COVER PAGE

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TITLE PAGE[1]

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[1] References are found in renewal application dated Jun 28, 2001.

Final Report

Brief Synopsis of Accomplishments in relationship to the proposed research

This award was funded at <51% of the proposed budget. The scope of the work was limited commensurately, ***focusing on the second of two hypotheses that were proposed for testing,*** viz., ***that transpiration rate determines the extent to which suc accumulates in the GC wall, providing a mechanism for regulating stomatal aperture size.*** That hypothesis received strong support because the suc content of the GC apoplast fell 7-fold on lowering of the VPD. Progress in other related areas (suc uptake mechanism, physiological effect of external suc, and especially the kinetics of ABA accumulation in the guard-cell wall) has also been made.

Summary Statements of Progress in All Areas

Here, accomplishments since submission of the previous proposal renewal are keyed to publications, as available. (Some projects spanned more than one funding period.)

1. Ewert MS, Outlaw WH Jr, Zhang SQ, Aghoram K, Riddle KA (2000) Accumulation of an apoplastic solute in the guard-cell wall is sufficient to exert a significant effect on transpiration in *Vicia faba* leaflets. Plant Cell Environ 23: 195–203 (+ cover photograph)

Mannitol—a model apoplastic solute supplied at the [suc] in the leaf apoplast—moves apparently unimpeded throughout the leaf apoplast and becomes sufficiently concentrated in the GC wall to diminish transpiration. On the basis of this and other work (Lu et al. 1995; 1997), ***we hypothesized that apoplastic solute accumulation is one mechanism by which plants "measure" the transpiration rate.*** (Attribution of Support: DOE.)

2. Popova LP, Outlaw WH Jr, Aghoram K, Hite DRC (2000) Abscisic acid—an intraleaf water-stress signal. Physiol Plant 108: 376–381

Pre-existing foliar ABA is a source for ABA accumulation by GCs upon imposition of water stress, as indicated by ABA accumulation in GCs of a detached leaflet in which ABA biosynthesis is blocked. Thus, ABA is an intra-leaf water-stress signal. This conclusion has been corroborated by Zhang and Outlaw (2001a) using intact plants without ABA-synthesis inhibition. (Attribution of Support: primary support from DOE and secondary support from NSF.)

3. Aghoram K, Outlaw WH Jr, Bates GW, Cairny J, Pineda AO, Bacot CM, Epstein LM, Levenson CW (2000) *Abg*1—a novel gene up-regulated by abscisic acid in guard cells of *Vicia faba* L. J Exp Bot 51: 1479–1480

A GC transcript up-regulated by ABA was isolated, and properties of the putative protein were studied. This research, directed toward elucidating how GCs respond to stress, has special importance because we subsequently discovered that **abg*1*** ***has similar expression characteristics*** (e.g., sensitivity to ABA, time course) ***in GCs and mesophyll cells*** (Aghoram K, Outlaw WH Jr, Zhang SQ, Meng FX, Levenson CW, unpublished). (Attribution of Support: DOE.)

4. Outlaw WH Jr, Zhang S (2001) Single-cell dissection and microdroplet chemistry. J Exp Bot 52: 605–614

This invited review for a special issue described methods of single-cell analysis and provided examples to illustrate the utility of data thus obtained. (Attribution of Support: DOE.)

5. Zhang SQ, Outlaw WH Jr, Aghoram K (2001) Relationship between changes in the guard-cell abscisic-acid content and other stress-related physiological parameters in intact plants. J Exp Bot 52: 301–308 [One of the articles featured on the cover page]
6. Zhang SQ, Outlaw WH Jr (2001a) The guard-cell apoplast as a site of abscisic acid accumulation in *Vicia faba* L. Plant Cell Environ 24: 347–355

7. Zhang SQ, Outlaw WH Jr (2001b) Gradual long-term water stress results in abscisic acid accumulation in the guard-cell symplast and guard-cell apoplast of intact *Vicia faba* L. plants. J Plant Growth Reg 20: 300-307
8. Zhang SQ, Outlaw WH Jr (2001c) Abscisic acid introduced into the transpiration stream accumulates in the guard-cell apoplast and causes stomatal closure. Plant Cell Environ 24:1045-1054

