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Hormonal Interactions and Stomatal Responses

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ABSTRACT

Both environmental and hormonal factors and their interactions affect stomatal behavior. Methodologies for identifying hormonal interactions affecting stomatal function are reviewed. Although there is abundant evidence that abscisic acid (ABA) closes stomata, evidence that the other classical plant hormones (auxins, cytokinins, ethylene, gibberellins) in isolation alter stomatal response often comes from exogenous applications to detached epidermes and leaves, rather than correlation of endogenous concentrations with stomatal conductance (g_s). Evidence for hormonal interactions comes from isolated tissues with exogenous hormones supplied at nonphysiological concentrations, or from variation in stomatal response to xylem ABA concentration *in planta*. The roles of hormonal changes in causing stomatal closure following changes in soil environment are considered. Although soil drying induces multiple changes in xylem sap composition, analysis of stomatal responses suggests a dominant role for increased endogenous ABA concentrations and relatively little evidence of roles for other hormones. A similar picture emerges from studies of soil compaction. Although soil flooding decreases ABA export from the root system, there is some evidence that apoplastic ABA accumulation elicits stomatal closure. Stomatal closure following nitrogen deprivation does not appear to involve ABA and may provide a suitable experimental system to investigate roles for other hormones. The availability of mutant or transgenic lines with altered hormone homeostasis or sensitivity provides opportunities to screen for altered stomatal behavior in response to different environments, and may provide new evidence that hormonal interactions are important in the control of stomatal behavior.

Key words: Stomata; Hormone interactions; Abscisic acid; Auxins; Cytokinins; Soil drying

INTRODUCTION

Drury (1969) introduced the concept of ''hormonal interactions'' to the literature, suggesting that a balance of hormone concentrations might control physiological processes. Recently, as exemplified by several papers in this volume, it has been shown that plant hormones are able to influence the biosynthesis or signal transduction of other hormones. To some extent, an investigation of the physiological significance of hormonal interactions to a given process will be driven by available technology and research perspective. Hormonal interactions and stomatal responses are unlikely to be studied at the level of guard cell hormone biosynthesis, as quantification of guard cell hormone concentrations is rarely achieved (but see Zhang and others 2001), and may be of limited relevance to stomatal response due to the occurrence of extracellular hormone receptors (Anderson and others 1994). Much

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progress has been made in the study of intracellular signal transduction when guard cell protoplasts and guard cells in isolated epidermes are supplied with exogenous hormones, and is reviewed elsewhere (for example, McAinsh and others 2000). This review takes a physiological perspective in questioning whether the balance of hormone concentrations is important to stomatal behavior, in both isolated systems and whole plants.

Because stomata regulate the exchange of CO_2 and water vapor between the leaf and the atmosphere, control of stomatal conductance (g_s) is essential for both resource acquisition and to prevent desiccation. Stomata respond to many changes in their aerial (external) environment such as light, CO_2 concentration, temperature and evaporative demand. Guard cells also respond to changes in their internal environment (the leaf apoplast) such as ionic composition and hormone concentrations. Microinjection of hormones into individual guard cells has also shown that stomata can perceive increases in symplastic hormone concentrations (Schwartz and others 1994). Given the diversity of factors that stomata can sense, interactions between hormones represent only part of the control of stomatal behavior. Of more importance to the plant is whether plant hormonal status determines stomatal response to the environment.

In assessing the importance of hormonal interactions, and hormone x environment interactions, to stomatal behavior, different experimental systems for the study of stomatal responses are introduced in the Methodology section. Stomatal responses to the classical plant hormones (abscisic acid, auxins, cytokinins, ethylene, gibberellins) in isolation are reviewed later in the Hormonal Effects and Interactions section, as physiological activity of a single hormone suggests it may interact with other hormones to influence stomatal behavior. Hormonal interactions, and interactions between plant hormonal status and environmental variables are also considered in this section. Stomatal behavior is most critical when plants experience environmental change, thus case studies assess the roles of observed changes in plant hormone status to stomatal response. Changes in the edaphic environment are considered, reflecting the progress made in understanding how root-to-shoot signalling affects g_s. Many of the examples in this review consider stomatal response to ABA because stomatal responses to other hormones have received less attention and in many situations (for example, Figure 1), stomatal responses to ABA are of a greater magnitude than responses to other hormones.

Methodology

Manipulation and Measurement of Hormone Concentrations in Intact Plants

Foliar spraying or soil application of plant hormones can modify stomatal behavior in intact plants and may be agriculturally useful. Although such applications avoid potential artefacts of isolated plant parts, endogenous hormone concentrations are not always measured and will depend on hormone uptake, transport and catabolism. However, soil application of various ABA concentrations has generated differences in xylem ABA concentration, demonstrating that ABA can quantitatively explain the effects of soil drying on g_s (Zhang and Davies 1990; Tardieu and others 1996).

Although transgenics and mutants with altered hormone homeostasis and sensitivity allow stomatal physiologists to explore hormonal interactions, their use has been somewhat limited. For example, the ability of indole-3-acetic acid (IAA) to antagonize ABA-induced stomatal closure (Snaith and Mansfield 1982b) (see Figure 2) could be explored by submitting auxin overexpressing plants and wild-type (WT) plants to soil drying and comparing stomatal sensitivity to xylem ABA concentration between lines. Care must be taken to ensure that stomatal responses are compared on a similar basis, such as leaf age, if the transgenics/mutants show profound changes in plant development (for example, changes in leaf morphology or initiation).

Grafting theoretically allows leaf and xylem hormone composition to be manipulated independently, which can be important in studies of root-to-shoot signalling. For example, the importance of root-sourced ABA in mediating drought-induced stomatal closure has been addressed by reciprocal grafting of WT and ABA-deficient genotypes and comparing the stomatal responses and ABA concentrations of the graft combinations (Holbrook and others 2002).

