Increased Accumulation of Cuticular Wax and Expression of Lipid Transfer Protein in Response to Periodic Drying Events in Leaves of Tree Tobacco[1][W]

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Cuticular wax deposition and composition affects drought tolerance and yield in plants. We examined the relationship between wax and dehydration stress by characterizing the leaf cuticular wax of tree tobacco (*Nicotiana glauca* L. Graham) grown under periodic dehydration stress. Total leaf cuticular wax load increased after each of three periods of dehydration stress using a CH_2Cl_2 extraction process. Overall, total wax load increased 1.5- to 2.5-fold, but composition of the wax was not altered. Homologous series of wax components were classified into organic groups; *n*-hentriacontane was the largest component (>75%) with alcohols and fatty acids representing <10% of the entire wax load. An increase in density, but no change in the three-dimensional shape, of leaf wax crystals was evident under low-kV scanning electron microscopy after each drying event. Leaves excised from plants subjected to multiple drying events were more resistant to water loss compared to leaves excised from well-watered plants, indicating that there is a negative relationship between total wax load and epidermal conductance. Lipid transfer proteins (LTPs) are thought to be involved in the transfer of lipids through the extracellular matrix for the formation of cuticular wax. Using northern analysis, a 6-fold increase of tree tobacco *LTP* gene transcripts was observed after three drying events, providing further evidence that LTP is involved in cuticle deposition. The simplicity of wax composition and the dramatic wax bloom displayed by tree tobacco make this an excellent species in which to study the relationship between leaf wax deposition and drought tolerance.

As we attempt to cultivate additional acreage of marginal land while water resources become limited, yield will be increasingly influenced by the effects of periodic dehydration stress. Many characteristics of the leaf affect drought tolerance, such as leaf water relations, osmotic adjustment, cell membrane stability, cuticular wax characteristics, and epidermal conductance. In this work we have focused on one aspect of drought tolerance, cuticular wax character, composition, and load, as it relates to epidermal conductance after stomatal closure. The cuticle, a thin, continuous, extracellular membrane that covers the aerial surfaces of plants, provides a protective barrier between the plant and its environment and functions primarily as a barrier to water loss. During stomatal closure, which occurs in response to periodic drying or drought stress, epidermal conductance becomes the primary mode of water vapor loss (Hall and Jones, 1961). The chemistry and the load of cuticular wax affect the epidermal conductance rate and influence the ultrastructure and topology of the leaf (Baker, 1982). In turn, the topology of the leaf affects the plant's interactions with biotic and abiotic components of its environment. Ultimately, both the rate of epidermal conductance and the topology of the leaf affect yield (Johnson et al., 1983; Premachandra et al., 1994; Sánchez et al., 2001).

Comprehending the genetic and environmental factors that control the biosynthesis and composition of waxes will improve our understanding of epidermal conductance (Bianchi, 1995; von Wettstein-Knowles, 1995; Post-Beittenmiller, 1996). Cuticular waxes consist of homologous series of very-long-chain fatty acids, alcohols, aldehydes, alkanes, esters, and cyclic organic compounds. Great strides have been made recently in comprehending the metabolic pathways involved in cuticular deposition through studies of the *bloomless* and *eceriferum* mutants (Jenks and Ashworth, 1999). Hydrophobicity of the cuticle is dependent upon the relative composition of the hydrocarbon, alcohol, and aldehyde fractions of the cuticular wax (Grncarevic and Radler, 1967; Bianchi, 1995). The relative composition of each fraction is unique to each species and affects the rate of epidermal conductance, as well as the physical structure of the surface wax (Bianchi, 1995).