This series of papers reported the quantitative pattern of ABA localization in GCs upon various kinds of water stress (whole plant, whole root, partial root, soil, hydroponic, long term, short term, etc.). In brief, first, ***ABA accumulates in GCs when part of the root is stressed***, although the water status of the shoot is unaffected initially. In my opinion, **this was the final required test of the idea that stressed roots signal GCs via ABA in the transpiration stream.** (As discussed below, ABA emanates from stressed roots but whether, or to what extent, ABA is removed from the leaf apoplast or blocked in its passage to GCs was unknown.) Second, ***on stressing of roots, ABA accumulates in the GC apoplast, first, from foliar sources, and second, from the transpiration stream***. Localization in the apoplast has importance because some ABA receptors are internal, whereas others face the apoplast. Third, ***introduction of ABA into the transpiration stream allowed the study of ABA in the absence of other potential signals*** (i.e., without any change in the water status). The method also permitted a quantitative appraisal because uncertainties in the concentration of ABA and the mass of ABA delivered naturally via the transpiration stream were avoided. **ABA that is introduced into the transpiration stream accumulated in the GC apoplast, proving that the concentration around the GC is greater than the concentration arriving in the leaf.** I interpret this accumulation as a result of evaporation from the GC wall, which is hypothesized to provide ***a transpiration-dependent means of amplifying the [ABA] arriving in the transpiration stream***. This variable amplification may explain apparently variable sensitivity to ABA. Last, ***stressing the whole plant results in the accumulation of ABA in the GC symplast and the GC apoplast.*** Overall, it is concluded that **root-source ABA and ABA from other leaf cells accumulates in the GC apoplast and provides recent information about the whole-plant water status. ABA accumulation in the symplast is an indicator of foliar stress.** A complete interpretation of these physiological data awaits understanding of the different ABA receptors and the signal transduction networks with which each interfaces. *Selected results will be amplified later in this report* (Attribution of support: DOE and National Key Basic Research Special Funds (G 1999011700) of the People's Republic of China. The Chinese support was simply in providing release time (but not financial support) for Professor Zhang to conduct this research, all of which was done in Tallahassee.)

9. Outlaw WH Jr, De Vlieghere-He X (2001) Transpiration rate—an important factor controlling the sucrose content of the guard-cell apoplast of *Vicia faba* L. Plant Physiol 126: 1716-1726

As reported with previous DOE support (Lu et al. 1995; 1997), the suc contents of the GC apoplast and the GC symplast increase during the photoperiod. We developed the hypothesis that suc accumulation in the GC apoplast results from evaporation from the GC wall. In the previous application, we proposed to test that hypothesis. That work has been completed. In brief, we found that ***the suc content of the GC apoplast is dramatically lower if plants are transpiring less***. To my knowledge, this is the only known molecular mechanism that accounts for diminution of stomatal aperture size in response to high VPD. I do not, however, think that it operates to the exclusion of hydraulic effects or other undiscovered molecular mechanisms (see Experimental Plan). *Selected results will be amplified later in this report.* (Attribution of Support: DOE.)

10. Outlaw WH Jr, Du Z, Meng FX, Aghoram KA, Riddle KA, Chollet (2002) Requirements

for activation of the signal-transduction network that leads to regulatory phosphorylation of leaf guard-cell phospho*enol*pyruvate carboxylase during fusicoccin-stimulated stomatal opening. Arch Biochem Biophys 407: 63-71 [COVER PHOTOGRAPH KEYED TO THIS ARTICLE IN DECEMBER ISSUE]

Previous DOE-supported work (Du et al. 1997) indicated that GC PEPC is phosphorylated when GCs are stimulated with FC (a fungal toxin that causes stomatal opening). Phosphorylation renders PEPC relatively insensitive to inhibitory cytosolic [mal$^-$]s. Conversely, PEPC is dephosphorylated when GCs are exposed to ABA. The present study focuses on parameters that affect PEPC phosphorylation, of particular interest because PEPC phosphorylation is controlled by the abundance of an unregulated highly specific protein kinase in the C_4 system. In brief, a short exposure to FC is sufficient to cause stomatal opening. The high-phosphorylation status is maintained through ***continuous phosphorylation in open stomata*** (i.e., not just during stomatal opening when mal$^-$ accumulates). To investigate whether downstream events moderate the phosphorylation status, we supplied external Cl^-, a permeable anion that prevents GC mal$^-$ accumulation that otherwise stabilizes cytosolic pH. ***External Cl^- decreased the phosphorylation status by ~70–85%. External suc, which supports a small aperture-size increase in the absence of K^+, did not cause GC PEPC phosphorylation***, again supporting a downstream (post-H^+-ATPase) moderation of PEPC phosphorylation. Most basic elements described above were completed earlier, but portions of the project were completed during the current funding period. I decided to expand the scope of the research before publication; therefore, the final aspect of this paper will address cytosolic-pH control of PEPC phosphorylation. The correlative PEPC data have been collected, and the pH-imaging studies, which will complete this contribution, are in progress, though not an explicit part of this proposal. (Attribution of support: DOE.)