Irrespective of whether hormone concentrations are manipulated by exogenous or endogenous means, it may be necessary to measure hormone concentrations in a plant compartment that is relevant to the observed stomatal response. Ideally, this would be the hormone concentration adjacent to the receptors. However, guard cell receptor location is known only for ABA, both intracellular (Schwartz and others 1994) and extracellular (Anderson and others 1994). Although measurement of guard cell ABA concentration (Zhang and others 2001) is relevant to intracellular ABA receptors, there can be no assurance that measurement of

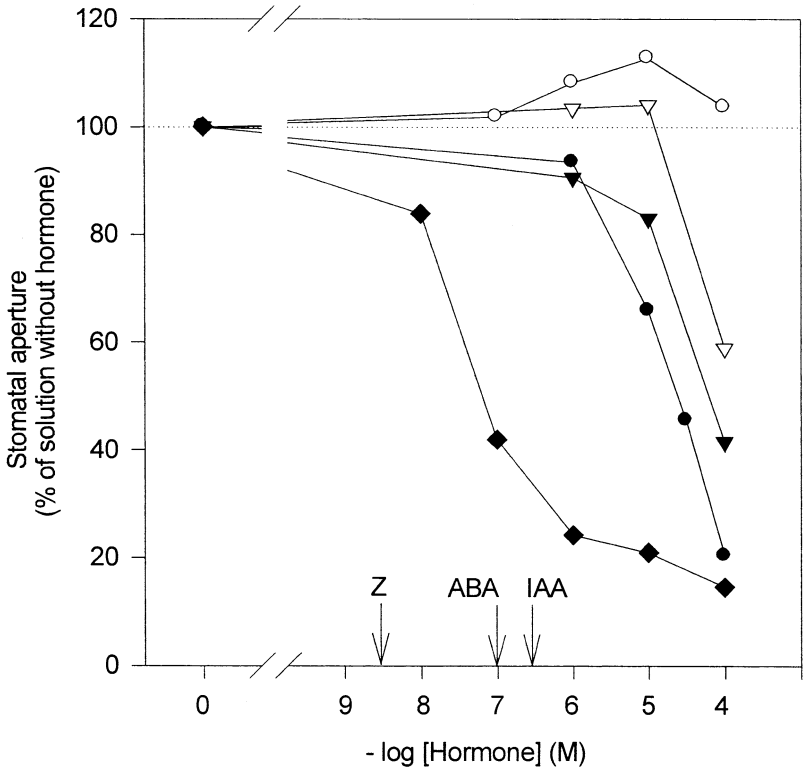

Figure 1. Stomatal aperture of *Commelina communis* when epidermal strips were floated on solutions containing ABA (◆), the naturally occurring auxin IAA (○), the synthetic auxin NAA (●), the naturally occurring cytokinin zeatin [Z] (▽) and the synthetic cytokinin kinetin (▼). Strips were incubated at 25°C in CO_2-free air at a light intensity of 100–280 μmol $m^{-2}s^{-1}$. Hormone-solutions were made up in a buffer containing 50 mM KCl and 10 mM MES at pH 6.15. Data were re-drawn fromTrejo and others 1993 (◆), Snaith and Mansfield 1982a (○), Snaith and Mansfield 1984 (●) and Blackman and Davies 1983 (▽,▼). Arrows on the x-axis indicate representative xylem hormone concentrations for cotton (from Hartung and others 1992).

other guard cell hormone concentrations (if technically possible) would be physiologically meaningful, if other hormone receptors were extracellular. Accordingly, xylem sap is often assayed for hormones as it is assumed to be in direct contact with the leaf apoplast. Xylem ABA concentration was shown to be a better predictor of stomatal response than bulk leaf ABA concentration during soil drying (Zhang and Davies 1990). Representative xylem hormone concentrations (Hartung and others 1992) for cotton (a species in which several hormones have been measured in the same xylem sap samples) are plotted in Figure 1 to provide a reference point with which to compare hormone dose-response curves in isolated epidermes.

Detached Leaf Transpiration Bioassays

One approach to identifying hormonal interactions has been the gravimetric measurement of transpiration in detached leaves supplied with solutions of defined hormonal composition via the xylem. This can be useful in assessing the physiological significance of changes in xylem sap hormone composition, but depends on the accuracy with which xylem hormone concentrations are determined. Xylem sap collected from leaves will more closely reflect apoplastic sap adjacent to the guard cells than sap collected from the root system, due to gains or losses in xylem solutes during long-distance transport in the stem (Jokhan and others 1999). Leaf xylem sap can be obtained by growing plants in a whole plant pressure chamber (Passioura and Munns 1984) or by pressurizing entire detached leaves in a Scholander-type pressure chamber. If xylem sap must be collected from the root system, sap should flow at rates equivalent to the whole plant transpiration rate, because the concentrations of many xylem constituents increase exponentially with decreasing sap flow rate (Else and others 1995; Schurr and Schulze 1995). Sap collection from detopped root systems under root pressure alone will overestimate true xylem sap concentration (Schurr and Schulze 1995).

In testing the effects of a hormone on stomatal behavior using detached leaves, it is useful to determine whether guard cells are responding to a concentration or a flux of that hormone. Concentration is the number of molecules in a given volume while flux is the concentration multiplied by the flow rate (of the transpiration stream). Altering the temperature and VPD surrounding detached leaves can vary the hormone flux at a given concentration. A 3-fold variation in ABA flux had no effect on the restriction of conductance at a given concentration, whereas the same flux achieved by increasing the ABA concentration was able to further decrease conductance (Trejo and others 1995).

