 h approx. 2 cm above the ligule, and the cut ends of the leaves placed in 15-ml test tubes containing 3 ml of water or aqueous solution of ABA. The tubes in the test-tube racks were then moved to the growth chamber (22° C, 1200 μmol photons·m^{-2}·s^{-1} at the plant level and 50% RH). After 3 h incubation the leaf-surface temperature was recorded by thermography. Thereafter, the lights were turned off and the leaves were incubated in darkness (22° C, 50% RH) for another 3 h and another thermal image was taken. For the desiccation treatments, both ends of the detached leaves were attached to a piece of cardboard with tape, and the cardboard was placed vertically in the growth chamber set at 22° C, 1200 μmol photons·m^{-2}·s^{-1} and 50% RH. The leaf-surface temperature profile was recorded 40 min after leaf excision.

Measurements of ABA levels and leaf water potential. Flag-2 or Flag-3 leaves were cut off from the greenhouse-grown plants at 16:00 h and the leaf water potentials measured with a pressure chamber (Model 1000; PMS Instrument Co., Corvallis, Ore., USA). Immediately after taking the measurement the detached leaves were frozen in liquid N_2, freeze-dried in a Dura-Dry freeze-dryer (Model TDS-3-S; FTS Systems, Stone Ridge, N.Y., USA), weighed, ground in a test tube with a glass rod, and extracted twice with acetone containing 1% acetic acid and 100 μg·ml^{-1} butylated hydroxy-toluene (pH 5.6) for 2 h at 4° C. For the third extraction, 20% water was added to the extraction solution. The extracts were filtered through Whatman No. 1 filter paper. The three filtrates were combined and the acetone evaporated under N_2. Immunological enzyme-linked immunosorbent assay (ELISA) analysis of ABA levels was performed in Immulon-2 96-well microtiter plates (Dynatech Laboratories, Alexandria, Va., USA). The plates were treated with short-wave ultraviolet light for 20 min, washed with distilled water, and dried. The wells were coated with 250 μl of buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3.08 mM NaN_3, pH 7.5) containing 10 μg·ml^{-1} of the antigen, Concanavalin A-ABA, prepared by linking the carboxylic acid moiety of ABA to an amino-acid residue of Concanavalin through a carbodiimide linkage (Schwalby et al. 1984; Newsom 1985). The plates were covered and left overnight at 37–40° C.

Competitive immunoassay samples were prepared by adding 50 μl of monoclonal antibodies to ABA (Idetek, San Bruno, Cal., USA) diluted 1:25 with phosphate-buffered saline solution (8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 0.2 g KCl, 0.2 g NaN_3 dissolved in 1 l of H_2O, pH 7.5) to either 1 ml of ABA standards (0.03–5 ng·ml^{-1}) or to plant tissue extract diluted with phosphate-buffered saline solution containing 1% bovine serum albumin (pH 7.5), and the mixtures incubated overnight in small test tubes. The next morning the wells of the microtiter plates were washed with the phosphate-buffered saline solution (pH 7.5) supplemented with 0.05% polyoxyethylene sorbitan monolaurate (Tween-20) as a surfactant, and 250 μl of competitive assay sample was added to each well. After 2–3 h incubation at room temperature the content of the wells was discarded and the wells washed two or three times with phosphate-buffered saline supplemented with Tween-20. The wells were then filled with 250 μl of a solution of alkaline-phosphatase-labeled anti-mouse antibodies diluted 1:1000 with the phosphate-buffered saline solution containing 0.5% bovine serum albumin. After 2 h incubation at room temperature the wells were washed with distilled water, and the 250 μl of the enzyme substrate, p-nitrophenol phosphate (1 mg·ml^{-1}), was added to each well. The reaction could be slowed by adding 3 N NaOH (50 μl) after 30 min. The absorbance at 405 nm was read 1–2 h later with a EIA reader (Model Titertek Multiscreen MC; Flow Laboratories, McLean, Va., USA). Levels of ABA which could be quantified using this procedure were 0.039–5 ng·ml^{-1}.

