Chapter 4
Dynamic Stomatal Changes

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Abstract Stomatal pores regulate CO_2 uptake and water loss from leaves. Stomatal responses are dynamic by nature and often lag behind the faster changing environmental conditions as is common in tree canopies. Even under constant conditions, gas exchange of angiosperms occasionally shows cycling fluctuations, called stomatal oscillations. They are interpreted as an effect of feedback control failing to achieve stable regulation and thus demonstrate that stomata not only respond to external factors, but also to the environment inside the leaf. The processes which translate transpiration into turgor are called the physiological gain. The physical processes and environmental conditions which control stomatal aperture, stomatal conductance and transpiration are called the physical gain. More research on the physiological gain is needed in order to understand these processes. In order to overcome the epidermal backpressure, guard cell turgor has to reach a certain threshold level, although guard cell swelling anticipates the opening. When the pore opens, the relation between pore area and stomatal conductance determines the physical gain. In contrast to the Fick's first law of diffusion, this relation is not linear, but convex shaped, with a rapid increase of conductance just after opening and much less effect of aperture changes at large apertures. The high and abruptly changing gain at smallest pore openings can promote overshooting oscillatory responses, as supported by microscopic observations of stomatal apertures. A review of the literature suggests that stomatal movements are metabolically active responses of guard cells to local water status. A full understanding of the mechanisms, however, is complex because stomatal movements result from the interaction of two processes that are difficult to separate experimentally: hydraulic effects, and active osmotic adjustment of guard cells and epidermal cells. Hydropassive

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movement, resulting from an unbalance of turgor pressure between guard cells and the surrounding epidermis, may also occur. An example of hydropassive movement is the so-called Iwanoff effect or Wrong Way Response (WWR), i.e. a fast opening response followed by a slow closure, that occurs as a response to a steep increase in the leaf to air difference in water vapor pressure and may last 2.5–38 min depending on the species and the experimental conditions. An additional 10–60 min may be required for completing the closing response. In contrast to the rather slow osmoregulatory negative feedback, hydraulic responses act fast, starting within seconds and completing within minutes, and have been suggested as a key mechanism in stomatal oscillations. In a plant displaying oscillations, movements of individual stomata are more or less synchronized on a very small scale within a leaf (1–2 mm). The nature of the synchronizing mechanism is not clear. Synchronization can also occur among leaves, ultimately leading to concerted cycling of gas exchange of entire plants. Comprehensive models of stomatal behaviour based on the mechanisms operating in and around stomatal guard cells are still missing, and may help explaining gas exchange response to stressors. Studies with the air pollutant of most concern to forests, i.e. ground-level ozone, suggest that stomata show a transient decrease of stomatal conductance upon exposure and are sluggish in responding to further stimuli.

4.1 Introduction

Ever since vascular plants started the conquest of land, the interior of photosynthesizing organs had to be maintained in a state of sufficient hydration by evaporational barriers which limit transpiration, while at the same time putting minimal constraints on CO_2 supply for photosynthesis. The solution was stomatal pores which in response to a multitude of environmental factors perform the task of adjusting leaf conductance to an optimal compromise between the needs of water conservation and photosynthetic carbon gain. As the multi-factorial microclimatic conditions, which determine optimal leaf conductance, are usually in permanent fluctuation, stomatal responses are dynamic by nature. Due to the slow rates of movements, stomatal responses often lag behind the faster changing environmental conditions. All this makes the stomatal response in natural environments transitory and fugitive, most often far from the optimum and equilibrium state which at best can be observed under constant laboratory conditions.

We now know a great deal about gas exchange of trees (see Thomas and Winner 2002), although most insight comes from young trees and steady-state measurements. Eddy-correlation measurements provide an integrated assessment of canopy-level gas exchange, but cannot untangle the leaf-level dynamics as a response to fluctuating environmental stimuli. Tree canopies are very dynamic environments where all physical parameters vary with space and time (Zhang and

Xu 2000; Wang and Jarvis 1990), for example, variable light may represent two thirds of the incident light in forest canopies (Pearcy 1990). The ability to adjust gas exchange to rapid changes in environmental stimuli is an index of successful adaptation of trees.

Stomatal opening is driven by the accumulation of K^+ salts and sugars in guard cells, which is mediated by electrogenic proton pumps in the plasma membrane and/or metabolic activity. Opening responses are achieved by coordination of light signalling, light-energy conversion, membrane ion transport, and metabolic activity in guard cells. Great progress has been made in elucidating the signal transduction pathways by which stomatal guard cells respond to changes in light intensity and CO_2 concentration (Assmann and Shimazaki 1999; McAinsh et al. 2000; Assmann and Wang 2001; Hetherington 2001; Vavasseur and Raghavendra 2005), and short-term changes in hydraulic variables such as humidity (Mott and Parkhurst 1991; Monteith 1995; Oren et al. 1999), xylem hydraulic conductance (Saliendra et al. 1995; Cochard et al. 2002; Brodribb and Holbrook 2004; Powles et al. 2006), and soil water status (Fuchs and Livingston 1996; Comstock and Mencuccini 1998). Substantial progress has been made in elucidating the mechanisms leading to stomatal closure (Pei and Kuchitsu 2005; Schroeder et al. 2001). Briefly, as a response to a sudden exposure to a stressor, production of reactive oxygen species (ROS) in guard cells increases. This leads to suppression of plasma membrane H^+ and Ca^{+2}, adenosine 5′-triphosphatases, and perturbations in membrane polarization and ion permeability, particularly to Ca^{+2}. The ultimate result is loss of osmotic substances and a decrease in stomatal pore width. The whole cascade of events may be completed within 5–10 min (Pei and Kuchitsu 2005), although 10–60 min may be required for complete stomatal closure.

