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Studies on chloroplastic factors responsible for drought tolerance in rubber plants (*Hevea brasiliensis*)

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Abstract

Four clones of natural rubber plants (clone RRII 105, RRIM 600, GT 1 and PR 255) were grown in large poly bags under three different light conditions namely, 100, 70 and 30% sunlight. One set of plants was drought stressed for three weeks by withholding irrigation during the rain free summer season and a second set was kept as irrigated controls at all light levels. The malondialdehyde (MDA)/chlorophyll ratio in drought affected plants was determined as an indicator of drought tolerance/ susceptibility. Photosynthetic oxygen evolution rate was measured at different light intensities (LED). High light grown plants responded to measurement light intensity better than low light grown plants in the relatively stress tolerant clone RRIM 600. The apparent quantum yield of oxygen evolution and light compensation point were lesser in the low light grown plants than the open light plants. The degree of drought mediated inhibition in photosynthetic O_2 evolution was lower in low light grown plants than open light plants indicating protection from photoinhibition in shaded plants. The analysis of chloroplast protein profile showed that the sun exposed plants with concomitant drought stress induced a novel 23 kDa chloroplast stress protein. LC/MSMS analysis revealed that the protein was a small chloroplast heat shock protein (sHSP). These findings indicate that the sHSPs may play a role in drought tolerance as the more tolerant clones expressed relatively increased amounts of this protein.

Key words: Light, drought, *Hevea brasiliensis*, photosynthesis, small chloroplast heat shock proteins (sHSP).

Introduction

On a global basis, drought coincident with other environmental stresses such as high temperature and high light intensity poses a major constraint to plants survival and productivity (Boyer 1982). The natural rubber plant (*Hevea brasiliensis*) is mainly cultivated in tropical and sub-tropical belts. During summer months young plants in the field face many environmental stresses and succumb to the cumulative effects of abiotic stresses such as drought, high light and high temperature. Drought combined with high solar light intensity has been reported as major environmental constraint for establishing rubber cultivation in areas such as the North Konkan (Jacob *et al.*, 1999). High intensity solar radiation concomitant with soil moisture deficit leads to an imbalance between light and dark reactions of photosynthesis and causes an increased diversion of electrons for the production of active oxygen species in the leaves of rubber plants (Jacob *et al.*, 1999).

In general most of the damaging effects of irradiation and moisture stress to green leaves occur at the chloroplast membrane and enzyme level (Oquist *et al.*, 1995). The ability to utilize higher or lower photon flux density (PFD) is mainly dependent on structural modulations in leaves and photosynthetic apparatus resulting from growth light conditions. The PS II, thylakoid membranes and electron transport components are the main targets of photoinhibition due

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to the formation of excess active oxygen species during adverse climatic conditions (Demmig-Adams and Adams 1992, Halliwell and Gutteridge 1999). In mature rubber plants the photosynthetic contribution of shaded leaves to the total carbon balance has been suggested to be higher than fully exposed leaves during drought period (Devakumar *et al.*, 1999). Drought is a multidimensional stress affecting plants at various levels of growth and development. Most of the field grown plants tolerate environmental stresses through many metabolic adaptations at the cellular level. The response of plants such as rubber to drought is more complex due to the interactive actions at the whole plant level with factors like water relations, stomatal behavior and photosynthesis and cellular processes like the induction of certain growth hormones, dehydrins, free radical scavenging compounds, enzymes and specific stress response proteins.

Tolerance to abiotic stresses has mainly been achieved through engineering for increased cellular levels of osmotically-active solutes (such as proline, glycinebetaine, mannitol, trehalose, fructans, etc.). However the induction of stress proteins in tolerant lines of a variety of crop species has been widely reported (Vierling 1991; Zhu, 2001). Furthermore an increased level of stress proteins in some plants have been previously correlated with enhanced tolerance for drought and light stress (Adamska 1997, Hutin *et al.*, 2003). Currently little information is known regarding the effect of light and drought on the protein profiles of chloroplasts of leaves from rubber plants. This study aims to characterize the effects of drought and high light on photosynthetic rate and stress responsive protein expression in the chloroplast of young rubber plants and implication of such stress proteins in drought tolerance in young rubber plants.