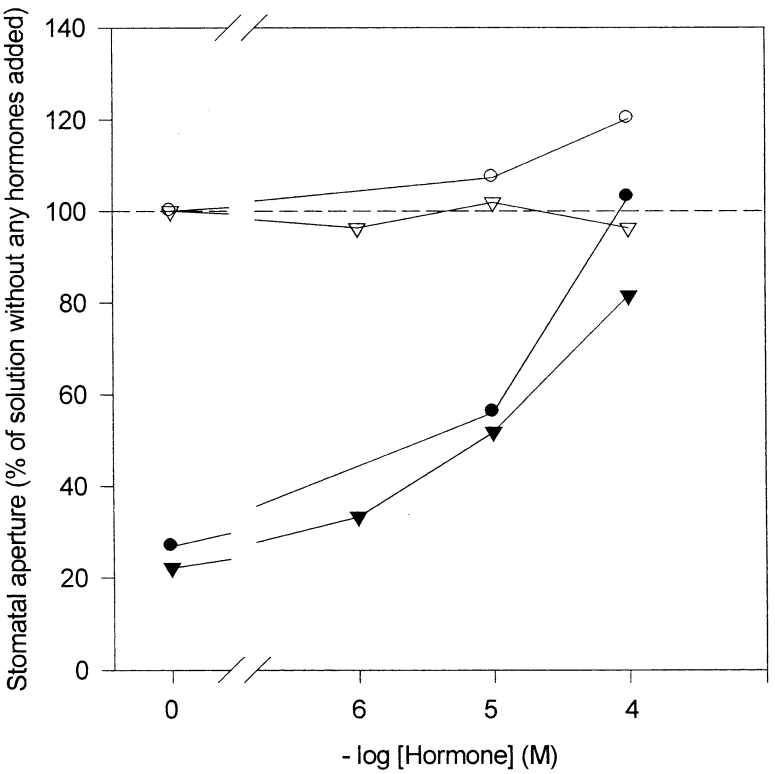

Figure 2. Antagonism of ABA-induced stomatal closure by IAA in *Commelina communis* epidermal strips incubated with (●) or without (○) 1 μM ABA, and by zeatin in *Zea mays* leaf pieces incubated with (▼) or without (▽) 100 μM ABA. All tissues were incubated at 25°C in CO_2-free air. Epidermal strips were incubated in a buffer containing 100 mM KCl and 10 mM MES at a light intensity of 160 μmol $m^{-2}s^{-1}$. Leaf pieces were incubated in distilled water at a light intensity of 130 μmol $m^{-2}s^{-1}$. Data were re-drawn from Snaith and Mansfield 1982b (●), and Blackman and Davies 1983 (▼,▽).

Although similar analyses have not been applied to other hormones, this example suggests that measuring hormone concentration is valuable in interpreting stomatal responses.

An advantage of feeding hormones via the transpiration stream to detached leaves is that any signal is ''processed'' (for example, by xylem parenchyma uptake within the petiole, or mesophyll catabolism) en route to the guard cells. The apparent decrease in stomatal sensitivity of detached *Commelina* leaves to ABA (relative to ABA dose-response relationships determined in epidermal strips) could be reconciled on the basis of a single stomatal response to epidermal ABA concentration (Trejo and others 1993). Although the use of detached leaves allows ''signal processing'' to be considered in the stomatal response to xylem-supplied hormones, this processing may be dissimilar in detached and attached leaves. Detaching leaves under water releases xylem tension thus increasing leaf water potential, which can increase the rate of catabolism of xylem-supplied ABA (Jia and Zhang 1997) and also decrease apparent stomatal sensitivity to applied ABA (Tardieu and Davies 1992). The greater sensitivity of intact leaves to xylem ABA concentration *in vivo* when compared to detached leaves supplied with synthetic ABA (Correia and Pereira 1995) may result from increased leaf water potential in the latter. Leaf detachment may also perturb phloem flow from the leaf thus changing the rate of hormone re-export from leaves and thus leaf hormone status.

Epidermal Strip Bioassays

The response of stomatal aperture to hormonal interactions has been facilitated (at least in certain species such as *Commelina communis, Vicia faba* and *Arabidopsis thaliana)* by the ability to peel epidermal strips from leaves and incubate them on solutions of defined hormonal composition. A variation of this technique is to float leaf pieces on solutions and remove the epidermis after incubation for microscopic measurement of stomatal aperture (Blackman and Davies 1983; Trejo and others 1993). Use of epidermal strips allows hormone concentrations to be known at the guard cell apoplast whereas the effects of exogenous hormones on endogenous concentrations can vary according to hormone uptake, transport and breakdown. Another advantage of using epidermes is that environmental conditions known to affect stomatal behavior (for example, temperature, CO_2 concentration) can be tightly controlled. This can be especially important if exogenous hormone applications are suspected of having a direct effect on photosynthetic metabolism (Meyer and Genty 1999), which might modify leaf internal CO_2 concentration and thus indirectly

affect g_s. However, the advantages of precisely controlling conditions during incubation of epidermal strips may hinder the identification of hormonal interactions affecting stomatal behavior, and in some cases can result in stomatal behavior that is atypical of *in vivo* response.

Optimizing the incubation solution to maximize stomatal aperture for ease of measurement, or using an incubation solution on the basis of previous work, may ensure that only a subset of the stomatal responses found in nature are encountered. For example, in CO_2-free air, stomata of *Commelina communis* are relatively insensitive to IAA concentration (Figure 1), yet increasing IAA concentrations abolishes CO_2-induced stomatal closure (Snaith and Mansfield 1982a).

Another choice, which might restrict the range of stomatal responses encountered, concerns the selection of experimental material. Often, the youngest fully expanded leaf of plants of a defined age is used to ensure reproducibility of hormonal effects on stomata. However, stomatal sensitivity to hormones varies with leaf age (for example, Blackman and Davies 1984; Willmer and others 1988) and stomatal behavior over the life of a leaf will integrate a range of responses and potentially hormonal interactions. Bulk leaf hormone ratios change as leaves age (Guinn and Brummett 1993) and may influence stomatal behavior if bulk leaf concentrations reflect hormone concentrations at the receptor sites. For example, promotion of stomatal opening by 10 μM kinetin in pea epidermal strips was only apparent 2 weeks after full leaf expansion (Incoll and Jewer 1987), suggesting that stomata might become sensitive to cytokinins only once leaf concentrations decrease.

Even though care is usually taken to define the age of experimental material, the hormonal relations of this material are often not measured (but see Willmer and others 1988 who showed that decreased stomatal sensitivity to ABA with leaf aging in *Commelina communis* was independent of bulk leaf ABA concentrations). Leaf hormonal relations may assist in interpreting stomatal response when epidermal strips are taken from plants subjected to different environmental conditions (for example, well watered vs drought stressed). Assaying leaf (or better still, epidermal) hormonal relations may help interpret the variability in hormone response seen when different investigators subject similar material to similar hormone treatments.

Although the epidermal strip technique has provided much information on *potential* hormonal interactions affecting stomatal behavior, the challenge has been to identify their physiological significance *in vivo*. Because epidermes can be obtained from *Arabidopsis thaliana,* the range of hormone sensitivity mutants in this species should assist the identification of hormonal interactions that might influence stomatal behavior. However, many sensitivity mutants were identified on the basis of seedling germination tests (McCourt 1999) where cross-resistance to several hormones is encountered. It will be interesting to see whether the stomatal sensitivity of these mutants agrees with their assigned sensitivity classification.