As soon as methods for continuous observations of stomatal responses were available, scientists found that even under constant conditions, gas exchange occasionally showed cycling fluctuations, a baffling observation as it does not reconcile well with the idea of an optimal stomatal aperture. Stomatal oscillations were soon interpreted as an effect of feedback control failing to achieve stable regulation and as such, demonstrated that stomata not only respond to external factors, but also to the environment inside the leaf which is affected by the diffusional streams through stomatal pores, thus forming a negative feedback loop.

The aim of this chapter is to summarize the present state-of-knowledge and future prospects about dynamic stomatal changes, with a focus on stomatal oscillations.

4.2 Stomatal Oscillations

The interest in these peculiar responses has led to a large number of published observations from a diverse range of species (Barrs 1971) both from monocotyledonous and dicotyledonous angiosperms. In gymnosperms, there is only one casual observation (Stålfelt 1928) of uncertain quality. Other authors only found damped

oscillations in conifers (Phillips et al. 2004). Apparently stomatal oscillations have never been observed in *Pteridophyta*.

Stomatal oscillations were observed in plants with various stomatal anatomies ranging from the simple anomocytic type (without specialized subsidiary cells, Barrs 1968; Kaiser and Kappen 2001; Marenco et al. 2006) to plants with more complicated stomatal complexes with an apparatus of several subsidiary cells (Nikolic 1925; Brun 1961). Oscillations were also observed in *Gramineae* type stomata (Raschke 1965; Brogårdh and Johnsson 1973; Prytz et al. 2003). Stomatal oscillations may occur in herbaceous plants (Ehrler et al. 1965; Shaner and Lyon 1979; Santrucek et al. 2003; Yang et al. 2003; Wallach et al. 2010), grasses (Florell and Rufelt 1960; Raschke 1965; Johnsson 1973; Johnsson et al. 1979), shrubs (Ehrler et al. 1965; Shirazi and Stone 1976a; Rose et al. 1994; Kaiser and Kappen 2001; Marenco et al. 2006) and trees (Levy and Kaufmann 1976; Elias 1979; Reich 1984; Naidoo and von Willert 1994; Herppich and von Willert 1995; Zipperlen and Press 1997; Steppe et al. 2006). Therefore it can be concluded that stomatal oscillations may occur in any clade of angiosperms, irrespective of life form and stomatal anatomy.

Stomatal oscillations were most often observed under laboratory conditions for the simple reason that under fluctuating outdoor conditions oscillatory responses cannot be easily discerned from responses to environmental fluctuations. Nonetheless, a number of observations in the field (Elias 1979; Hirose et al. 1994; Dzikiti et al. 2007) show that stomatal oscillations do not only occur under artificial lab conditions but can be of relevance for real life situations of plants.

Oscillations can be observed on different spatial scales, ranging from movements of individual stomata to whole tree fluctuations of gas-exchange and stem-flux. The temporal and spatial resolution of the applied methods is determined by the degree to which responses of individual stomata are integrated. Most observations were made at leaf level by measuring gas-exchange of leaves or parts of leaves. Leaf patches, often separated by veins, may however show independent dynamics, with phase shifted oscillations (Cardon et al. 1994). This variation can be detected by chlorophyll fluorescence imaging which visualizes effects of different CO_2 supply on the photosynthesizing tissue (Cardon et al. 1994; Siebke and Weis 1995; West et al. 2005). Using chlorophyll fluorescence parameters as a proxy for stomatal apertures, however, only allows qualitative inferences as long as the causal chain stomatal aperture → conductance → C_i → fluorescence yield is not quantified. Another method to determine spatial differences in transpiration is thermography of leaf temperature, which responds to transpirational cooling (Prytz et al. 2003; West et al. 2005). The spatial resolution of these imaging methods is not so much restricted by pixel resolution as by thermal conduction and CO_2-diffusion blurring the image. Nonetheless they may approach sub-millimeter resolution and thus offer the most spatially inclusive and comprehensive measurement of stomatal actions. However, even the smallest discernible area contains many stomata which may include significant variation. The degree of variation among stomata is little known as only a few reports of directly observed aperture oscillations exist (Kaiser and Kappen 2001).

Integrating measurements hide the variation in amplitude and frequency between individual stomata, leaf patches or different leaves. Therefore they cannot answer central questions of the mechanism of stomatal oscillations: How do individual stomata get synchronized to a degree that periodic oscillations become observable on a higher scale? At what level does variation among stomata prevent coordination and has a damping effect on oscillations?

4.2.1 The Mechanism of Stomatal Oscillations

Stomatal oscillations disclose the action of negative feedback loops in aperture regulation, which under certain conditions produce an unstable response (Cowan 1972). Feedback controlled systems have one or more inputs from signal sources conveying information on the state of the parameter to be controlled. Dependent on the magnitude of this input, an output is produced, which has an effect on the state of the controlled parameter. In negative feedback loops, the effect of this output is inverse to the deviation of the controlled parameter, thus having a stabilizing effect. In positive feedback the output is positively related to input, thus enforcing deviations and having a destabilizing effect. The dynamics of feedback control is determined by some basic properties. The degree of regulation, called feedback gain, is the amplification a signal receives when being translated into regulatory output. A high gain promotes oscillations. All processes in the feedback loop, the sensing of the system state, the generation of an output and the response of the controlled parameter to this output usually do not occur instantaneously but with a certain lag, which introduces delays and possibly overshooting responses and oscillatory cycling. Feedback loops may also consist of a mixture of negative and positive feedback acting with separate kinetics.

