Discovering New Mechanisms of Abiotic Stress Tolerance in Endemic Australian *Nicotiana* Species

Richard A. O'Brien

BBioCons

Supervisor: Professor Brian J. Atwell

Co-supervisor: Dr Andrew P. Scafaro

Department of Biological Sciences

Faculty of Science and Engineering

Macquarie University NSW Australia

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Research

Submission date: 9 January 2016

Declaration

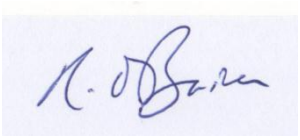
I certify that the material of this thesis has not been previously submitted as part of the requirements for a higher degree to any other university or institution.

This thesis contains no material previously published or written by any other person. I certify that all information sources and literature used are indicated in the thesis.

I wish to acknowledge the following assistance with the research detailed in this report:

- Dr Brian J. Atwell, my supervisor, whom assisted with method development and editing my drafts;
- Dr Andrew P. Scafaro for assistance in genetic analysis;

All other research described in this report is my own original work.



Note to examiners

This thesis is written in the form of a journal article for Plant Physiology. The majority of the author guidelines (Appendix II) have been followed, except for minor deviations detailed here and where the guidelines clash with Macquarie University thesis formatting requirements. All figures and tables have been presented at the appropriate places in the text to enhance readability.

Acknowledgements

I would like to thank my supervisor Dr. Brian J. Atwell for his unwavering support and guidance.

To all my friends I thank you for making this journey very enjoyable and interesting. To the writing group I had been lucky to be a part of; Belinda, Fonti, Katie, Elisse and Louise, I am grateful for their constructive discussions, general encouragement and friendship. I would also like to thank Najeeb, Aaron and Sayed for their companionship and words of advice.

The ‘Emma’ lab led by Michael Gillings with Louise Chow and Amy Asher. I would like to extend my gratitude for laboratory access and the use of their fine equipment.

I would like to thank my family. In particular, my sister Lisa who gave advice and support during the course of the Masters program.

Finally, I thank Macquarie University for their support and for making this research possible.

Table of Contents

Abbreviations

Base pair	**bp**
Deoxyribonucleic acid	**DNA**
Polymerase chain reaction	**PCR**
RCA	**Rubisco activase**
CO_2	**Carbon dioxide**
SE	**Standard error**
O_2	**Oxygen**
EC	**Electrical conductivity**
g_m	**Mesophyll conductance**
g_s	**Stomatal conductance**
HSPs	**Heat shock proteins**
TFs	**Transcription factors**
NAR	**Net assimilation rates**
RGR	**Relative growth rates**
LAR	**Leaf area ratio**
Rubisco carboxylase/oxygenase	**ribulose-1, 5-bisphosphate**
cDNA	**Complementary DNA**

Abstract

Global climate change is predicted to cause more frequent extreme weather events over the next century. These events will exacerbate various abiotic stresses, including drought and high temperatures, each of which can inhibit growth and threaten survival of natural and managed plant systems. This study postulates that wild Australian relatives of *Nicotiana* from very arid sites might reveal unique characteristics that confer tolerance of hot environments. This may serve high implications in managing agricultural systems and understanding plant species that are sensitive to increased heat. A multi-disciplinary approach investigated heat tolerance at plant, leaf, cell and gene levels. Physiological analysis including growth analysis and gas exchange, suggested that *N. megalosiphon* and *N. tabacum* L. tolerated heat stress best among the five species tested. However, solute leakage from leaves of *N. megalosiphon* did not reveal superior cell membrane integrity under heat stress. A preliminary molecular analysis of a gene known to be vulnerable to heat-stress, Rubisco activase (RCA), revealed some putative amino acid changes between *N. tabacum* L. and *N. benthamiana*. This could imply divergent evolution between these two species, particularly in *N. tabacum* L. which has been highly selected for hot environments under cultivation.

Department of Biological Sciences, Macquarie University, New South Wales, Australia (Email: richard.obrien@students.mq.edu.au).

Running title: Abiotic stress tolerance in Australian *Nicotiana* spp.

Keywords: Abiotic stress, heat, gas exchange, membrane integrity, Rubisco activase

1 Introduction

Climatic change, agricultural productivity and abiotic stress tolerance

The world faces grave challenges in maintaining agricultural productivity as climate impacts impose ever greater abiotic stresses (Clay, 2013). The predicted effects of supra-optimal temperatures on agriculture production are of particular concern, with reliable evidence that ambient temperatures are rising inexorably. Modelled projections for the worst greenhouse gas emission scenario forecast global mean temperatures to increase from 1.1˚C to 6.4˚C from 1990 to 2100 (Change, 2007). Under the most benign greenhouse gas emission scenario, the prediction is 1.1˚C to 2.9˚C. However, since 2000 the trajectory for global gas emissions has exceeded those that have been predicted in the aforementioned highest gas emission scenario (Change, 2007). With these predictions, apprehensions around agricultural productivity are rising, with an increase of just 1.5˚C predicted to have significant negative effects on crop yields worldwide (Hasanuzzaman et al., 2013).

Traditionally, relationships between agriculture, climate and population densities have largely determined the 'carrying capacity' of regions (Rosenzweig and Hillel, 2008). Today, in light of climate change, there is more international concern on how these variables will play out in agricultural production on a worldwide basis (Rosenzweig et al., 2014). Plant breeding, improved management and biotechnology have kept pace with rising demand for food to date but indications are that this equilibrium cannot be expected to continue (Fischer et al., 2012).

Abiotic stress is the primary limitation to crop production worldwide, with an estimated 50% of major crops being affected annually because of non-optimal growth conditions (Bray et al., 2000). Heat stress can result in morphological, biochemical, physiological and molecular level changes, which thus alters development and reduces yield (Wang et al., 2000).

1.1 Exploiting wild species to understand abiotic stress tolerances

Mining genetic diversity with particular reference to rice

The domestication of modern crop species has seen sharp declines in their genetic diversity (Sakuma et al., 2011). Hence there is a recent emphasis on conserving and investigating the genetic diversity of agricultural plants and their wild progenitors. Here alleles with a practical application can be conserved: abiotic stress tolerance genes are prime candidates for such an approach (Zamir, 2001; Tester and Bacic, 2005). Identifying genotypic variation in relatives

of crop species is now more plausible due to dramatic changes in the costs and efficiency of genotyping. Thereby, gene banks are now acquiring rapidly growing databases of genetic information that may be used to enhance crop diversity (McCouch et al., 2012). Genetic diversity might range from interspecific sources to variants within species (Hamrick and Godt, 1996). On the intraspecific level, the term 'hidden diversity' is used to describe the unknown genetic variation of individual relatives and is best illustrated in rice genetics. Outcrossing to create new hybrids now takes the interest of crop breeders. In primary rice gene pools, the strategic backcrossing of three elite *Oryza* cultivars with 203 *Oryza* landraces, breeding lines and other commercial strains revealed efficient phenotypes conferring increased salinity and drought tolerance (Ali et al., 2006). These landraces harbour a suite of abiotic stress tolerance genes that reflect the abiotic stresses of local niches they occupy. For instance, the wild Australian species *O. australiensis*, which has low genetic cross compatibility with the domestic *O. sativa*, exhibits physiological and molecular tolerance to heat that is probably region-specific (Scafaro et al., 2009). Mining genetic diversity has been successfully researched in rice cultivars and local varieties of *Oryza* species making it a suitable model species when considering cultivar outcrossing hybridisation with wild progenitor relatives.

Diversity of Solanaceae and Nicotiana

Over 3000 plant species across 93 genera belong to the family Solanaceae, many of which are important economically (Mueller et al., 2005). Within the family is the potato (*Solanum tuberosum*), ornamental plants such as petunias (*Petunia*), a large variety of fruit-bearing vegetable plants such as the tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*) the peppers (*Capsicum annuum*) as well as tobacco. With such a wide range of crop species, Solanaceae is regarded the third most important taxon in economic terms, the most valuable vegetable crop taxon and the most diverse regarding agricultural utility (Mueller et al., 2005). In addition, model species belonging to the family Solanaceae have produced valued scientific conclusions. Examples include the study of fruit development in the tomato and pepper (Julie Gray et al., 2012; Mounet et al., 2012), tuber development in the potato (Abelenda et al., 2011), anthocyanin pigment studies in petunia (Cavallini et al., 2013) and the analysis of plant defence mechanisms, both biotic and abiotic, seen in tomato and tobacco (McDowell and Woffenden, 2003; Jada et al., 2014). The Solanaceae pose interesting

questions due to vast phenotypic diversity alongside many highly conserved and shared genes across the family.

The genus *Nicotiana* is the sixth largest genus within the Solanaceae and is represented by 76 species worldwide (Marks and Ladiges, 2011). For the purposes of this paper, widely distributed Australian species of *Nicotiana* and an exotic species native to South America (*Nicotiana tabacum* L.), have been investigated. Regions in Australia inhabited by 'wild' tobacco have experienced a succession of climatic changes throughout geologic history (Bally et al., 2015). It is estimated that primary progenitors of wild Australian *Nicotiana* arrived about 20 Ma during a period of warm and wet environmental conditions (Byrne et al., 2008). This was followed by a period (10–6 Ma) characterised by aridification and the development of additional geographic barriers. These processes encouraged the observed genetic diversification to develop about 1–0.8 Ma (Byrne et al., 2008). The distribution of wild and cultivated species are shown in Figure 1.

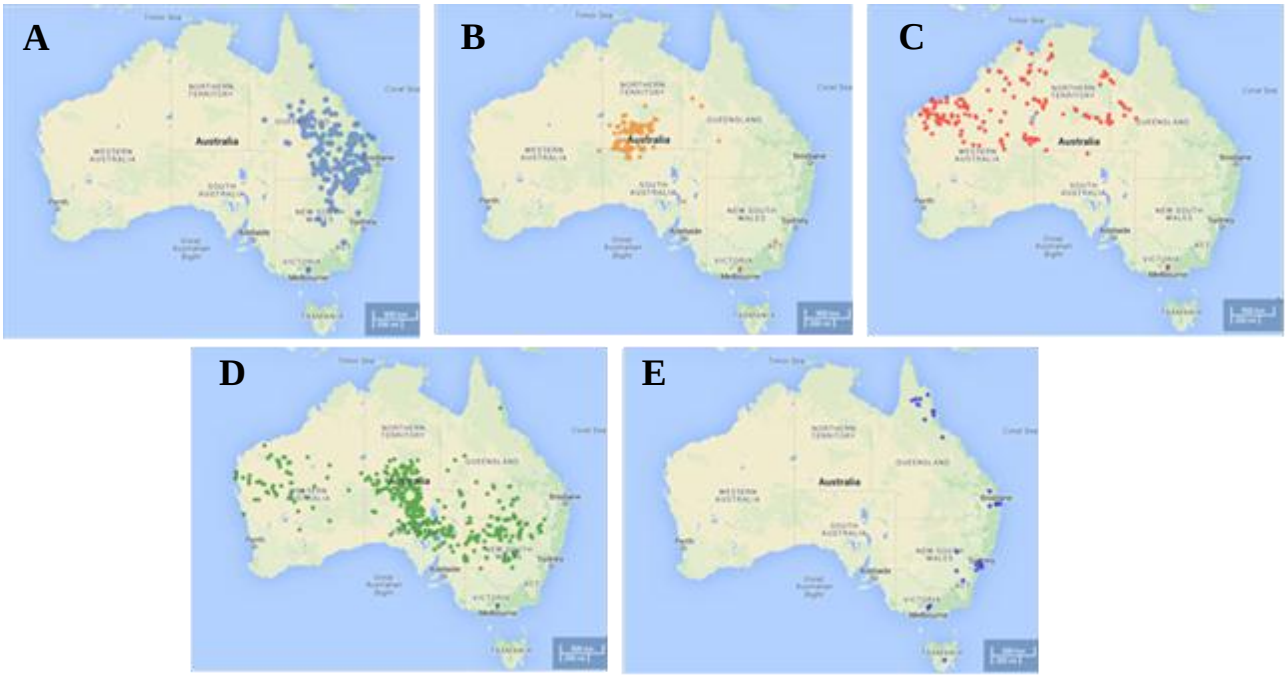


Figure 1 Species distribution of (A) *N. megalosiphon*, (B) *N. gossei*, (C) *N. benthamiana*, (D) *N. simulans* and (E) *N. tabacum* L. (Atlas of Living Australia).

Commercial tobacco (*N. tabacum* L.) was the first genetically modified plant, making it a model plant species for genetic modification (Hoekema et al., 1983). Modern tobacco originated in the highland Andes and was likely a result of hybridization of two other species, *N. sylvestris*, a member of the section Tomentosae, *N. tomentosiformis* and possibly *N.*

otophors. Cultivated tobacco is an allopolyploid species (n = 24) and shares its basic chromosome number n = 12 with other solanaceous species such as tomato, potato, pepper and eggplant. The species is most likely the result of a tetraploidization event (Lim et al. 2004). *N. tabacum* L. plays an important experimental role in revealing phenotypic diversity, tissue cultures hybridisation and ploidy manipulation (Lewis, 2011). It is a robust, fast-growing large-leafed plant that suits many physiological studies well.

Nicotiana benthamiana, an Australian species, is also used extensively in plant research as a model species. It was used initially for targeted RNA-induced gene silencing in order to probe gene function (Waterhouse and Helliwell, 2003). Because of its arid zone environmental niche, it has been included in this study to broaden the genetic comparisons within the genus.

The distribution of *N. gossei* is restricted to ranges of southern Northern Territory and north-western South Australia where high temperature extremes occur. Preferred soil profile is fertile, often sandy. *N. gossei* occupies micro-niches that are sheltered by rocks or upper slopes (Latz and Green, 1995).