Details of Selected Progress

Most of the work was focused on the accumulation leaf apoplastic solutes in the GC wall because of physiological parameters (e.g., the rate of photosynthesis for [suc]) and environmental parameters (e.g., soil-water availability for [ABA]). This idea is contrary to the notion that leaves have flumes, essentially areas of discontinuity of the bulk-water apoplastic space (Canny 1995a), for which we find no evidence. Therefore, this Final Report will emphasize 2 key findings.

Elevation of the GC-wall [suc] is a result of high transpiration rate. Mesophyll-derived leaf apoplastic suc is the source of GC apoplastic suc (Lu et al. 1997) during midday (Lu et al. 1995) in *Vicia faba*, an apoplastic phloem loader (Delrot et al. 1983; Kühn et al. 1999). Suc becomes concentrated in the GC apoplast because this is the distal point in the evaporative pathway. Accordingly, ***the extent to which suc accumulates in the GC apoplast was hypothesized to be controlled by two interacting physiological parameters: (a) leaf apoplastic [suc], which is a function of photosynthesis rate and phloem-transport rate from the leaf*** (Ntsika & Delrot 1986; Lohaus et al. 1995), ***and (b) transpiration rate***, addressed below. One figure from Outlaw & De Vlieghere-He (2001) is selected. These data indicate that GC apoplast [suc] is a factor in the stomatal-closing response to increasing VPD.

In a landmark study, Mott and Parkhurst (1991) found that the stomatal response to humidity is consistent with sensing of the transpiration rate rather than that of RH *per se*. A reanalysis (Monteith 1995) of 52 published sets of measurements and subsequent work (Jarvis et al. 1999) support this conclusion. As alluded to, we (Lu et al. 1997) therefore hypothesized that transpiration-linked accumulation of GC apoplastic suc is a mechanism by which plants respond to high transpiration rate. To test that hypothesis, we maintained *Vicia faba* plants at 60% RH (control conditions) or shifted them to 90% RH, which decreased the transpiration rate nominally 4-fold. At various times, the GC apoplast suc contents, the GC symplast suc contents, the whole-leaf suc contents, and the bulk-leaf apoplast [suc] were determined. Various control studies proved that the results were not consequences of [suc] changes at the whole-leaf level or

affected by artifacts resulting from dew formation at the high RH, leaf heterogeneity, or sample sizes used in histochemistry. The effects on GC suc of an RH shift during the photoperiod, when stomata are open, are shown in Fig. 1. In these experiments, all plants were maintained at 60% RH until 1100 h; then, some plants were shifted to 90% RH. At 1100 h in control plants, the GC apoplast suc content was 743 ± 107 fmol·GCpair^{-1}; the stomatal aperture size, 7.4 ± 1.6 μm; and the GC symplast suc content, 441 ± 71 fmol·GCpair^{-1}. These values were not significantly different from those at 1100 h in plants that were later shifted to 90% RH or from those in control plants at 1300 h. Shifting to 90% RH during the photoperiod corresponded to a decrease in the GC apoplast suc content to 96 ± 120 fmol·GC-pair^{-1} ($P < 0.01$), an increase in stomatal aperture size to 9.0 ± 1.8 μm ($P < 0.01$), and an increase in the GC symplast suc content to 832 ± 92 fmol·GCpair^{-1} ($P < 0.01$). Thus, in this set of experiments, the decrease in transpiration rate resulted in a decrease of the [suc] of 155 mM in the GC apoplast. Unexpectedly, GC symplastic [suc] increased along with the decline in the GC apoplastic [suc]. Fortuitously, the higher GC symplastic [suc] at lower transpiration essentially provided an explanation for earlier differences in quantitation between my lab and that of Tallman and of Zeiger. It is interesting to speculate (Outlaw & De Vlieghere-He 2001) that the increased GC [suc] in the symplast results from activation of suc symport.