The cuticular waxes are arranged in distinct layers, which are chemically different and physically

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separated from one another (Jetter et al., 2000; Knoche et al., 2000). Waxes embedded in the cutin are classified as intracuticular waxes and on the surface as epicuticular waxes (Jetter et al., 2000). Epicuticular waxes, as defined by their ability to be mechanically removed from the surface of a tissue, appear to consist of the longest chain alkanes (C_{29} and above; Jetter et al., 2000; Vogg et al., 2004). Intracuticular waxes, the waxes that are left over after mechanical removal of the epicuticular waxes, can be chemically removed with an organic solvent such as chloroform (Jetter et al., 2000). Mechanical removal of the epicuticular wax significantly improves the rate of water permeability through the cuticle in *Lycopersicon esculentum* and *Prunus avium* fruits (Knoche et al., 2000; Vogg et al., 2004), but the majority of the rate of epidermal conductance is determined by the intracuticular waxes (Vogg et al., 2004). The rate of diffusion of water and other organic solutes through these waxes is determined by the polar pathways in each layer of the cuticle (Riederer and Schreiber, 1995; Baur et al., 1999). Furthermore, wax load has been shown to influence the rate of epicuticular conductance in some instances, but not others. An increase in wax load was correlated with lower conductance in nonirrigated sorghum (*Sorghum bicolor*) cultivars (Premachandra et al., 1992), and reduced wax load was correlated with increased conductance in sorghum *bloomless* mutants (Jenks et al., 1994). Alternatively, cuticular wax load was not correlated with epidermal conductance in pea (*Pisum sativum*; Sánchez et al., 2001).

To date, it is not known how wax is deposited in the cuticle. Neinhuis et al. (2001) have recently proposed that cuticular wax deposition is a mechanical self-assembly process whereby epicuticular wax crystals associate with the cuticular water current and are deposited on the surface of the cuticle. While this may account for some wax deposition, it is assumed that there is also a biological aspect to cuticle deposition. Lipid transfer proteins (LTPs) are thought to be involved in this process. LTPs are small, basic, soluble proteins that are characterized by their ability to transfer lipids between natural and artificial membranes in vitro (Kader, 1996). They are involved in plant defense, with strong evidence supporting their mutual involvement in cuticle deposition. LTPs are induced by pathogens (Molina and García-Olmedo, 1993; Guiderdoni et al., 2002; Park et al., 2002), display antimicrobial activity (Nielsen et al., 1996; Molina and Garcia-Olmedo, 1997; Kristensen et al., 2000), and are involved in systemic resistance signaling (Maldonado et al., 2002). Furthermore, many *LTP* genes are induced by drought stress and/or water deficit (Colmenero-Flores et al., 1997; Jang et al., 2004), and some are up-regulated when exposed to both pathogens and dehydration stress (Jung et al., 2003). Several studies have confirmed that LTPs are secreted and that many members of this large family are predominately expressed in the epidermis (Sterk et al., 1991; Fleming et al., 1992; Thoma et al., 1993; Pyee et al., 1994; Canevascini et al., 1996; Smart et al., 2000). The tertiary structure of LTP allows it to form complexes with various lipids (Douliez et al., 2000; Silva et al., 2005), and LTP has been identified as the major protein present in the cuticular wax of broccoli (*Brassica oleracea*; Pyee et al., 1994). Currently, there is still no direct evidence that LTP functions in intracuticular or epicuticular wax deposition. In this work, we have focused on the regulation of LTP in tree tobacco (*Nicotiana glauca* L. Graham).

Tree tobacco has at least five highly similar members of the type 1 *LTP* gene family, which are differentially regulated (Cameron, 2001). Due to the high similarity among members of the family, traditional techniques such as northern analysis or reverse transcription-PCR are not adequate to determine the pattern of regulation of each isoform. Therefore, a generic probe was designed that hybridizes to homologous genes in the family. In tree tobacco, as in other plants, *LTP* is predominantly expressed in the epidermis and is induced under drought stress (Smart et al., 2001; Cameron et al., 2006). Here, we demonstrate that there is a dramatic induction of *LTP* concomitant with increased wax deposition in tree tobacco exposed to periodic drying events. This provides further evidence that LTP is involved in cuticular wax deposition and is important in the development of drought tolerance.