N. megalosiphon distribution is limited to central and south-eastern Queensland and northern New South Wales over a range of temperatures (Symon, 2005).

Exploitation of the genetic diversity found within the Solanaceae and *Nicotiana* is important for the ongoing improvement of commercial Solanaceae cultivars as well as research into genetic function in higher plants (Cao et al., 2013).

1.2 Physiological heat stress responses in plants

Development

The responses of plant species to environmental stresses are often seen in developmental changes (Poorter and Remkes, 1990). Initially seed germination is affected, where increased temperatures reduce germination rate, plant emergence, seedling vigour and reduced radicle length in various crop species (Piramila et al., 2012). For example, germination of wheat was restricted when exposed to increased temperatures where cell death in seed embryos occurred (Stone, 2001). Temperature can have substantial developmental effects on leaf size, thickness and number (Raven et al., 2005). Reductions in net assimilation rates (NAR) and the

morphological symptoms of heat stress ultimately reduce relative growth rates (RGR) and distort leaf development, reduce branch number, increased leaf senescence and abscission and inhibit root and shoot growth (Hasanuzzaman et al., 2013). Partitioning of metabolites in order to tolerate increased temperatures is a strategy employed by heat-tolerant plant species (Smith and Dukes, 2013). For example, the assimilate partitioning of dry matter to plant roots under increased heat has evolved to enhance the acquisition of water, although it is probably in part a drought response (Leport et al., 1999). There is evidence suggesting that assimilate partitioning to roots in perennial cotton is significantly higher than in annual cotton cultivars (De Souza and Da Silva, 1987). This shows preferential accumulation of biomass in the roots of perennial species that survive longer periods of heat than their annual counterparts. Reproductive tissues of plants are considered the most sensitive to heat stress, where slight elevations of temperature can impair reproductive function (Barnabás et al., 2008). Periods of heat stress can determine the production of flowers and thus fruit quantity in crop species, where meiosis processes in both male and female reproductive organs are severely affected (Barnabás et al., 2008; Yun-Ying et al., 2008).

Photosynthesis

A major effect of excessive heat on plants is impaired photosynthetic function (Farooq et al., 2009). The chloroplast and specifically the photochemical reactions in the stroma associated with the thylakoid lamellae, are considered the primary locations of heat-induced injury (Wang et al., 2009). Under heat stress, disorganisation of these elements – thylakoids and granal stacks – disrupts photosynthesis severely (Marchand et al., 2005; Rodríguez et al., 2005). Additionally, a reduction in efficacy of photosynthetic pigments can be attributed to increased temperature (Havaux and Tardy, 1999). Thus, the efficiency and activity of photosystem II (PSII) is severely affected by high temperatures (Morales et al., 2003).

Stomatal closure during increased heat stress is a defence mechanism used to reduce water loss but will inevitably increase leaf temperature (Boyle et al., 2015). Indirectly, this limits the efficiency of photosynthesis because CO_2 is not available for photosynthesis and O_2 accumulates in the sub-stomatal cavity (Flexas et al., 2012). Here an imbalance can occur between reactive oxygen species and antioxidant defence which can lead to photorespiration and, in the case of increased light intensity (often coupled with increased heat), photo-chemical damage (Kasahara et al., 2002). Stomatal closure has relatively transient effects on

photosynthesis when compared with the irreversible damage that can be induced by heat. For example, observed reductions in photosynthetic rates as temperatures increase from 25 to 45˚C in *Vitis vinifera* leaves were attributed to reductions in photosynthesis due to stomatal closure at 15-30% (Greer and Weedon, 2012). When stomata respond strongly to heat, stomatal resistance rather than mesophyll resistance dominates the reduction in photosynthesis (Wahid et al., 2005; Scafaro et al., 2011).

Other factors believed to restrict photosynthetic rates include the reduction in the levels of soluble proteins such as Rubisco binding proteins (RBP) and both the large and small subunits of Rubisco (Demirevska-Kepova et al., 2005). The synthesis of starch and sucrose under heat stress is also compromised via the reduced activity of invertase, ADP-glucose pyrophosphorylase and sucrose phosphate synthase under heat stress (Sumesh et al., 2008).

Temperature response of mesophyll conductance

A critical element of photosynthetic potential is the diffusion of CO_2 from intercellular spaces to the stroma of chloroplasts where CO_2 carboxylation occurs: this is described as mesophyll conductance (g_m; Raven et al., 2005; Tholen and Zhu, 2011). Increased temperatures may limit photosynthesis because of changes in the rate of CO_2 diffusion (Tholen and Zhu, 2011). To date, there is conclusive evidence that photosynthetic rates are positively correlated with g_m in rice and transgenic plants (Hanba et al., 2004; Scafaro et al., 2011). Positive effects of temperature correlated with g_m in *Nicotiana* spp. until 35˚C (Bernacchi et al., 2002). Further, slight declines in g_m were observed from 28 to 38˚C in the tropical rainforest tree species *Eperua grandiflora* (Bernacchi et al., 2002; Pons and Welschen, 2003). Increased leaf temperature has also been shown to limit g_m as seen in spinach leaves (Yamori et al., 2006). Under ambient conditions where no apparent stresses confound photosynthetic rates, g_m and thus CO_2 diffusion can differ greatly between plant species naturally and in transgenic plants (Zhu et al., 2010). For example, the aquaporin NtAQP1 from *N. tabacum* L. positively increases *in vivo* mesophyll conductance when upregulated in transgenic *Xenopus oocytes* (Flexas et al., 2006). Additionally, the overexpression of aquaporin HvPIP2;1, derived from barley, increased g_m and CO_2 assimilation in leaves of transgenic rice plants (Hanba et al., 2004). Consistent with these findings, those plants which integrate the overexpression of these genes demonstrate increased efficiency of g_m.

Cell membrane integrity

Solute leakage from excised tissues is a well-established method for estimating membrane permeability in relation to abiotic stresses (Whitlow et al., 1992). Electrolyte leakage as a consequence of membrane dysfunction, as indicated by electrical conductivity (*EC*), shows the severity of damage as a result of heat stress (Dexter et al., 1932; Saelim and Zwiazek, 2000). Reductions in photosynthesis and subsequently growth and development, at above-optimal temperatures can be partially ascribed to decreased stability of cell membranes (Gupta, 2007). Membrane permeability has been used to determine heat tolerance in species as diverse as wheat, pasture species, *Brassica* sp. and cotton (Schaff et al., 1987; Hossain et al., 1995; Bajji et al., 2002). Electrolyte leakage measurements also demonstrate that correlations exist with antioxidative enzyme synthesis, membrane acyl lipid concentrations and stomatal resistance (Lauriano et al., 2000; Sreenivasulu et al., 2000; Väinölä and Repo, 2000). Hence, the measurement of electrolyte leakage is a valuable criterion in assessing physiological heat stress responses in plant species.

1.3 Improving gene level responses to heat and other abiotic stresses

There has been a growing interest in increasing heat stress tolerance with the insertion or upregulation of abiotic stress tolerance genes (Bhatnagar-Mathur et al., 2008). Candidate genes include those for osmoprotection, functional and structural stress-induced proteins, water and ion movement, signal perception and transduction, scavenging of free-radicals and the accumulation of compatible solutes (Bhatnagar-Mathur et al., 2008; Todaka et al., 2012). These proteins include heat shock proteins (HSPs), dehydrins and late embryogenesis proteins (LEA) and acid phosphatases (Chaves et al., 2003). The improvement of these stress tolerance mechanisms may integrate small RNAs (smRNAs) as regulatory elements, as well as transcription factors (TFs), which often control multiple genes (Sunkar et al., 2007; Golldack et al., 2011).

HSPs are vital elements in mediating heat stress responses by acting as molecular chaperones (Wiech et al., 1992). Here accumulation of a variety of HSPs are required to minimise tissue and cell damage due to heat (Kotak et al., 2007). It is reported in rice that the HSP gene *OsHSP1* is induced not only from heat but other abiotic stresses such as salt and osmotic stress (Moon et al., 2014). The overexpression of this gene in transgenic *A. thaliana* did confer increased heat tolerance but no tolerance to salt and osmotic stresses was observed,

leading to the conclusion that *OsHSP1* is part of a co-expression network of functional genes regarding heat stress. Further analysis confirmed that the upregulation of *OsHSP1* in rice activated a cascade of transcription events, 39 genes were co-expressed, leading to several increased abiotic stress tolerances (i.e. salt and osmotic stress tolerance; Moon et al., 2014).

The responses of plants to abiotic stresses are regulated by a multitude of signalling pathways that initiate gene transcription and associated downstream machinery (Wang et al., 2003). Transcription factors (TFs) – sometimes called sequence-specific DNA-binding factors – are important proteins that bind to specific sites on DNA sequences, thereby regulating rates of transcription and thus mRNA production (Latchman, 1997). Genes such as *OsHSP1* require these TF precursors in the form of heat stress transcription factor upregulation (Hsfs; Todaka et al., 2012). An example of this type of regulatory system is seen with *DREB2* in transgenic *A. thaliana*, where it acts in response to heat stress (Mizoi et al., 2012). Additionally, the NAC regulon is a TF associated with heat and osmotic stress responses in *A. thaliana* and certain grasses (Nakashima et al., 2009). Transgenic *A. thaliana* plants overexpressing *OsDREB2A* influenced the upregulation of *DREB2A* targeted genes which led to improved tolerance to heat (Matsukura et al., 2010). Further, the functionality form of *ZmDREB2A* was successfully inserted into transgenic *A. thaliana* conferring increased heat stress tolerance (Todaka et al., 2012).

DnaJ proteins serve as molecular chaperones, alone or in alliance with Hsp70, to carry out essential cellular processes such as protein folding, assembly/disassembly and degradation (Hennessy et al., 2005). They are integral to ensuring cellular protein homeostasis under normal and stress conditions (Wang et al., 2015). An example of a DnaJ protein related to heat tolerance is AtDjA2 and AtDjA3. Here the production of these proteins in *A. thaliana* has shown improved thermotolerance (Li et al., 2007). Further, a mutation found in the DnaJ protein TMS1 improves thermostability of pollen tubes in *A. thaliana* (Yang et al., 2009). LeCDJ1 and *SlCDJ2* are both DnaJ proteins associated with the chloroplast and have shown improved heat stress tolerance when expressed in transgenic tomato plants (Kong et al., 2014; Wang et al., 2015). In both cases these DnaJ proteins were found to protect Rubisco activity under heat stress.

Other examples of abiotic stress tolerance genes expressed in transgenic plant hosts include: *DREB1C* sourced from *Medicago truncatula* used in China Rose (*Rosa chinensis*) showing superior performance under freezing stress (Chen et al., 2010). *DREB1* sourced from soybean

(*Glycine max*) caused significant salt tolerance to be expressed in alfalfa (*Medicago sativa*) hosts (Jin et al., 2010) and *DREB1A* derived from *A. thaliana* used in tobacco to exhibit high tolerance to drought and freezing conditions (Kasuga et al., 2004).

Translation under heat stress can also be inhibited when the up-regulation and synthesis of chaperones is inadequate to prevent damage from abiotic stresses. Other physiological responses that are encountered under severe abiotic stress include exclusion, or compartmentalisation of ions by efficient transporter and symporter systems, accumulation of compatible osmotica (sucrose, proline, glycine betaine, trehalose, mannitol, myo-inositol; Jan et al., 2013) and engagement of oxygen free radical scavenging mechanisms.

1.4 Calvin Cycle enzymes Rubisco and Rubisco activase (RCA)

Diminished activity of key Calvin Cycle enzymes inevitably causes reduced carbohydrate availability in plants under heat stress (Ashraf and Harris, 2013). Large impacts including limited photosynthetic rates under heat stress are mainly due to the inactivity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco), phosphoenolpyruvate carboxylase (PEPCase), NADP-malic enzyme (NADP-ME), fructose-1, 6-bisphosphatase (FBPase) and pyruvate orthophosphate dikinase (PPDK; Farooq et al., 2009).

Rubisco catalyses the fixation of atmospheric CO_2 during photosynthesis and therefore is central to the production of organic compounds (Lin et al., 2014). A slow turnover is established under oxygenase activity of Rubisco under heat stress, leading to diminished photosynthetic capacity. Rubisco activase (RCA) is an 'auxiliary' Dark Reaction protein that is essential for Rubisco activity. Its structure is highly conserved across plant taxa. RCA is classed as an AAA+ protein and participates in removing naturally occurring sugar-phosphate inhibitors from both active and inactive sites of Rubisco (Kurek et al., 2007). This enables carbon fixation during photosynthetic biochemical pathways and maintains catalytic efficiency of Rubisco (Whitney et al., 2011). When plants are exposed to super-optimal temperatures, the efficiency of Rubisco and RCA diminishes (Parry et al., 2013). The ability of RCA to reactivate carboxylation sites of Rubisco through ATP-driven processes is impeded under increased heat stress (Portis Jr, 2003).

Plants naturally adapted to more extreme environments (i.e. increased temperatures) have shown base-pair variations in RCA which are associated with increased affinity, turnover rate

or specificity for CO_2 (Parry et al., 2013). Further, genetic modifications of RCA in *A. thaliana* has shown homologues of the gene that confer increased heat stress tolerance (Kurek et al., 2007) and faster photosynthetic induction has been observed in transgenic rice plants overexpressing RCA via maize transgenes (Fukayama et al., 2012). It is these natural variations and genetically engineered configurations of RCA that are of interest to increasing heat stress tolerance in crop species with the use of biotechnology.