Materials and Methods

Plant material and growth condition

Budded stumps of four clones of Hevea namely RRII 105, RRIM 600, GT1 and PR 255 were planted in large (35 x 65 cm) size polythene bags. The plants were grown under different light conditions (twenty plants per treatment) namely, open sunlight (100% light), partial shade (70% light) and deep shade (30% light). Different light intensities were achieved using shade nets of varying thickness erected 3 meters above the top whorl of the plants. One set of plants in each light condition was drought stressed by withholding irrigation for three weeks during rain free summer season and a second set was kept as irrigated controls.

Measurement of Photosynthetic O2 Evolution

The rate of photosynthetic oxygen evolution by leaf discs of the freshly harvested leaf (n=8) was measured at 25°C with a Clark type oxygen electrode (Hansatech LD2/2, King's Lynn, UK). The measurement light (LED) was achieved using a Hansatech LH 36 light source. To avoid any CO_2 limitation 5% CO_2 was generated in the closed chamber by a bicarbonate/ carbonate buffer (pH 9.2).

Additionally an experiment was conducted to find out the apparent quantum yield of oxygen evolution (mol of O_2 evolved per mol of photon incident on the leaf) and light compensation point. The leaf disc was first acclimatized to dark for five minutes and the rate of dark respiratory oxygen uptake by the leaf disc was measured. The leaf disc was then exposed to different light intensities (50, 100, 200, 400, 600 μmol m^{-2} s^{-1}) using an LED source (LH 36, Hansatech, UK) for 5 minutes each and photosynthetic oxygen evolution was measured at 25°C. (Walker 1988). A linear regression analysis was done between the rate of oxygen evolution (dependent variable) and light intensity (independent variable) for each leaf sample. The apparent quantum yield of photosynthetic oxygen evolution (mol of oxygen evolved per mol of photon incident on the leaf disc) was calculated from the slope of the regression equation. The photosynthetic light compensation point (which is the light intensity at which the rate of photosynthetic oxygen evolution and the rate of respiratory oxygen uptake are equal) was also calculated from the same regression models.

Estimation of chlorophyll and malondialdehyde

Leaf chlorophyll content was estimated using Arnon's method (Arnon 1949). The lipid peroxidation product malondialdehyde(MDA) was estimated according to Heath and Packer (1968).

Chloroplast protein profile

Type II chloroplasts were isolated from leaves and chloroplast polypeptides were prepared from those samples. The polypeptides were resolved in 12% SDS-PAGE and stained with coomassie brilliant blue (Laemmli, 1970).

Mass Spectrometry

The 23 k Da protein band was harvested from the

gel using a sterile scalpel and placed into a microfuge tube. Gel pieces were washed and digested overnight at 37°C with trypsin according to Heazlewood *et al.*, (2003). Peptides were extracted from the overnight digest by adding an equal volume of acetonitrile and shaking for 15 minutes at 8000 rpm on an orbital shaker. The supernatant was removed and 20µl of a solution containing 50% acetonitrile and 5% formic acid was added to the gel pieces, and agitated for 15 minutes at 8000 rpm on an orbital shaker. Supernatant was removed and the previous step repeated. Supernatants were pooled and solvent evaporated using a speedvac (Thermo Savant) for 20 - 30 minutes until nearly dried. Peptides were hydrated in 16 µl of 5% acetonitrile and 1% formic acid prior to mass spectrometric analysis. Samples were analyzed using an Agilent 1100 series capillary LC system with a 0.5x50mm C18 reverse phase column coupled to a QSTAR Pulsar i LC/MS/MS system (Applied Biosystems) equipped with the Ionspray source running Analyst QS software (v1.1). Peptides were eluted from the column using a 5 - 80% acetonitrile gradient in 0.1% formic acid at 8µl/min. An information-dependent acquisition method for data acquisition was used with rolling collision energy for automated collision energy determination based on the ion m/z (Sciex/AB). The method used a 1s TOF-MS scan and switched to MS/MS for a 2s information-dependent acquisition of the product ion for ions of 2+, 3+ and 4+ with greater than 30 counts. Data was exported and analyzed using Mascot (Matrix Sciences) against the MSDB database with search parameters using a peptide tolerance of ±2 Da and an MS/MS tolerance of ±0.8 Da, allowing up to 1 missed cleavage for trypsin, a variable modification of Oxidation (M) and the instrument type set to ESI-QUAD-TOF.