Understanding the basis of water use efficiency in plants is an important step toward manipulating the plant's physiology to improve drought tolerance, increase yield, and ultimately reduce the costs associated with irrigation. Conventional breeding programs often focus on improving drought tolerance only to see a decline in yield or realize improved yields at the expense of drought tolerance (Boyer, 1992; Passioura, 1994). For instance, glaucousness, the light-scattering effects of the wax, has been used as an indicator of wax load in some species. Although fairly easy to observe by eye, correlating the amount of glaucousness displayed with wax load, yield, or water use efficiency has had mixed results (Johnson et al., 1983; Jefferson et al., 1989; Febrero and Araus, 1994; Premachandra et al., 1994; Febrero et al., 1998). Tree tobacco can serve as an excellent model for these studies. Native to the arid regions of South America, tree tobacco has been used as a model system to study stomatal physiology (Thomas et al., 1991) and guard cell gene expression (Smart et al., 2000, 2001), but it also displays an extensive wax bloom, making it an interesting subject for the study of cuticular wax deposition. Furthermore, the composition of the wax is relatively simple, consisting primarily of *n*-hentriacontane (Cameron, 2001). In this study, we have characterized the response of tree tobacco to periodic dehydration stress by quantifying total wax load, analyzing the constituents of the wax under periodic dehydration stress, and correlating wax load with desiccation tolerance in leaves detached from plants that were subjected to periodic dehydration stress.

RESULTS

Effect of Drought Stress on Leaf Surface Ultrastructure

In an attempt to simulate conditions of varying water availability that plants might experience in the field, tree tobacco was subjected to multiple periodic drying events. Treated plants continued to grow at approximately the same rate as controls; stem height and leaf size were comparable (data not shown). Periodically dried plants produced a prominent wax bloom relative to plants that were always well watered (Fig. 1). The increase in epicuticular wax uniformly covered the adaxial surface of the fully expanded leaves. Although wax blooms were obvious after one drying event, the glaucous appearance became increasingly apparent to the naked eye on plants that were exposed to multiple drying events (data not shown). The presence of globular crystals was the most prominent feature of the tree tobacco leaf surface ultrastructure (Fig. 2). Epicuticular wax crystals formed with approximately equal density across the adaxial and abaxial surfaces of the leaf, with the exception of the guard cells, which displayed a noticeably lower density of wax crystals than pavement cells. A greater density of wax crystals was observed on leaves of plants that had been exposed to successively more periodic drying events, although the shape and form of the crystals remained unchanged (Fig. 2, A–C). Under high magnification (2,000×), it was possible to observe patches of crystals that were present in higher density than in surrounding areas, but these patches did not appear to form in any uniform pattern on the leaf (data not shown). Under low magnification (200×) no obvious differences or changes in the number, shape, or size of individual cells were observed in leaf discs from either periodically dried or well-watered plants (data not shown). Leaf discs that were examined after immersion in CH_2Cl_2 for 30 s displayed no wax crystal structures, verifying that the globular crystals observed by scanning electron microscopy (SEM) on the surfaces of untreated leaves were composed of epicuticular wax that was effectively removed by solvent extraction (Fig. 2D).

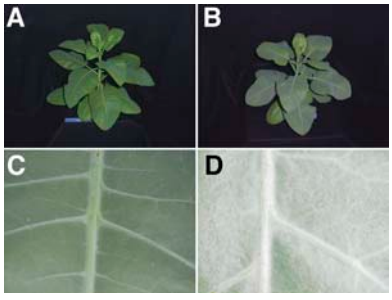

Figure 1. Tree tobacco plants exposed to periodic drying events. A, Well-watered plant. B, Plant exposed to three periodic drying events. C, Close-up of the adaxial surface of a fully expanded leaf from the plant in A. D, Close-up of the adaxial surface of a fully expanded leaf from the plant in B.

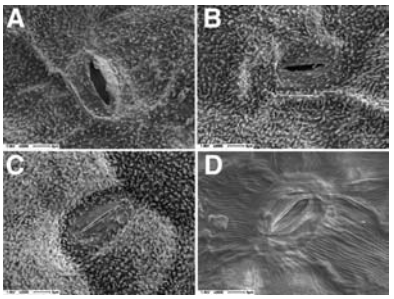

Figure 2. SEM of the adaxial surfaces of leaves from three tree tobacco plants after exposure to one drying event (A), two drying events (B), three drying events (C), and after removal of the CH_2Cl_2-soluble wax fraction (D). Magnification ×2,000. Bar = 5 μm.

