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The apoplastic antioxidant system and altered cell wall dynamics influence mesophyll conductance and the rate of photosynthesis.

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Abstract

Mesophyll conductance (g_m), the CO_2 diffusion from substomatal cavities to the carboxylation sites in the chloroplasts, is a highly complex trait driving photosynthesis (A_N). However, little is known concerning the mechanisms allowing its dynamic regulation. The apoplast is considered as a "key information bridge" between the environment and the cells. Interestingly, most of environmental constraints affecting g_m cause also apoplastic responses, cell wall (CW) alterations and metabolic rearrangements. Since CW thickness is a key determinant of g_m, we hypothesize that changes in this parameter should also influence g_m. We study the relationship between the antioxidant apoplastic system and CW metabolism with the g_m responses in tobacco plants (*Nicotiana sylvestris* L) under two abiotic stresses (drought and salinity) combining *in vivo* gas-exchange measurements with antioxidant activities, CW composition and primary metabolism analyses. Stress treatments imposed substantial reductions in A_N (58-54%) and g_m (59%), accompanied by a strong antioxidant enzymatic response at the apoplastic and symplastic levels. Interestingly, apoplastic but not symplastic peroxidases were positively related to g_m. Leaf anatomy remained mostly stable, however, the stress treatments significantly affected the CW composition, specifically pectins, which showed significant relationships with A_N and g_m. The treatments additionally promoted a differential primary metabolic response, and specific CW related metabolites including galactose, glucosamine and hydroxycinnamate showed exclusive relationships with g_m independent of the stress. These results suggest that g_m responses can be attributed to specific changes in the apoplastic antioxidant system

and CW metabolism, opening more possibilities to improve photosynthesis with breeding / biotechnological strategies.

Introduction

Photosynthetic rates (A_N) are mainly determined through the leaf CO_2 diffusion properties (stomatal, g_s, and mesophyll conductance, g_m) and biochemically by the often-coupled operation rate of photochemistry, Rubisco and the Calvin cycle. Traditionally, g_s (conductance of CO_2 from the atmosphere to the substomatal cavity) has been considered as the most important photosynthetic limitation, especially during drought and/or salt stresses. However, in recent years g_m (CO_2 conductance from substomatal cavities to the carbotoxylation sites in the chloroplastic stroma) has been recognized as another important photosynthetic limitation – being of similar magnitude to g_s (Flexas *et al.,* 2009, 2012; Galle *et al.,* 2009; Hu *et al.,* 2010; Galle *et al.,* 2011). It is widely assumed that g_m is a highly complex trait which integrates the diffusion of CO_2 through several "barriers" of different nature along the mesophyll including the apoplast, plasma membrane, cytoplasm, chloroplast membrane and stroma – where RuBisCo carbolyxation occurs. However, the exact nature of the factors influencing g_m is still not yet fully understood (Evans *et al.,* 2009; Flexas *et al.,* 2012).

The apoplast is defined as the entire compartment beyond the plasmalemma. It therefore includes the cell wall (CW), and it is considered to constitute "an information bridge" between the environment and the cells (Pignocchi and Foyer, 2003). Evolutionary based anatomical studies of plants have suggested that CW thickness and the surface of chloroplasts exposed to the mesophyll air spaces (*Sc/S*) are the traits determining the largest fraction of the variability observed in g_m since increasing CW thickness results in decreasing g_m while increasing *Sc/S* results in increasing g_m (Terashima *et al.,* 2011; Tosens *et al.,* 2012, 2016; Tomas *et al.,* 2013; Carriqui *et al.,* 2015). While the role of *Sc/S* is understood as a means to shorten the pathway of CO_2 to the carboxylation sites of the chloroplastic stroma, the manner in which CW thickness affects g_m is likely more complex. The pores within CWs are approximately an order of magnitude larger than the CO_2 molecule (Evans *et al.,* 2009), however, little is currently known concerning how the biochemical properties of the apoplast can influence CO_2 diffusion. In addition, CW exhibit highly changing dynamics both their chemical composition and physical structure (Bellincampi *et al.,* 2014; Houston *et al.,* 2016). In this sense, the role of the apoplast and CW responses in pathogen-resistance, stress-tolerance and improving crop yield has been widely described (Diezt, 1996; Hernández *et al.,* 2001; Miedes *et al*., 2014; Houston *et al.,* 2016 and references therein). However, to our knowledge, no broad-scale studies regarding a possible role of CW in the alteration of g_m behaviour have been described, although a possible link between CW

metabolism and g_m has recently been postulated (Gago *et al.,* 2016) and the effects of changes in specific CW components, such as pectins, on g_m have been demonstrated (Ellsworth *et al.,* 2018).

The environmental constraints that typically reduce g_m (e.g. drought and salinity; Flexas *et al.,* 2012) also induce oxidative stress responses and thus reactive oxygen species (ROS) accumulation in plant cells (Hernández *et al.,* 2001; Faize *et al.,* 2011). The crucial role of ROS levels and the activity of different apoplastic enzymes in relation to responsiveness to biotic/abiotic stress, as well as their key role in CW remodelling, have been also widely studied (O'Brien *et al.,* 2012; Tenhaken, 2015). For instance, several studies demonstrated that proteins which relay information from the extracellular environment to the intracellular compartment are controlled by apoplastic ROS levels (Dietz, 1996; Pignocchi and Foyer, 2003). Furthermore, the activity of enzymes such as NADPH oxidases, peroxidases (POX) or superoxide dismutases (SOD), which also contribute to apoplastic ROS production, increased in response to either biotic or abiotic stress (Díaz-Vivancos *et al.,* 2006; You and Chan, 2015). Different studies (De Pinto and De Gara, 2004; Passardi *et al.,* 2004; Podgórska *et al.,* 2017) have described that apoplastic POXs are involved in the cross-linking between CW matrix components as well as lignin polymerization. In turn, ascorbic acid (ASC), the most abundant low-molecular-weight antioxidant of the apoplast, could have a regulatory effect on POX-dependent wall stiffening since it removes hydrogen peroxide (H_2O_2) (Ros-Barceló *et al.,* 2006). Moreover, it has been recently suggested that changes in the apoplastic ASC redox state influence the photosynthetic response to high light stress in tobacco (Karpinska *et al.,* 2018). Finally, changes in the pH of the apoplast, which is usually acidic, could alter protein functionality as well as the solubilisation capacity of CO_2 in liquid media (Flexas *et al.,* 2006; Sade *et al.,* 2014). Indeed apoplastic alkalization has been characterized as a general response to various stresses such as drought or salinity (Pitann *et al.,* 2009).

Despite mounting evidence suggesting a relationship between biochemical activity of the apoplast and/or CW dynamics with g_m, a mechanistic link between the CW composition/structure modification and CO_2 diffusion remains elusive. Moreover, to the best of our knowledge there are no reports directly relating the apoplast activity to mesophyll conductance to CO_2.