α-Amylase assay. Barley (*Hordeum vulgare* L. cv. Himalaya) caryopses were received from Washington State University

Agronomy Club (Pullman, USA). Each caryopsis was cut in half transversely and α-amylase production by the embryoless half was measured according to Jones and Varner (1967). Gibberellic acid (GA_3) was used in 1 μM concentration and ABA at 0.1 mM concentration.

Measurements of ABA metabolism. Flag-2 leaves were cut at the ligule, wiped with 70% ethanol, rinsed in sterile water, and the cut end cut once more under sterile water. For each experiment three sterilized leaf blades (about 20.5 cm long) were placed in three sterile 25-ml test tubes with the proximal ends of the leaves immersed in 2 ml of sterile 10 mM K-phosphate buffer containing 177.6 kBq RS [G-^{3}H]abscisic acid (Amersham, Arlington Height, III., USA), specific activity 2.55 TBq·mmol^{-1}. After 8 h incubation at 22° C and 1200 μmol photons·m^{-2}·s^{-1} the cut ends of the leaves were rinsed in water and each leaf ground separately in liquid N_2 in a test tube with a glass rod. The resulting powder was extracted twice at 4° C in 3 ml of 80% aqueous methanol containing 100 μg·ml^{-1} of butylated hydroxytoluene (pH = 8.0), and centrifuged at 8000·g for 10 min. The supernatants from both extractions were combined and filtered through Whatman No. 1 filter paper. The pellet was freeze-dried in a Dura-Dry freeze-dryer (Model TDS-3-S; FTS Systems), combusted in a Packard Oxidizer (Model 306; Packard Instruments Co., Downers Grove, III., USA), suspended in 10 ml of Ready-Solv HP scintillation fluid (Beckman Instruments, Fullerton, Cal.) and its radioactivity determined in a Tri-Carb Liquid Scintillation Spectrometer (Model 4640; Packard Instruments Co.). Three milliliters of *n*-hexane and 3 μl of 6 N NaOH were added to the combined supernatant, and the mixture centrifuged at 8000·*g* for 10 min. This procedure was repeated twice. An aliquot of the lower hexane fraction was suspended in 10 ml of Ready-Solv HP and its radioactivity determined in a Tri-Carb Liquid Scintillation Spectrometer. The upper methanol/aqueous fraction was dried under N_2, resuspended in 100 μl of original extraction solution and subjected to thin-layer chromatography on Silica gel 60 F-254 plates (E. Merck Industries, Darmstadt, FRG) using toluene:ethyl acetate:acetic acid (50:30:4, by vol.) as the developing solvent. The plates were dried overnight and divided into 31·5 mm^2 strips which were individually scraped from the plate, eluted overnight in 200 μl of 80% methanol, suspended in 10 ml of Ready-Solv HP, and counted in a Tri-Carb Liquid Scintillation Spectrometer. Abscisic acid standards were chromatographed on the same plate with the methanolic plant extracts.

Volumetric measurements of transpiration. At approx. 10:00 h flag-2 and flag-3 leaves from mutant and wild-type barley plants grown in the greenhouse were cut with a sharp razor blade 2 cm above the ligule. The cut ends of the leaf blades were placed in 15 ml test tubes containing 7 ml of water or ABA solution. Test tubes were placed vertically in test-tube racks and the racks placed in the growth chamber set at the conditions described above. Twenty four hours later the leaves were removed from the tubes, and their area measured with a Li-Cor (Neb., USA) Portable Area Meter (Model LI-3000). Thereafter, the volume of the water remaining in the test tubes was determined and the transpiration rate per unit area calculated.