By investigating diverse arid-zone germplasm from the genus *Nicotiana*, processes that underpin heat tolerance were explored. This was done by examining the evidence for four hypotheses as follows:

1) Rates of growth and development in desert *Nicotiana* species are maintained at temperatures at or above 35˚C, when compared with *N. tabacum*;
2) Are genotypic differences in heat tolerance manifested in variations in membrane leakiness;
3) Is variation in heat tolerance reflected in differences in photosynthetic capacity among genotypes;
4) Do nucleotide polymorphisms in the heat-labile photosynthetic enzyme, Rubisco activase (RCA), indicate evolution of this gene to more heat-tolerant forms.

Using an ecological approachit is hoped to discover abiotic stress tolerance genes in wild Australian tobacco and harness these for use in the production of *N. tabacum* hybrids that perform better in extreme environmental conditions. These genes are likely to be conserved across the genus *Nicotiana*, but even slight differences in the gene sequence could lead to beneficial changes in heat stress tolerance phenotypes.

2 Materials and Methods

Plant Material

Seed stock of native Australian *Nicotiana* species was obtained from Olive Pink (Alice Springs Botanic Gardens, NT) and the Australian Plant Genetic Resource Information System (AusPGRIS). The following species were grown in the glasshouse: *N. tabacum* L. (cultivated line “White Burley”), *N. gossei, N. simulans, N. megalosiphon* and *N. benthamiana.* The commercial cultivar *N. tabacum* was available from Macquarie University, Sydney.

Cultivation

Approximately 100-150 seeds of *N. gossei*, *N. simulans*, *N. tabacum* L., *N. megalosiphon* and *N. benthamiana* were germinated on Petri dishes with absorbent cotton and filter paper wetted with growth enhancing supplements (gibberellic acid 0.5 mg/l, $CaCl_2$ 73mg/l). Petri dishes were incubated at 28˚C under a 12 h-photoperiod (700-800 µmol m^{-2} s^{-1}) in growth cabinets (TRIL-1175; Thermoline Scientific Equipment, Smithfield, NSW, Australia). Seedlings were transplanted from Petri dishes and grown in a mixture (2:1) of fine-textured krasnozem (sourced from Robertson, NSW) and river sand in 15 cm diameter pots. A 1-cm layer of vermiculite was spread on the soil to maintain soil moisture. After germination and 14 d growth, plants were fertilized every 7-10 d with a soluble commercial fertilizer (0.25-0.5 g / L; Aquasol, Yates, Australia). 14 d after plants germinated pots were moved to the glasshouse where daily temperatures were set at 30˚C in naturally lit glasshouses and 22˚C at night.

Water/irrigation regimes consisted of daily watering so that plants subject to experimentation were not put under water stress. No silting occurred though tobacco has relatively poor stomatal control and wilts readily when deprived of water.

Growth analysis

Growth experiments in growth chambers were carried out on *N. megalosiphon*, *N. gossei* and *N. tabacum* L. After 35 days from germination, seedlings were randomly allocated to control and heat treatments (30 and 38˚C respectively during the day and 22˚C at night). Heat treatments followed an initial acclimation period of 2 days at 34˚C. Plants were harvested weekly during a three-week interval beginning 35 days after germination. Harvest zero (H0) after 35 days growth was made prior to heat commencing, providing a reference point for subsequent harvests. At all harvests, leaf number, leaf area (LA) and shoot (stem and leaf) and root dry weights were recorded. Roots were washed clean of soil and fresh samples were dried at 70˚C for at least 48 h and re-weighed. Leaf area was measured using a LI-3100C Area Meter (Lincoln, NE, USA). A total of six plant replicates per species per heat treatment at each harvest were analysed. As biomass sampling is destructive the same plant could not be harvested at each harvest date. Therefore 6 plants were randomly selected for biomass harvest. Biomass data from each harvest was sorted according to size between harvests and

paired to minimise the chance of negative results. Relative growth rates (*RGR*) were calculated for each heat treatment and each harvest using the following equation:

$$RGR = [(ln\ W_2 - ln\ W_1) / (t_2 - t_1)]$$

Where *RGR* is net gain biomass (*W*) per increment in time in days (*t*). The calculation is averaged over a time interval t_1 to t_2 and biomass increases from W_1 to W_2

Leaf area ratios (*LAR*) and specific leaf areas (*SLA*) were calculated between harvests as:

$$LAR = ½ [(A_1 / W_1) + (A_2 / W_2)]$$

$$SLA = ½ [(A_1 / W_{s1}) + (A_2 / W_{s2})]$$

Where '*W*' represents harvest week number, '*A*' the area of fresh leaves and 'W_s' represents dry shoot biomass. Net assimilation rates (*NAR*) were then calculated between harvests. The calculation was as follows:

$$RGR = NAR^* LAR$$

14d after the growth experiment had proceeded additional plants were moved from both the 30˚C and 38˚C treatments to a 36˚C growth cabinet for 7d. Thus, plants acclimated for 14d at 30˚C daytime temperature were shocked at 36˚C for 7 days. The plants pre-conditioned for 14d at 38˚C were analysed in parallel; these plants were considered to be acclimated to heat. Six replicates for each species were measured using growth analysis variables (i.e. RGR, LAR and SLA).

Heat-treated plants – gas exchange

Nicotiana gossei, N. tabacum L. *and N. megalosiphon* and were heat treated at temperatures of 35/22˚C and 40/22˚C (day/night). Ambient treatments were set at 30/22˚C (day/night). All treatments were inclusive to 12-h photoperiod, CO_2 levels were ambient (400 ppm) and quantum flux for the growth cabinets averaged 700-800 µmol m^{-2} s^{-1}. After three to four months' growth, plants were exposed to heat and gas exchange measurements undertaken over a two-week period. Elevated heat treatments at 35˚C were initiated after two days of acclimation at 34˚C. For 40˚C treatments, a day of 38˚C acclimation was carried out after 2 days of acclimation at 34˚C.

Net photosynthetic rates (A_{max}), stomatal conductance (g_s), Instantaneous Transpiration Efficiency (*ITE*) and intercellular CO_2 (C_i) were recorded using LI-6400 portable gas exchange system (LiCor, NE, USA). The CO_2 sample chamber was set at 400 ppm, photosynthetic photon flux density (PPFD) 1500 µmol m^{-2} s^{-1} and air flow at 500 µmol s^{-1}. Measurements were only taken when relative humidity (RH%) was between 50 - 80%. Leaf vapour pressure deficit (VPD) was kept under 3.0 kPa to ensure stomata remained open. All gas exchange measurements were taken in growth cabinets (TRIL-1175; Thermoline Scientific Equipment, Smithfield, NSW, Australia) where heat treatments took place. LiCor IRGA block temperature was set according to the heat treatments (i.e. 30, 35 and 38˚C). Data acquisition was carried out between 10:00 to 15:00 to ensure plant peak photosynthesis occurred.

Nitrogen (N) analysis

Nicotiana bethamiana, N. gossei, N. megalosiphon, N. simulans and *N. tabacum* L. were analysed for nitrogen levels in ground leaf laminae. Tissues were sampled when plants had six fully developed leaves (mid- to late-vegetative development). Plants had been grown in ambient conditions in glasshouses 30/22˚C (day/night). Three leaf discs (14-mm diameter) were excised from interveinal leaf lamina and were dried at 70˚C for 48 h. This was repeated three times for each species (n = 3). Nitrogen concentrations of ground samples were determined with a Leco Model 900 CHN analyzer (LECO Instruments, St. Joseph, Michigan, USA). Photosynthetic rates were re-calculated on an N basis to investigate the efficiency of

photosynthesis on a molar N basis. Rates were calculated from A_{max}, *SLA* and N levels and to give A_{max} based on N. Standard errors of each variable was added to show aggregate SE.

Electrolyte leakage and cell membrane integrity assay

Two leaf discs (14-mm diameter) were excised from the interveinal lamina of the youngest fully expanded leaf (4^{th} leaf from the apical meristem) with eight leaves sampled per treatment per species (n = 8). Tissue sampling was carried out between 13:00 and 14:30 when discs were placed immediately into distilled water and taken to the laboratory. Discs were triple rinsed (~ 3 min) with distilled water to remove electrolytes from damaged cells and then placed into 15-mL glass vials containing 2 mL of Milli Q (MQ) water. Vials were sealed with non-absorbent cotton to prevent evaporation. Incubation was carried out using controlled temperature water baths (Thermo Line, Scientific Equipment) set at 30, 35, 40, 45, 50 and 55˚C. Samples were left to cool to room temperature (25˚C) before osmolality (mOsmoles) was measured using a Fiske One-Ten Osmometer (Fiske Associates, Norwood, MA, USA). Electrical conductivity data acquisition was carried out (with discs *in situ*). Initial electrical conductivity (IEC_t) was measured using an ECT calibrated conductivity meter (Oakton Instruments, Veron Hills, IL, USA). Measurements (IEC_t) were measured after samples were diluted with MQ water (1:3) then multiplied by three for original IEC_t. Discs were then autoclaved at 110˚C for 15 min and then left to cool to room temperature (25˚C) then measured to give final electrical conductivity (FEC_t) and final mOsmoles. Data were then collated and used to calculate relative electrical conductivity (REC_t) as follows:

$$REC_t = (IEC_t / FEC_t) * 100$$

REC_t was plotted against temperature and a four-parameter sigmoid curve fitted using regression analysis in Sigmaplot 11.0. Temperature differences were observed at the species level where 50% leakage occurred. This was calculated using the equation:

$$f = y_0 + a/\{1+exp[-(x-x_0)/b)]\}$$

Where x = temperature (˚C), y_o = asymptotic REC as temperature decreases indefinitely, a = asymptotic increase in REC_t that occurs as x approaches infinity, x_o = temperature (˚C) at which the absolute growth is maximal and b = relative growth rate at x_o.

An Arrhenius plot was derived from all data points where natural log (ln) of osmolality was plotted against the inverse temperature ($1/T$) to reveal melting points of the plasma membranes (Wright, 2003).

Molecular Analysis (Experiment 1)

RNA was extracted from five Nicotiana species (*N. tabacum L., N. megalosiphon, N. gossei, N. benthamiana* and *N. simulans).* Tissue samples for molecular analysis of the Rubisco activase (RCA) gene were taken when six fully leaves had fully developed (mid- to late-vegetative stage). Tissue from the 4th leaf from the apical meristem (≥ 100 mg) was immediately ground in liquid nitrogen. In an initial experiment, RNA was extracted using RNeasy Plant Mini Kit (QIAGEN). QIAshredder columns were used to separate cell-debris from supernatant containing RNA. RNeasy Mini spin columns were used to isolate RNA from unwanted supernatant. Conversion of RNA to cDNA (up to 2.5 µl in a 20 µl cDNA synthesis reaction) was then followed using SuperScript VILO cDNA Synthesis Kit (Invitogen). PCR was then carried out, using 2.0 µl of cDNA template in a 25 µl reaction solution, using PCR Master Mix (Promega Corporation, Madison, WI) protocols. Primary gene specific reverse and forward primers were designed from published *N. tabacum* L. sequence and used to amplify the Rubisco activase (RCA) gene from wild Australian *Nicotiana* samples, as shown in Table 1.

Table 1. Forward and reverse primer design derived from *N.tabacum* L. RCA gene sequence (note overlapping of specific regions was carried out using different primer sets to test primer effectiveness and improve sequence resolution). Primers denoted with an * were utilised in experiment 2 focusing on specific regions known to have more single nucleotide polymorphisms, based on RCA studies of *Oryza australiensis* (Scafaro et al., unpublished data).

Primer	Code	Sequence
RCA *N. tabacum* forward 1	Nf43	3’ GGTCAAAGACCATGGCTACC 5’
RCA *N. tabacum* reverse 1	Nr472	3’ GCAGGAGCGATGTAGAATCC 5’
RCA *N. tabacum* forward 2	Nf763	3’ GAGCTGGTAGAATGGGTGGA 5’
RCA *N. tabacum* reverse 2	Nr1457	3’AGAGCCTTCAGCAACAGGAA 5’
RCA *N. tabacum* forward 3	Nf412*	3’ CCCAAGGTCTTCGCCAGTACA 5’
RCA *N. tabacum* reverse 3	Nr682*	3’ ATCAACTTGGCTGGCTCTCC 5’
RCA *N. tabacum* forward 4	Nf651*	3’ AGTGGAAATGCAGGAGAGCC 5’
RCA *N. tabacum* reverse 4	Nr851*	3’ TGTCGGGTTGTCAGCAATGT 5’
RCA *N. tabacum* forward 5	Nf1036*	3’ TGTTCCTGCTGAAGACGTTG 5’
RCA *N. tabacum* reverse 5	Nr1217*	3’ CTCAAAAGTTGGTCCGT 5’
RCA *N. tabacum* forward 6	Nf412*	3’ CCCAAGGTCTTCGCCAGTACA 5’
RCA *N. tabacum* reverse 6	Nr851*	3’ TGTCGGGTTGTCAGCAATGT 5’
RCA *N. tabacum* forward 7	Nf651*	3’ AGTGGAAATGCAGGAGAGCC 5’
RCA *N. tabacum* reverse 7	Nr1217*	3’ CTCAAAAGTTGGTCCGT 5’

For PCR, an initial step of 95˚C for 2 min was followed by 30 cycles of 95˚C for 30 s, 52˚C for 30 s and 72˚C for 2 min. The final non-cyclical step was set at 72˚C for 5 min after which PCR products were incubated at 4˚C. It is noted that the second molecular analysis experiment followed the PCR protocols as described for the first experiment with exception to annealing temperatures which were optimised for specific primer annealing (i.e. 46 and 48˚C).