Results and Discussion

The chlorophyll content per gram fresh weight of the leaf decreased under drought conditions in all the clones except in RRIM 600. The photo-oxidation of chlorophyll was highest in RRII 105 followed by PR 255 and GT 1 and the least in RRIM 600. Under shade condition total chlorophyll content increased in GT 1 and PR 255 (Table 1). The lipid peroxidation product malondialdehyde was significantly increased in droughted plants indicating a state of oxidative stress. However, shade grown plants accumulated comparatively lesser MDA than their open light grown counterparts when stressed. The MDA/chlorophyll ratio in young plants was a typical reflection of the degree of susceptibility to drought (Table 1). The droughted plants of RRII 105, a drought susceptible clone, recorded the highest MDA/chl ratio (50.5) whereas RRIM 600, a tolerant clone, recorded the lowest ratio (26.5).

The photosynthetic oxygen evolution rates of unstressed leaves were studied in two clones, namely the drought susceptible clone (RRII 105) and the drought tolerant clone (RRIM 600) under different light intensities. When the growth light intensity declined the rate of oxygen evolution also decreased (Fig. 1). In open light grown plants the rate of oxygen evolution progressively increased when the measurement light was increased. The light saturated level reached at around 300- 400 μmole $m^{-2}sec^{-1}$ (LED). The plants grown under partial shade (70% light) did not show any significant differences in photosynthetic rate compared to the 100% light grown plants. The deep shaded plants (30% light) did not respond to high measurement light (Fig. 1). The light saturation level was reached very early at around 200 μmol $m^{-2}sec^{-1}$ (LED) in the shaded plants. These results show the adaptive nature of the photosynthetic apparatus to various growth light conditions. In low light grown plants there was a limitation of the light driven electron transport rate and reaction center components to give maximum photolysis (Anderson and Barber, 1996).

Under drought condition the photosynthetic oxygen evolution activity was drastically inhibited in open light grown plants of RRII 105 but was less inhibited in RRIM 600 plants (Fig. 2). The extent of drought induced inhibition was lesser in shaded plants than full sunlight exposed plants. The degree of susceptibility of the popular clone RRII 105 to drought

Table 1. The MDA/chl ratio in leaves of young rubber plants grown in sun and partial shade (70% light) and with or without irrigation.

		RRII 105		RRIM 600		GT 1		PR 255	
		Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade
Total chl (mg/g Fwt)	Irrigated	3.7 ± 0.2	3.8 ± 0.04	3.5 ± 0.05	3.7 ± 0.03	3.6 ± 0.17	4.0 ± 0.15	3.2 ± 0.05	3.5 ± 0.11
	Drought	2.4 ± 0.02	3.3 ± 0.11	3.3 ± 0.08	3.01 ± 0.05	2.9 ± 0.1	3.1 ± 0.11	2.9 ± 0.2	3.2 ± 0.07
MDA (µM/g)	Irrigated	78 ± 10	71 ± 5.1	65 ± 8.5	65 ± 14	103 ± 2	59 ± 10	86 ± 5.9	62 ± 3.7
	Drought	124 ± 5.6	101 ± 4.6	87 ± 3	94 ± 8	123 ± 10	91 ± 3.4	99 ± 9.2	92 ± 5.4
MDA/chl ratio	Irrigated	21	24	19	17.5	28.5	14.5	27	18.4
	Drought	50.6	30.6	26.5	31	41	29	32.5	28.1