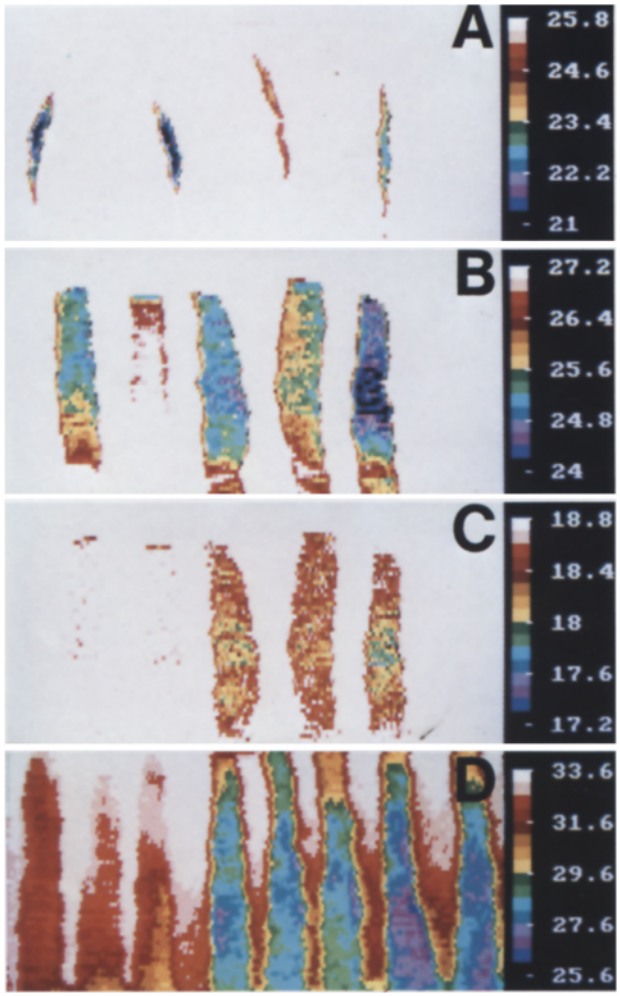

Fig. 1A–D. Thermal images of "cool" and wild-type barley subjected to different treatments. **A** Response of "cool" and wild-type barley seedlings to 1 mM ABA spray. *From left to right:* "cool" mutant sprayed with ABA, "cool" mutant control, wild-type sprayed with ABA, wild-type control. Each color represents a temperature difference of 0.3° C. **B** Excised leaves of "cool" and wild-type barley were incubated in light for 3 h. The cut ends of the leaves were immersed in 3 ml of water or ABA solution. *From left to right:* wild-type control, wild-type fed with 0.1 mM ABA solution, "cool" mutant H_2O control, "cool" mutant fed with 0.1 mM ABA, "cool" mutant fed with 1 mM ABA. Each color represents a temperature difference of 0.2° C. **C** The effect of 3 h of darkness on the same leaves as depicted in **B**. Each color represents a temperature difference of 0.1° C. **D** The effect of 40 min of desiccation on excised leaves of wild-type barley (*three leaves on the left*) and "cool" barley (*five leaves on the right*). Each leaf was cut from a different plant and left without water supply for 40 min. Each color represents a temperature difference of 0.5° C

Results

Ten thousand M2 barley seedlings were screened by thermography to determine if they respond to a foliar application of 1 mM ABA by a reduction in leaf transpiration and an increase in leaf temperature. Six plants were suspected as having ABA-insensitive transpiration. These plants were grown to maturity and their M3 progeny subjected to another thermographic screening. The M3 seedlings grown from the seeds collected from five potential mutants responded to ABA spraying with the increase of the leaf temperature, characteristic of the