In this work, the relationship between apoplastic metabolism (enzymatic and non-enzymatic antioxidants), CW dynamics and g_m responses to abiotic stress were studied in tobacco plants. Different treatments -salt, drought and drought recovery - were imposed to widen the response range between these traits and as well to "uncouple" the frequently proportional responses between stomatal and mesophyll conductances (Galle et al., 2009; Flexas et al., 2013); in order to exclusively associate the metabolic apoplast responses and CW compounds to g_m behavior. The aim of this work is to improve our knowledge of g_m and photosynthesis beyond the traditional anatomical and functional approaches.

Results

Stress treatments effects on leaf gas-exchange, anatomy and antioxidant responses at the apoplastic and symplastic levels

Both salt and drought stress treatments significantly decreased A_N to a similar extent, being accompanied by decreases of g_s, g_m and ETR (Figure 1). Photorespiration (P_R) and V_{cmax} were not significantly reduced in the salt treatment, however under drought the lowest values for both parameters were recorded (Table S1). Re-watering of drought-stressed plants promoted a significant recovery of photosynthesis (Figure 1a) mostly linked to an increase of g_m, (120% from drought conditions values), which reached similar values to those from pre-stress conditions. ETR and g_s that show slighter increases (ranging from 16 - 42 %, respectively) meanwhile V_{cmax} remained constant (Figure 1; Table S1). Drought induced a larger decline of RWC than salinity that was not fully restored following the recovery treatment (Table S1). The LMA displayed no significant change under stress treatments, but on drought-recovery was significantly higher than in control and salt stressed plants (Table S1). P_R and V_{cmax} were unaffected by saline treatment but were both reduced by 40% during drought and its subsequent recovery treatment (Table S1). Few significant changes were observed in leaf anatomy between treatments; leaf thickness, upper and lower epidermis thickness, palisade thickness and the porosity percentages of palisade and spongy tissues remained constant, meanwhile in the drought treatment only a significant increase of spongy mesophyll thickness was observed (Table 2).

Despite the similar decrease in photosynthesis under both drought and salinity (Figure 1a), a principal component analysis (PCA) revealed that the enzymatic antioxidant responses of the apoplast and symplast were critically different between both treatments (Figure 2a). The two first components of the model explain 37.9% and 22.15% of the variance, respectively; stress treatments are separated from control well-watered treatment along the component 1 (PC1), but differences between both stresses were observed in component 2 (PC2). Interestingly, loadings representation of each antioxidant measured in this experiment revealed that apoplastic and symplastic antioxidant activities (Figure 2b) grouped differentially in PC2 in the positive and negative sectors, respectively, indicating that each stress exhibited a different antioxidant response. Apoplastic POX and SOD occupied the highest values in the positive side of the PC1, while the total and reduced ascorbate contents of the apoplast occupied the most negative values of this axis, differentiating control and stress treatments along PC1 (Figure 2). Indeed, salt and drought stress treatments resulted in a general increase of the enzymatic antioxidant response in both compartments, although some differences were found between treatments (Table 1); in the apoplastic fraction all the enzymatic activities measured increased significantly in response to drought, but only POX activity increased in response to salinity. Similarly, we only observed a significant rise in the apoplastic H_2O_2 in response to drought treatment. However,

in the symplastic fraction an increase in antioxidant activities was only observed in response to salinity.

We also examined the ascorbate content and redox state changes, in both apoplastic and symplastic fractions, in response to salt, drought and drought recovery treatments. Although no significant changes were observed in apoplastic ASC content, both stresses decreased the ascorbate redox state. This decreased in ascorbate redox state was partially reverted following the drought recovery treatment (Table S2). Symplastic ascorbate changes were more marked, with both total (ASCt) and reduced (ASC) ascorbate content significantly increasing in response to both drought and recovery treatments. In addition, the ascorbate redox state also decreased under the stress treatments, especially under drought, was due to the increase in oxidized ascorbate (DHA) content (Table S2). Plants recovering from drought-stress exhibited the highest ASC content, resulting in an increase in the ASC redox state compared with drought-stressed plants (Table S2).

Exploring the relationship between the apoplastic and symplastic antioxidant responses and leaf gas-exchange physiology

In order to ascertain whether antioxidant enzymes activities correlate with the three potential limitations to photosynthesis (i.e. g_s, g_m and the rates of photochemistry/biochemistry, here represented by ETR), we performed different regression analyses between these traits pooling together data from all treatments (Table S3). Since stress situations induce decreases in the photosynthetic process and increases in antioxidant system response, all relations found between these parameters were inverse relationships, with 42% of them being significant (Table S3). Interestingly, we found that apoplastic POX and SOD activities as well apoplastic H_2O_2 content correlated significantly with g_m (Figure 3). Apoplastic POX also correlated significantly with g_s and A_N, and SOD also correlated with A_N. However the correlation coefficients were generally higher between these enzyme activities and g_m (Table S3). At the symplastic level, SOD and catalase (CAT) activities also correlated significantly with A_N, g_s and g_m, but POX did not. This may be associated to the fact that POX is more related to CW modification in response to stress than the other enzymes (Table S3). On the other hand, much fewer and generally milder correlations were found, either at the symplastic or apoplastic levels, between photosynthetic parameters and the ascorbate pool sizes and redox state (Table S4).

Cell wall dynamics in relation to gas-exchange

The enzymatic activities of the apoplast are highly related with the response of the CW to growth, developmental stage or biotic/abiotic stress responses (Sarkar *et al.,* 2009; O´Brien *et al.,* 2012). Consequently, after detecting significant changes in the activity of apoplastic enzymes and significant correlation of these with g_m, we analysed the anatomical characteristics and CW component changes in response to the treatments. Regarding the anatomical parameters measured, only a significant increase of spongy mesophyll thickness fraction was observed in leaves of drought plants compared with control plants. However, any significant change was observed in all the other parameters measured including leaf thickness and epidermis, palisade or spongy thickness (Table 2). The percentage of the CW, as alcohol insoluble residue (AIR), obtained on a fresh weight basis was significantly higher in drought and recovery plants than in control and salt-stressed plants (Table 3). In general, drought promoted an increase in all the CW components while salinity induced increases in most components except for hemicelluloses which were decreased. Plants having recovered after drought additionally maintained increased levels of cellulose and hemicellulose, but pectin contents were restored to control levels (Table 3). By contrast, no significant changes were found in the levels of the CW-bound phenolic compounds *p*-coumaric and ferulic acid in response to the treatments (Table 3).