Double stranded DNA extraction (Experiment 2)

Genomic DNA was successfully extracted from five *Nicotiana* species (*N. tabacum L., N. megalosiphon, N. gossei, N. benthamiana* and *N. simulans*). DNA was extracted using a bead-beating method (Yeates and Gillings, 1998; Gillings, 2014). Plant material was finely chopped (approximately 200 mg) with a sterile scalpel and added to a lysing matrix E tube.

CLS-VF buffer (800 µl) and protein precipitation solution (200 µl) was added to plant material and processed with a FastPrep FP120 (BIO 101 Savant) machine for 30 s at 5.5 m/s until fully homogenized. Samples were then centrifuged (Eppendorf 5417C) at 14,000 × *g* for 5 min. Protein precipitation, binding, washing and subsequent elution of DNA in TE buffer were as previously described (Yeates and Gillings, 1998). DNA was stored at -20˚C.

PCR purification protocols

All positive PCR amplicons were purified using either the Qiaquick or MinElute PCR Purification Kit (Qiagen), depending on the intensity of the gel bands (produced during electrophoresis) in preparation for cloning and sequencing.

Agarose gel electrophoresis and images

PCR products were separated on 1.5-2% agarose gels placed in Tris-borate – EDTA (TBE) buffer (Sambrook and Russell, 2001). DNA samples were loaded 10:4 with bromophenol blue loading dye (0.45 M Tris-borate, 0.01 EDTA, 40% sucrose, 0.25% bromophenol blue). A 100 and 1 kb-base pair ladder (Crown Scientific) was used over both experiments. Gels were run for 50-80 min in TBE at 110 V. Gels were then stained with GelRed^{TM} (Biotium) and DNA visualized under UV light. Gel images were captured using Gel Logic 2200 PRO camera and Carestream MI computer software. Genetic material in bands was excised and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Spectrophotometer (Eppendorf Biophotometer 6131) was used to calculate DNA concentrations and quality throughout RNA extraction, cDNA synthesis and dsDNA purification processes.

DNA sequencing

Sequencing was performed using the appropriate primers in the forward and reverse direction. PCR product purification and PCR product sequencing instruments utilised by Macrogen included a 6 Applied Biosystems 3730xl and 9 ABI 3700 (Macrogen, Seoul, Korea). Sequences were aligned and manually checked for quality and read errors using Geneious and Clustal 2.1 multiple sequence alignment software (version 8.1.3, Biomatters LTD, New Zealand; Edgar, 2004).

Statistical Analysis

Statistical analysis software Minitab 17 (Janke and Tinsley, 2005) utilised one and two-way analysis of variance (ANOVA) via general linear model analysis regarding LiCor data and electrolyte leakage and cell membrane integrity assays with significance set at $p < 0.05$. Sigmaplot 11.0 software was utilized to produce figures and graphs for presentation.

Genetic sequences were computed in BLAST (Altschul et al., 1990) to confirm regions of the RCA homologues derived from *N. tabacum* L. via NCBI (National Center for Biotechnology Information). . Additionally, sourced RCA homologues derived from NCBI were used to make comparative analysis of various RCA alignments between Solanaceae and *Oryza* spp.

3 Results

3.1 Growth Analysis

Final growth experimentation included species *N. tabacum* L., *N. gossei* and *N. megalosiphon.*

3.1.1 *RGR, NAR* and *LAR*

Relative growth rates and its components (*NAR* and *LAR*) were investigated at different temperatures over five weeks with three consecutive weekly harvests at 0, 7, 14 and 21 days after heat treatment began (H0 to H1, H1 to H2 and H2 to H3 respectively). Two-way ANOVA of *RGR* showed that species differences were significant throughout the growth experiment with *RGR* ranging from 0.429 d^{-1} (H0 to H1) to -0.283 d^{-1} (H2 to H3) in *N. megalosiphon* and *N. tabacum* L. respectively (Fig. 2). There were significant differences between temperature treatments and the interaction between species and temperature treatment (Fig. 2).

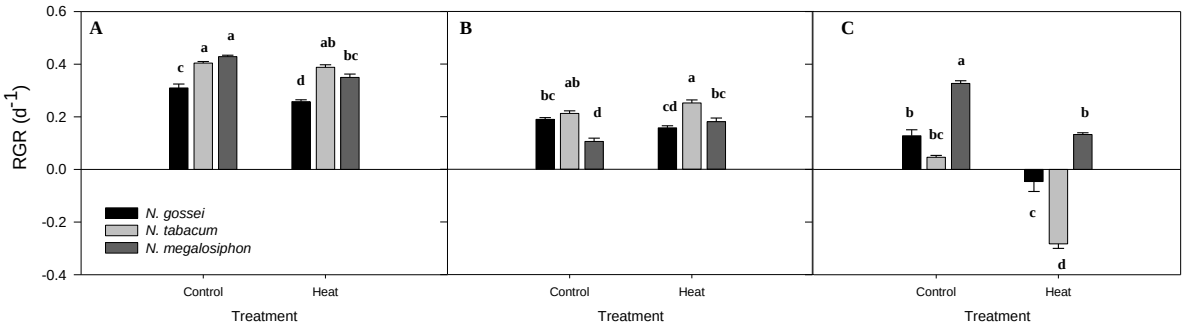


Figure 2 *RGR* (d^{-1}) for *N. gossei, N. tabacum* and *N. megalosiphon* (*n* = 6). (A) Shows *RGR* 5 – 6 weeks after sowing, i.e. first week of heat treatment (H0 to H1) , (B) 7 to 14 days of heat treatment (H1 to H2) and (C) 14 to 21 days of heat treatment (H2 to H3). 'Control' and 'Heat' were set at 30/22°C and 38/22°C respectively (day/night). Means that do not share a lower case letter are significantly different (Tukey's HSD). Bars show SE (mean).

The lowest *NAR* (g cm^{-2} d^{-1}) reflected the negative growth rates of *N. tabacum* L. during the last week of the growth experiment at -1.93 (H2 to H3; Fig. 3). While *N. megalosiphon* had the highest *NAR* at H0 to H1 (2.94 g cm^{-2} d^{-1}). Two-way ANOVA showed a difference between species in NAR at all harvests. Differences were also found between temperature treatments throughout the growth experiment. The interaction between species and treatment also showed differences across harvests, except H2 to H3 as shown in Figure 3.

Both the highest and lowest *LAR* (cm^{-2} g^{-1}) were observed in *N. megalosiphon* 297 cm^{-2} g^{-1} (H0 to H1) and 112 cm^{-2} g^{-1} (H2 to H3), respectively. *LAR* showed significantly different responses between species across harvest periods of the growth experiment. There was a significant effect of the interaction between temperature and species during the first harvest period and not proceeding harvests (H0 to H1: $F_{2,35} = 15.61$, $p < 0.001$)as shown in Figure 3.

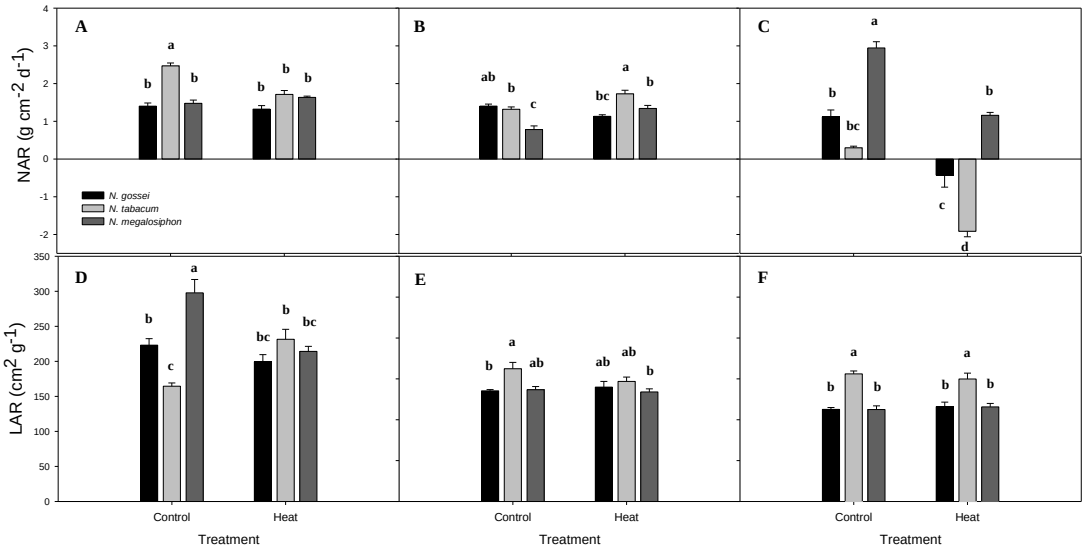


Figure 3 *NAR* (g cm^{-2} d^{-1}) and *LAR* (cm^{-2} g^{-1}) for *N. gossei, N. tabacum* L. and *N. megalosiphon* (*n* = 6). *NAR* at (A) H0 to H1, (B) H1 to H2 and (C) H2 to H3 harvest periods. *LAR* at (D) H0 to H1, (E) H1 to H2 and (F) H2 to H3 harvest periods. Temperatures and statistics as described in Figure 2.

3.1.2 Does preconditioning to 38˚C induce heat tolerance?

Plants preconditioned to 38˚C did not show differences in *RGR* in relation to changes in temperature and the interaction between species and temperature treatment. There were significant differences between species ($F_{2,35}$ = 14.43, p < 0.001). There were also differences between species and in the interaction between species and heat for *NAR* ($F_{2,35}$ = 13.23, p < 0.001; and $F_{2, 35}$ = 3.46, p = 0.044 respectively), but there was no effect of heat). *LAR* analysis showed a small but significant effect in the plants pre-conditioned at 38˚C. Further analysis using one-way ANOVA of *RGR, NAR* and *LAR* showed significant differences between species when both temperature pre-treatments were pooled (*RGR*: F_5 = 7.12, p < 0.001; *NAR*: F_5 = 6.92, p < 0.001; and *LAR*: F_5 = 3.12, p = 0.022). Differences between species regarding responses are shown in Figure 4.

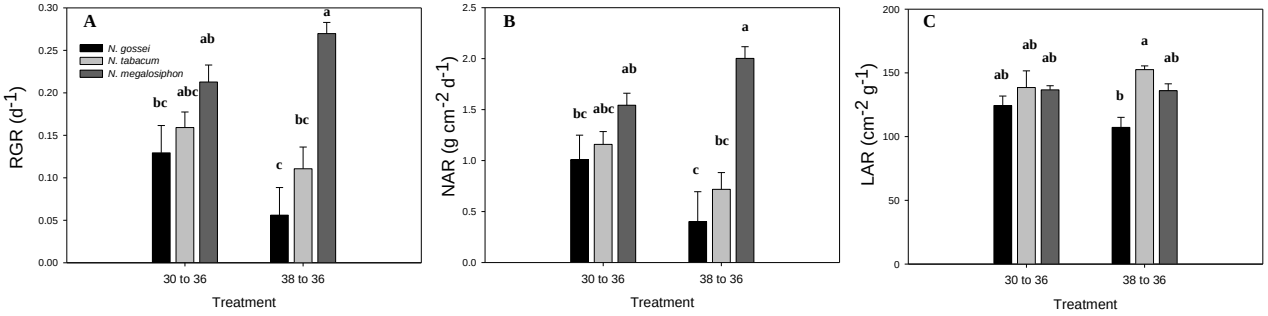


Figure 4 *RGR* (A), *NAR* (B) and *LAR* (C) for species preconditioned for two weeks at 30˚C and 38˚C and then treated at 36˚C for 7 days. Species include *N. gossei, N. tabacum* and *N. megalosiphon* (*n* = 6). Means that do not share a lower case letter are significantly different (Tukey's HSD). Bars show SE (mean). One-way ANOVA post hoc analysis (Tukey's HSD) has been made within responses (i.e. *RGR*, *NAR* and *LAR*).

3.1.3 Is *RGR* correlated with *NAR* and *LAR*?

Mean *RGR* from each species, harvest period and treatment (including preconditioned data) were pooled and plotted against corresponding mean values for *NAR* and *LAR* with a linear regression analysis performed. Figure 5 (A) shows a scatter plot of the mean values of *RGR* (d^{-1}) vs. *NAR* (g cm^{-2} d^{-1}). A positive linear correlation is defined by y = 5.789x + 0.037 (R^2 = 0.89). Mean values of *RGR* vs. *LAR* (cm^{-2} g^{-1}) show no linear association (y= 124.92x + 134.21; R^2 = 0.27), as shown in Figure 5 (B).

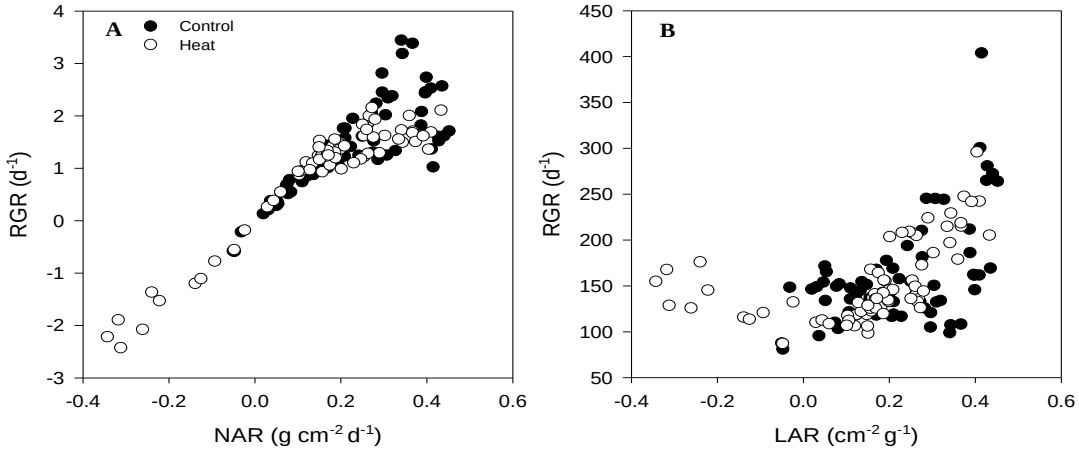


Figure 5 *RGR* plotted against corresponding values for *NAR* and *LAR*. Control, heat and plants preconditioned to heat (from Figures 2 - 4) are included in both figures.