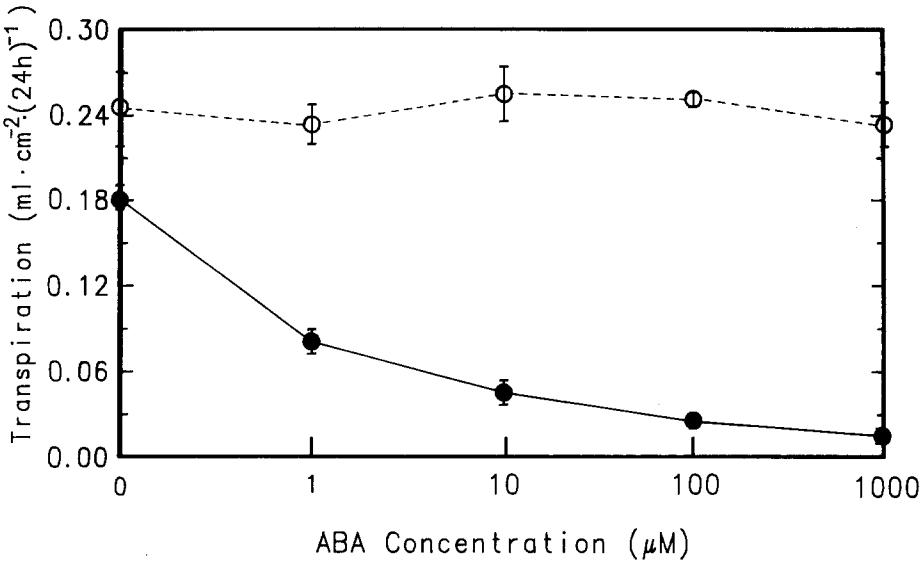

Fig. 2. The effect of ABA on the transpiration of the "cool" (o) and wild-type barley (•). Each point is the average of six leaves. Bars = ±SD. When no bars are given the SD is smaller than the symbol used

wild-type. However, the leaf transpiration of the entire progeny from one plant was completely unaffected by ABA. The leaves of these plants remained consistently cooler than the leaves of the wild-type barley. Because of this characteristic the identified mutant was named "cool" barley mutant. When epidermal strips and nail-polish imprints of leaves were examined through a light microscope, no differences in the stomatal morphology or cuticule thickness between the mutant and wild-type were detected, except that the stomatal pore was always visible on the leaves of the "cool" barley. Frequency of stomata also did not differ visibly between the two plant types. "Cool" barley plants had slower growth, two to three week delay in anthesis, significantly reduced stature at maturity and slightly hypochromic and leathery leaves. When provided with ample irrigation, the leaves of the "cool" mutant showed some delay in senescence compared to the leaves of the wild-type. As expected, even short interruptions in water supply caused rapid dessication of the mutant barley. Detached leaves of the "cool" barley lost most of their water within a short time and could be easily crumbled between fingers.

As indicated above, mutant plants could be readily visualized on the thermography screen as being significantly cooler than wild-type barley (Fig. 1 A). The average temperature of the leaf surface of the ABA-treated and untreated mutant was about 21.6° C, while the temperature of ABA-treated wild-type was 24° C. The temperature of the second leaf of untreated wild-type was 22.5° C which was still warmer than that of the mutants. This difference in temperature indicated that the transpiration rates of the leaves of the mutant barley were always higher than those of the wild-type. Figure 1 B shows the temperature differences of the detached leaves of wild-type and mutant barley fed for 3 h in light with water or ABA solution (1 mM and 0.1 mM). While the transpiration rates of the wild-type plant were reduced by 0.1 mM ABA, which made them at least 2° C warmer, the leaves of the mutant plant showed no reduction in transpiration even if supplied with 1 mM ABA. After 3 h, the lights in the growth chamber were turned off. The thermal image taken after 3 h of darkness showed that all leaves excised from the mutant plants continued to transpire at a higher rate than leaves from the wild-type plant (Fig. 1 C). As in light, in darkness ABA at 0.1 and 1 mM had no effect on the transpiration of leaves of the mutant plants. When the detached leaves of "cool" and wild-type barley were allowed to wilt in light at 22° C for 40 min without water supply, the temperature of the wild-type leaves was about 5° C warmer than that of the leaves excised from the mutant plant (Fig. 1 D). The same experiment was conducted for 2 h with the leaves weighed before and after the desiccation treatment. The six detached leaves of the mutant lost 68.4% ± 7.1 (SD) of their weight as a result of this treatment, while the six leaves of the wild-type lost only 19.7% ± 5.2 (SD). Figure 2 shows the results of longer-term volumetric measurements of ABA effects on leaf transpiration in "cool" and wild-type barley. In this experiment the aqueous solutions containing different ABA concentrations were fed through the cut proximal ends of the leaves for 24 h. In wild-type barley 1 mM ABA virtually stopped transpiration, while 1 μM ABA reduced it by 56% (Fig. 2). In dramatic contrast to these