In leaves, two separate negative feedback loops could be involved in regulation of stomatal aperture (Fig. 4.1). The first one is the feedback loop which keeps intercellular CO_2 (C_i) concentration at sufficient levels for photosynthesis. This loop is formed via guard cell sensitivity to CO_2 and aperture response to photosynthetically decreased C_i, thus allowing higher diffusional influx of CO_2 into the leaf, which then increases C_i. The other feedback loop balances leaf hydration: guard cells respond to transpirational water loss with stomatal closure and thus decrease transpiration.

The relative contribution of each of these interacting feedback loops has been analyzed experimentally only in a few cases. Reducing C_i towards the CO_2 compensation point and thus preventing feedback related to C_i fluctuations did not prevent oscillations (Bravdo 1977) nor the period of oscillations (Marenco et al. 2006). The (difficult) inverse experiment, keeping transpiration constant and allowing C_i fluctuations, apparently has not been performed, therefore it is not known if oscillations based on CO_2-feedback alone can develop. The prominent role of hydraulic relations in oscillations is obvious in most studies on stomatal oscillations where multiple parameters were measured. Hydraulic fluctuations

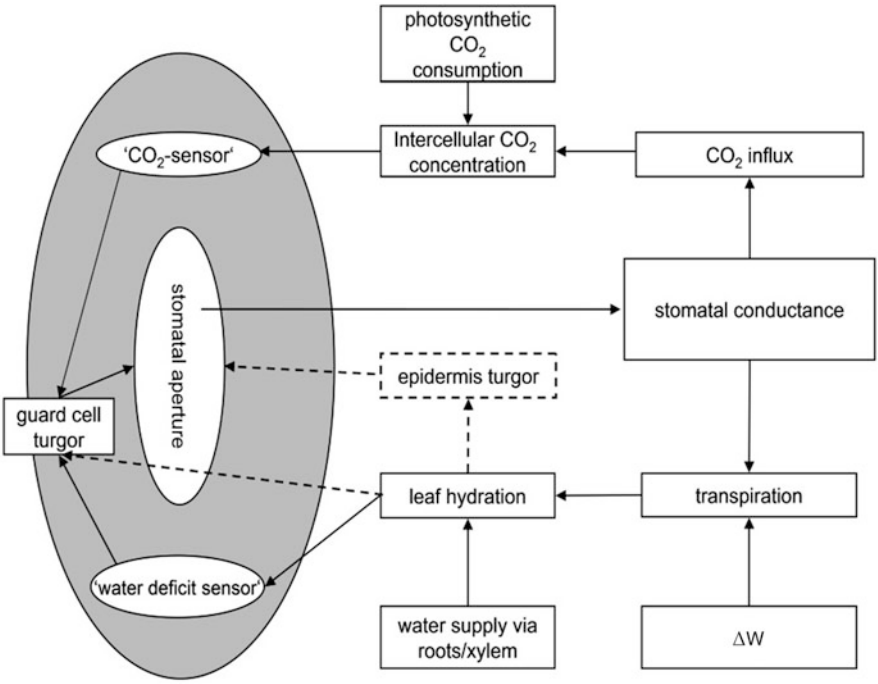

Fig. 4.1 CO_2 and water-related feed-back loops in stomatal regulation of gas exchange. Stomatal aperture governs stomatal conductance, which controls both CO_2 influx and transpiration. CO_2 influx, together with photosynthetic CO_2 uptake, affect intercellular CO_2-concentration which is sensed by guard cells. This feedback loop keeps CO_2-concentration in the mesophyll at sufficient levels for photosynthesis. Transpiration, driven by leaf to air concentration gradient of water vapour (ΔW) and controlled by stomatal conductance affects leaf hydration, directly impacts epidermal and guard cell turgor (*dashed lines*) and results in 'hydropassive' movements. This rapid effect leads to increased stomatal opening upon increased transpiration (and vice versa), thus forming a positive feedback-loop. A putative 'water deficit sensor' perceives leaf hydration and elicits active stomatal osmoregulation leading to changes in guard cell turgor and aperture. This active response of guard cells constitutes a negative feedback loop, keeping transpiration below a threshold level

affect any parameter related to water status like transpiration, leaf thickness, trunk diameter and sap flow. In contrast, as fluctuations in photosynthesis remain comparably small, it can be concluded that stomatal oscillations are mainly caused by instabilities in the water-related feedback loop with a possible additional contribution of CO_2-related feedback. The feedback mechanism involved in oscillations therefore appears to be identical to the mechanisms involved in regulation of leaf water loss. Unfortunately, these mechanisms are not well understood although examined and heatedly debated over decades (Buckley 2005). One of the points of dispute was whether air humidity could also be sensed directly, i.e., without transpiration through the stomata necessarily being involved. The claim for this so-called feed-forward response was bolstered by observations of a

disproportionally strong closing response to dry air (Schulze et al. 1972; Farquhar 1978). If only negative feedback via transpiration sensing was involved, transpiration should gradually approach a maximum with increasing leaf to air difference in mole fraction of water vapor (ΔW). Contrary to this expectation, in some cases, transpiration at highest ΔW decreased again, which could not be explained by feedback regulation alone. These observations led to the proposition of a feed-forward response and to a search for mechanisms providing the claimed direct sensitivity to air humidity outside the leaf without proportional transpirational water loss. Many of the proposed mechanisms involved water loss of stomata through cuticles (Farquhar 1978; Maier-Maercker 1983; Grantz 1990), but experimentally such a mechanism could not be confirmed (Meidner 1986; Kerstiens 1997).

The idea of feed-forward lost momentum after it was shown that stomata are sensitive to changes in transpiration when ΔW was kept constant, but not to changes in ΔW under constant transpiration (Mott and Parkhurst 1991). A reanalysis of existing data (Monteith 1995) and further experiments (Franks et al. 1997) questioned the general existence of a true feed-forward response (e.g., direct sensitivity to external humidity) and offered alternative explanations for the earlier observations. The current evidence supports feedback-response of stomata to effects of transpiration on leaf water status (Buckley 2005).