3.2 Developmental Analysis

3.2.1 Shoot-to-root ratio

The developmental response of the plants was investigated at different temperatures during the course of three consecutive one week harvests after 7, 14 and 21 days of heat treatment (H1, H2 and H3 respectively). Shoot-to-root ratios and leaf number were both recorded each time.

Two-way ANOVA on shoot-to-root ratio differed significantly for species at all harvests. Species and temperature interactions in H2 and H3 were significant ($F_{2,35}$ =12.71, $p < 0.001$; and $F_{2,35}$ = 3.63, $p = 0.039$ respectively) but were not significant during H1(Fig. 6).

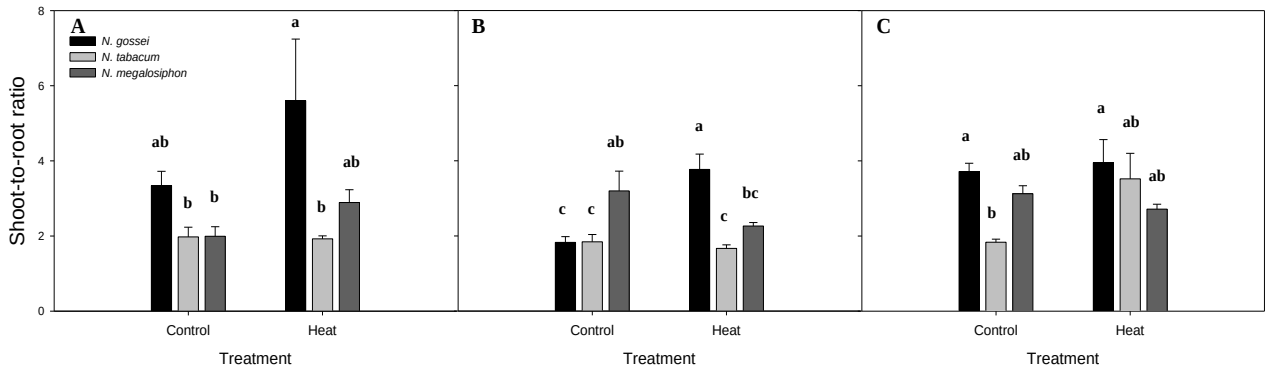


Figure 6 Shoot-to-root ratios for species *N. gossei, N. tabacum* L. and *N. megalosiphon* (*n* = 6). (A) Five weeks after sowing and 7 days treatment (H1), (B) 14 days treatment (H2) and (C) 21 days treatment (H3). Means that do not share a lower case letter are significantly different (Tukey's HSD). Bars show SE (mean).

3.2.2 Leaf development and *SLA*

Mean leaf number was highest in *N. megalosiphon* for all harvests under control and heat treatments. The highest recorded mean leaf number was *N. megalosiphon* in low temperatures during the last harvest (28°C).The fewest leaves were in *N. tabacum* L. at high temperatures during the third and last harvest (~ 4). Mean leaf number differed significantly (two-way ANOVA) for species and temperature treatment at all harvests. The interaction between species and treatment was statistically significant at H1 but not the proceeding harvests (H1: $F_{2,35}$ = 6.18, $p = 0.006$). One-way ANOVAs were used to test the difference between species within heat treatments i.e. control (30˚C) and heat (38˚C) temperature treatments and were all

highly significant. Comparisons between species within temperature treatments are shown in Figure 7.

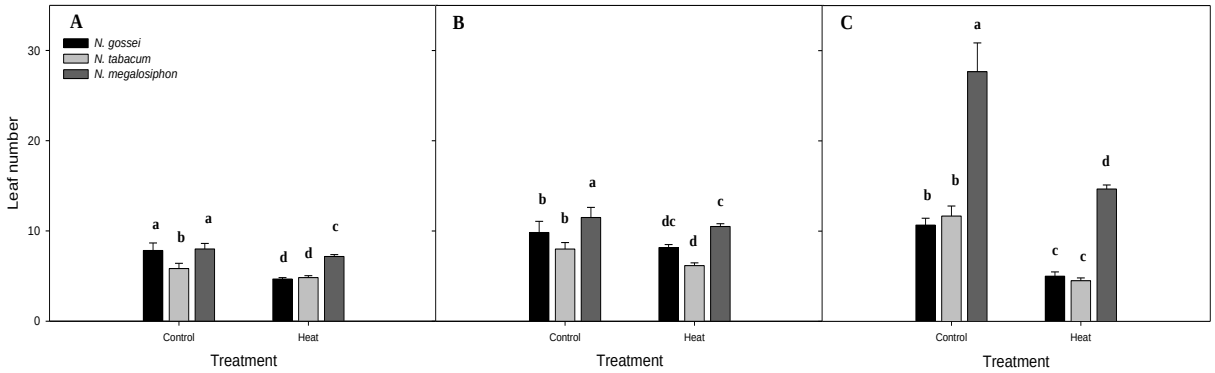


Figure 7 Numbers of leaves for *N. gossei, N. tabacum* and *N. megalosiphon* (*n* = 6). (A) H1, (B) H2 and (C) H3. Bars show SE (mean). Means that do not share a lower case letter are significantly different (Tukey's HSD). Comparisons have been made within control and heat treatments across harvests (one-way ANOVA). Bars show SE (mean).

There was no statistical significance found regarding *SLA* ($cm^{-2}\ g^{-1}$) and species and the interaction between species and treatment during H1. There was a difference found in H1 for temperature treatment ($F_{1,35} = 10.17$, $p = 0.003$). The highest recorded mean *SLA* were *N. tabacum* L. and *N. megalosiphon* in control treatments during H2 (265.84 and 264.74 respectively). These were significantly different from heat-treated plants when tested for differences between treatment (two-way ANOVA; $F_{2,35} = 26.54$, $p < 0.001$). Mean *SLA* was highest in *N. tabacum* L. for the last harvest under control and heat temperatures (183.57 and 191.12 respectively). A difference between species was found here between *N. tabacum* L. (heat) and *N. megalosiphon* (control) as shown in Figure 9 (two-way ANOVA; $F_{2,35} = 8.31$, $p = 0.001$).

Nicotiana species subject to growth experimentation had observed differences particularly when comparing heat treatment with control treatments as shown in Figure 8.

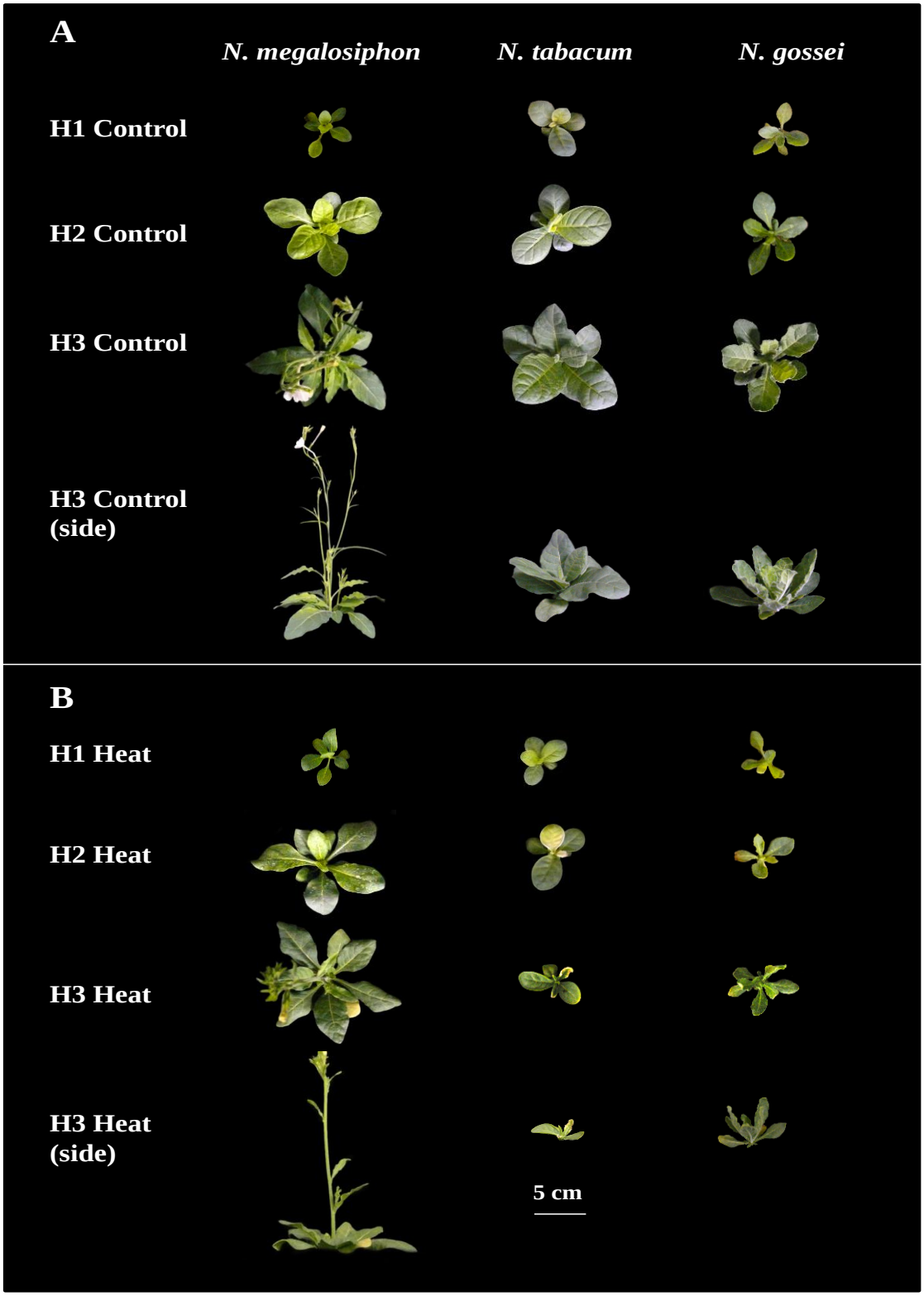


Figure 8 *Nicotiana* species subject to growth experimentation (refer to materials and methods for experiment protocols). Figure 8 (A) represents control treatment (30˚C) and (B) heat treated plants (38˚C).

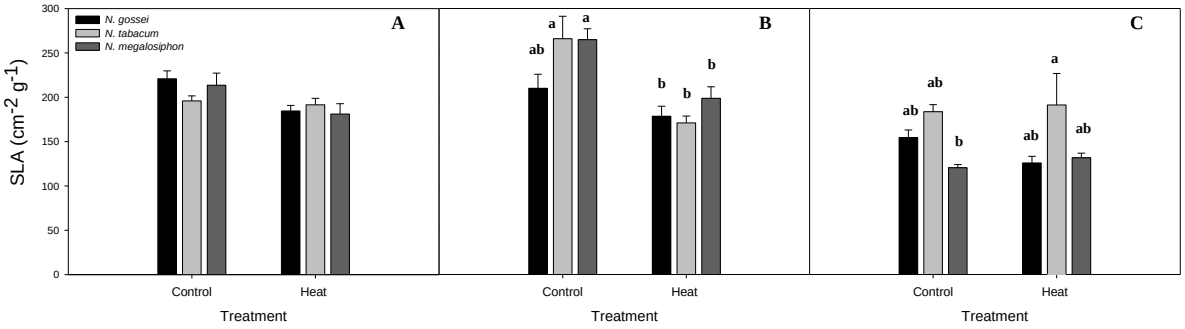


Figure 9 *SLA* for *N. gossei, N. tabacum* and *N. megalosiphon* (*n* = 6). (A) H1, (B) H2 and (C) H3. Bars show SE (mean). Statistics as described in Figure 7. No lower case letters are allocated to (A) as no means were significantly different.

3.3 Gas Exchange

Photosynthetic rates (A_{max}) under three heat regimes were tested in *N. gossei, N. tabacum* L. and *N. megalosiphon*, with intracellular activity (C_i) and Instantaneous Transpiration Efficiency (*ITE*) at 30, 35 and 40˚C. A_{max} varied significantly between species and temperature treatment ($F_{2,160}$ = 19.64, p < 0.001; $F_{1,160}$ = 22.78, p < 0.001 respectively). To understand species effects in more detail, one-way ANOVA on temperature and species showed differences (F_2 = 11.82, p < 0.001; F_2 = 5.32, p = 0.008; and F_2 = 10.40, p < 0.001 respectively). *Nicotiana megalosiphon* had significantly higher mean photosynthetic rates compared with *N. gossei* and *N. tabacum* at 30˚C (26.3 µmol m^{-2} s^{-1}). At 35˚C, *N. megalosiphon* (20.9 µmol m^{-2} s^{-1}) photosynthesised significantly faster than *N. gossei* (16.1 µmol m^{-2} s^{-1}) but not to *N. tabacum* (19.4 µmol m^{-2} s^{-1}) while at 40˚C, rates in *N. megalosiphon* (21.4 µmol m^{-2} $s^{-}1$) were significantly higher than *N. gossei* (11.1 µmol m^{-2} s^{-1}) and *N. tabacum* (15.0 µmol m^{-2} s^{-1}) as shown in Figure 10 (A).