Next, in order to know if these CW changes could affect the g_m functionality and thereby A_N, we explored the relationship between the functional gas-exchange traits (A_N, g_s, and g_m) versus the CW components changes. First, given that CW is traditionally associated as a structural trait, we observed a significantly correlation between the total CW content (as the sum of cellulose, hemicellulose and pectins) versus structural traits such as LMA (Figure 4a), but no relationship was found either for A_N or g_m (Figure 4b, c). This observation highlights that the total CW content is more closely related to the general leaf anatomical structure than to the physiological responses to stress. However, when we considered the relationship between each major CW components (namely cellulose, hemicelluloses and pectins) individually or to the respective ratios of their levels, several significant relationships were identified (Figure 5; Table S5). Pectin content was strongly and negatively correlated with both A_N and g_m (Figure 5a, b; Table S5), while cellulose was inversely correlated with g_s (Table S5). By contrast, significant positive correlations were found between the hemicellulose/pectin ratio and both A_N and g_m, (Figure 5c, d) while the cellulose/hemicellulose ratio exhibited a negative correlation to g_m (Table S5), suggesting that changes in the ratios of the major CW polysaccharides affect g_m and thus A_N.

Drought and salt effects on primary metabolic profiles

We next performed metabolic profiling of primary metabolites assaying 4-6 biological replicates per treatment and annotating 52 different metabolites from several different compound classes such as amino acids, organic acids and TCA cycle intermediates, sugars and sugar-alcohols (Table S6). First, we observed via PCA analysis that the imposed stress treatments significantly affected leaf primary metabolites (Figure 6). Clearly, the saline treatment was highly different from the other treatments as demonstrated by their separation on PC1 suggesting a specific metabolic response to deal with this stress. Interestingly, control and drought treatments were mostly separated along component 2, while the recovery treatment showed an intermediate position between them (Figure 6a).

Additionally, to facilitate the visualization we plotted the loadings of the three metabolites with the highest absolute scores per component and additionally those most intimately related with CW (Figure 6b). PC1 was mostly driven by TCA cycle intermediates such as malate, fumarate or succinate and sugars such as glucose, galactose and the organic acid threonate while some metabolites related to protection (proline), membrane and protein stabilization (trehalose) and antioxidant secondary metabolism precursors (tyramine or spermidine) were observed amongst the negative values of PC1 (Figure 6b). For PC2 it should be noted that TCA cycle intermediate such as pyruvate in a positive sense and several CW related metabolites such as galactose and hydroxycinnamic acid, as well as sugar-alcohols, as well as phenolic precursors and membrane stabilizers such as tryptophan, galactinol and raffinose were observed in the negative sector of PC2 (Figure 6b).

The changes in the levels of primary metabolites are clearly visualized in a fold-change heat-map of each treatment with respect to control conditions (Figure 7; Table S6). Salinity invoked dramatic changes in primary metabolism with significant increases in the photorespiratory intermediates (glycine and serine), osmoprotectors (proline) and secondary metabolite precursors related to antioxidant responses such as tyrosine and spermidine alongside certain sugars related to protection, membrane and protein stabilization such as sucrose and trehalose. Moreover, this treatment promoted a significant reduction in the content of several metabolites, including the TCA cycle intermediates succinate, malate and fumarate (Figure 7). Drought treatment induced changes in common with the salinity treatment, but some specific responses were also noted namely the accumulation of the amino acids alanine, glycine and tryptophan as well as erythritol, galactinol, glycerol-2-P and hydroxycinnamic acid. The plants exposed to stress followed by a recovery period showed an intermediate position between drought and control plants yet revealed a significant decrease in the *N*-acetylserine (NAS) content (Figure. 7).

For further exploration of the role of primary metabolism in driving the physiological responses measured we performed a least partial square (PLS) model combined with variable importance for projection (VIP) as a criteria to elucidate metabolite relevance from the models (Gago *et al.,* 2016). This approach allowed us to reveal the putative metabolic networks behind the observed physiological parameters (as response variables) A_N, g_s and g_m, but not for ETR.

Inspection of the root mean square error of prediction (RMSE(P)) and coefficient of multiple determination (R^2) bias-corrected *via* cross validation indicated that the smallest value for A_N was one component, whereas for g_s and g_m six and four components were obtained, respectively. In Table 4 we present the top 19 most important metabolites identified by the VIP annotation of the PLS sparse regression modeling explaining each variable. Only three metabolites are common for the three physiological traits, homoserine, glutamine and malonate; however, A_N and g_m share another four metabolites (glycine, hydroxycinnamic acid, erythritol and glycerol-2-P), while A_N and g_s share three metabolites (glycerate, pyruvate and tryptophan). Both g_s and g_m shared three main TCA cycle metabolites, namely fumarate, malate and succinate as well as threonate (an ascorbate derivative).

Interestingly, around 21% of the metabolites which significantly correlated with g_m were CW-related and three of them (galactose, gamma-aminobutyric acid (GABA) and glucosamine) were specific for this parameter. Several respiratory metabolites displayed clear correlations with g_s, as did sucrose and trehalose which can act as osmoprotectants under stress and β-alanine, raffinose and *myo*-inositol which can act as membrane stabilizers.

Discussion

Mesophyll conductance is recognized as a highly complex trait which driven by several structural, chemical and biological factors that can limit photosynthesis dynamically and in a similar magnitude to g_s and photobiochemistry (Flexas *et al.,* 2012). The results obtained from the three independent methodologies that we present here suggest that dynamic changes in apoplastic metabolism and CW composition could lead alterations in g_m and thereby A_N in response to abiotic stresses. Drought and salt stress have already been documented to trigger important alterations within the photosynthetic process and to induce oxidative stress which can lead to biochemical imbalances resulting in the reduction of plant growth and productivity (Flexas *et al.,* 2006; Chaves *et al.,* 2009; Acosta-Motos *et al.,* 2017). Stomatal closure and decreased g_m are also recognized as the primary effect of drought and salt stress on carbon assimilation (Flexas *et al.,* 2008). In the present study, both stresses imposed significant reductions in photosynthesis mostly through g_s and g_m limitations, whereas photosynthetic recovery following drought was mostly driven by the recovery of g_m rather than that of g_s or photobiochemistry (ETR and V_{cmax}). These results (Figure 1; Table S1), are similar to previous reports

in tobacco and other plant species (Flexas *et al.*, 2009; Galle *et al.,* 2009, 2011; Hu *et al.,* 2010). Indeed, they agree with several works describing the importance of g_m in driving photosynthetic response either to stressors or to environmental cues (Flexas *et al.,* 2012; 2016). It is noteworthy that salinity imposes a physiological water deficit, as observed in the RWC data, but also includes ion toxicity (Chaves *et al.,* 2009). Here, salinity treatment resulted in the lowest g_s values in agreement with the observation that salt stress alters the balanced relationship between guard cell and apoplastic ion content (Santelia and Lawson, 2016). The leaf anatomical properties were neither influenced by salinity nor water stress. Indeed, only spongy mesophyll thickness showed significant increases under drought, a trend previously observed in fully developed leaves under a similar sustained physiological stress in several grapevine cultivars (Tomás *et al.,* 2014). In general, the significant g_m changes observed can therefore not be associated to general leaf morpho-anatomical rearrangements.