Characteristics of the Leaf Cuticular Waxes

The CH_2Cl_2-soluble fraction of the cuticular wax consisted primarily of *n*-hentriacontane (C_{31}) and homologous series of alcohols (C_{24}, C_{26}, and C_{28}) and fatty acids (C_{16}, C_{18}, C_{20}; Fig. 3; Supplemental Table I). *n*-Hentriacontane was the primary component, representing at least 75% of the total wax load of fully expanded leaves. Some components of the cuticular wax could not be identified, usually because their concentrations were too low. The unidentified components represented 9% to 15% of the total wax load. Fatty acids and alcohols were minor components of the wax, and proportions varied between samples in different experiments. Even when an entire series of homologous compounds were combined, neither the fatty acids nor the alcohols ever represented greater than 10% of total wax load (Fig. 3). To determine if the wax profile of individual leaves was dependent upon the age or position of the leaf on the plant, wax was extracted from every leaf larger than 4 cm in length from one plant. A difference in total wax load was the only notable difference between extracts from the series of leaves. We observed 60% less total wax load on the 12th leaf down from the shoot tip than on the sixth leaf from the top (4.4 $\mu g\ cm^{-2}$ versus 11.5 $\mu g\ cm^{-2}$), although wax loads on the fifth, sixth, seventh, and eighth leaves from the top were consistently similar (ranging from 10.1–11.5 $\mu g\ cm^{-2}$). Based on these data we decided to use young, fully expanded leaves for all subsequent wax analyses.

Effect of Multiple Drying Events on Total Leaf Wax Load and Composition

Total wax load was dependent upon the number of drying events. The total leaf wax load on young, fully expanded leaves increased, on average, approximately 2.5-fold after exposure to three periodic drying events (Fig. 4). The major increase in wax load occurred after the first drying event. After exposure to periodic

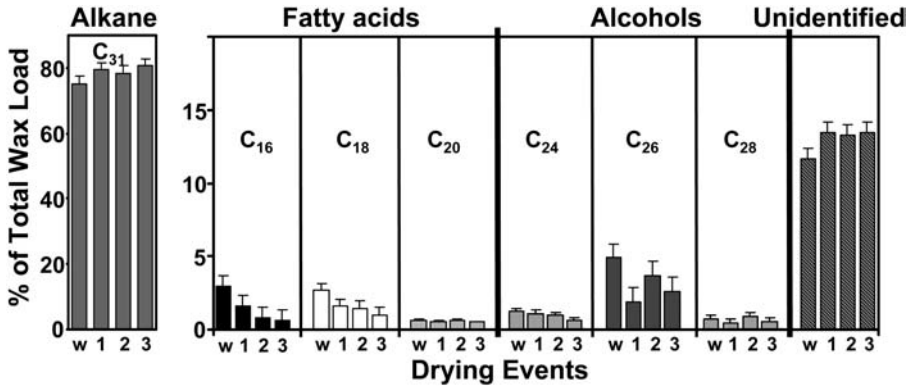

Figure 3. Relative abundance of fatty acids, alcohols, alkane, and unidentified components in the CH_2Cl_2-soluble fraction of the cuticular wax of tree tobacco plants exposed to multiple drying events. Sampling of leaves was performed from one individual after each periodic drying event, and the experiment was repeated twice. Each bar represents the mean of two experiments. Error bars indicate the SE. All components are identified by chain length. w, Well-watered plants harvested before dehydration stress was applied.

drying events, the relative abundance of *n*-hentriacontane remained fairly constant (77%–85%; Fig. 3) and was not correlated with an increase in total wax load (data not shown). The relative abundance of the fatty acid and alcohol fractions of the wax fluctuated in both the well-watered and periodically dried plants, yet we did not observe any significant correlations between abundance of wax components either with each other or with total wax load (data not shown).

Leaf Resistance to Water Loss after Exposure to Periodic Drying Events

To test the relative desiccation tolerance of leaves produced under different watering regimes, we excised young, fully expanded leaves from plants that either had been periodically dried or were always well watered. Those excised leaves were then exposed to a desiccation treatment, and the rates of weight loss were measured. Immediately after excision, leaves excised from well-watered plants weighed more than leaves excised from plants subjected to three to five drying events. After 6 h of drying, weights of leaves from periodically dried or well-watered plants were statistically identical in each experiment ($P < 0.05$; data not shown). Leaves from well-watered plants exhibited greater actual weight loss over 6 h of desiccation when compared with leaves from periodically dried plants (data not shown).