Hormonal Effects and Interactions

Abscisic Acid

Although stomatal closure is almost universal in response to endogenous or exogenous ABA, some plants show ABA insensitivity such as the ABA-insensitive mutants *abi1* and *abi2* in *Arabidopsis* (Leymarie and others 1998). Even ABA-deficient mutants and transgenics, which show increased g_s, show a normal stomatal response to exogenous ABA (for example, Donkin and others 1983). However, the sensitivity of stomata to ABA varies considerably according to environmental factors such as temperature, leaf water potential and atmospheric [CO_2]; ionic composition of epidermal strip incubation solutions and the presence of other hormones (reviewed in Dodd and others 1996).

Because stomata show by far the greatest response to ABA of any applied hormone in many bioassays (Figure 1), effects of other hormones are often considered in terms of their ability to alter ABA-induced stomatal closure. Incubation of *Commelina* epidermes in solutions containing 10 μM or 100 μM IAA (Snaith and Mansfield 1982b) or 50 μM benzyladenine (Das and others 1976), and incubation of *Zea mays* leaf pieces in solutions containing 10 μM or 100 μM zeatin or kinetin (Blackman and Davies 1983) antagonized ABA-induced stomatal closure (Figure 2). However, supply of 0.1–10 μM kinetin to detached barley leaves (Cooper and others 1972) and supply of 0.1-10 μM zeatin to detached lupin *(Lupinus cosentinii)* leaves (Henson and others 1989) did not reverse ABA-induced stomatal closure. The relevance of these potential interactions to *in vivo* g_s might be tested by submitting auxin and cytokinin overproducing transgenics and WT plants to gradual soil drying and analyzing stomatal sensitivity to xylem ABA concentration.

ABA-insensitive and-deficient mutants allow exploration of the role of ABA in stomatal responses to environmental stimuli such as light, CO_2 concentration and evaporative demand. The decreased

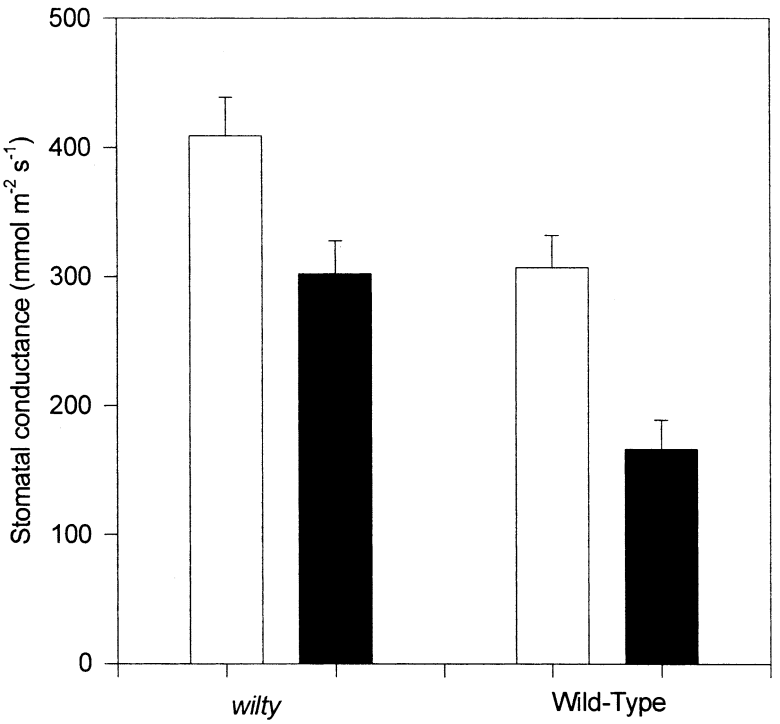

Figure 3. Stomatal conductance of the youngest fully expanded leaflets of *wilty* and WT pea plants grown at 0.5 mM (filled bars) and 5.0 mM (hollow bars) N for 4 weeks. Data are means ± S.E. of at least 5 plants. Unpublished data of I.C. Dodd.

leaf water potential ($_{leaf}$) of such mutants suggests that comparison of stomatal responses with WT plants should try to avoid confounding of $_{leaf}$ by growing the lines at different evaporative demands, or using epidermal strip bioassays where medium water potential can be controlled. In epidermal strips, stomata of the ABA-deficient *wilty* pea showed WT responses to both light and CO_2 concentration (Donkin and others 1983). Leaf conductance of the ABA-insensitive *Arabidopsis thaliana (abi1* and *abi2)* mutants was sensitive to CO_2, but responses were attenuated compared to WT plants (Leymarie and others 1998). Wild-type, ABA-deficient *(aba1)* and ABA-insensitive *(abi1* and *abi2) Arabidopsis* plants all showed stomatal closure as evaporative demand increased (Assmann and others 2000), apparently excluding a role for changes in ABA concentration or flux as mediators of stomatal response to atmospheric humidity. Taken together, these results suggest that stomata of ABA-deficient, ABA-insensitive and WT plants respond similarly to the environment, even though g_s of the mutants is usually higher (Figure 3).

Auxins

Although the synthetic auxin NAA can inhibit stomatal opening of *Commelina communis* at high concentrations (Snaith and Mansfield 1984)(see Figure 1), stomata generally open in response to naturally occurring IAA. Stomatal sensitivity to applied IAA can depend on CO_2 concentration (Snaith and Mansfield 1982a), the source of epidermis (adaxial versus abaxial) (Pemadasa 1982) and incubation solution composition (Pemadasa 1982; Dunleavy and Ladley 1995). Cotton xylem sap contains 150–200 nM IAA (Hartung and others 1992), yet no attempts have been made to determine whether environmental perturbation alters xylem IAA concentration and thence stomatal response. As far as is known, there have been no measurements of g_s in auxin over-expressing transgenic lines.