No statistically significant differences (two-way ANOVA) were observed in intracellular activity (C_i) in relation to species, temperature or the interaction between species and temperature as shown in Figure 10 (B). The same applied to stomatal conductance (g_s), which did not differ across genotypes and treatments as shown in Suppl. 1 (B).

Photosynthetic rate and transpiration rates were used to calculate *ITE* (the ratio of the rate of carbon assimilation to the rate of transpiration) as shown in Figure 10 (C).

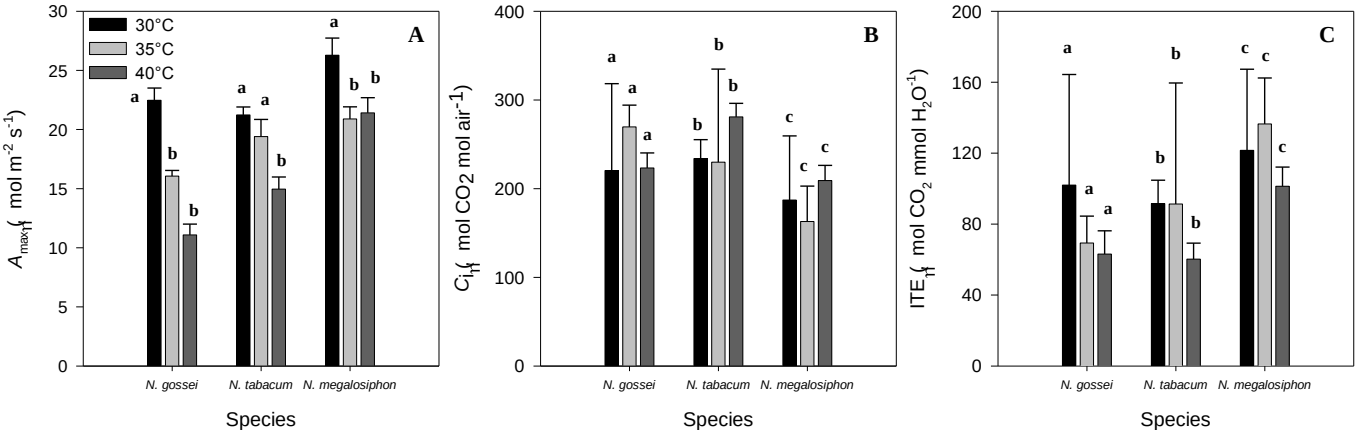


Figure 10 Daytime temperatures of 30, 35 and 40˚C were imposed and the response of three species was measured for (A) photosynthetic rate (µmol m^{-2} s^{-1}), (B) C_i (µmol CO_2 mol air^{-1}) and (C) *ITE* (biomass per unit water). Plants were subject to day/night photoperiods 12/12 hrs with light intensity 700-800 µmol m^{-2} s^{-1} in growth cabinets with an average relative humidity of 70%. Means that do not share a lower case letter are significantly different (Tukey's HSD). All columns represent mean averages +/- SE ($n \geq 4$).

3.4 Nitrogen analysis

Nitrogen concentrating analysis was carried out with *N. benthamiani, N. simulans*, *N. gossei, N. tabacum* L. and *N. megalosiphon*. Due to data regarding gas exchange measurements being exclusive to *N. gossei, N. tabacum* L. and *N. megalosiphon* A_{max} based on N concentration remained on these three species.

Nitrogen (N) analysis (one-way ANOVA) revealed differences between species ($F_4 = 16.83$, $p > 0.001$). *Nicotiana gossei* had significantly higher N percentage (per unit dry mass) than the other four species (4.5% N); all other species had between 2.8 and 3.5% N as shown in Figure 11 (A). In Figure 11 (B), photosynthetic rates were re-calculated on an N basis, with *N. megalosiphon* having the highest rates (20.0 µmol CO_2 mol^{-1} N s^{-1}) and *N. gossei* shows the lowest rates (9.7 µmol CO_2 mol^{-1} N s^{-1}).

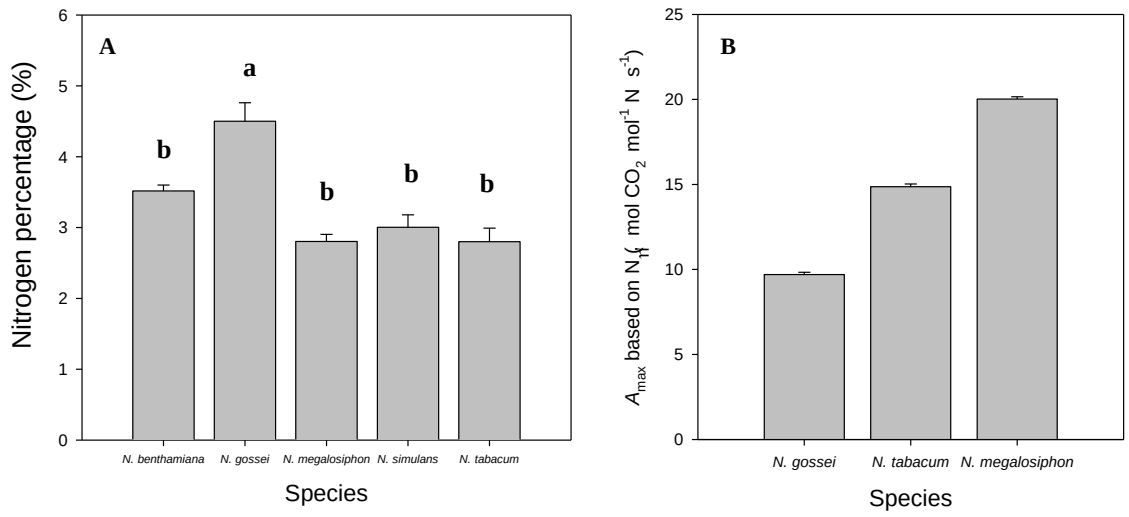


Figure 11 (A) Nitrogen concentration of *N. benthamiana, N. gossei, N. megalosiphon, N. simulans* and *N. tabacum* L. (*n* = 3). Plants were grown in ambient conditions 30/22˚C (day/night) with 70% relative humidity. Means that do not share a lower case letter are significantly different (Tukey's HSD). Bars show SE (mean). (B) Photosynthetic rates at 30˚C based on N (*n* = 3).

3.5 Electrolyte and solute leakage

All *Nicotiana* species were included in electrolyte and solute leakage experimentation.

Osmolality

Initial electrolyte leakage rates per unit of fresh weight (mOsmoles g^{-1}) showeddifferences between species, temperature treatment and the interaction between species and temperature treatment as shown in Figure 12 (A). A *post hoc* analysis of temperature treatments showed differences between 45, 50 and 55˚C means (mOsmoles g^{-1}). One-way ANOVA within temperature treatments 45 and 50˚C in relation to species showed statistical significance (F_4 = 9.65, $p < 0.001$; and F_4 = 11.58, $p < 0.001$ respectively). Within 45˚C treatments, *N. tabacum* L. showed the lowest mean average of mOsmoles/g (131.8 mOsm g^{-1}) with no other difference found between species. The 50˚C temperature treatment showed one difference in average means of mOsm/g where *N. simulans* had the lowest amount of electrolyte leakage (275.0 mOsm g^{-1}) as shown in Figure 12 (A). Figure 12 (B) shows final osmolality. Two-way ANOVA shows differences between species, temperature treatments and the interaction between species and temperature treatments. Final osmolality used solely for Relative Electrical Conductivity (*REC*) as shown in Figure 13. When initial leakage was made a

percentage of final leakage a similar trend was observed (Suppl. 1.3) as for initial leakage rate.

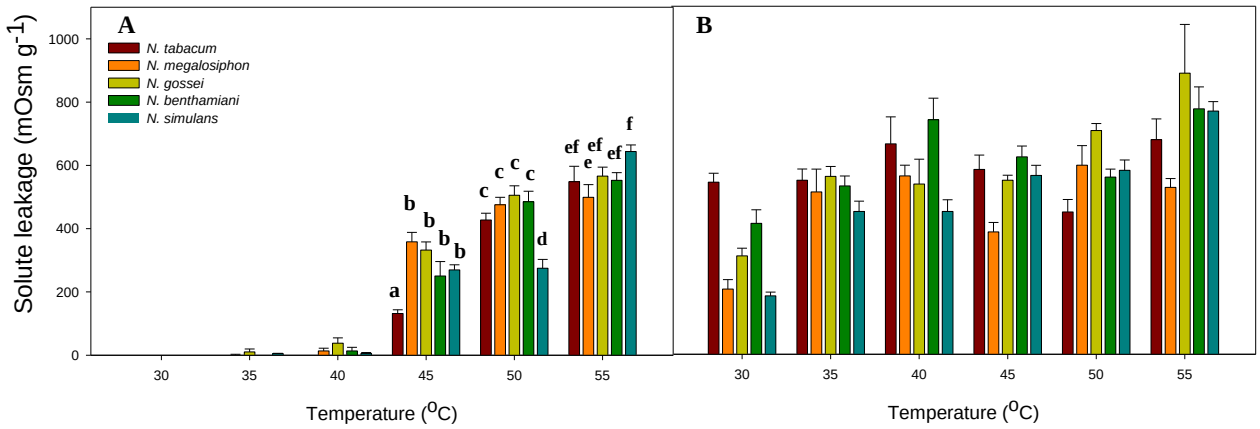


Figure 12 (A) Leaked electrolytes after a two-hour incubation at 30, 35, 40, 45, 50 and 55˚C in five species (*N. tabacum* L., *N. megalosiphon, N. gossei, N. benthamiana* and *N. simulans n* = 8). (B) Autoclaved samples at 110˚C for 15 min after initial water bath treatments (2 h) at temperatures mentioned above. Columns represent solute mean averages. Fig. 11 (A) comparisons have been made within different temperature treatments (i.e. one-way ANOVAs). Means that do not share a lower case letter are significantly different (Tukey's HSD). Bars show SE (mean).

Relative electrical conductivity (REC_t)

Fig. 12 (A) shows *REC* at 45˚C where *N. megalosiphon* showed the highest mean *REC* (70.4%) which was significantly different from the lowest mean shown in *N. tabacum* L. (30.0%). At 50˚C *N. benthamiana, N. tabacum* L. and *N. megalosiphon* all had average mean *REC* significantly higher than of *N. simulans* as shown in Suppl. 1.4 (A). The temperature at which 50% REC_t (T_{50}) occurred was lower for *N. megalosiphon* (43.6˚C) when compared to all other species. The highest temperature at which T_{50} occurred was *N. simulans* (48.2˚C) as shown in Suppl. 1.4 (B).

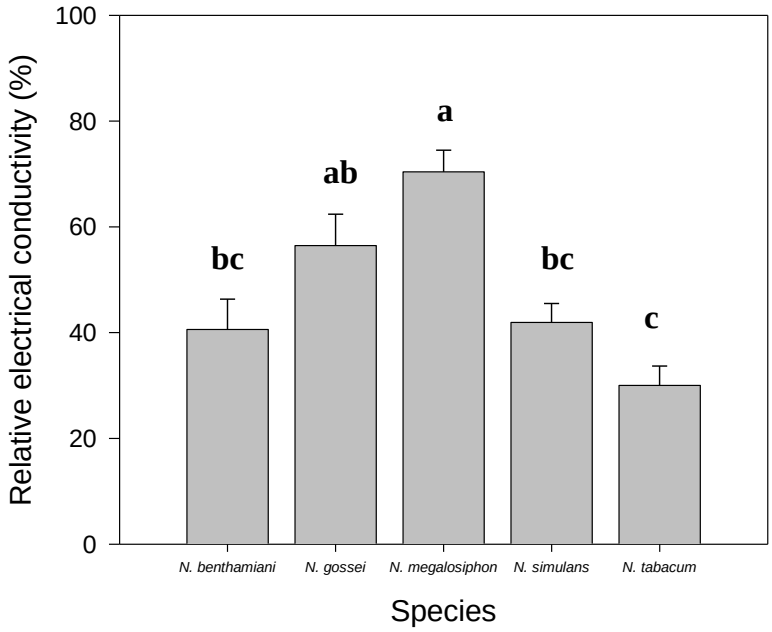


Figure 12 Relative electrical conductivity (%) at 45˚C. Data compiled in this figure have been derived from Figure 13 as it shows the most pronounced differences between means. Columns represent *REC* mean averages +/- SE (*n* = 8). Means that do not share a lower case letter are significantly different (Tukey's HSD).

REC_t was plotted against temperature treatments with four-parameter sigmoid curves fitted (Table 2). The extent of membrane leakage was not significant between *Nicotiana* species in temperature treatments 30, 35, 40 and 55˚C (Fig. 13).

Table 2 Fitted equations to the relationship between increased water bath temperatures and 50% relative electrical conductivity (*REC*) for *Nicotiana* species.