Numerous previous studies have demonstrated that decreased A_N under salinity or drought is accompanied by increased generation and accumulation of ROS. To avoid ROS-induced damage and induce plant stress tolerance, common and/or stress-specific antioxidant responses have been postulated (You and Chan, 2015). We observed a general increase of the antioxidant response being these changes stress-dependent. Drought involved a significant accumulation of H_2O_2 and a greater response of apoplastic enzymatic activities, while salt stress was more related to changes in the symplastic enzymatic activities. The redox state of the ascorbate pool decreased in both fractions following both stresses indicating oxidative stress alterations; however, these changes were considerably higher in response to drought (Table 1; Figure 2; Table S2). Likewise, different alterations in CW composition have been described in response to biotic and abiotic stress (Houston *et al.,* 2016). Drought and salinity tolerance have been mainly associated with increases in pectin levels and alterations in cellulose biosynthesis. In addition, increased abundance of proteins associated with the modification of branching degree of hemicellulose or pectin and the secondary wall reinforcement by lignin deposition, have been also described as common responses leading to abiotic stress tolerance (Le Gall *et al*., 2015; Tenhaken, 2015; Houston *et al.,* 2016). Here, a general increase of cellulose and pectins content was observed for both stresses, while following drought recovery plants exhibited intermediate values between stress and control conditions (Table 3).

Metabolic rearrangements have been also described in response to both saline and drought stresses (Krasensky and Jonak, 2012; Obata and Fernie, 2012). Stress treatments induced a strong metabolic response with 29% and 23% of metabolites displaying significantly altered levels for saline and drought treatments, respectively. Interestingly, similar to the antioxidant response, the primary metabolite changes showed differentiated responses between the treatments in the PCA analysis (Figure 6). The stresses shared some common response patterns such as the accumulation of osmolytes, compatible solutes, increases of secondary antioxidant metabolite precursors and decreases in the levels of TCA cycle intermediates (malate and fumarate) as well in asparagine or homoserine.

All these changes are typically described in the literature related to plant stress responses (Obata and Fernie, 2012; Brunetti *et al.,* 2013). Moreover, a range of specific responses was also observed for each stress. In brief, only drought showed significant accumulation of sugar-alcohols as galactinol and metabolites related to antioxidant responses as precursors of phenolic compounds (tryptophan or hydroxycinnamic acid) (Zhao *et al.,* 1998; Hoffman *et al.,* 2004; Vincent *et al.,* 2005), membrane stabilization (glycerol-2-P) (Sui *et al.,* 2007; Van den Ende, 2013), or erythritol related to terpenoid biosynthesis (Rohdic *et al.,* 2000). In response to salinity we observed a metabolic shift to deal with osmotic stress, an increase of several amino acids, specifically proline, a known osmoprotectant, as well as sugar (sucrose), membrane and protein stabilizers (trehalose) and polyamine precursors such as spermidine with important antioxidant properties; these general trends were also observed previously under saline treatments in a range of different species (Obata and Fernie, 2012; Kissoudis *et al.,* 2015; Del-Saz *et al.,* 2016) (Figure 7; Table S6).

The fact that drought and salt stress levels (using g_s as physiological indicator) imposed a similar decline of photosynthesis but with different responses in antioxidant activities and leaf primary metabolism, together with the uncoupled behaviours of g_s and g_m during the drought recovery treatment (as described previously by Gallé et al. 2009) allowed us to expand the variation range of g_m and cell wall composition. Moreover, our results are based mostly on correlation data from independent methodologies and biological levels, providing key insights into the complex traits regulating the observed dynamic responses of g_m to environmental changes.

Interestingly, in the present study g_m (and A_N) was significantly and negatively correlated with apoplastic POX and SOD activities, H_2O_2 content, and pectin abundance. The role of apoplastic metabolism as well as the effect of ROS on cellular growth and the modification of CW properties have been well documented (Hernández *et al.,* 2001; Pignocchi and Foyer, 2003; Tenhaken, 2015). Apoplastic H_2O_2 increases in response to salinity or drought have been linked to CW stiffening and lignification process mediated by the POX activity (Passardi *et al.,* 2004), while changes in the apoplastic ASC metabolism could have a regulatory effect via the removal of H_2O_2 (De Pinto and De Gara 2004; Ros-Barceló *et al.,* 2006). In addition, POX activity could promote cell elongation via CW loosening via the oxidative cleavage of polymers such as xyloglucan and pectin (Tenhaken, 2015; Schmidt *et al.,* 2016). Wheat cultivars tolerant to drought were shown to increase the content of pectins rhamnogalacturonan I and II in response to drought potentially by promotion of the formation of hydrated gels which ameliorate cell damage (Leucci *et al.,* 2008). Pectins have been described as the filling material for the open space in the cellulose/hemicellulose networks and have been argued to be the most complex CW polysaccharide with roles in expansion, strength, porosity, adhesion and intercellular signaling (Tenhaken, 2015; Houston *et al.,* 2016). Indeed, they are hydrophilic compounds with important capacities to retain water within the cell wall altering its porosity, permeability and enzyme activity (Cathala *et al*. 2001; Panchev *et al*. 2010), a major issue that could

significantly affect CO_2 diffusion through the CW. Pectin accumulation under stress (Table 3), could on one hand increase the CW lattice thickness (thus, enlarging the CO_2 pathway) but on the other hand these hydrophilic matrix would help to the CO_2 solubilization and its movement through all CW pores. However, the positive relation observed with the hemicellulose/pectin ratio strongly suggests the involvement of CW composition in regulating g_m (Fig. 5).

In keeping with this theory, data from resurrection plants described hemicelluloses and pectins (pectin-arabinans, arabinogalactan proteins and arabinoxylans) to be the major CW contributors to ensure flexibility and facilitating rehydration in these plants (Moore *et al.,* 2013). Additionally, considering the quick CW responses to stress, we propose that they should be related to primary metabolic rearrangements (Verbancic *et al.,* 2018) rather than general leaf anatomical changes (Table 2; Tomas *et al.,* 2014). Thus, it seems likely that the CW response could be related to g_m as was previously postulated following the observation of the relationship between cell wall sugars and g_m in a recent multi-species meta-analysis (Gago *et al.,* 2016). In this way, a modelling approach based on PLS combined with VIP allowed us to identify the most important primary metabolites associated with g_m. We observed that several metabolites importantly and exclusively associated with g_m belonged to metabolic routes linked to CW metabolism such as the pathways of galactose, glucosamine and hydroxycinnamic acid which are related to synthesis, maintenance and degradation of hemicelluloses and pectins (Chen *et al.,* 2013; Franková and Fry, 2013) or to potential growth signaling molecules such as GABA (Renault *et al.,* 2010).