Epidermal conductance was measured after stomates were closed. To be absolutely sure that stomates were closed, we did not begin recording the rate of weight loss associated with epidermal conductance until 150 min after excision. Plants subjected to multiple drying events displayed a slower rate of weight loss (Fig. 5).

Leaves from plants never subjected to periodic dehydration stress exhibited dramatic weight loss when placed in a dehydrating environment. In two experiments, leaves excised from well-watered plants lost greater than 10% of their weight in the first 60 min, compared with less than 5% for leaves excised from periodically dried plants (data not shown). After 60 min, the rate of weight loss slowed. In contrast, leaves from periodically dried plants exhibited a slow, steady weight loss over time.

Tree Tobacco *LTP* Gene Expression Pattern in Leaves of Plants Exposed to Periodic Drying

RNA was extracted from the same leaves that were used for wax analysis in order to assay expression of tree tobacco *LTP* (*NgLTP*). *NgLTP* mRNA accumulation was 3-fold higher in plants exposed to a single drying event and 5-fold higher in plants that had been subjected to three drying events compared to well-watered plants (Fig. 6).

DISCUSSION

Tree tobacco, considered a drought-tolerant species, is a good model system to explore the relationship between cuticular wax production and periodic dehydration stress. Unlike many plants, it produces leaf cuticular wax that is remarkably simple in composition, and it responds to periods of dehydration stress by dramatically up-regulating wax production. The glaucous appearance visible by eye (Fig. 1) corresponded well with the density of the wax crystals observed by SEM (Fig. 2) and with the cuticular wax load determined by gas chromatography (GC) analysis of

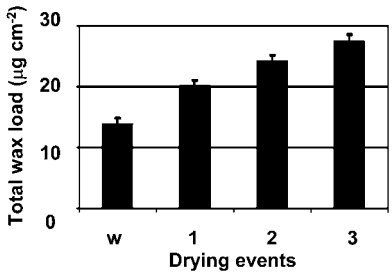

Figure 4. Total wax load in leaves of tree tobacco plants exposed to periodic drying events. The CH_2Cl_2-soluble fraction of the wax was extracted from fully expanded leaves exposed to periodic drying events. Bars represent the mean of two experiments. Error bars indicate the SE. w, Well-watered plants harvested before dehydration stress was applied.

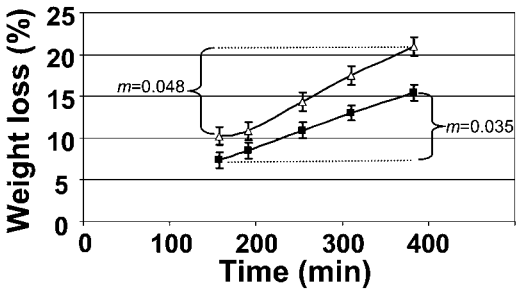

Figure 5. Rate of actual weight loss due to epidermal conductance from leaves excised from periodically dried (■) or well-watered (△) plants. Data represent one of three replicated experiments. Three leaves per plant from each of four well-watered and periodically dried plants were excised and immediately placed in a 30°C incubator. Leaves were weighed after excision at approximately 150 min, 180 min, and at hourly increments thereafter. The percent of water loss was determined relative to the original leaf weight. Error bars indicate the mean SD across all time points. m = slope of the line.

CH_2Cl_2 extracts (Fig. 3). Glaucousness has also been strongly correlated with wax load and crystal formation in pea, wheat (*Triticum aestivum*), and sorghum (Jordan et al., 1983; Bianchi and Figini, 1986; Jenks et al., 1992; Clarke et al., 1993; White and Eigenbrode, 2000). Furthermore, environmental conditions do not affect the composition of the leaf cuticular wax of growth chamber-grown tree tobacco. Our studies confirm that the wax from growth chamber-grown plants is essentially no different from the wax of tree tobacco plants growing in their native habitat in Argentina (Zygadlo et al., 1994) or in a garden plot at the University of Bonn (Meusel et al., 1999). Tree tobacco is also an excellent plant to use for plant molecular genetics. It is easy to transform with *Agrobacterium tumefaciens*; grows well in tissue culture, growth chamber, and greenhouse settings; and is self-fertile. The simplicity of tree tobacco wax, ease of obtaining large epidermal peels, and ability to manipulate gene expression are logical reasons to use tree tobacco as a model system for the study of the mechanisms of cuticular wax synthesis and deposition and their integration with genetic and physical responses to drought stress.