Species	*n*	R^2	Equation	P - value
N. benthamiana	8	0.98	y = 4.4066 + 95.7125/(1 + exp (-(x - 46.2765/2.3733))	0.0132
N. gossei	8	0.98	y = 4.3714 + 75.0264/(1 + exp (-(x - 43.7280/1.7693))	0.0312
N. megalosiphon	8	0.99	y = 4.7432 + 78.5135/(1 + exp (-(x - 43.2856/1.0609))	0.0218
N. simulans	8	0.97	y = -3.0726 + 106.8508/(1 + exp (-(x - 48.2375/5.2826))	0.0412
N. tabacum	8	0.99	y = 5.2502 + 90.2894/(1 + exp (-(x - 47.0395/2.2193))	0.0098

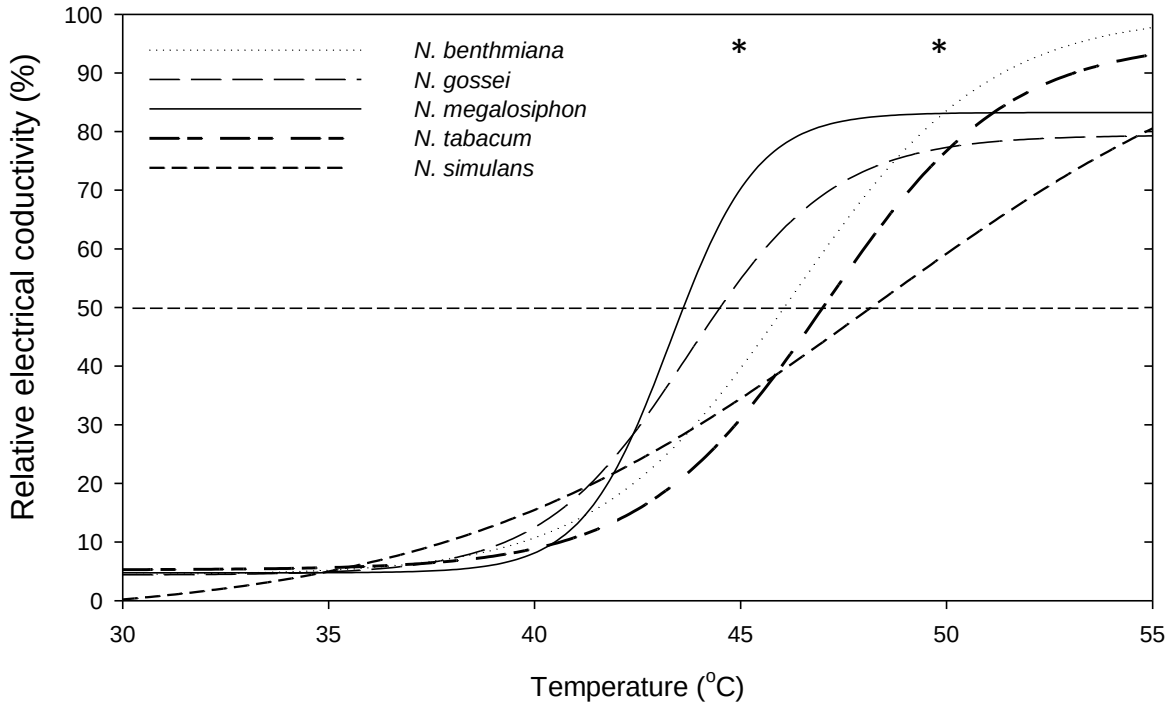


Figure 13 Relative electrical conductivity (%) of *Nicotiana* cultivar *N. tabacum* and wild Australian tobacco species (*N. benthamiana, N. gossei, N. megalosiphon* and *N. simulans*; *n* = 8). The dashed horizontal line intersecting sigmoid curves represents temperatures where 50% leakage occurred. Asterisks (*) represents temperatures at which there are significant differences between *Nicotiana spp.* relative to conductivity.

3.6 Genetic Analysis

Nucleotide sequence via reverse transcription (Experiment 1)

RNA was extracted from five *Nicotiana* species (*N. tabacum* L., *N. megalosiphon, N. gossei, N. benthamiana* and *N. simulans*) with cDNA synthesis and PCR of specific regions carried out using primers as described in Table 1. Of the samples, all five produced amplicons of the expected size for RCA by gel electrophoresis. Of these positive amplicons, two species (*N. tabacum* L. and *N. benthamiana*) were subsequently confirmed as RCA via DNA sequence analysis (BLAST). Amplicons are shown in Figure. 14 (note identical amplicons of *N. tabacum* L. were confirmed in gels not represented in this thesis).

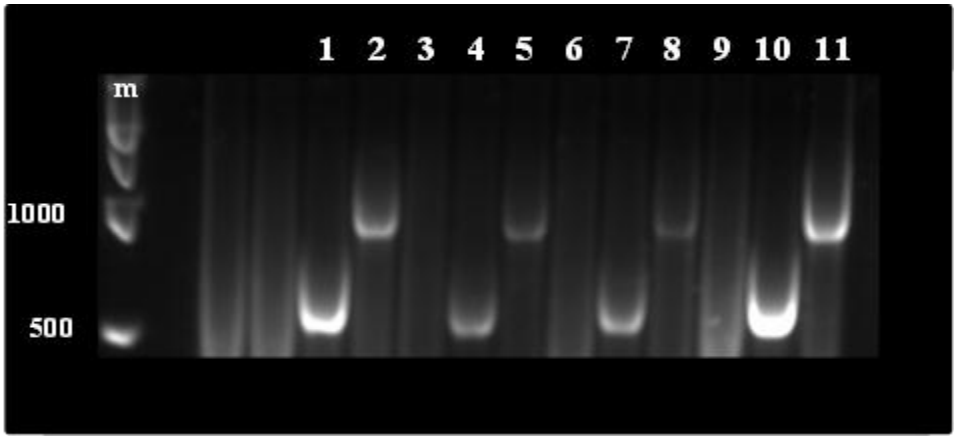


Figure 14 Representative gel showing amplicons of RCA. Lane labels are as follows: *m* = 1 kb ladder. Gene fragments are noted as independent lines where lanes 1, 4, 7 and 10 represent the region Nf763 (forward) to Nr1457 (reverse) for species *N. simulans, N. gossei, N. megalosiphon* and *N. benthamiana* respectively. Lanes 2, 5, 8 and 11 represent the region Nf43 to Nr1457 for species *N. simulans, N. gossei, N. megalosiphon* and *N. benthamiana* respectively. Lanes 3, 6 and 9 yielded no product from regions Nf43 to Nr1457 in *N. gossei, N. megalosiphon* and *N. benthamiani*.

After nucleotide sequencing was carried out, one species (*N. benthamiana*) had a region that was successfully sequenced and translated into amino acid sequences then aligned with the *N. tabacum* L. RCA homologue which showed two amino acid differences as shown in Figure 15.

```
                  **************************************************.*.****************
N.tabacum         GNDFSTLYAPLIRDGRMEKFYWAPTREDRIGVCTGIFRTDNVPAEDVVKIVDNFPGQSIDFFGALRARVY
N.benthamiana     GNDFSTLYAPLIRDGRMEKFYWAPTREDRIGVCTGIFRTDNVPAEDVVKIADSFPGQSIDFFGALRARVY
                  1.......10........20........30........40........50........60........70
```

Figure 15 Amino acid sequence alignment of a conserved region between 995 bp and 1428 bp from *N. tabacum* L. and *N. benthamiana*. Alignments are based on the *N. tabacum* L. RCA homologue.

Nucleotide sequence via genomic DNA (Experiment 2)

Genomic DNA was successfully extracted from five *Nicotiana* species (*N. tabacum* L., *N. megalosiphon, N. gossei, N. benthamiana* and *N. simulans*; Fig. 15A) with PCR the specific regions carried out as shown in Table 1. Of these primer sets, three produced amplicons which were considered for sequencing (Fig. 16B, C and Suppl. 1.5B). Amplicons associated

with the region Nf412 to Nr851 were chosen for sequencing as this region was of most variable of the RCA gene based on comparisons between *O. sativa* and *O. australiensis*. These DNA fragments were represented by all species tested. Amplicons are shown in Figure. 16 and Suppl. 1.5 (A). Amplicons specific to regions defined by Nf1036 to Nr1217 for *N. megalosiphon* are shown in Suppl. 1.5 (B).

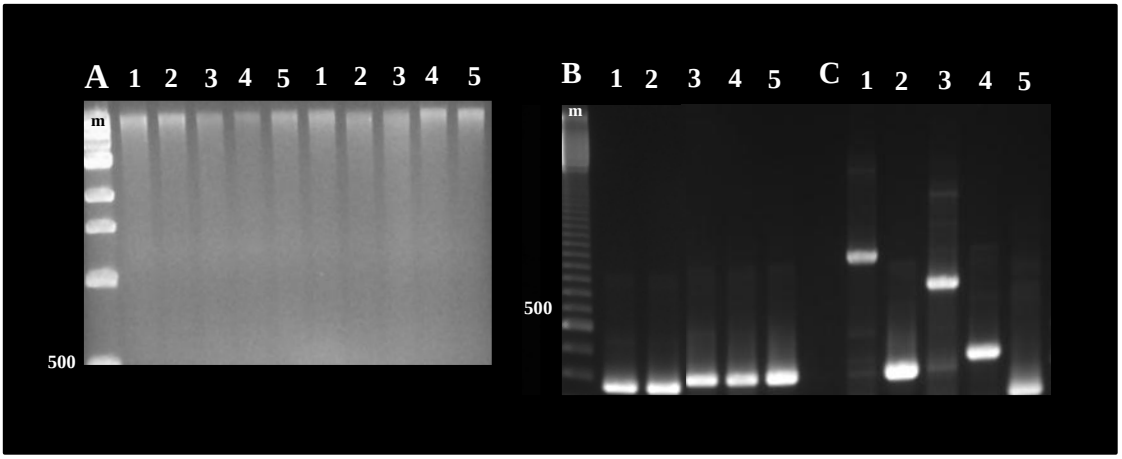


Figure 16 (A) DNA extractions of *Nicotiana* species. Lanes represent species *N. benthamiana* (1), *N. simulans* (2), *N. gossei* (3), *N. tabacum* L.(4) and *N. megalosiphon* (5). Representative gels showing final RCA fragment purity (B) Nf412 to Nr851, (C) Nf651 to Nr851. B and C show *N. benthamiana, N. simulans, N. gossei, N. tabacum* and *N. megalosiphon* represented in lanes 1-5.

Reliable alignments of PCR fragments were made for Nf412 to Nr851 using amplifications of genomic DNA. All five *Nicotiana* species tested aligned with 100% homology as shown in Figure 17.

```
                  **************************************************************************************************************************************************************
N. benthamiana    TGCAGAACTCATTTGGTTCATCCTATTGGTGTGACCCATCCGAAATAAGTATCTTCCAAATAAATGTGAGAATTCCAATGAATCCCTAATTGTCTTTTTTTGTTAGCCTATCGATAATTCCCTAAATTAGACCTGCTTAATCTAGAACAG   150
N. gossei         TGCAGAACTCATTTGGTTCATCCTATTGGTGTGACCCATCCGAAATAAGTATCTTCCAAATAAATGTGAGAATTCCAATGAATCCCTAATTGTCTTTTTTTGTTAGCCTATCGATAATTCCCTAAATTAGACCTGCTTAATCTAGAACAG   150
N. megalosiphon   TGCAGAACTCATTTGGTTCATCCTATTGGTGTGACCCATCCGAAATAAGTATCTTCCAAATAAATGTGAGAATTCCAATGAATCCCTAATTGTCTTTTTTTGTTAGCCTATCGATAATTCCCTAAATTAGACCTGCTTAATCTAGAACAG   150
N. tabacum        TGCAGAACTCATTTGGTTCATCCTATTGGTGTGACCCATCCGAAATAAGTATCTTCCAAATAAATGTGAGAATTCCAATGAATCCCTAATTGTCTTTTTTTGTTAGCCTATCGATAATTCCCTAAATTAGACCTGCTTAATCTAGAACAG   150
N. simulans       TGCAGAACTCATTTGGTTCATCCTATTGGTGTGACCCATCCGAAATAAGTATCTTCCAAATAAATGTGAGAATTCCAATGAATCCCTAATTGTCTTTTTTTGTTAGCCTATCGATAATTCCCTAAATTAGACCTGCTTAATCTAGAACAG   150
                  1.......10.........20.........30.........40.........50.........60.........70.........80.........90.......100.......110.......120.......130.......140.......150

                  **************************************************************************************************************************************************************
N. benthamiana    AACGTGCAATCCTTGAATATCTGAAATTGTCTAAGTGGAAATAGCTTTCTTATCATTCAATGAGCATCTTGTATTTCATAAAAAATTGGGGGCAATATAATCCTTACGTAAGGGCCATCCTATCCAACTTTCAGGCATTAAGATACGTTTC   300
N. gossei         AACGTGCAATCCTTGAATATCTGAAATTGTCTAAGTGGAAATAGCTTTCTTATCATTCAATGAGCATCTTGTATTTCATAAAAAATTGGGGGCAATATAATCCTTACGTAAGGGCCATCCTATCCAACTTTCAGGCATTAAGATACGTTTC   300
N. megalosiphon   AACGTGCAATCCTTGAATATCTGAAATTGTCTAAGTGGAAATAGCTTTCTTATCATTCAATGAGCATCTTGTATTTCATAAAAAATTGGGGGCAATATAATCCTTACGTAAGGGCCATCCTATCCAACTTTCAGGCATTAAGATACGTTTC   300
N. tabacum        AACGTGCAATCCTTGAATATCTGAAATTGTCTAAGTGGAAATAGCTTTCTTATCATTCAATGAGCATCTTGTATTTCATAAAAAATTGGGGGCAATATAATCCTTACGTAAGGGCCATCCTATCCAACTTTCAGGCATTAAGATACGTTTC   300
N. simulans       AACGTGCAATCCTTGAATATCTGAAATTGTCTAAGTGGAAATAGCTTTCTTATCATTCAATGAGCATCTTGTATTTCATAAAAAATTGGGGGCAATATAATCCTTACGTAAGGGCCATCCTATCCAACTTTCAGGCATTAAGATACGTTTC   300
                  .......160.......170.......180.......190.......200.......210.......220.......230.......240.......250.......260.......270.......280.......290.......300

                  **************************************************************************************************************************************************************
N. benthamiana    AAGCGTGGATGATTATCATAAGAGATTCCCAACATATCATATGATTCTCGTTCTTGAAAATCCACACTTTTCCAAACCCAGAAAACAGACGGAATTCTAGGATTCCTCCTGGAGGCAAATACTTTTATGCATACCTCCTCTGGTTGATCC   450
N. gossei         AAGCGTGGATGATTATCATAAGAGATTCCCAACATATCATATGATTCTCGTTCTTGAAAATCCACACTTTTCCAAACCCAGAAAACAGACGGAATTCTAGGATTCCTCCTGGAGGCAAATACTTTTATGCATACCTCCTCTGGTTGATCC   450
N. megalosiphon   AAGCGTGGATGATTATCATAAGAGATTCCCAACATATCATATGATTCTCGTTCTTGAAAATCCACACTTTTCCAAACCCAGAAAACAGACGGAATTCTAGGATTCCTCCTGGAGGCAAATACTTTTATGCATACCTCCTCTGGTTGATCC   450
N. tabacum        AAGCGTGGATGATTATCATAAGAGATTCCCAACATATCATATGATTCTCGTTCTTGAAAATCCACACTTTTCCAAACCCAGAAAACAGACGGAATTCTAGGATTCCTCCTGGAGGCAAATACTTTTATGCATACCTCCTCTGGTTGATCC   450
N. simulans       AAGCGTGGATGATTATCATAAGAGATTCCCAACATATCATATGATTCTCGTTCTTGAAAATCCACACTTTTCCAAACCCAGAAAACAGACGGAATTCTAGGATTCCTCCTGGAGGCAAATACTTTTATGCATACCTCCTCTGGTTGATCC   450
                  .......310.......320.......330.......340.......350.......360.......370.......380.......390.......400.......410.......420.......430.......440.......450

                  *******************************************
N. benthamiana    ACACCATCCTCTATTCTCGTAAGATGATACACACTAGCTAACAGC   495
N. gossei         ACACCATCCTCTATTCTCGTAAGATGATACACACTAGCTAACAGC   495
N. megalosiphon   ACACCATCCTCTATTCTCGTAAGATGATACACACTAGCTAACAGC   495
N. tabacum        ACACCATCCTCTATTCTCGTAAGATGATACACACTAGCTAACAGC   495
N. simulans       ACACCATCCTCTATTCTCGTAAGATGATACACACTAGCTAACAGC   495
                  .......460.......470.......480.......490.....
```