These data allow two significant conclusions: (1) GC apoplastic [suc] is sufficiently high to diminish stomatal aperture size, which corroborates previous DOE-supported research, ***and (2) transpiration-linked [suc] changes provide a means for the plant to respond to transpiration rate.*** In addition, we hypothesize that transpiration-linked [suc] changes alter GC gene expression and provide a means for the plant to acclimate. Support for these statements follows. From the aqueous cell-wall volume of *Vicia* GCs (Ewert et al. 2000), we calculate that the [suc] in the GC apoplast changed by ~0.16 *m*, equivalent to a $\Delta\Psi s = 0.4$ MPa (Michel 1972). Similar calculations for the GC symplast, yielding $\Delta\Psi s \approx 0.2$ MPa, are imprecise because the volume of the GC symplast increases with stomatal aperture size. (That is, part of the increase in suc content of the GC symplast simply maintains the concentration there as the volume increases.) There are various estimates for $\Delta\Psi s$ (GC)·μm^{-1} (aperture) for *Vicia faba*. Hsiao (1976, table 4.6) cites a

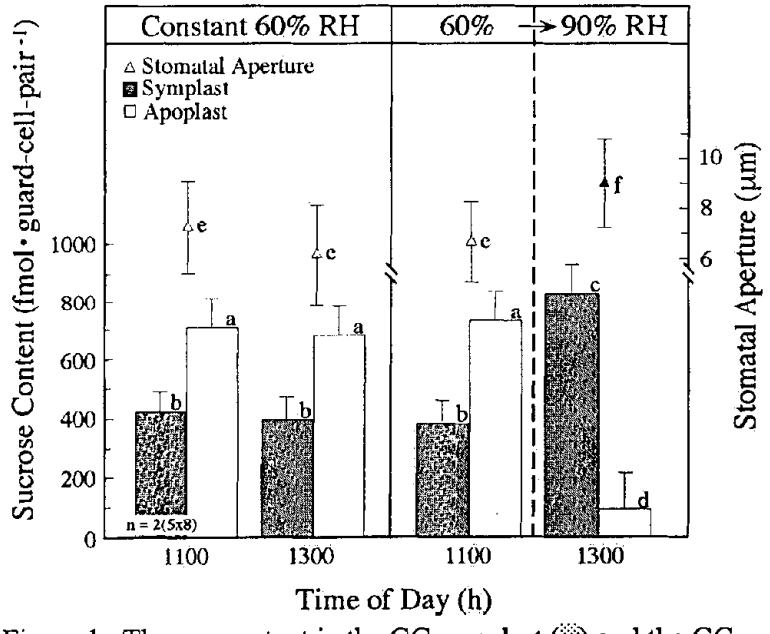

Figure 1. The suc content in the GC symplast (▩) and the GC apoplast (□) at 1100 h and at 1300 h. The control plants (left panel) were maintained at constant 60% RH. RH-shifted plants (right panel) were shifted from 60% RH at 1100 h to 90% RH, which required ~ 10 min. The experiments were conducted in duplicate; five leaflets were sampled in each experiment for each datum (n = 80 GC pairs per column). Corresponding stomatal aperture sizes (n > 180 GC pairs for each column) are represented by triangles (Δ, plants at 60% RH; ▲, plants at 90% RH). Data labeled by identical alphabetic characters are not significantly

range of 0.12–0.2 MPa·μm^{-1} from four studies, and Poffenroth et al. (1992, table III) use a range of 0.05–0.16 MPa·μm^{-1}, derived from three different studies, in model calculations. Use of the midpoint of these values, 0.125 MPa·μm^{-1}, provides a perspective on the importance of suc fluctuations to stomatal aperture size. Thus, the change in GC apoplast [suc] predicts a change in stomatal aperture size of ~3 μm (less if calculated with the factors of Franks et al. (2001), but more if GC symplastic [suc] is considered). In summary, ***decreasing the transpiration rate coincides with reciprocal changes in the GC suc pools that are sufficient to increase stomatal aperture size.*** In addition, the ***[suc]s in the GC apoplast under high transpiration exceed those required to alter gene expression*** (for general reviews of sugar-regulated gene expression, see Gibson 2000; Koch et al. 2000; Pego et al. 2000; Smeekens 2000).