Other metabolites traditionally associated to protein and membrane stabilization such as raffinose or lipid precursors such as glycerol (Kwon *et al.,* 2012) were also linked to g_m. In this sense, changes in membrane properties could directly affect their permeability to solutes, water, protons and as well the own activity of transmembrane proteins (Watkins *et al.,* 2011). Likewise, lipid peroxidation could alter bulk membrane permeability or modify the functional abilities of transmembrane proteins (Carruther and Melchior, 1986; Watkins *et al.,* 2011), including the activities of aquaporins (facilitating CO_2 diffusion through membranes), and carbonic anhydrases (accelerating CO_2 solubility in each cell compartment) (Flexas *et al.,* 2006; Pérez-Martín *et al.,* 2014). Indeed the activities of either/or both of these could be altered either by direct oxidation and formation of secondary radicals (Ye and Steudle, 2006) or indirectly through lipid membrane oxidation, which would consequently reduce g_m. According to this, it has been described that abiotic stress conditions which result in increased H_2O_2 levels, promote a low abundance of aquaporins at the plasma membrane in *Arabidopsis thaliana*, which can be prevented by high catalase abundance (Boursiac *et al.,* 2008; Schmidt *et al.,* 2016).

In summary, the results of the present study demonstrate that short-term drought and salinity stresses induce dynamic changes in apoplastic antioxidant enzyme activities, cell wall composition and leaf primary metabolism and some of these changes are associated with variations in photosynthesis and, most specifically, with mesophyll conductance to CO_2 (g_m). More interestingly, it suggests that both apoplastic metabolism modifying the physical and chemical composition of the CW as well the metabolic pathways related to CW synthesis/degradation could influence g_m in response to stress situations. Despite the fact that the exact mechanisms underlying these connections remain unknown and thus merit further investigation, we hypothesize that stomatal closure and ROS accumulation in response to early water or salinity stresses, may eventually lead to different adjustments of apoplastic metabolism thereby altering the physical and biochemical CW composition properties (such as the increase in pectin contents and CW-related metabolites changes), leading to a more viscous and complex CW lattice which would affect g_m.

Overall these results open perspectives which allow us to evaluate previously unexplored aspects of g_m, with previous work being largely concentrated on anatomical parameters and transmembrane protein activities. The generation of inducible CW mutants (to reduce/avoid abnormal growth and pleiotropic effects traditionally observed in conventional cell wall mutants) offers important opportunities to understand this topic. For example, Arabidopsis *gae1 gae6* deficient pectin mutants (lacking the glucuronate 4-epimerases (GAEs) which catalyze the synthesis of UDP-$_D$-galacturonate acid from UDP-$_D$-glucuronic acid) showed brittle leaves because of the lower pectin content indicating important changes in the cell wall elasticity (Bethke et al. 2016). In the light of our results, future approaches combining inducible mutants and g_m dynamic change treatments would help to improve our understanding about the relationships between cell wall composition and gas-exchange functionality. In this sense, improving g_m is considered essential for agricultural parameters such as productivity and water use efficiency (Flexas *et al.,* 2013; Gago *et al.,* 2014), presenting more opportunities for breeding and biotechnological strategies for improving our crops.

Experimental Procedures

Plant material, growth conditions and experimental design

Tobacco seeds (*Nicotiana sylvestris* L.) were germinated in vermiculite at 25 ºC under dark conditions. After a week, tobacco seedlings were transferred to pots with a 3:1 mix of peat:perlite, and placed in a growth chamber at 23/25ºC (200 µmol m^{-2} s^{-1} and 16/8 h day/night photoperiod) for the duration of the experiment. Plants were irrigated to full substrate capacity daily and fertilized weekly with Hoagland's solution until the onset of the treatments.

The experiment was carried out with 8-week-old tobacco plants randomly divided in different sub-sets per each treatment established: control (plants watered to substrate capacity), salt treatment (plants watered with a solution 250 mM $NaCl_2$ during 6 days), drought treatment (plants watered with approximately 60% less water than control during 6 days), and recovery (plants re-watered after drought to full substrate capacity for 4 days). The drought treatment was imposed by withholding water until moderate water stress (g_s < 100 mmol m^{-2} s^{-1}; Galle *et al.,* 2009). When the desired g_s level was reached, pot weight was maintained by adding the amount of water lost daily. The experiment was repeated twice, gas-exchange and chlorophyll fluorescence parameters were measured for all plants. In the first experiment we measured the apoplastic and symplastic enzymatic activities and apoplastic H_2O_2 content in control, salt- and drought-treated plants. Based on previous results we include a recovery treatment from drought in the second experiment in order to widen the response range; we further explored the relationship between *in vivo* gas-exchange with apoplast metabolism, measuring the apoplastic and symplastic ascorbate content, CW main components composition and primary metabolism. Plants of each subset were grown under identical environmental conditions, leaf sampling for the different analytical techniques was performed using the second and third fully expanded young leaves when counting from the apex.

Plant water status and leaf mass per area

The leaf relative water content (RWC) was determined as (FW-DW)/(TW-DW) x 100, where FW, TW and DW are fresh, full turgor and dry leaf weights, respectively. FW was determined immediately after excising leaves from plants at midday; TW was obtained after keeping the leaves in distilled water 24 h in darkness at 4ºC until they reached a constant weight and DW was subsequently determined after keeping samples for minimum 48 h at 70ºC in an oven until constant weight. Leaf pieces were also photographed before drying to calculate the leaf area using the ImageJ software. Leaf mass area (LMA) was calculated from leaf dry weight and leaf area.

Gas-exchange and chlorophyll fluorescence measurements

An infrared gas analyser (Li-6400-40, Li-Cor Inc., Nebraska, USA) coupled with a chlorophyll fluorimeter (Li-6400-40 2 cm^2 chamber, Li-Cor) was used to measure gas-exchange and chlorophyll fluorescence in fully expanded leaves (3^{rd} or 4^{th} fully expanded leaves from the apex) from 2 h after start the photoperiod up to midday. Conditions in the cuvette were set to: block temperature 25ºC, air vapour pressure deficit (VPDa) ca. 1.5 kPa, 400 µmol CO_2 mol^{-1} air and a light-saturating photosynthetic active radiation (PAR) of 1000 µmol m^{-2} s^{-1} (90-10% red-blue light).