IAA-induced stomatal opening can be an indirect effect of auxin-induced ethylene production. Compounds such as aminoethoxyvinylglycine (AVG) which inhibits ACC synthase and α-aminoisobutyric acid which inhibits ACC oxidase (Merritt and others 2001) or silver ions which block ethylene action (Levitt and others 1987) prevented auxin-induced stomatal opening in *Vicia faba* epidermes. Addition of ACC (the immediate precursor of ethylene) restored auxin-induced opening in a concentration-dependent manner when AVG was also present in the incubation solution (Merritt and others 2001). Further confirmation that ethylene is responsible for auxin-induced stomatal opening would come from incubating epidermal strips from ethylene-insensitive *Arabidopsis* mutants in auxin, and detecting no stomatal response. Alternatively, ethylene-independent effects of auxin on stomatal behavior might be probed using a high auxin, low ethylene transgenic (Romano and others 1993),

Table 1. Stomatal Effects of Cytokinins on Light-induced Stomatal Opening in Different Experimental Systems, and Xylem Cytokinin Concentrations

Species	Treatment	Effect (% of control)	Reference
Epidermal strip bioassay			
Commelina benghalensis M	10 μM BA/K	92/104	Das and others 1976
Anthephora pubescens M	1 μM BA/K/Z	106/137/110	Jewer and Incoll 1980
Commelina communis M	10 μM K/Z	83/104	Blackman and Davies 1983
Commelina benghalensis M	10 μM K/Z	142/155	Santakumari and Fletcher 1987
Vicia faba	10 nM Ade/K/KR	151/95/246	Morsucci and others 1991
Transpiration bioassay			
Avena sativa M	30 nM BA/Z	146/125	Biddington and Thomas 1978
Avena sativa M	36 nM BA/51 nM Z	219/265	Badenoch-Jones and others 1996
Lupinus cosentinii	0.1–100 μM Z	No effect	Henson and others 1989
Lupinus albus	10 μM BA	No effect	Correia and Pereira 1995
Gossypium hirsutum			Radin and others 1982
High nitrogen plants	2–10 μMK	No effect	
Low nitrogen plants	2–10 μMK	No effect	
Xylem sap concentration			
Oryza sativa M	4.6 nM Σ cytokinins		Bano and others 1993
Avena sativa M	37.5 nM Σ cytokinins		Badenoch-Jones and others 1996
Urtica dioica	1–4 nM Σ cytokinins		Beck 1996
Lupinus angustifolius	25.4 nM Σ cytokinins		Jameson and others 1987
Foliar spraying			
Lycopersicon esculentum			Bradford 1983
Well-drained	100 μM BA	114	
Flooded	100 μM BA	514	
Vitis vinifera			Stoll and others 2000
Partial rootzone dried plants	89 μM BA	Increased g_s	

Ade = adenosine, BA = benzylaminopurine, K = kinetin, KR = kinetin riboside, Z = zeatin, ZR = zeatin riboside. Monocotyledonous species are indicated by placing 'M' after the species name

produced by crossing a high auxin line with an ethylene deficient line.

Cytokinins (CKs)

Stomatal sensitivity to applied CKs has been previously reviewed (Incoll and Jewer 1987) and varies widely according to the species (for example, Badenoch-Jones and others 1996), the CK applied (Biddington and Thomas 1978) and leaf age (Blackman and Davies 1984). Selected observations are summarized in Table 1. In isolated epidermes, micromolar CK concentrations are usually required to stimulate stomatal opening, but stomata have responded to CK concentrations as low as 10 nM (Morsucci and others 1991). Xylem CK concentrations typically range between 1–50 nM according to the species (Table 1), growth conditions and perhaps most importantly, sap flow rate during collection (Beck 1996). When supplied via the xylem to detached leaves, nanomolar CK concentrations stimulate transpiration of some monocotyledonous species, yet much higher CK concentrations apparently have no effect on dicotyledonous species (Table 1). The apparent insensitivity of stomata to CKs in some studies may occur because endogenous CK concentrations are already optimal for stomatal opening. Another explanation is that CKs most effectively promote stomatal opening in aging leaves (Blackman and Davies 1984) and most investigators have used the youngest fully expanded leaf.

Although detached leaves can be unresponsive to high concentrations of CKs fed via the transpiration stream (for example, Henson and others 1989), spraying similar concentrations onto attached leaves can promote stomatal opening (Bradford 1983; Stoll and others 2000) (see Table 1). No direct comparison of the efficacy of these two methods of CK application is available. However, foliar application of CK to leaves may alter CK concentrations at the receptor sites, whereas supplying similarly high concentrations to detached leaves via the xylem may be ineffective due to rapid CK metabolism by the mesophyll. Such data question the physiological

Table 2. Stomatal Effects of Ethylene (C_2H_4) and Compounds Affecting Ethylene Production

Species	Treatment	Effect	Reference
Epidermal strip bioassay			
Vicia faba	100 μM AVG	No effect	Tissera and Ayres 1986
	10 μM $CoCl_2$	No effect	Tissera and Ayres 1986
Vicia faba	0.3% Ethrel	⇑aperture	Levitt and others 1987
Vicia faba	12.5 μM ACC	12% ⇓aperture	Merritt and others 2001
Vicia faba	50 – 250 μM ACC	13% ⇑aperture	Merritt and others 2001
Transpiration bioassay			
Lycopersicon esculentum	1 μM ACC	No effect	Bradford and Hsiao 1982
Rhizosphere applications			
Lycopersicon esculentum	0.3% Ethrel	No effect	Woodrow and Grodzinski 1989
Glycine max	10 μM AVG	No effect	Taylor and others 1988
Aerial fumigations			
Glycine max	0.1 μL $L^{-1}C_2H_4$	Decreased g_s	Gunderson and Taylor 1988
Gossypium hirsutum	5.1 μL $L^{-1}C_2H_4$	Decreased g_s	Taylor and Gunderson 1986a
Lycopersicon esculentum	5.1 μL $L^{-1}C_2H_4$	No effect	Taylor and Gunderson 1986
Pisum sativum	5.1 μL $L^{-1}C_2H_4$	No effect	Taylor and Gunderson 1986

ACC (1-aminocyclopropane-1-carboxylic acid) is the immediate precursor of ethylene. AVG (aminoethoxyvinylglycine) inhibits ACC synthase activity. Cobalt chloride ($CoCl_2$) inhibits ACC oxidase activity. Ethrel (2-chloroethylphosphonic acid) liberates ethylene

relevance of changes in xylem CK concentration to stomatal behavior, unless such changes in xylem CK concentration can alter mesophyll catabolism and thus influence CK penetration to the guard cells.