The GC-wall [ABA] can be higher than that of the transpiration stream. ABA synthesized by water-stressed roots is exported to the shoot (Davies & Zhang 1991; Jackson 1993; 1997) where it lowers stomatal conductance and provides a mechanism for avoiding water stress (Tardieu 1996). Although this overall outline is well established, questions remain about the importance of the [ABA] in the xylem (Gowing et al. 1993a), the amount of ABA arriving at the leaf (Gowing et al. 1993b; Jia & Zhang 1999), removal of ABA from the apoplast (Hartung et al. 1998), and influences of the chemical environment, particularly pH (Gollan et al. 1992; Schurr et al. 1992; Wilkinson & Davies 1997; Wilkinson 1999).

The movement of ABA and other solutes through the leaf apoplast is poorly understood. On the one hand, Canny (1995a) concluded that the parenchyma cell walls normally are not part of the apoplastic flow space. Accordingly, water leaves the apoplast and enters the symplast at flumes, which are located in or near the termini of fine veins. This interpretation implies that the delivery of apoplastic solutes to the epidermis is either by symplastic transport processes (Canny 1995b) or by apoplastic diffusion from sumps, i.e., solute accumulation sites at flumes. On the other hand, the rapid accumulation of petiole-fed mannitol in the GC wall (Ewert et al. 2000) is best explained by direct flow of the apoplastic solution to the GC wall, a pathway that is supported by various studies (refs. in Ewert et al. 2000). Unlike mannitol, however, imported ABA can be metabolized or sequestered by leaf cells, as mentioned above. Specifically, ABA in the transpiration stream has been reported not to be as effective in causing stomatal closure as ABA applied directly to epidermal peels (refs. in Wilkinson & Davies 1997). Finally, the cellular site at which ABA accumulates in GCs is important because, as mentioned several times above, receptors bind to extracellular ABA (Anderson et al. 1994; also see MacRobbie 1997) and intracellular ABA (Allan et al. 1994; Schwartz et al. 1994; Wang et al. 1998). For this part of the Progress Report, I have selected one figure from Zhang & Outlaw (2001c). These data show that ABA delivered to a leaf of an intact plant by petiolar infusion accumulates in the GC apoplast. For brief general background, please refer to the summary statements, above, for Items 5–8.

As background for Fig. 2, we conducted a series of preliminary experiments with infusion solution that had been formulated to mimic xylem sap (see Atkinson et al. 1992; Mühling & Sattelmacher 1995; 1997; Mimura et al. 1996) with 1 μM ABA (indicative of stress, but less than the maximum values obtained with the pressure bomb; Zhang & Outlaw 2001b). The experiments indicated that the delivery system does not result in detectable stress-related hydraulic signals or other chemical signals. Thus, ***"root-source" ABA could be studied quantitatively and in isolation from other root signals.*** In addition, ***the protocol avoided the ambiguity that arises when extruded-sap ABA concentrations and transpiration rates are used to estimate the relevant xylem ABA concentrations and rates of ABA delivery to the leaf*** (see Jokhan et al. 1996; 1999). The experimental protocol included measurements of whole-leaf ABA content (ABA was not metabolized over the 1-h time course) and leaf apoplastic ABA content (a portion of infusion ABA was apparently taken up by the leaf symplast).

Leaf conductance and GC apoplastic [ABA] were strongly correlated (Fig. 2), whereas the GC symplastic ABA pool and leaf conductance were essentially unrelated (Inset, Fig. 2). Note that

Fig. 2 is not a dose-response curve *per se* as conductance was decreasing and the GC apoplastic ABA content was increasing. However, the $t_{1/2}$ for ABA-induced stomatal closure in epidermal peels of *Vicia faba* is only ~5 min (Raschke et al. 1975), indicating that, for present purposes, Fig. 2 is a good approximation of a dose-response curve. Thus, the half-saturation of the decline in conductance was ~1 μM external ABA (Fig. 2). This value is typical of that required to elicit 50% of the full ABA response on stomatal aperture size in *Vicia* epidermal peels (cf. 4 μM, Horton 1971; 0.2 μM, Kondo & Maruta 1987; both at the same pH used here).