The mechanism by which changes in transpiration translate into stomatal responses, however, is still not identified. A number of possible mechanisms have been proposed, either involving localized transduction processes, which are confined to the guard or adjacent cells, or spatially distributed mechanisms involving other leaf tissues. A locally confined sensing mechanism could be developed through transpiration-dependent accumulation of substances in the apoplast of guard cells (Lu et al. 1997; Zhang and Outlaw 2001). Evaporation from the guard cell apoplast should induce a local accumulation of apoplastic solutes. This effect has been confirmed for sucrose (Outlaw and De Vlieghere He 2001). Evidence against such strictly local sensing mechanisms in or at individual guard cells comes from the observation that blocking the transpiration of a single stoma has no effect on its humidity response (Kaiser and Legner 2007). Only after additionally blocking adjacent stomata could closure in dry air be observed. Sensing of transpiration therefore is not located at individual guard cells but appears to be a function of the local tissue, integrating the transpiration of several stomatal pores on a sub-millimeter spatial scale. These results support the general notion, derived from gas exchange and water status measurements that stomatal responses are controlled by local tissue leaf water potential. For a discussion see Buckley (2005).

Understanding the involved mechanisms is difficult due to the fact that stomatal movements are a result of an interaction of hydraulic effects, and active osmotic adjustment of guard cells and epidermal cells. Both processes, acting simultaneously but with different kinetics, are hard to separate experimentally. The simplest conceivable mechanism would be a direct drawdown of guard cell turgor by an increase in transpiration, without active osmotic adjustment. This does not reconcile, however, with the mechanical relations between guard and epidermal

cells. Stomatal aperture is regulated by the balance of turgor pressures between guard cells and the surrounding epidermis. One crucial feature of this counterbalance is the so called *mechanical advantage* of epidermal cells over guard cells (DeMichele and Sharpe 1973; Sharpe et al. 1987; Franks et al. 1998). This means that a change in epidermal turgor has a larger effect on aperture than a similar change in guard cell turgor. A shift in water status similarly affecting epidermal and guard cell turgor will therefore cause a so-called hydropassive movement. Such hydropassive movements have been detected for any possible perturbation of the balance between water supply and loss, irrespective if the cause is a change in leaf water supply (Iwanoff 1928; Powles et al. 2006) or altered transpiration rate due to an increase in ΔW (Kappen et al. 1987; Kaiser and Legner 2007). The typical stomatal response to a steep increase in ΔW is a fast hydropassive opening response (also called Iwanoff effect or Wrong Way Response, WWR), starting almost immediately and finished within a few minutes, followed by a more or less delayed closing response (Fig. 4.2a). The hydropassive opening response further increases transpiration and acts as positive feedback within the control loop. These hydraulic processes therefore have a tendency to destabilize the regulatory loop, making the concept of a negative feedback regulation of transpiration based on purely hydraulic processes implausible.

Nonetheless, there were some attempts to develop hydraulic models explaining the observed stomatal responses on the basis of transpiration-induced micro-gradients between mesophyll, epidermis and guard cells (e.g. Farquhar 1978; Dewar 1995; Eamus and Shanahan 2002). These models require intricate additional assumptions, like variable flow resistance in the hydraulic continuum, to describe the biphasic stomatal response to a step change in humidity (Buckley and Mott 2002) which lack experimental support. Therefore, the most parsimonious hypothesis for stomatal response to transpiration is a metabolically active response of guard cells to local water status (for a detailed discussion see Buckley 2005).

The sensing mechanisms leading to the 'physiological' response to transpiration are not well understood. They could involve osmo-sensing (Yoshida et al. 2006) or mechano-sensitive channels (Zhang et al. 2007), which are triggered by hydraulic disturbances in guard cells or the adjacent tissues. Another possibility could be a local perturbation of the chemical composition of the apoplastic solution (Harris et al. 1988; Zhang and Outlaw 2001), possibly involving pH and its interaction with partitioning and redistribution of abscisic acid (Wilkinson and Davies 2008). As it is not yet known if abscisic acid (ABA), pH, mechanical stresses or other proximal effectors transduce transpiration-related changes into leaf water relations into active stomatal responses, there is no reason to delve into intracellular details of signal transduction. In this field, much more recent progress has occurred than in the question of transpiration sensing by guard cells. How little this research field is settled is demonstrated by recently proposed mechanisms for transpiration sensing, which are fundamentally different from the existing models (Peak and Mott 2011; Pieruschka et al. 2010).

We will now try to identify properties of the stomatal feedback system which favor oscillations. In feedback-controlled systems, oscillations are promoted by a

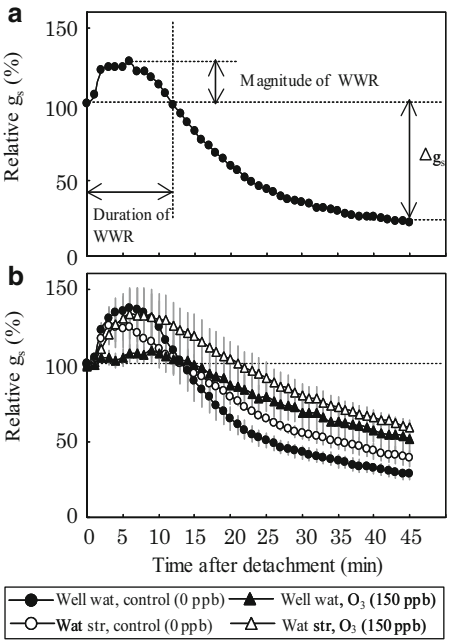

Fig. 4.2 Time course of *Phaseolus vulgaris* stomatal conductance (g_s) after severing a leaf at time 0. *Graph A* shows an example of Wrong Way Response (*WWR*), a transient increase of g_s after severing, followed by a decrease with increasing leaf water stress. *Graph B* shows that water stress and ozone (O_3) exposure resulted in stomatal sluggishness: longer WWR duration and lower degree of g_s decrease (Δg_s) relative to the controls (Hoshika et al. 2012)