Perhaps the most convincing demonstration that endogenous CKs increase g_s and plant transpiration *in vivo* is provided by plant transformation with bacterial *ipt* (isopentenyl transferase, which catalyzes *de novo* CK biosynthesis) or *zmp* (which encodes a protein capable of cleaving CK glucosides into active forms) genes (for example, see Wang and others 1997; Pospisilova and others 1998). However, such transformations can also alter the concentrations of other hormones, which might affect g_s. Tobacco *ipt* and *zmp* transformants showed decreased and increased leaf IAA levels, respectively, yet both showed increased g_s and increased leaf CK concentrations (Pospisilova and others 1998), suggesting that CKs and not auxin caused the observed stomatal phenotype.

Several factors (change of CK concentration achieved, age of leaf, leaf surface measured) determine whether such transgenics show altered stomatal behavior. Because the level of *ipt* expression can be modulated by the effectiveness of constitutive or inducible promoters, the range of CK concentrations achieved in such transformants provides a means of correlating CK concentrations with stomatal responses. Adaxial g_s of *zmp* transgenics was increased in all leaves measured but changes in abaxial g_s were variable such that leaf conductance was only altered in certain leaves (Pospisilova and others 1998). Because CK-overproducing transgenics show delayed leaf senescence, the best evidence of a specific CK effect on stomata would come from comparing young transgenic and WT leaves of a similar physiological age.

One complication in studying stomatal responses to CK overproduction is that many transformants have severely decreased root mass (Hewelt and others 1994) causing leaf wilting which might directly close stomata, thus masking CK effects. Grafting CK-overproducing scions on WT rootstocks allowed CK effects to be determined in non-water-stressed leaves (Pospisilova and others 1998). Another complication is that some CK overproducing lines show lower (40–50%) leaf ABA concentrations (Synkova and others 1999) which might also be expected to promote stomatal opening. For these reasons, the stomatal responses of such transgenics are best analyzed in terms of whether stomatal sensitivity to xylem ABA concentration differs from WT plants. Such experiments would also test whether the CK antagonism of ABA-induced stomatal closure observed in epidermal strips (Das and others 1976) and leaf pieces (Blackman and Davies 1983) occurs in CK-overproducing lines.

Ethylene

Ethylene is unique amongst the classical plant hormones in being gaseous. Because ethylene is a byproduct of fossil fuel combustion, mean atmospheric ethylene concentrations in polluted city

environments can reach 40 μL L^{-1} (Altuzar and others 2001), several fold higher than an ethylene concentration (5.1 μL L^{-1}) causing stomatal closure in several sensitive crop species (Taylor and Gunderson 1986). In natural systems the ethylene concentrations that plants are exposed to will depend to a large extent on ethylene emissions by their neighbors.

Stomatal responses to ethylene depend very much on the species and the way in which plant ethylene evolution is modified (Table 2). Epidermal strip experiments show that modifying enzyme activity in the ethylene biosynthesis pathway has no effect on stomatal response of *Vicia faba* (Tissera and Ayres 1986), whereas increasing ethylene synthesis (via application of Ethrel or ACC) has variable effects. Xylem sap contains the ethylene precursor ACC (1-amino-cyclopropane- carboxylic acid) at concentrations ranging between 0.3 and 2.0 μM (Bradford and Yang 1980; Gomez-Cadenas and others 1996; Else and Jackson 1998). Supplying detached leaves with ACC via the transpiration stream allows the physiological significance of stress-induced changes in xylem ACC concentration to be evaluated, but a single report showed no stomatal response to 1 μM ACC in tomato (Bradford and Hsiao 1982). Similar experiments in species showing pronounced stomatal closure in response to aerial fumigation with ethylene (Taylor and Gunderson 1986) might be worthwhile. However, modification of plant ethylene synthesis by addition of 10 μM AVG (which inhibits the enzyme ACC synthase) to the rhizosphere had no consistent effect on g_s in *Glycine max,* a species with ethylene-responsive stomata (Taylor and others 1988).

Transgenic plants provide another tool for discerning any effect of ethylene on stomatal behavior. When grown at various levels of soil compaction, tomato plants with an antisense gene for ACC oxidase ($ACO1_{AS}$) and decreased leaf ethylene evolution showed wild-type g_s in young expanded leaves (Hussain and others 1999). $ACO1_{AS}$ plants also show delayed leaf senescence (John and others 1995) thus aged leaves might be expected to show higher g_s than WT plants, but this might be an indirect effect of differences in internal leaf CO_2 concentration.

Gibberellins

Gibberellic acid (GA_3) can enhance stomatal opening in *Commelina* epidermal strips (10 μM) (Santakumari and Fletcher 1987) and enhance transpiration when supplied via the xylem to detached barley leaves (150 μM) (Livne and Vaadia 1965). However, few studies have examined effects of gibberellins, let alone their interactions with other hormones. Early work with *Commelina* epidermal strips showed that 500 μM GA_3 did not antagonize ABA-induced stomatal closure (Tucker and Mansfield 1971; Ogunkanmi and others 1973), but the incubation solutions used also showed that CKs and IAA did not antagonize ABA-induced stomatal closure (compare Figure 2). Transpiration rates of gibberellin-deficient tomato leaves did not differ from WT plants (Cramer and others 1995), suggesting that variation in endogenous gibberellin concentrations is not important in stomatal control.