Table 1. ABA concentrations and mid-day water potential of flag-2 or flag-3 leaves of "cool" and wild-type barley grown in the greenhouse. Each value is the mean of three leaves ±SD

	ABA ($\mu g \cdot (g\ DW)^{-1}$)	Leaf water potential (MPa)
Wild-type	1.53±0.27	−0.80±0.06
"Cool" mutant	1.70±0.44	−0.82±0.04

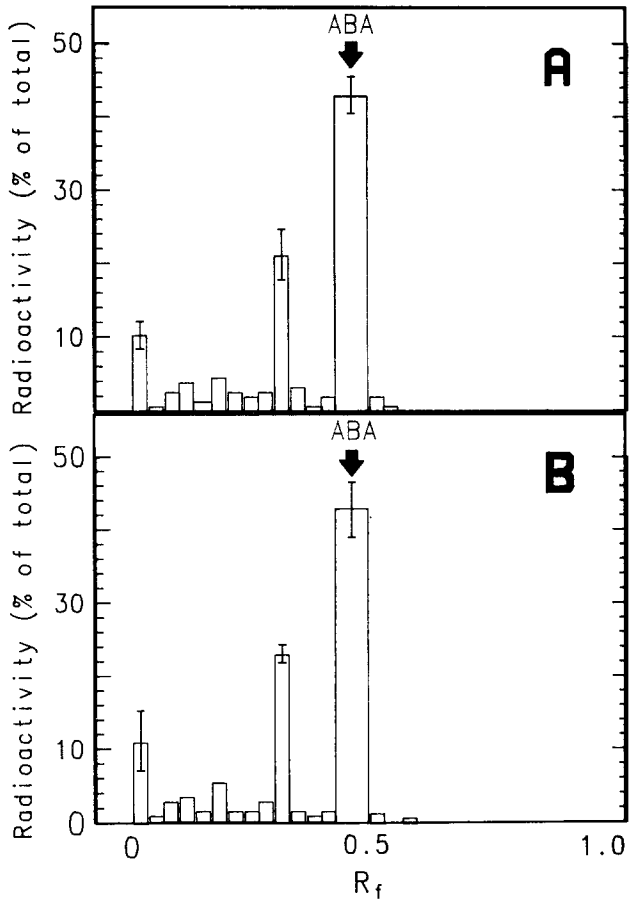

Fig. 3A, B. Distribution on a thin-layer chromatogram of radioactivity in ABA and its catabolites from extracts of excised leaves of the wild-type (**A**) and "cool" (**B**) barley. The total radioactivity taken up by the leaves of the wild-type barley was 58 kBq and 61 kBq for the leaves of "cool" barley. The recovery of total leaf radioactivity in the methanolic plant extract was 97%. Each histogram is the average of three leaves. Bars = ±SD

results, ABA had no effect on transpiration of the mutant barley. Even in the absence of ABA, the mutant leaves transpired 37% more water than wild-type leaves.