high feedback gain, and delays in the response to changed input with involvement of positive feedback. Feedback gain in negative regulation of transpiration is the degree of regulation of the causal chain: transpiration → physiological (osmotic) activity of stomata → turgor → aperture → stomatal conductance (g_s) → transpiration. The processes which translate transpiration into turgor determine the physiological gain, whereas the following translation into stomatal aperture, g_s and transpiration is governed by physical processes and environmental conditions, which can be summarized as the physical gain. The overall gain is the product of physiological and physical gain. The physiological gain is somewhat obscure, as the underlying physiological events are hardly understood and there is a lot of variability induced by plant species, acclimation responses, diurnal variations and large stoma to stoma variability. Therefore, the physiological gain at the current state of knowledge can only be addressed in a "black box" approach without much prospect to better understand its influence on oscillations. The physical gain, however, is better understood and has some clear effects on the susceptibility to oscillations. First, it depends on the relation between guard cell turgor and aperture, which has a sigmoidal or convex shaped relation (Franks et al. 1998). This relation is strongly dependent on the epidermal backpressure. Notably, in order to overcome the given epidermal backpressure, guard cell turgor has to reach a certain threshold level. As a consequence, at the lowest range of the physiologically possible turgor pressures, the pore is simply closed and any osmotic activity below the opening threshold has no effect on leaf diffusion resistance, and the total gain of the feedback loop is zero. The opening threshold may also lead to a delay in opening,

as demonstrated in *Sambucus nigra*, where guard cell swelling was observed for up to 30 min before the pore initially opened (Kaiser and Kappen 2001). As soon as the pore has opened, the relation between pore area and gs determined the physical gain. In contrast to widespread notions, based on a simple application of Fick's first law of diffusion, this relation is not linear, but convex shaped, with a rapid increase of conductance just after opening of the pore and much less effect of aperture changes at large apertures (Kaiser 2009). The reasons for this non-linearity can be found in the three dimensional shape of the pore and additional mesophyll resistances to water vapor diffusion (Kaiser 2009). Consequently physical gain is variable and changes discontinuously within the available physiological range of turgor pressures. It is zero at small pressures, leaps to maximum gain at initial opening and gradually decreasing again, as the pore opens further (Fig. 4.3). The high and abruptly changing gain at smallest pore openings should promote overshooting oscillatory responses.

This view is supported by microscopic observations of stomatal apertures of *Sambucus nigra* during oscillations (Kaiser and Kappen 2001), which revealed that most stomata were closed completely in the troughs of the oscillations and opened only slightly during their respective maxima. Similar observations of oscillations in another four species (Fig. 4.4 and Kaiser, unpublished) confirmed that stomata always cycled between the completely closed and slightly opened state. During the troughs of oscillations, Marenco et al. (2006) estimated that 22 % of the stomata were open. Gas-exchange measurements of oscillation often show very small, minimal conductance, also indicating temporary complete closure (e.g. Rose and Rose 1994; Steppe et al. 2006). Some measurements, on the other hand, appear to contradict this view as oscillations occur at a rather high g_s, indicating on average significantly opened pores (Hirose et al. 1994; Santrucek et al. 2003). The integrating gas exchange signal, however, may hide a lot of variation between individual stomata and asynchronously oscillating leaf patches (Cardon et al. 1994). Each individual pore may completely close in the troughs, but at any time there are enough open pores to maintain a high leaf conductance (Kaiser and Kappen 2001). In summary, experimental evidence indicates that intermittent, complete closure is the typical mode of stomatal oscillations, which is in accordance with the idea that the high and discontinuously changing gain at small apertures, promote oscillations.

Delays in feedback loops contribute to oscillating behavior. For the case of stomata, the time required to perceive transpiration and produce osmotic activity of guard cells introduces a significantly lagging response. The lag is difficult to determine, as physiological responses of stomata to changes in humidity are always intermixed with the hydropassive wrong way opening response. Typical lag times, defined as the time span between switching to high ΔW and the reversal of the initial transient opening response range between 2.5–4 min in *Phaseolus vulgaris* (Meidner 1987), 5–8 min in *Xanthium strumarium* (Mott 2007), 8–20 min in *Sambucus nigra* (Kaiser and Legner 2007) and 8–38 min in *Vicia faba* (Kappen et al. 1987; Assmann and Gershenson 1991; Kaiser and Legner 2007). An additional 10–60 min may be required for completing the closing response. The lag times for opening are similar, as opening speed as an energy-requiring process is