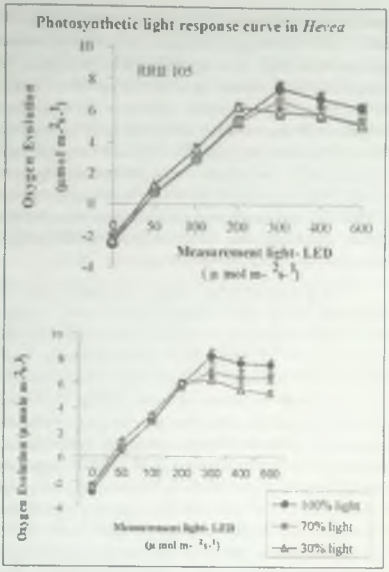

Fig.1. Photosynthetic light response curve in young plants of *Hevea*. Plants were grown under different light intensities (100, 70 and 30% sunlight). The measurement light was provided by LED source. For details regarding the measurements please see materials and methods.

has been well characterized in previous reports (Annamalainathan *et al.*, 2005, Alam *et al.*, 2005). The percentage inhibition in photosynthetic O_2 evolution in drought stressed leaves as compared to the respective controls was as low as 33 % in 30% light grown plants and as high as 51% in open light grown plants. These results demonstrate how partial shade can protect young rubber plants against photoinhibition and can sustain photosynthetic activity during water deficit condition.

The light compensation point progressively declined as the growth light intensity decreased in RRII 105 and RRIM 600 (Table 2). This was attributed to less dark respiration in the shade grown plants. The apparent quantum yield of oxygen evolution also decreased in shaded plants (Table 2). This result revealed that the light use efficiency of shade grown plants were better under low measurement light than high light (Schiefthaler et al., 1999).

Table 2. The light compensation point and quantum yield of oxygen evolution in different light grown plants.

Growth light	Light compensation point (μmol/m2/s1)		Q.Y of O2 evolution (mol O2/mole photon)	
	RRII 105	RRIM 600	RRII 105	RRIM 600
Open	23.6 ± 3.2	26.2 ± 2.5	0.023 ± 0.0012	0.026 ± 0.002
70%	12 ± 2	15 ± 3	0.019 ± 0.0013	0.021 ± 0.001
30%	11 ± 4	11 ± 3	0.018 ± 0.0018	0.018 ± 0.001

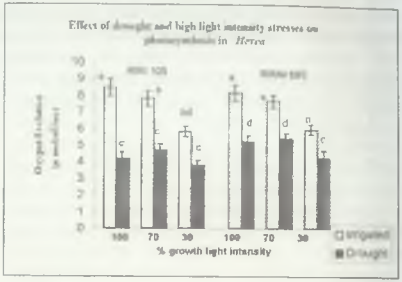

Fig. 2. The photosynthetic oxygen evolution rate in irrigated and drought stressed young plants of Hevea. Plants were grown under different light intensities (100, 70 and 30% sunlight). For other details regarding the drought imposition and measurements please see materials and methods.

In the present study we have observed a consistently over-expressing 23 kDa protein in chloroplasts of rubber plants experiencing drought concomitant with high sunlight intensity (Fig 3). This band was excised and digested using trypsin and analyzed by LC-MS/MS. Data obtained by mass spectrometry were used to identify the protein using the non-redundant protein database (MSDB) available through the Mascot search engine (http://www.matrixscience.com/). This search analysis produced a number of significant cross species matches (Table 3) revealing that the protein was a small chloroplast heat shock protein (sHSP). A total of six different peptides from the induced 23 kDa protein successfully matched several sHSPs from tobacco, petunia and tomato (Table 4).