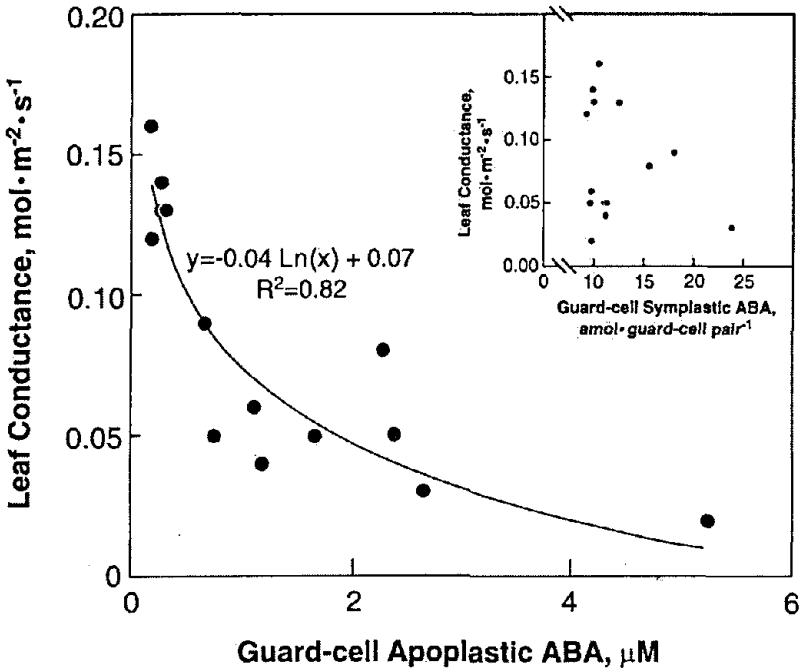

Figure 2. The relationship between leaf conductance and GC apoplast [ABA] during a 1-h of petiolar infusion of 1 μM ABA. The inset shows the corresponding GC symplast ABA content.

After 1 h of infusion, the average maximum GC apoplast [ABA] exceeded 3 μM. All of several lines of argument indicate that this concentration is higher than that in the leaf apoplast, consistent with ***evaporative deposition of ABA in the GC wall.*** First, and simplest, if all infused ABA remained in the leaf apoplast, the average ABA concentration there would be ~1.35 μM (infused ABA·(apoplast volume)$^{-1}$ + preexisting ABA). Thus, this most conservative case indicates an amplification of >2x. Second, on the basis of the leaf apoplast ABA concentration, the amplification was >6x. In a previous investigation, the ratio (GC-apoplast ABA concentration)·(leaf-apoplast ABA concentration)$^{-1}$ was higher, up to 13.5x (Zhang & Outlaw 2001a). Two features explain the apparent decreased amplification in this study: (a) the apoplastic-sap ABA concentration was probably overestimated, being influenced by recently infused ABA (1 μM) in this study, and (b) stomatal aperture size, and hence evaporation, was less in this study. Third, ***the most reasonable estimate of amplification under these conditions is ~9x, which is based on the estimate that three-fourths of infused ABA was taken up into the leaf symplast.*** Reference values for this best estimate come from experiments in which dilute solutions of mannitol were fed to leaflets (Ewert et al. 2000). In those experiments, the average [mannitol] in the GC wall was 4.6–6.7x the average concentration in the leaf apoplast after 3–5 h (Ewert et al. 2000). In summary, despite imprecision in quantification, ***root-source ABA does accumulate in the GC wall, which is the distal point in the evaporative pathway.*** Our data do not permit distinction between the possibilities that ABA is restricted to the GC apoplast and that ABA taken up by the GC symplast is rapidly metabolized. However, as discussed

elsewhere (Zhang & Outlaw 2001b), symplastic accumulation of ABA would only be favored in a part of the plant that experiences water stress.

The accumulation of ABA in the GC wall is reminiscent of the accumulation of suc there, as discussed in the previous section. (This similarity and our experience with ABA compartmentation in the leaf (Harris et al. 1988; Harris & Outlaw 1990; 1991) directed our research efforts to include ABA.) In the case of suc, high transpiration rate coincides with high GC-wall [suc], and decreasing the transpiration rate results in diminished GC-apoplast [suc] and larger stomatal aperture sizes (Outlaw & De Vlieghere-He 2001). Thus, as shown in the previous section, transpiration-linked accumulation of suc is one means by which plants respond to the rate of transpiration. **No simple explanation is likely to be complete.** I hypothesize that ***transpiration-linked ABA accumulation would provide another and*** **water-status-dependent means to sense the transpiration rate** ***when the xylem-sap ABA concentration is sufficiently high.*** If this is true, stomata should have apparently higher sensitivity to root-source ABA when stomata are open and the air is dry because higher evaporation would tend to concentrate the ABA more. It is important to emphasize, however, that our interpretation does not call for an obligate role for ABA in humidity sensing (see Assmann et al. 2000). In addition to a role in humidity sensing under some conditions, ***concentrating ABA in the GC apoplast also potentially confers GC-specific regulation of ABA-responsive genes.***