After stabilization of net CO_2 assimilation (A_N) and steady-state fluorescence (F_s), an intense light flash (8000 μmol m^{-2} s^{-1}) was used to determine the maximum fluorescence (F_m') and the real quantum efficiency of the photosystem II:

(1) $$\Phi_{PSII} = \frac{(F_m' - F_s)}{F_m'}$$

Photosystem II electron transport rate (ETR) was calculated as:

(2) $$ETR = \Phi_{PSII} \cdot PAR \cdot \alpha \cdot \beta$$

Where PAR is the quantum flux density of photosynthetically active radiation incident on the leaf, α is the leaf absorbance and β is the partition of absorbed PAR between photosystems I and II. The product $\alpha \cdot \beta$ was determined from the relationship between ϕPSII and ϕCO_2 under non-photorespiratory conditions through low O_2 light-curves (Valentini et al. 1995, Flexas et al. 2007). Day respiration (R_{day}) was considered as half the dark-adapted mitochondrial respiration (R_{dark}) after minimum 30 min under dark conditions (Niinemets et al. 2005). When determining R_{dark}, the maximum efficiency of PSII (F_v/F_m) was simultaneously determined by chlorophyll fluorescence (Genty *et al.,* 1999).

CO_2 photosynthetic response curves (A_N-C_i) were performed varying atmospheric CO_2 concentration (C_a) in the gas-exchange cuvette in six steps (400, 300, 200, 100, 75 and back to 400 μmol CO_2) designed to focus only on the CO_2-limited range to estimate the maximum velocity of Rubisco carboxylation (V_{cmax}). CO_2 leakage curves during A_N-C_i were determined and corrected as in Flexas et al. (2007). For all measurements, the chloroplastic CO_2 concentration (C_c) and g_m were estimated following Harley et al. (1992), and V_{cmax} following the model of Farquhar et al. (1980), considering the temperature dependence of kinetic parameters of Rubisco described on a C_c basis by Bernacchi et al. (2002).

Isolation of apoplastic washing fluids (AWFs)

Immediately after the gas exchange measures, fully expanded young leaves were sampled for the isolation of apoplastic washing fluids. The apoplastic washing fluids (AWFs) were isolated by vacuum infiltration/centrifugation technique in the presence of 50mM TRIS-acetate buffer pH 6.0 (infiltration buffer) containing 0.2 M KCl and 1 mM Cl_2Ca as described Hernández et al. (2001).

To assay the main enzymatic activities in the apoplast, the AWFs were concentrated using Centricon YM-10 filter devices (Amicon, Millipore) and pre-purified by chromatography on Sephadex G-25

NAP columns (Amersham, BioSciences) equilibrated with the infiltration buffer. Contamination by cytoplasmic constituents during the extraction was assessed by measuring the levels of catalase, an enzyme used as a specific peroxisomal marker (as Hernández *et al.,* 2000), which was always less than 0.15% with regard to that found in the cytosolic fraction. The enzymatic activity data presented here were corrected by the percentage of contamination caused by the cytosolic constituent.

Leaf Enzyme Extraction

Leaf residues following AWF extraction were subsequently homogenized with a mortar and pestle containing ice-cold 50 mM TRIS-acetate buffer pH 6.0, containing 0.1 mM EDTA, 2 mM cysteine, 2% (w/v) polyvinylpolypyrrolidone (PVPP), and 0.2% (v/v) Triton X-100. The "symplastic" homogenate was centrifuged at 14,000 rpm for 20 min and the supernatant fraction was filtered as described above.

Antioxidative metabolism and oxidative stress parameters

Enzymatic activities were analysed in both apoplastic and symplastic fractions. The activities of catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7), and superoxide dismutase (SOD, EC: 1.15.1.1) were measured as described in Díaz-Vivancos et al. (2008). NADH oxidase/peroxidase was measured by monitoring the decrease in absorbance at 340 nm due to NADH oxidation as described Hernández *et al.,* (2007). Protein was estimated according to Bradford (1976).

Total (ASCt) and reduced ascorbate (ASC) contents were assayed in both apoplastic and symplastic fractions as described Queval & Noctor (2007). For the apoplastic fraction, leaves pieces were infiltrated with cold metaphosphoric acid (2%, w/v), and handled and centrifuged as above. The supernatant was adjusted to between pH 5.5 and pH 6.0 by the addition of 1M KH_2PO_4. Leaf residues following AWF extraction were used to obtain the symplastic homogenized and measured as above.

Reduced ascorbate was measured by the change in absorption at 265 nm, where ascorbate was determined via oxidation to DHA in the presence of ascorbate oxidase (Díaz-Vivancos *et al.,* 2013).

Apoplastic H_2O_2 concentration was determined immediately after AWFs isolation, based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (Bellincampi *et al.,* 2000). In this case, 5 mM KCN was added to the AWFs extraction buffer.

Cell wall component determination

For cell wall analysis, independent biological replicates of each treatment (3-4) were collected next day, after the dark period to minimize the starch content in the leaf. The second and third from the apex fully expanded young leaves that were not employed in the previous samplings were used. Finally, these samples were boiling in absolute ethanol until bleaching them. Then, samples were cleaned in acetone shaking for 30 min twice in order to eliminate any alcohol soluble part. Next further air-dried and homogenized by dry milling. The resulting crude CW material or alcohol insoluble residue (AIR) was used for the polysaccharides and bound phenolic compounds analyses.

Firstly, 3 mg of each AIR were hydrolysed with 2M trifluoroacetic acid (TFA) for 1 hour at 121ºC in order to obtain non-cellulosic CW components (mainly hemicellulose and pectins). Samples were centrifuged for 10 min at 13000 rpm, then, supernatant was recollected and kept at 4ºC and pellet was cleaned in distilled water followed by two washes in acetone and finally air-dried. The dry fraction (cellulose fraction) was hydrolysed with 200 µl sulphuric acid (72%) for 1 hour at room temperature and subsequently diluted with distilled water to 6 ml (0.25M sulphuric acid) and heated to 121ºC for 2 hours to obtain the total sugar corresponding to the cellulose fraction. Total sugar from both, non-cellulosic and cellulosic fractions, was determined by the phenol sulphuric colorimetric method (Dubois *et al.,* 1956) using glucose equivalents as standard in a Varioskan-Lux spectrophotometer (Thermo Scientific). Total sugar obtained from the non-cellulosic fraction corresponded to soluble free-sugar and was considered to represent hemicellulose. Uronic acids (representing pectins) were quantified from the soluble 2 M TFA fraction by the colorimetric method (Blumenkratz & Asboe-Hansen, 1973) using 2-hydroxydiphenyl as reagent and galacturonic acid as standard in a Varioskan-Lux spectrophotometer (Thermo Scientific).

For phenol analysis, AIR (ca. 50-100 mg) was suspended in water (10 ml) and hydrolysed by adding 10 ml 2N NaOH, incubating for 1h at 80°C and 14h at room temperature. Hydrolysates were neutralized with H_3PO_4 and filtered under vacuum. Samples were extracted in triplicate with 40 ml ethyl-acetate. The combined organic phases were washed with water three times to remove traces of acid and concentrated to dryness in a rotary evaporator at 40°C. Dry residues were re-diluted in 5 ml 80 % MeOH. For HPLC analysis, each aliquot was filtered through a membrane filter (Technochroma OlimPeak, Reg. Cellulose 13 mm filter diameter, 0.22 µm pore diameter) and injected in a column (Macherey Nagel CC 250/4 Nucleosil 100-5 NH2-RP, 5 µm particle size, 250 mm length) thermostated at 40°C. Detection was performed using a photo-diode array detector and chromatograms were monitored at 300 nm. Peak identification was accomplished based on spectral and mobility data of pure standards. Quantitation was accomplished based on pure standards and reference curves for ferulic acid and *p*-coumaric acid.