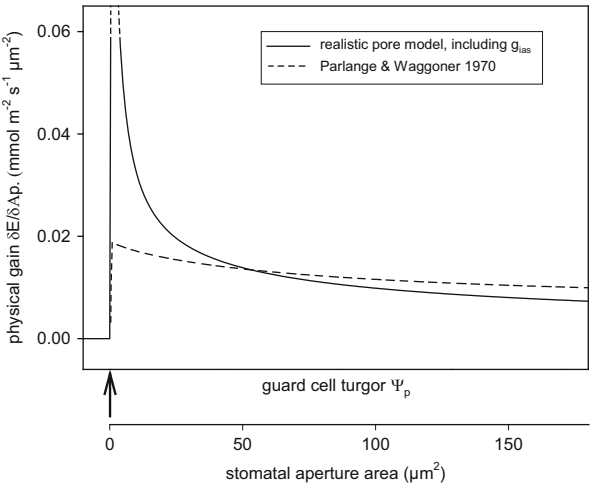

Fig. 4.3 Schematic representation of the dependence of physical gain of the stomatal feed-back loop on aperture area. Physical gain here is defined as δE/δAperture, which is the unit change of transpiration (E) per unit change in aperture. Gain was calculated using the widely used formula describing the relationship between aperture and stomatal conductance given by Parlange and Waggoner (1970) or a realistic pore model including a detailed pore geometry and mesophyll resistances (Kaiser 2009). As aperture area is, in first approximation, linearly related to guard cell turgor pressure (ψ_p), the graph can also be read as $\delta E/\delta\psi_p$ vs. ψ_p. At the ψ_p value indicated by the arrow, guard cell turgor is sufficient to overcome the backpressure of the epidermal cells, leading to rapid opening from zero to maximum gain (The figure is redrawn from Kaiser 2009)

often slower than the closing response. These delays in signal transduction can delay the response to changed transpiration to the extent that it coincides with the opposite phase of the cycle and this feedback becomes positive.

A destabilizing component of positive feedback is also introduced by the hydropassive response, which tends to open pores further upon increasing transpiration and vice versa. The gain of this feedback, like the gain of negative feedback, also depends on the relation between aperture and g_s, and is largest at small apertures. This effect should further increase instability of response of nearly closed stomata. In contrast to the rather slow osmoregulatory negative feedback, hydraulic responses act fast, starting within seconds and completing within minutes. This effect is known to speed up any response in dry air (Assmann and Grantz 1990; Kaiser and Kappen 2000), and has been suggested as a key mechanism in stomatal oscillations (Cox 1968).

Low air humidity, therefore, has a dual effect on the development of oscillations: an increased water vapor gradient proportionally increases transpiration and thus the physical gain of the feedback loop and, second, the aperture at which the target transpiration is attained is shifted to smaller apertures, where a larger effect of aperture changes on g_s further increases the gain.

In a plant displaying oscillations of gas exchange, movements of individual stomata are more or less synchronized. Obviously mechanisms exist which

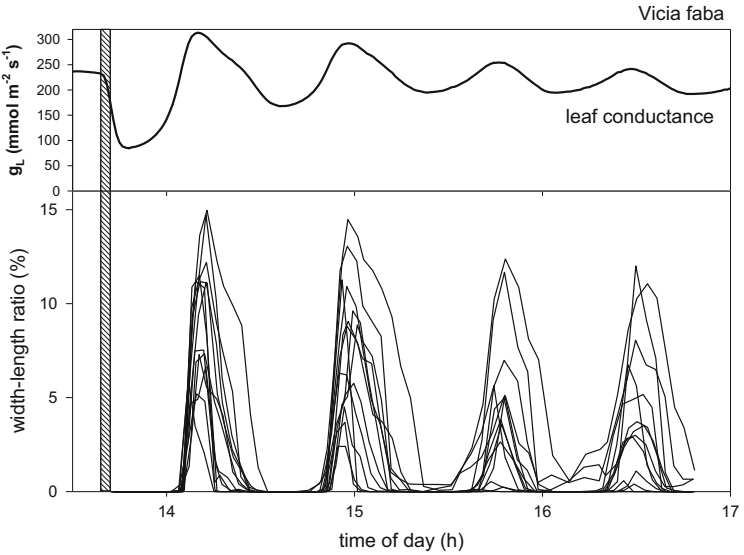

Fig. 4.4 Typical stomatal oscillations in *Vicia faba*. Simultaneous recordings of leaf conductance and stomatal opening (length:width ratio) of 12 randomly selected stomata. Oscillations were elicited by darkening the leaf for 3 min (*hatched bar*). No apertures were recorded before 14 h. The leaf was kept at 25 °C, a photon flux density of 145 µmol mol m^{-2} s^{-1} and ΔW of c. 25 mmol mol^{-1} (Data author and holder: H. Kaiser)

synchronize the individual oscillators. Synchronization has been observed on many different scales, between neighboring stomata (Kaiser and Kappen 2001), and on leaf patches measuring a few mm (Siebke and Weis 1995; West et al. 2005) within leafy organs (Teoh and Palmer 1971). Even entire trees can oscillate synchronously (Herppich and von Willert 1995; Dzikiti et al. 2007).

Due to a lack of detailed microscopic observations, the minimum number of synchronized stomata needed in order to sustain oscillations is unknown. In *Sambucus nigra*, out-of-phase oscillations were observed in stomata with a distance of less than 2 mm (Kaiser and Kappen 2001). More recent experiments revealed a tight synchronization when the distance between stomata was less than 1 mm, gradually decreasing with increasing distance (Kaiser, unpublished), which indicates synchronizing mechanisms acting on a very small scale. This view is supported by observing the effect of blocked transpiration of selected stomata on their humidity response (Kaiser and Legner 2007): the transpiration of a single pore affected active closure of other stomata within an area of 0.5 mm^2. In *Helianthus annuus*, oscillations on only one face of the leaf were observed, without transmitting to the other surface (Nagarajah 1978). Similar evidence comes from Mott et al. (1993) who found different patchy patterns of stomatal opening on the two faces of leaves of *Xanthium strumarium*. Additionally, Mott (2007) found no response of stomata on one face of the leaf, if only the other face was subjected to dry air, despite a substantial decrease in epidermal turgor on the treated face of

the leaf. This lack of synchronization between the two leaf surfaces points to a localization of feedback mechanisms in the epidermis rather than in the mesophyll.