The sHSPs are a class of the HSP superfamily initially identified through their response to elevated temperatures (Vierling 1991). While present in most eukaryotes, the sHSP class appears to be most prevalent in higher plants. The chloroplast sHSPs are a subclass of the of the sHSP family, with subclasses also present

Table 3. Significant Mascot cross species hits that provided novel peptide matches.

Hit*	Accession	Protein ID	Species	Mr (kDa)	Mowse	Coverage (%)	NP**
1	T02018	heat shock protein 26a, chloroplast	*Nicotiana tabacum*	26.6	135	15	5
2	S16004	heat shock protein 21	*Petunia hybrida*	26.8	126	9	3
3	T06324	heat shock protein 21, chloroplast	*Lycopersicon esculentum*	26.2	125	11	4

*Hit refers to the Mascot rank in order of score; Accession refers to the SwissProt identity for the matched protein; Mr is predicted molecular mass based on amino acid sequence; Mowse is the total protein score given to the match by Mascot; Coverage is percentage of the protein matched; **NP is number of peptides matched.

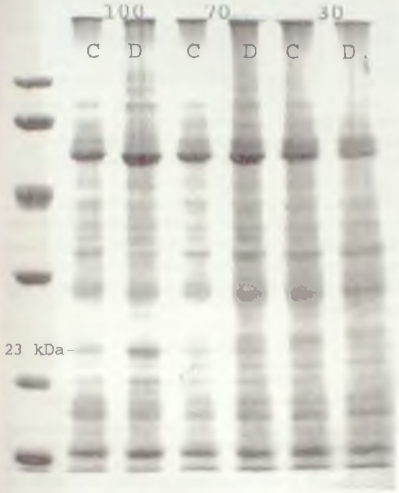

Fig.3. The chloroplast protein profile of young Hevea plants (clone RRIM 600). The plants were grown under different light intensities (100, 70 and 30 % solar radiation) with (C) or without (D) irrigation for 21 days during summer season. The stress protein 23 kDa is indicated on the right side. The molecular weight markers (STD) are indicated in the left side.

in the endoplasmic reticulum, the mitochondrion and the cytosol. The chloroplast sHSPs have been reported in a variety of plant species, including a 26 kDa HSP from tobacco (Lee *et al.*, 1998) and 21 kDa HSPs from tomato, Arabidopsis and soybean (Suzuki *et al.*, 1998). The sHSPs are present within the chloroplast as large oligomers containing 9 or more subunits and are actively synthesized during heat stress (Suzuki *et al.*, 1998). Moreover, a great deal of evidence indicates that sHSPs play a role in tolerances to a variety of biotic and abiotic stresses as well as key developmental processes (Vierling 1991; Heckathorn *et al.*, 2004).

In chloroplasts the sHSPs have been implicated in protecting this organelle from photoinhibitory and oxidative stresses by preventing protein aggregation and stabilizing the thylakoid membrane (Török et al., 2001). Heckathorn et al., (1998) have demonstrated that the chloroplast sHSP plays a direct role in stabilizing the photosystem II (PSII) oxygen-evolving complex (OEC) proteins during heat stress and thereby promotes the maintenance of PSII electron transport. The chloroplast small heat shock protein also implicated in protective mechanism in plants experiencing oxidative stress by undergoing oxidation-dependent conformational changes in the molecular structure. More recently it has been reported that chloroplast sHSPs also protect photosynthetic electron transport from the inhibitory effects of heavy metals (Heckathorn *et al.*, 2004). Thus, sHSPs appear to be general stress proteins in chloroplasts that are involved in maintaining function and survival of this organelle during stress or facilitating recovery from stress.