Anatomical measurements

For anatomical measurements, small leaf tissue pieces (2x1 mm) were cut between the main veins from the same leaves used for gas-exchange measurements. Leaf material was quickly fixed under vacuum with glutaraldehyde 4% and paraformaldehyde 2% in a 0.1 M phosphate buffer (pH 7.4). Four samples were taken per treatment. Afterwards, samples were sent to the Microscopy Service of the University of Murcia for processing them. Briefly, samples were post-fixed in osmium tetroxide buffer 1%, dehydrated in a graded series of ethanol, embedded in Spurr's resin and solidified in an oven at 60ºC for 48h.

Then, semi-thin cross-sections of 1 μm were cut with an ultramicrotome (Leica UC6, Vienna, Austria) and dyed with 1% toluidine blue. Semi-thin preparations were observed at 200×magnifications under an Olympus BX60 light microscopy (Olympus, Tokyo, Japan) and photographed with a digital camera Moticam 3 (Motic Electric Group Co., Xiamen, China).

From light microscopy images leaf thickness (Tleaf), upper and lower epidermis thickness, palisade and spongy mesophyll thickness, and the fraction of the mesophyll occupied by intercellular airspaces in palisade and spongy parts were measured using IMAGEJ software. Ten measurements were made for each type of anatomical characteristic.

Primary metabolite profiling by gas chromatography–mass spectrometry (GC-MS)

Leaf samples (4-6 biological replicates) (avoiding veins and the midrib) were collected at midday for all plants and treatments, immediately frozen in liquid nitrogen and stored at -80ºC. Tissue grinding was performed under frozen conditions using liquid nitrogen and lately weighed ca. 50 mg, this material was extracted in 1.4 ml of 100% methanol following well-established previous methodology (Lisec *et al.,* 2006). Gas chromatography-time of flight-mass spectrometry (GC-TOFMS) analyses was carried out exactly as described by Lisec et al. (2006). Metabolites were identified by comparison with database entries of standards (Kopka *et al.,* 2005; Schauer *et al.,* 2005). Metadata information about the parameters employed for the GC–MS and LC–MS annotation as well an overview of the metabolite reporting list is shown in Methods S1.

Statistics

The data were analysed using one-way ANOVA with the Tukey's post-hoc test (0.05) using the statistical software package SPSS 20 (SPSS Inc., Chicago. USA). All regression analyses and plots were performed employing data from the same leaf and replicate. We used the modified Thompson τ

technique to check for data outliers in the datasets. Principal component analysis (PCA) was employed to explore the primary metabolic profiles between treatments and species. Data were previously normalized by the median, centred and scaled. These analyses were performed using the free software R (2017) libraries LATTICE and PCAMETHODS. For the Partial Least Squares sparse regression (PLS) the missing values were imputed by a random forest imputation method implemented in the library (Gromski *et al.,* 2014). For the PLS regression we used the *pls* package in R environment with the add-on function implementing the variable importance in projection (VIP) for single-response orthogonal scores *plsr* models (Wehrens & Mevik, 2007).

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Author contributions

M.J.C.M. and J.G. conceived the research plans, processed and interpreted the results. M.J.C.M., J.A.H., P.D.V and A.B. grew the plants and carried out the apoplastic extraction and the measurements of enzymes and non-enzymatic antioxidants. J.G carried out the gas exchange measurements and the metabolic analysis. A.R.F. supervised the metabolic analysis. M.J.C.M. performed cell wall analysis and anatomical measurement. P.B., and G.L. performed phenolic determination. M.J.C.M, J.G., J.F. and A.R.F. write the article with contributions of all the authors; M.J.C.M. agrees to serve as the author responsible for contact and ensures communication.

Conflicts of interest

The authors declare no conflicts of interest.

Supporting information

Methods S1 Parameters used for peak annotation in GC-MS analysis and metabolite reporting list.

Table S1 Gas-exchange parameters measured in leaves of tobacco plant (*Nicotiana sylvestris* L) under control, salt, drought and recovery conditions.

Table S2 Ascorbate contents at apoplastic and symplastic fractions.

Table S3 Correlation coefficients between the antioxidant enzymes activities and the apoplastic H_2O_2 content related with the physiological carbon metabolism parameters.

Table S4 Correlation coefficients of ascorbate metabolism related to the physiological carbon metabolism parameters.

Table S5 Correlation coefficients for physiological carbon parameters (A_N, g_s, g_m, and ETR) related to the cell wall components.

Table S6 Relative metabolite changes from the three stress treatment compared to control.

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Table 1 Antioxidant enzymatic activities as well as hydrogen peroxide content at apoplast and symplast levels in leaves of tobacco plant (*Nicotiana sylvestris* L.) under control, salt or drought conditions. Data are shown as average values from 6-8 biological samples ± SE. Different letters means statistical differences by Tukey´s test ($p<0.05$).

APOPLAST				
	POX (μmol min^{-1} g^{-1} DW)	**SOD** (Unit g^{-1} DW)	**NADH $_{ox\text{-}pox}$** (μmol min^{-1} g^{-1} DW)	**H_2O_2** (nM g^{-1} DW)
Control	292.9 ± 18.9 c	23.7 ± 2.9 b	153.5 ± 30.6 b	460.4 ± 80.2 b
Salt	401.9 ± 17.7 b	30.8 ± 1.4 ab	71.3 ± 14.9 b	491.5 ± 99.8 b
Drought	540.7 ± 18.2 a	42.3 ± 3.7 a	440.2 ± 66.9 a	938.5 ± 119.1 a
SYMPLAST				
	CAT (mmol min-1 g-1 DW)	**POX** (μmol min-1 g-1 DW)	**SOD** (Unit g-1 DW)	
Control	272.2 ± 82.5 b	6575.6 ± 511.8 b	1076.5 ± 86.9 b	
Salt	2007.6 ± 279.9 a	10358.1 ± 1100.8 a	1604.2 ± 183.3 a	
Drought	1179.9 ± 334.7 ab	8152.6 ± 678.4 ab	1403.4 ± 96.6 ab	

POX; Peroxidase, SOD; Superoxide dismutase, NADH ox-pox; NADH oxidase-peroxidase, CAT; Catalase, H_2O_2; Hydrogen peroxide.

Table 2 Leaf anatomical parameters measured from light microscopic images of semi-thin section of tobacco plant leaves (*Nicotiana sylvestris* L) under control, salt, drought and recovery conditions. Values are means from three biological replicates ± SE. Letters denote a significant difference ($P<0.05$).