Tree tobacco leaf cuticular wax is dominated by a very high proportion (approximately 75%) of a single lipid component, *n*-hentriacontane (Fig. 3). Baker (1982) determined that, in some species, the dominant wax component determines the ultrastructure of the wax crystals. The positive correlation observed in this study between the wax load and the density of the wax crystals strongly supports Baker's studies. Previous characterization of leaf wax load in tree tobacco led to the conclusion that cuticle deposition ceased with leaf blade expansion (Skoss, 1955). However, if wax deposition completely ceases in mature leaves, then the older leaves on plants exposed to periodic drying events should be glossy and the younger leaves should be glaucous with corresponding higher amounts of cuticular wax. We never observed an obvious difference in glaucousness between leaves, indicating that older leaves do not lose the capacity for further wax deposition and can still respond to environmental conditions. The lower wax load observed on older leaves may be associated with degradation of the cuticular wax as it is exposed to environmental conditions.

Tremendous variation in wax load between species has been reported. Mean wax load on well-watered, chamber-grown tree tobacco leaves was 10 μg cm^{-2} and was 26 μg cm^{-2} on leaves periodically dried three times. Sorghum and cotton (*Gossypium hirsutum*) leaf cuticular wax loads were in the range of 100 to 300 μg cm^{-2} (Premachandra et al., 1994; Bondada et al., 1996), while averages of less than 25 μg cm^{-2} have been reported for rice (*Oryza sativa*), oat (*Avena sativa*), willow (*Salix* spp.), hybrid poplar (*Populus* spp.), and Arabidopsis (*Arabidopsis thaliana*; Bengtson et al., 1978; O'Toole et al., 1979; Hietala et al., 1995; Jenks et al., 1995; Cameron et al., 2002). An optimal amount of wax per unit area was observable in sorghum; further wax deposition did not significantly increase resistance to water loss (Jordan et al., 1984). Similar plateaus may be identified in other species, although we did not observe this phenomenon in tree tobacco.

Drought tolerance is the inherent ability of a plant to withstand periodic or continual dehydration stress. One method plants employ to mitigate the effects of drought is to control water loss associated with epidermal conductance. A comparison of leaf water loss rates, a method used to measure the rate of epidermal conductance (Jordan et al., 1984), is considered an effective screening technique to identify drought-tolerant cultivars in wheat (Clarke and McCaig, 1982). Wax composition is a significant factor in influencing the water permeability coefficient, which then influences

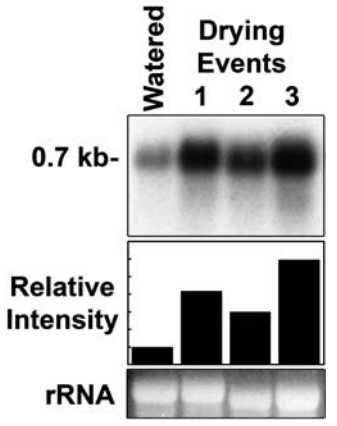

Figure 6. Northern analysis of leaves of tree tobacco exposed to periodic drying events using *NgLTP1* as a probe. Each lane contains 24 μg of total RNA extracted from fully expanded leaves of tree tobacco plants either well watered or exposed to periodic drying events. Hybridization was quantified by phosphorimagery, and relative signal intensity is depicted in the graph below the autoradiograph. An image of the 25S rRNA in the ethidium bromide-stained gel is shown as a measure of approximate equal loading of the gel. Approximate size of the band on the autoradiograph is indicated and was estimated based on migration of bands in a 0.24 to 9.6 kb RNA ladder (Invitrogen Life Technologies).