The nature of the synchronizing mechanism acting on this small scale is not clear. Based on the assumption that stomata respond to variations in local water potential in the epidermis or the adjoining layers of mesophyll (Buckley 2005), local transpiration could affect larger areas by gaseous diffusion within the intercellular spaces. Additionally, gradients of water potential could be equalized by symplastic and apoplastic flows of water. Another possible mechanism providing lateral synchronization could be the generation of a chemical signal by epidermal or mesophyll cells which is spread by diffusion or mass flow within the tissue. Only one of these possibilities, the hydraulic coupling of the tissue, has solid experimental support. Streaming dry air to a small region of a leaf, which was otherwise kept humid, led to hydropassive opening in a distance of up to 0.4 mm (Mott and Franks 2001), which demonstrates that positive hydraulic feedback of pore transpiration also affects the responses of adjacent stomata. A spatial model of hydraulically connected stomata (Haefner et al. 1997) showed agreement with observed patch formation and dynamics.

Lateral transmission of hydraulic disturbance within this small scale network of hydraulically coupled stomata relies on cell to cell water transport. The hydraulic interaction of different regions of the leaf (Buckley and Mott 2000) most likely involves water transport in xylem vessels, which are able to transmit water potential changes to distant regions of the leaf due to their low resistance compared to extravascular pathways (Sack and Holbrook 2006). Interactions between hydraulically coupled leaf patches, forming a higher level network, may allow for pattern formation and synchronization (Johnsson 2007). Hydraulic resistance in itself is highly dynamic and its fluctuation appears to play a role in the development of stomatal oscillations. In *Helianthus annuus*, Marenco et al. (2006) found periodic xylem embolism and refilling corresponding with the fluctuations in transpiration, with highest percentage of embolised vessels at peak transpiration. Embolism occurring under increasing transpiration further impairs leaf water status, and amplifies hydropassive opening. This not only boosts positive feedback, but also synchronizes responses within the area supplied by the affected vessel.

Synchronization can also occur between leaf organs, ultimately leading to concerted cycling of gas exchange of entire crop plants (Cox 1968; Marenco et al. 2006) or trees (Steppe et al. 2006; Dzikiti et al. 2007). The responses in *Citrus sinensis* (Dzikiti et al. 2007) are in good agreement with a water balance model considering water reservoirs and hydraulic resistances within the entire plant. The role of cavitations in the generation of whole plant oscillations, however, is still hypothetical and needs further research (Marenco et al. 2006).

Stomatal oscillations promise insight into the stomatal control system; therefore many attempts have been made to construct models that will allow testing their assumptions (Johnsson 2007). Any modeling of complex systems faces the dilemma of choosing between a simple and manageable but possibly nonrealistic model, and the futile attempt to comprehensively describe all sub-processes. Earlier attempts were optimistic in that they focused on the hydraulic processes which are

comparably easy to formalize. These first modeling approaches described the leaf in terms of hydraulic capacitors connected by flows with corresponding hydraulic resistances (Cowan 1972; Shirazi et al. 1976b; Delwiche and Cooke 1977), assuming no short-term, active adjustment of guard cell osmotic potential in response to transpiration. These models were valuable in that they enhanced the understanding of physical processes within the stomatal hydro-mechanic apparatus. They treated the leaf as a "lumped model" consisting of one guard cell and one of each of the interacting components, epidermal cells, xylem, etc. As a spatio-temporal dynamic was obvious from observations of patchy oscillatory behavior, models of hydraulically interacting stomata were developed (Rand et al. 1982; Haefner et al. 1997), which were based on known hydraulic interactions, and included stomatal variability and its influence on pattern formation (Laisk et al. 1980). These models successfully simulated patchy stomatal coordination and dynamics similar to those occurring in real leaves. However, the active regulation of guard cell osmotic pressure in response to local leaf water status, and the spatial and temporal dynamics of leaf hydraulic resistances involved in stomatal oscillations were not satisfactorily accounted for.

4.3 Rapid Transient Variation of Stomatal Conductance Under Ozone Exposure

A very interesting example of rapid stomatal responses to environmental stimuli is the rapid transient decline of g_s (RTD, Fig. 4.5) induced by ozone (Vahisalu et al. 2010). Ground-level or tropospheric ozone (O_3) is the gaseous pollutant at present of most concern for forest health (Serengil et al. 2011). Ozone is also used as a tool to induce ROS production and investigate their effects. RTD coincided with a burst of ROS in guard cells of 11 Arabidopsis ecotypes (Vahisalu et al. 2010). Mutants deficient in various aspects of stomatal function revealed that the SLAC1 protein, essential for guard cell plasma membrane S-type anion channel function, and the protein kinase OST1 were required for the ROS-induced fast stomatal closure. The recovery of g_s occurred even during O_3 exposure (Fig. 4.5) and stomata did not respond to additional O_3 pulses until a resting period for the guard cells allowed them to sense and respond to O_3 again (Vahisalu et al. 2010).

The temporary desensitization of stomata may be a cause of the sluggish responses to environmental stimuli observed after O_3 exposure (Paoletti and Grulke 2010). Sluggishness is defined as a delay in stomatal response to changing environmental factors relative to controls (Fig. 4.2), and has been demonstrated in different plant physiognomic classes (Paoletti and Grulke 2010). Sluggishness results from a longer time to respond to the closing signal and slower rate of closing. Sluggish stomatal responses to light variation with O_3 exposure were first postulated in Norway spruce using a transpirational assay, i.e. by measuring water