There was a tight association existing between the appearance of the sHSPs and growth light intensity. The expression level of the protein was very low in 70% light grown plants and absent in deeply shaded (30% light) plants (Fig. 3). There was also clonal variation observed in the level of expression of the stress protein. Among the four clones studied, RRIM 600 showed prominent expression, which is also a drought tolerant clone (Fig 4) as evidenced from the present study and previous report (Alam *et al.*, 2005), followed by the clones, RRII 105, GT 1 and PR 255. Given the extensive research undertaken in the area of stress response in plants, the relationship between drought tolerance in young rubber plants and the induction of an HSP was not surprising. However, this new finding in Hevea could be further tested in more numbers of stress tolerant and susceptible clones. Similarly, the extent of expression of sHSP and its correlation with (MDA/chl)

Table 4. Parent ion and corresponding peptide matches from Mascot search results.

Hit 1: T02018 heat shock protein 26a, chloroplast - common tobacco

Observed Ion (m/z)	Charge (z)	Mr (expt)	Mr (calc)	Delta	Score	Peptide
455.7221	2	909.4296	909.4266	0.0030	39	FDMPGLSK + Oxidation (M)*
544.3267	2	1086.6389	1086.6437	-0.0048	(25)	NGVLFISIPK*
544.8253	2	1087.6361	1086.6437	0.9924	49	NGVLFISIPK
615.3541	2	1228.6937	1228.6914	0.0023	20	VSVEDDLLVIK*
461.2307	3	1380.6704	1380.6595	0.0109	30	FDMPGLSKDEVK + Oxidation (M)*

Hit 2: S16004 heat shock protein 21 - garden petunia

Observed Ion (m/z)	Charge (z)	Mr (expt)	Mr (calc)	Delta	Score	Peptide
455.7221	2	909.4296	909.4266	0.0030	39	FDMPGLSK + Oxidation (M)
608.3518	2	1214.6891	1214.6758	0.0133	37	VSVEDDVLVIK
556.3136	3	1665.9189	1665.8937	0.0252	50	VSVEDDVLVIKGEHK*

Hit 3: T06324 heat shock protein 21, chloroplast - tomato

Observed Ion (m/z)	Charge (z)	Mr (expt)	Mr (calc)	Delta	Score	Peptide
455.7221	2	909.4296	909.4266	0.0030	39	FDMPGLSK + Oxidation (M)
521.7202	2	1041.4259	1040.4267	0.9992	37	QMIDTMDR + 2 Oxidation (M)*
544.3267	2	1086.6389	1086.6437	-0.0048	(25)	NGVLFISIPK
544.8253	2	1087.6361	1086.6437	0.9924	49	NGVLFISIPK

Each Hit corresponds to the list outlined in Table 3. Observed Ion corresponds to the ion or peptide analyzed by the mass spectrometer; Charge refers to the observed ions charge; Mr (expt) is the extrapolated mass of the observed ion or peptide; Mr (calc) is the mass of the matched peptide; Delta is the difference in mass between Mr (expt) and Mr (calc); Score is the Mowse score for the peptide; Peptide is the amino acid sequence of the matched trypsin peptide. The bracket that surrounds some values in the score column indicates that this value was not included in the final protein Mowse score due to redundancies. The asterisk (*) identifies the novel ions or peptides from each protein hit.

ratio also needs to be worked out in more number of drought tolerant and susceptible clones of Hevea. Once a strong correlation could be established (MDA/chl) ratio can be taken as yet another surrogate expression of intrinsic drought tolerance in Hevea plants.

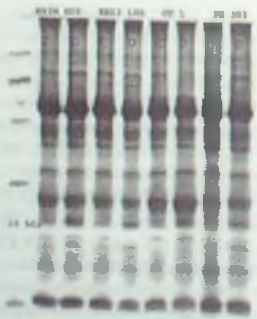

Fig. 4. The chloroplast protein profile of young Hevea plants. The plants were grown under open light condition (100 % solar radiation) with (C) or without (D) irrigation for 21 days during summer season. The stress protein sHSP (23 kDa) is indicated on the right side

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