	Leaf thickness (µm)	Upper epidermis thickness (µm)	Lower epidermis thickness (µm)	Palisade mesophyll thickness (µm)	Spongy mesophyll thickness (µm)	% Porosity palisade	% Porosity spongy
Control	235.6 ± 4.0 a	31.0 ± 1.7 a	21.9 ± 1.8 a	79.1 ± 4.3 a	112.8 ± 3.7 b	18.3 ± 1.6 a	38.5 ± 1.0 a
Salt	244.5 ± 14.3 a	23.6 ± 0.3 a	20.1 ± 0.5 a	85.4 ± 3.1 a	117.5 ± 8.5 ab	26.33 ± 5.0 a	39.2 ± 3.0 a
Drought	275.7 ± 11.9 a	29.1 ± 1.4 a	23.2 ± 2.8 a	91.8 ± 6.3 a	138.9 ± 4.2 a	28.5 ± 3.1 a	45.4 ± 5.2 a
Recovery	239.7 ± 6.5 a	29.5 ± 2.4 a	22.33 ± 3.8 a	80.1 ± 7.6 a	114.9 ± 5.4 ab	22.7 ± 2.5 a	38.9 ± 1.8 a

Table 3 Percentage of the cell wall obtained as alcohol insoluble residue (AIR) based on fresh weight and the main cell wall components content of leaves of tobacco plant (*Nicotiana sylvestris* L) under control, salt, drought and recovery conditions. Data are shown as average values from 4-6 biological samples ± SE. Different letters means statistical differences by Tukey´s test ($p<0.10$).

	% AIR extracted	Cellulose (µg glc mg^{-1} AIR)	Hemicellulose (µg glc mg^{-1} AIR)	Pectins (µg gal mg^{-1} AIR)	Coumaric acid (µg mg^{-1} AIR)	Ferulic acid (ng mg^{-1} AIR)
Control	13.71 % ± 1.5 b	103.01 ± 10.91 b	353.45 ± 16.91 ab	74.61 ± 3.80 c	0.271 ± 0.010 a	15.14 ± 1.70 a
Salt	8.28 % ± 0.3 c	133.27 ± 2.75 ab	321.21 ± 4.16 b	85.88 ± 1.97 ab	0.308 ± 0.044 a	14.06 ± 0.62 a
Drought	18.16 % ± 0.8 a	146.76 ± 2.78 a	369.24 ± 9.03 a	87.07 ± 1.94 a	0.297 ± 0.015 a	11.37 ± 0.04 a
Recovery	18.75 % ± 0.3 a	141.72 ± 6.64 ab	373.03 ± 9.57 a	76.04 ± 3.63 bc	0.346 ± 0.004 a	12.27 ± 0.74 a

Table 4 Metabolites with the higher ViP (Variable Importance for the Projection) values obtained from Partial Least Square sparse regression modelling outputs for each trait. ViP ranked metabolites is a representation of the most important metabolites for the models explaining each trait. Colour scales are different per each parameter, green indicates higher significance than warmer colours.

Metabolites	A_N	g_m	g_s
Glutamine	3.30	6.59	13.20
Homoserine	2.42	4.25	18.27
Malonate	3.52	6.14	0.76
Hydroxycinnamic_acid	3.23	6.57	
Glycerol-2-P	1.65	4.75	
Glycine	1.04	4.08	
Erythritol	0.06	4.78	
Pyruvate	2.02		2.56
Glycerate	1.76		0.16
Tryptophan	1.57		15.57
Threonate		4.17	0.02
Fumarate		6.58	0.52
Succinate		5.92	0.60
Malate		5.79	0.64
Galactose		6.39	
gamma-aminobutyric acid (GABA)		6.14	
Glucosamine		4.68	
Proline		5.62	
Ribose		5.06	
Fructose		4.84	
Aspartate		4.67	
Mannitol		4.12	
Sucrose			5.32

Raffinose			3.35
Myo-inositol			3.31
Tyrosine			1.45
Trehalose			1.44
Valine			1.36
N-acetylserine (NAS)			0.81
β-alanine			0.64
Serine			0.29

Figure legends

Figure 1 Net photosynthesis (A_N) (a), stomatal conductance to CO_2 (g_s) (b), electron transport rate (ETR) (c) and mesophyll conductance of CO_2 (g_m) (d), of tobacco plants (*Nicotiana sylvestris* L) under control, salt, drought and drought recovery conditions. Data are shown as average values from between 6-12 biological samples for treatment. Bars denote SE values. Different letter means significant differences between treatments by Tukey´s multiple comparisons´s test ($p<0.05$).

Figure 2 Principal component analysis of (a) the antioxidant response to salt and drought stress treatments of leaves of tobacco plants (*Nicotiana sylvestris* L.) at apoplastic (peroxidase (POX), superoxide dismutase (SOD), NADH oxidase-peroxidase ($NADH_{ox\text{-}pox}$) and ascorbate) and symplastic level (POX, SOD, catalase (CAT) and ascorbate) and (b) loadings representation of each of the antioxidants in the model.

Figure 3 Correlation analysis for the apoplastic activities peroxidase (POX) (a, b), superoxide dismutase (SOD) (c, d) and hydrogen peroxide content (H_2O_2) (e, f) related to net photosynthesis (A_N) and mesophyll conductance (g_m) respectively. Treatments are represented by forms (control: circle, salt: triangle and drought: square).

Figure 4 Total CW polysaccharides content (cellulose (µg glc mg^{-1} AIR) + hemicellulose (µg glc mg^{-1} AIR) + pectins (µg gal mg^{-1} AIR)) correlations with (a) leaf mass area (LMA g DW m^{-2}), (b) net photosynthesis (A_N), and (c) mesophyll conductance (g_m). Treatments are represented by forms (control: circle, salt: triangle, drought: square and drought recovery: diamond).

Figure 5 Pectins (µg gal mg^{-1} AIR) and the ratio hemicellulose/pectins correlations with (a, c) net photosynthesis (A_N) and (b, d) mesophyll conductance (g_m) respectively. Treatments are represented by forms (control: circle, salt: triangle, drought: square and drought recovery: diamond).

Figure 6 Principal component analysis of the primary metabolic profile (a) in leaves of tobacco plant (*Nicotiana sylvestris* L.) under the different treatments imposed (control: circle, salt: triangle, drought: square and drought recovery: diamond) and b) loadings representation of the three metabolites with absolute higher scores per component of the model and additionally those related with CW.

Figure 7. Fold-change heatmap of the primary metabolic profile of the saline, drought and recovery treatments respect to the control in in leaves of tobacco plant (*Nicotiana sylvestris* L). Dots means significant differences by Student´s test (black. $p<0.05$; green. $p<0.1$).