No significant differences in the amounts of the endogenous ABA were found in the leaves of the greenhouse-grown mutant and wild-type plants (Table 1). Also the mid-day water potential measured at 16:00 h, 8 h after the lights were turned on, was not significantly different between the wild-type and the mutant (Table 1). However, the average early-morning leaf water potential of three leaves from different plants, measured just before sunrise, was 0.53 MPa±0.03 (SD) for the mutant and 0.20 MPa±0.5 (SD) for the wild-type. Therefore, even when provided with ample irrigation, the "cool" barley experiences some water stress. One conceivable cause of the lack of sensitivity to ABA may be its rapid metabolism in the "cool" mutant. However, the metabolic profile of exogenous ABA showed a quantitative and qualitative similarity between the "cool" and wild-type barley (Fig. 3).

Table 2. The effect of ABA and GA_3 on α-amylase production by embryoless half-seeds of wild-type and "cool" barley. Each number is the average of three replicate treatments each using 10 half-seeds, ±SD. Average weights of 10 half-seeds were 0.63±0.01 g for the wild-type and 0.52±0.01 g for the "cool" mutant

	$A_{620\ nm} \cdot (g\ DW)^{-1}$		
	Control	GA_3 (1 μM)	GA_3 (1 μM)+ ABA (0.1 mM)
Wild-type	0.75±0.04	0.11±0.03	0.62±0.03
"Cool" mutant	0.82±0.06	0.11±0.05	0.62±0.20

In addition to causing stomatal closure, ABA is known to inhibit GA_3-induced α-amylase release from barley aleurone (Chrispeels and Varner 1966), and to block precocious embryo germination in many plants (for reviews see Walbot 1978; Quatrano 1987). While the stomata of the mutant were completely insensitive to ABA, no significant differences were observed in the degree of ABA inhibition of GA_3-induced α-amylase release from the aleurone layer of "cool" and the wild-type barley (Table 2). In half-seeds of both "cool" and wild-type barley the addition of 0.1 mM ABA to medium containing 1 μM GA_3 caused a 5.6-fold reduction in α-amylase release. Similarly, no differences were found in the ability of ABA to inhibit the precocious germination of immature embryos of the "cool" and the wild-type barley. In both genotypes the addition of 0.01 μM ABA to the culture medium substantially inhibited precocious embryo germination, while 0.1 μM ABA completely blocked it (data not shown).

Discussion

Several explanations can be given for the complete inability of "cool" barley stomata to respond to ABA. The increase in ABA metabolism could not

account for this lack of sensitivity, since the metabolism of exogenous ABA in the "cool" barley leaves followed the pattern similar to that of the wild-type (Fig. 3). A large reduction in endogenous ABA content can potentially decrease the tissue sensitivity to exogenous ABA. Alternatively, the lack of sensitivity of some plant mutants to a particular hormone was shown to be coincidental with the increase in the concentration of this hormone (Koornneef et al. 1984). However, the comparison of the levels of endogenous ABA in "cool" and wild-type barley did not show significant differences (Table 1). The unlikely possibility that the "cool" barley is different from the wild-type in the rate of ABA uptake into the guard cells can be discounted because even massive doses of ABA combined with different methods of its application were completely ineffective in closing mutant stomata. Therefore, it seems likely that the "cool" barley is deficient in the ability of its guard cells to respond to ABA or to any other treatment reported here. At this time it remains to be established whether the "cool" barley is a true ABA-receptor mutant, or the stomata of the "cool" barley have an anatomical or physiological lesion not directly related to ABA action, which prevents closure under any conditions. The latter possibility can explain the apparently unaltered ability of ABA to inhibit GA_3-induced α-amylase secretion (Table 2) and block precocious embryo germination in "cool" barley. If, however, the "cool" barley cannot regulate its transpiration because of a lesion in the stomatal ABA receptor, the existence of different ABA receptors in different tissues should be postulated. The unaltered water relations in the otherwise ABA-insensitive *Arabidopsis thaliana* mutant (Koornneef et al. 1984) seem to be consistent with this intriguing possibility. More research in this area may lead to a better understanding of hormone action in plants.

The successful use of thermography for the identification of the "cool" barley mutant has confirmed that this technique can be a powerful and efficient tool for studying stomatal physiology and other biological processes which cause temperature changes.

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