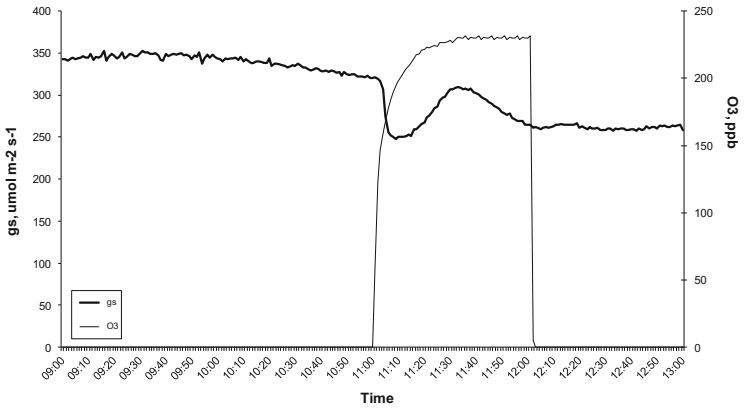

Fig. 4.5 Transient decline of stomatal conductance (g_s) in a *Helianthus annuus* leaf exposed to 230 ppb ozone (O_3). The recovery of gs occurred even during the O_3 exposure, suggesting a desensitization of stomata (Data authors and holders: H. Kaiser and E. Paoletti)

losses over time in detached needles (Keller and Häsler 1984). Delayed stomatal response following O_3 exposure has since been reported with changes in leaf to air vapour pressure deficits (Tjoelker et al. 1995; Kellomaki and Wang 1997; Grulke et al. 2007b), fluctuating photosynthetic photon flux density (PPFD) (Reich and Lassoie 1984; Reiling and Davison 1995; Paoletti 2005; Grulke et al. 2007b; Paoletti and Grulke 2010), and water stress (Reich and Lassoie 1984; Paoletti 2005; Mills et al. 2009; Grulke et al. 2007b; Paoletti et al. 2009; Hoshika et al. 2013). Drought stress itself, however, is able to induce stomatal sluggishness (Hoshika et al. 2013). Sluggish stomatal control over transpiration may increase water loss at the leaf level. At the crown-level, however, O_3 exposure reduced gas exchange and accelerated leaf shedding, thus compensating for sluggishness-increased water loss (Hoshika et al. 2012). Several mechanisms by which O_3 may induce sluggishness can be found in the published literature. Omasa (1990) reported a slight increase in permeability of epidermal cell membranes and alteration of the osmotic pressure after O_3 exposure that may modulate a balance in turgor between guard and subsidiary cells. Vahisalu et al. (2010) found that Ca^{2+}-dependent signalling and O_3-induced stomatal movements were independent, and noted that the temporary desensitization of the guard cells was due to blocked K^+ channels. Ozone may also delay stomatal responses by stimulating ethylene production and reducing stomatal sensitivity to ABA (Wilkinson and Davies 2010). Another cause of sluggishness may be O_3-induced lower rates of transpiration, which permit leaves to take longer to perceive the same change in water status or light variation.

4.4 Concluding Remarks

Stomatal regulation is the primary function for balancing the efficiency of water expenditure in relation to carbon gains (Cowan 1977). Optimization theory states that for each set of environmental conditions, an optimal stomatal conductance exists. It is immediately evident that g_s during stomatal oscillations is not at its optimum most of the time, either expending too much water in relation to carbon gain at peak conductance, or unnecessarily limiting carbon gain in the troughs. Nonetheless, modeling the effect of stomatal oscillations on time-averaged water use efficiency, Upadhyaya et al. (1988) identified conditions where oscillations slightly improved water use efficiency at reduced transpiration when compared to constant conductance. However, there is no experimental support for these observations. The marginally positive effect of a relatively rare phenomenon is unlikely to provide sufficient selection for this resulting complex feature.

Considering the prominent role of hydraulic positive feedback in stomatal oscillations, another hypothesis can be suggested. Hydropassive positive feedback in stomatal mechanics is a property only existing in seed plants and has not been found in ferns and mosses which appear to respond with hydropassive closure to increased transpiration (Brodribb and McAdam 2011). Positive hydraulic feedback evolved in seed plants along with a more sophisticated control of stomata through leaf water relations (McAdam and Brodribb 2012). Acceleration of stomatal opening as well as closing by hydropassive positive feedback enables larger and faster responses with the same metabolic effort. This allows a faster tracking of the dynamic environmental conditions resulting in an on average smaller deviation from the floating optimum. The metabolic costs necessary for dynamic stomatal movements (Vico et al. 2011) could be reduced due to hydraulic amplification of osmotic activity. Stomatal oscillations therefore may not in itself enhance efficiency of water use, but could be seen as a side effect of an aggressive tuning of feedback-regulation, which has evolved because it allows a faster response to environmental fluctuations.

The control of gas exchange by leaf stomata has broad implications for the response of terrestrial vegetation to changes in environmental conditions, including climate change (Hetherington and Woodward 2003). The feedback mechanism involved in oscillations appears to be identical to the mechanisms involved in regulation of leaf water loss. Unfortunately, there is still no consensus regarding the identity of the effectors involved in stomatal responses to hydraulic perturbations, nor regarding the biophysical mechanisms by which those effectors induce changes in stomatal conductance (Buckley and Mott 2002b; Meinzer 2002; Franks 2004; Buckley 2005). Although a vast amount of knowledge has been gathered on the intracellular events of guard cell signal transduction, these processes are both too complex and still too poorly understood to be described other than in a ‘black box’ approach. Moreover, the mechanism providing sensorial input of local leaf water relations into guard cell signaling is still obscure. Integrating these signaling events and metabolic actions merely as empirical functions into the models is

difficult as the response is highly variable depending on – among others – species, previous treatment, and circadian effects.

Similar to cellular processes, the higher levels of hydraulic interaction between stomata, leaf regions or different leaves or branches require a better understanding before these pivotal processes can be integrated into models of stomatal dynamics at the leaf or whole plant level. A prerequisite is to monitor rapid changes in plant gs by means of gas-exchange measurement devices with high-time resolution (Grulke et al. 2007a), ideally coupled with microscopical observation of individual stomata (Kappen et al. 1987; Kaiser and Kappen 2000, 2001).

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