epidermal conductance rates (Becker et al., 1986). Furthermore, as was recently observed with alfalfa (*Medicago sativa*) and Arabidopsis, alterations in cuticular wax accumulations and/or composition affect the plant's epidermal permeability and drought tolerance (Chen et al., 2003; Zhang et al., 2005). In this work we observed lower epidermal conductance in leaves excised from plants subjected to multiple drying events in comparison to leaves excised from well-watered plants. This reduction in epidermal conductance corresponds well with the increased cuticular wax load. The rate of epidermal conductance may also be dependent upon the number and size of preferred polar pathways that are located throughout the subcuticular and epicuticular layers. While analysis by GC-mass spectrometry did not reveal any dramatic compositional difference, and structural differences were not observed using low kV SEM, alterations in polar pathways were not examined in this study. Since tree tobacco does not modify its wax composition nor is the topology of the epicuticular wax surface altered when exposed to periodic dehydration stress, we suggest that the amount of wax, not wax composition, is an influencing factor in determining the rate of epidermal conductance in tree tobacco.

It was apparent that tree tobacco responded to dehydration stress in at least one other way. While plants subjected to periodic drying recovered, their leaves did not return to as hydrated a state as plants never subjected to periodic drying. Over time, this would affect growth rate and leaf size, as well as influence changes at the cellular and subcellular levels. An immediate difference in response to dehydration stress was also observed between the two sets of leaves. Leaves excised from plants subjected to multiple drying events lost less weight in the first 60 min of desiccation, suggesting that a plant adapts to dehydration stress by other physiological means not studied in this work. Alterations in growth rate and leaf size were not obvious in the 14 to 17 d of the experiments performed here, nor were differences noted in the size of individual cells when leaf samples were examined by SEM.

In this research, we provide strong correlative evidence that LTP plays a role in cuticle deposition. At least one member of the *NgLTP* gene family is up-regulated with drought stress, increased cuticular wax deposition results from periodic dehydration stress, and we observed increased accumulation of *NgLTP* transcripts in plants with greater deposition of cuticular wax. Individual members of the *LTP* families are up- and/or down-regulated under dehydration stress in wild tomato (*Lycopersicon pennellii*), rice, and pepper (*Capsicum annuum*; Vignols et al., 1997; Treviño and O'Connell, 1998; Jung et al., 2003). Due to our inability to resolve the expression patterns of the five known *NgLTP* genes at this time, neither down-regulation nor functional redundancy within the *NgLTP* gene family under dehydration stress has been examined, though it is known that *NgLTP* genes are differentially regulated due to other environmental factors. For instance, wounding, not dehydration stress, induced the expression of an *NgLTP2* promoter:β-glucuronidase fusion in transgenic Arabidopsis plants (Cameron et al., 2006). The high similarity between *NgLTP* isoforms suggests that there is at least some functional redundancy in the *NgLTP* gene family. Further research is needed to determine if the capability to produce a dramatic wax bloom under dehydration stress in tree tobacco is related to the size and complexity of the LTP gene family.

Here, we show that in tree tobacco there is a correlation between periodic dehydration stress, cuticular wax load, and reduced epidermal conductance. This strong correlation is not as obvious in other species. A similar correlation of multiple drying events to wax deposition was originally shown for seedlings from five oat cultivars (Bengtson et al., 1978), although a subsequent study found no significant correlation for two of the cultivars (Svenningsson and Liljenberg, 1986). No relationship was found between epidermal conductance and wax load in barley (*Hordeum vulgare*) cultivars that were subjected to dehydration stress (Larsson and Svenningsson, 1986). However, transpiration rates were significantly higher and yield significantly lower in *bloomless* cultivars of sorghum than in isogenic normal bloom cultivars of sorghum (Chatterton et al., 1975). The strong correlation of *NgLTP* expression with cuticular wax load and reduced epidermal conductance warrants further study of the molecular role of LTP in the development of drought tolerance.