Stomatal and Hormonal Responses to Edaphic Changes

Soil Drying

Although soil drying induces multiple changes in xylem composition (Gollan and others 1992), most interest has centered on the ability of drought-induced changes in xylem ABA concentration to explain stomatal closure. A number of comprehensive data sets from field and glasshouse studies indicate an excellent correlation between xylem ABA concentration and g_s in species such as barley (Borel and others 1997), maize (Tardieu and Davies 1992), sunflower (Tardieu and others 1996) and tobacco (Borel and others 2001). In these studies, xylem sap was collected from the same leaves in which g_s was measured. Irrespective of the site of sap collection, as the soil dries, the concentration of all xylem sap constituents should increase as transpirational fluxes decrease. Thus, any solute, irrespective of its effect on stomata, could give the negative exponential relationship demonstrated between xylem ABA concentration and g_s. Consequently, more rigorous tests of the physiological significance of ABA have been formulated. *Correlation* and *duplication* experiments have shown that soil application (Zhang and Davies 1990) or stem injection (Tardieu and others 1993) of synthetic ABA to well-watered plants generates a similar relationship between xylem ABA concentration and g_s to that found in droughted plants. *Deletion* and *re-instatement* experiments test the specificity of hormone action by manipulating endogenous hormone levels. Using an immunoaffinity column, Zhang and Davies (1991) removed ABA from xylem sap and in doing so, eliminated the antitranspirant activity of maize xylem sap, as assessed by a detached leaf transpiration assay. Given this evidence, is there a need to consider other factors in explaining drought-induced stomatal closure?

Several lines of evidence indicate that other factors (not necessarily hormonal) should be considered. Detached leaf transpiration studies have suggested the presence of other antitranspirant compounds in wheat and barley xylem sap (Munns and King 1988) because the antitranspirant activity of xylem sap could not be explained in terms of its ABA concentration. Time-course studies have shown that drought-induced stomatal closure of *Phaseolus vulgaris* can precede increases in xylem ABA concentration (Trejo and Davies 1991). Furthermore, the relationship between xylem ABA concentration and g_s can vary with time of day in peach (Correia and others 1997) and genotypically in *Nicotinia plumbaginifolia* (Borel and others 2001).

Alkalization of xylem sap is a common response to various environmental stresses (Wilkinson and Davies 2002) and supplying detached *Commelina* and tomato leaves with alkaline buffers (≥ pH 7) via the transpiration stream can close stomata (Wilkinson and Davies 1997; Wilkinson and others 1998). These alkaline buffers increased apoplastic pH, thus decreasing sequestration of ABA by mesophyll cells, causing increased apoplastic ABA concentrations, which closed stomata (Wilkinson and Davies 1997). Time course studies indicate that changes in xylem pH can precede changes in xylem ABA concentration in maize (Bahrun and others 2000) and thus initiate stomatal closure. Furthermore, alkalization of xylem sap may be responsible for the ''unexplained'' antitranspirant activity of some saps. However, in some species, xylem sap pH can acidify (*Ricinus communis*) (Schurr and Schulze 1996) or show no change (*Nicotinia plumbaginifolia*) (Borel and others 2001) in response to drought.

Diurnal variation in the sensitivity of g_s to xylem ABA is observed in some species under field conditions (Tardieu and Davies 1992; Correia and others 1995; 1997). Maize stomata showed a more pronounced response to xylem ABA in the afternoon (Tardieu and Davies 1992) which was attributable to $_{leaf}$ sensitizing stomata. However, neither $_{leaf}$ or leaf temperature could explain a more sensitive stomatal response of grape and peach leaves to ABA in the afternoon (Correia and others 1995, 1997). It was speculated that other hormonal factors such as auxin and CKs were involved, especially because xylem CK concentrations can vary diurnally (retain beta,β Fuβeder and others 1992).

Both xylem (Bano and others 1993; Duan and others 1996) and root and shoot (Goicoechea and others 1997) CK concentrations can decrease with decreasing soil water content. However, there was a poor relationship between xylem zeatin riboside concentration and g_s in *Vigna unguiculata*, compared to the excellent correlation between xylem ABA concentration and g_s (Duan and others 1996). Although decreased xylem CK flux and changes in xylem or leaf ABA:CK ratio have been implicated in stomatal closure of droughted plants (Wang and others 1994; Shashidhar and others 1996), such studies have not tested whether the stomatal response can be explained by ABA alone, using bioassays. Foliar spraying of 89 μM benzyladenine (BA) reversed soil drying-induced stomatal closure in grape when $_{leaf}$ had not changed (Stoll and others 2000). However, supplying 10 μM BA via the transpiration stream did not increase the g_s of detached lupin (*Lupinus albus*) leaves in which $_{leaf}$ had decreased (Correia and Pereira 1995). It is possible that the ability of exogenous CKs to reverse drought-induced stomatal closure is dependent on the degree of water stress experienced.

Some ABA underproducing transgenic lines of *Nicotinia plumbaginifolia* show a more sensitive stomatal response to xylem ABA compared to WT plants (Borel and others 2001). These authors speculated that decreased xylem CK concentrations were responsible (since greater root dehydration of ABA-deficient lines was required to achieve a comparable xylem ABA concentration). The availability of transgenic CK overproducing tobacco lines (Pospisilova and others 1998) combined with grafting studies would theoretically allow the independent manipulation of CK and ABA concentrations, as discussed above.

Although several authors have invoked hormonal antagonism of ABA-induced stomatal closure as an explanation for a variable relationship between xylem ABA concentration and g_s in droughted plants, *in planta* tests of these interactions have yet to occur. Measurement of apoplastic or guard cell ABA concentrations may provide an alternative explanation, obviating the need to invoke hormonal interactions.

Soil Compaction

Generally, there have been few measurements of hormone concentrations in xylem sap from plants growing in compacted soil. Paired measurements of xylem ABA concentration and g_s in the same leaf in compacted field-grown maize have shown a similar relationship to that found in droughted plants grown in noncompacted soil (Tardieu and others 1992). Soil compaction experiments with ABA-deficient barley and tomato mutants also suggest a unifying relationship between xylem ABA concentration and g_s regardless of genotype (Mulholland and others 1996, 1999). However, in these studies

Table 3. Summary of Hormonal Effects on Stomata and any Relevant Hormonal Interactions

	Epidermal strips		Detached leaves		Transgenic plants
	Main effect	Interaction ?	Main effect	Interaction ?	Main effect
Abscisic Acid (ABA)	⇓***	See below	⇓***		⇓ABA⇒ ⇑g_s***
Auxin (AUX)	⇑**	AUX ⇒ ⇓ ABA-R	⇑*		⇑ AUX ⇒?
Cytokinins (CK)	⇑**	CK ⇒ ⇓ ABA-R	⇑*	CK ⇒ ⇓ ABA-R	⇑ CK ⇒ ⇑ g_s*
Ethylene (ETH)	⇓ or ⇑*	AUX ⇒ ⇑ ETH-S	No effect		⇓ ETH ⇒ NE**
Gibberellins (GA)	⇑*		⇑*		⇓ GA ⇒ NE**