MATERIALS AND METHODS

Plant Material

Tree tobacco (*Nicotiana glauca* L. Graham) plants were grown in a 4:1 mixture of MetroMix 510 (Scotts) and perlite under 16-h-light:8-h-dark photoperiod at 120 to 165 μmol photons m^{-2} s^{-1} at 24°C in a growth chamber. Plants were fertilized with modified Hoagland's solution two out of every three waterings. Dehydration stress was applied by withholding water until the leaves were visibly wilted (approximately 2–3 d). The wilted plants were rewatered and allowed to recover, but were then subjected to subsequent periods of water deprivation, then rewatering. This drying treatment was applied to different sets of plants either once, twice, or three times in series before samples were collected. All experiments were completed within 17 d. Plants harvested before any periodic dehydration stress was applied are referred to as well-watered controls. Tissue samples were collected for RNA and wax extraction and SEM approximately 12 h after the final rewatering from either one or two fully expanded leaves (the second and/or third leaves longer than 4 cm which usually represented the seventh and eighth leaves from the top of the plant). Leaf discs (1 cm diameter) were cut from the leaves using a cork borer, and the adaxial leaf surface structure was examined using a JEOL scanning electron microscope at low kV (1.4–1.8 kV). The remaining leaf tissue was partially deveined, frozen in liquid N_2, and then used for RNA extraction. To determine if there were differences in wax load or composition among leaves of different ages, wax was extracted from four equal-sized leaf discs from every leaf longer than 4 cm (15–17 leaves total) collected from two replicate plants.

Leaf Desiccation Experiments

The rate of weight loss was determined for leaves excised from plants that were periodically dried or from well-watered plants as described (Qin and Zeevaart, 2002) with minor modifications. The experiment was repeated three times using periodically dried plants that had undergone between three and five drying events. Two or three young, fully expanded leaves were excised 12

to 24 h after the final watering. Leaves were immediately weighed, then either placed in a plastic storage container with desiccant (Drierite) or put on the racks of an incubator at 30°C. The leaves were weighed periodically over the course of approximately 6.5 h. We determined absolute weight loss by expressing the cumulative weight loss over time relative to the leaf weight immediately after excision. Since we assumed that only epidermal conductance occurred after 100 min, the rate of weight loss due to epidermal conductance was determined by expressing the cumulative weight loss over time relative to the leaf weight at the first weight recorded after the first 100 min of desiccation. A mixed-model ANOVA, blocked by experiment, was used to test for main effects of treatment on the weight loss per unit of time. All statistical analyses were performed using SAS version 9.0 (SAS Institute, 2004) at a significance level of $\alpha = 0.05$.

Cuticular Wax Analysis

Cuticular wax was extracted from freshly cut leaf discs with CH_2Cl_2 following the procedure of Cameron et al. (2002). Specifically, the surface lipids were extracted from four 12-mm^2 leaf discs cut from fully expanded leaves by immersing tissue in CH_2Cl_2 for 30 s. The extracts were evaporated under a stream of N_2 and the dried wax residues were prepared for GC by methylation with boron trifluoride (BF_3:MeOH) and by silylation with bis-(trimethylsilyl)-trifluoroacetamide. Samples were redissolved in 2,2,4-trimethylpentane for flame ionization detector-GC (Hewlett-Packard model 6890) analysis on a DB-5 capillary column (30 m) using helium as the carrier gas (2 mL min^{-1}). An initial temperature of 60°C was increased 15°C min^{-1} to 200°C, then 4°C min^{-1} to 300°C, and remained at 300°C for 5 min. Individual components were identified by their mass spectra and quantified relative to an internal standard of *n*-hexatriacontane (C_{36}). Total wax load was determined by summing the area under all the peaks after 10 min as a ratio to the area of the *n*-hexatriacontane standard.

A General Linear model (SAS Institute, 2004) was used to assess differences in the wax components among the different treatments based on a randomized complete block design, blocked by experiment. Correlation analysis, using PROC CORR (SAS Institute, 2004), was used to determine if correlations exist between individual wax components or groups of homologous components.

RNA Purification and Northern Analysis

RNA was isolated from leaf tissue using a modified hot borate method (Smart et al., 1998). An *Xba*I-*Hin*dIII fragment of D431, a cDNA clone representing *NgLTP1*, was radiolabeled with [α-^{32}P]dATP by the random primer method and used as a probe for northern analysis. Hybridizations were performed at 42°C using a 50% (v/v) formamide solution as described (Hentzen et al., 1996). Final wash conditions were 0.2× SSC/0.1% (w/v) SDS at 60°C. Hybridization was detected by autoradiography using Kodak XAR-5 film and quantified using a Molecular Dynamics PhosphorImager SI.

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