*Stomatal responses in epidermal strips, detached leaves and transgenic plants are indicated by an increase (⇑) or decrease (⇓) or no effect (NE) in stomatal aperture, transpiration and stomatal conductance (g_s). A subjective assessment of the strength of the evidence is given by the number of asterisks (*** indicating the most convincing evidence). Effects of plant transformations are given in terms of an increase (⇑) or decrease (⇓) in the hormone of interest affecting (⇒) stomatal conductance (g_s). Hormonal interactions are given in terms of one hormone affecting (⇒) the response to (-R) or synthesis of (-S) another hormone. Thus, CK ⇒ ⇓ ABA-R indicates that cytokinin decreases stomatal response to ABA; and ⇑ CK ⇒ ⇑ g_s indicates that increased cytokinin concentrations increases stomatal conductance.? indicates response unknown.*

xylem sap was collected under root pressure alone (which is decreased by ABA deficiency), which may not accurately reflect the xylem ABA concentration in the leaves in which g_s was measured, as discussed previously. Although ethylene has been implicated in the inhibition of leaf growth by soil compaction, transgenic tomato plants with an antisense gene for ACC oxidase showed WT stomatal conductance at various levels of soil compaction (Hussain and others 1999). Further measurements of hormone concentrations in leaves and xylem sap of plants growing in compacted soil might suggest a role for hormonal interactions in the control of stomatal response, but the evidence thus far points to a dominant role of ABA.

Soil Flooding

Attenuated stomatal closure of ABA-deficient mutants points to an involvement of ABA in the response to soil flooding (Jackson and Hall 1987), even though flooding decreases root export of ABA in tomato (Else and others 1995a). Redistribution of ABA from leaf mesophyll cells to the guard cell apoplast in response to a transient water deficit (Else and others 1995b), or perhaps a xylem sap pH signal (M.A. Else, personal communication), appears to be a likely candidate to initiate stomatal closure within hours of soil flooding. Over several days, leaf ABA accumulation may be important to maintain stomatal closure (Jackson and Hall 1987). Foliar spraying of flooded tomato plants with 44 μM BA + 29 μM GA_3 (Jackson and Campbell 1979) or 100 μM BA alone (Bradford 1983) partially reversed stomatal closure, suggesting a role for other hormones and possibly hormonal interactions. In contrast, incubation of leaf pieces from flooded pea plants in 0.5–50 μM zeatin or 1–100 μM kinetin (Zhang and Davies 1986), or leaf pieces from flooded bean (*Phaseolus vulgaris*) plants in 1–1000 μM zeatin, zeatin riboside, kinetin or BA (Neuman and others 1990) was unable to promote stomatal opening. The stomatal response to foliar spraying supports measurements showing that flooding could decrease xylem CK flux (Neuman and others 1990) and earlier measurements with bioassays showing that flooding could decrease xylem sap CK (Burrows and Carr 1969) and gibberellin (Reid and others 1969) activity. Further xylem sap measurements of CKs and gibberellins using physico-chemical techniques, combined with transpiration bioassay techniques, will be necessary to establish whether root-to-shoot hormone transport is important to the stomatal behavior of flooded plants.

Nitrogen Deprivation

It has been suggested that growth of nutrient-deprived plants is controlled by a centralized stress response system in which the hormonal balance of ABA and CKs is important (Chapin 1990); this balance may also affect g_s. However, such responses may not necessarily involve changes in xylem sap hormone concentrations, as supply of nitrate to detached leaves can stimulate CK production (Salama and Wareing 1979). For this reason, changes in xylem composition in response to N deprivation have been less comprehensively studied than other edaphic changes, even though N deprivation invariably causes stomatal closure.

Generally, N deprivation does not increase xylem ABA concentration or delivery when xylem sap is collected at appropriate flow rates (reviewed in Munns and Cramer 1996). However, N deprivation of *Capsicum annuum* increased xylem sap ABA concentration from 15–20 nM to 50–100 nM, when sap

was collected by pressurizing detached shoots in a Scholander-type pressure chamber (Dodd and others 2003). However, supplying 100 nM ABA to leaves detached from N-deprived plants induced only a small (*circa* 20%) inhibition of transpiration, whereas g_s of N-deprived plants declined by up to 70%. Increased xylem ABA concentration may not always be necessary for ABA-induced stomatal closure as N deprivation increased the sensitivity of detached cotton leaves to ABA supplied via the xylem (Radin and others 1982). However, this increased sensitivity was not observed in N-deprived *Capsicum* leaves fed 100 nM ABA (Dodd and others 2003). Detached N-deprived leaves showed a decreased transpiration rate (relative to N-supplied leaves) even when fed an optimal artificial xylem solution (low ABA concentration, pH 6), showing that factors residing within N-deprived leaves also mediate stomatal closure. Experiments with ABA-deficient mutants provide further evidence against ABA playing a major role in causing stomatal closure following N deprivation, as both *flacca* and WT tomatoes (Chapin 1990) and *wilty* and WT peas (Figure 3) showed stomatal closure following N deprivation.

N deprivation decreases xylem CK concentration and delivery (Beck 1996). Although supplying micromolar concentrations of kinetin to detached cotton leaves (from both N-supplied and N-deprived plants) had no effect in isolation, kinetin was able to reverse ABA-induced stomatal closure in N-deprived leaves (Radin and others 1982). It remains to be tested whether the nanomolar CK concentrations seen in cotton xylem sap have similar effects.

Future Prospects

Current efforts in stomatal physiology are focused on elucidating guard cell signal transduction pathways in response to single hormones. The use of common components in these pathways (for example, calcium, nitric oxide, hydrogen peroxide) by different hormones may provide a molecular basis for the hormonal interactions summarized in Table 3. Although these interactions have been identified using detached epidermes and leaves, there is little evidence that they are important to whole leaf behavior. The availability of transgenic and mutant lines with altered biosynthesis of, or sensitivity to, the classical plant hormones, when coupled with xylem sap hormone quantification, offers opportunities to remedy this deficiency. Such genetic tools will also assist in determining whether plant hormonal status determines stomatal response to environmental variables.

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