Figure 17 Sequence alignment of Nf412 to Nr851 in wild tobacco species (*N. benthamiana, N. gossei, N. megalosiphon, N. tabacum* L. and *N. simulans*).

With no polymorphisms observed in the amplified wild *Nicotiana* sequences (Fig. 17), it was concluded that the wild relatives, from the perspective of this very limited sequencing run, had no particular tolerance mechanisms. Therefore, commercial tobacco (*N. tabacum* L.), from which translated RCA has been fully sequenced, was used as a representative of the genus *Nicotiana* in a comparison with cultivated (*O. sativa*) and wild rice (*O. australiensis*) as shown in Figure 18. The wild *Oryza* has an established reputation for heat tolerance (Scafaro et al., 2011).

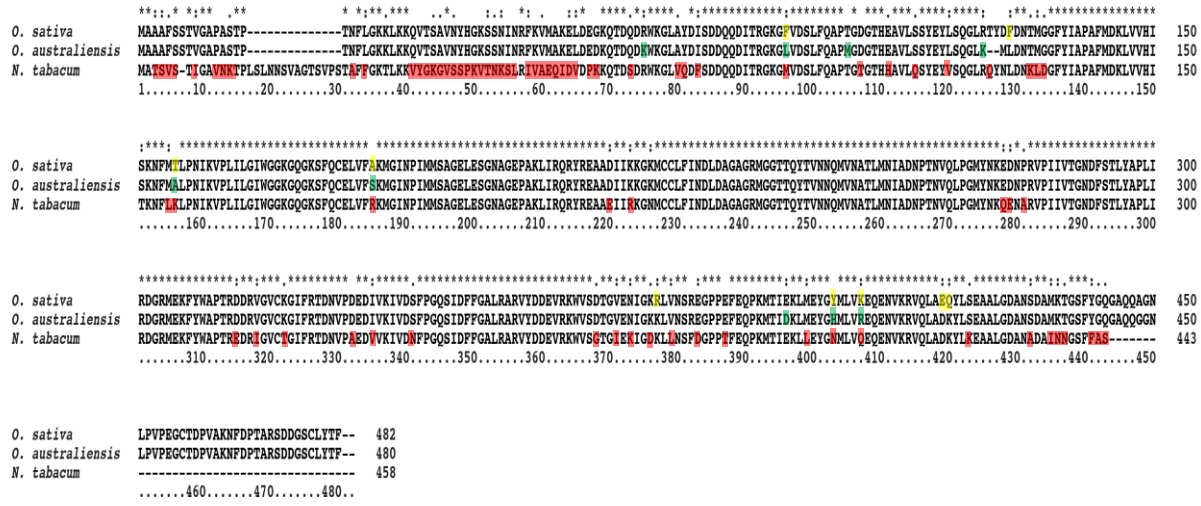


Figure 18 Amino acid sequence alignments of *Oryza sativa, O. australiensis* and *N. tabacum* L. Areas of alignments not marked with an asterisk (*) represent amino acid differences.

The diversity between RCA sequence of rice and cultivated tobacco was substantial and led to the question of whether the translated protein was only highly conserved within families, in this case the family Solanaceae. This is addressed in Figure 19 where the four published amino acid sequences for common solanaceous species are aligned and polymorphisms are identified. Substantial sequence diversity will be discussed below.

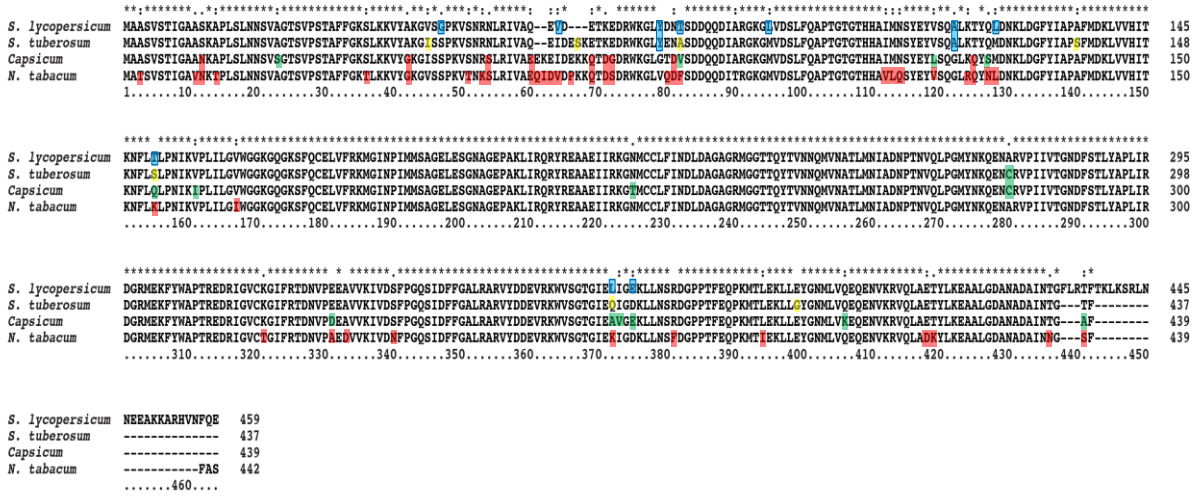


Figure 19 Amino acid sequence alignment of four solanaceous species: *Solanum lycopersicum, Solanum tuberosum L., Capsicum annuum* and *N. tabacum* L. Areas of alignments not marked with an asterisk (*) represent amino acid differences.

4 Discussion

Heat can induce a range of physiological responses, each potentially having an impact on whether a species can adapt to extreme temperatures. This study addresses these phenomena by testing four hypothesis — growth and development, photosynthesis, membrane leakage and differences in nucleotide sequences in RCA.

Physiological responses to heat

Among the five species, *N. tabacum* L. and *N. megalosiphon* generally displayed superior tolerance to heat stress. Significantly, *N. tabacum* L. has been selected for rapid leaf growth and high vegetative yield which explains its niche as a ‘sun’ plant and possibly, its heat tolerance. Surprisingly, *RGR* (and *NAR*) of *N. tabacum* L. during heat treatment (Figs 2 and 3) show gradually declining growth over several weeks at 38°C. The initial two weeks of heat treatment did not severely inhibit leaf or root growth in *N. tabacum* L but in the last phase of the experiment growth gave way to leaf senescence, death and evidently loss of biomass, suggesting that *N. tabacum* L. (and similarly *N. gossei*) do not survive long under heat stress. This species did not decrease biomass in order to increase survival rates as plant senescence occurred.

Nicotiana megalosiphon is a prostrate annual desert plant that endures full sun in its endemic range in the Australian desert (Symon, 2005). The high seasonal variability of these areas could have selected for the ephemeral life history in *N. megalosiphon*, as seen in similar annuals of the southern Gurbantunggut Desert of China (Wang et al., 2006) and other arid zones. During the second week of heat *N. megalosiphon* displayed the lowest *RGR* and *NAR* at 38°C. This slow growth rate coincided with initiation of flowering (Fig. 8), where it is hypothesized that resource allocation to the shifted differentiation of reproductive organs (Figs 2 and 3). The ability of *N. megalosiphon* to withstand heat stress throughout the duration of the growth experiment and into sexual maturity supports the case that it is a stress avoider as opposed to a stress tolerator (Blum, 1988). Rapid completion of the life cycle in *N. megalosiphon* may be a strategy to avoid severe heat damage. This is supported by a comparatively high A_{max}, indicative of rapid partitioning of carbohydrates to sinks. Alternatively, *N. gossei* had low rates of growth and photosynthetic rates under heat stress, in comparison to *N. tabacum* L. and *N. megalosiphon.* This may reflect its perennial habit and niche in crevices of rocky outcrops of Central Australia where direct sunlight, and thus increases in heat, are not experienced all day long (Symon, 2005).

N. megalosiphon plants preconditioned to 30˚C and 38˚C responded best to 36˚C regarding gas exchange easurements whereas *N. gossei* and *N. tabacum* L., both perennial plant species, did not benefit from the high-temperature pretreatment. This suggests that *N. megalosiphon* is better acclimated to higher temperatures (Fig. 4).

The correlation of *RGR* and *NAR* was notable (R^2 = 0.89), implying that the physiological aspects of carbon gain (i.e. photosynthesis or respiration) rather than leaf morphology drives increases in growth. This will be pursued further below in the investigation of photosynthesis and RCA sequence.

Development

Nicotiana gossei had the highest shoot-to-root ratios, suggesting that photoassimilates are more strongly allocated to leaves. Root systems play a critical role in whole-plant adaptation to heat stress (Huang et al., 2012). They found in creeping bentgrass, *Agrostis palustris* cultivar L-93, that increased root to shoot ratios were characteristic of heat tolerance. Further, it was postulated that the superior performance of L-93 under heat stress was due largely to

these morphological characteristics where fine root hairs and increased underground biomass underpinned higher water and macronutrient acquisition (Xu and Huang, 2001). *Nicotiana gossei* and *N. tabacum* L. had consistently fewer leaves than *N. megalosiphon* for the duration of the growth experiment at all temperatures (Fig. 7 and 8). *N. megalosiphon* also expressed the highest *LAR* measurements in 2 of the last 3 harvest periods in control and heat treatments. This might reflect a developmental pattern in *N. megalosiphon* that confers tolerance to heat stress, by some unknown mechanism, or simply a greater tolerance of the photosynthetic machinery of *N. megalosiphon* to heat as seen in Fig. 10.

Assimilate translocation under heat stress might be most favourably deployed in reproductive sinks, i.e. seed development to ensure survival. This was shown in *N. megalosiphon* which was the only plant to advance to adult stages in both control and heat treatments. *Nicotiana benthamiana* displays early development in its extreme desert niche (i.e. high temperatures of the Australian desert), which leads to accelerated maturity (Bally et al., 2015). Accelerated development in *N. megalosiphon,* as seen in precocious flowering, is consistent with its high photosynthetic rate under heat stress but perhaps with a reduced immunity to pathogens (Bally et al., 2015).

Gas Exchange and A_{max} relations to N

A_{max} responses to heat could not be attributed to stomatal behaviour because C_i and stomatal conductance (g_s) were closely matched across species. Photosynthetic rates were higher in *N. megalosiphon* in ambient conditions (30˚C) than when treated at 40˚C (26.3 and 21.4 µmol m^{-2} s^{-1} respectively). Similar differences between *Oryza meridionalis* and *O. sativa* have been observed, where *O. meridionalis*, with a lower leaf N content, had higher photosynthetic rates as heat increased (Scafaro et al., 2012). More rapid photosynthesis might relate to increased efficiency of biochemical pathways and in turn relate to the RCA homologues that were the subject of this study. There was a higher N concentration in *N. gossei* (4.5% N) compared with all other species. Therefore, not surprisingly, *N. gossei* had the lowest A_{max} on an N basis (9.7 µmol CO_2 mol^{-1} N s^{-1}) in contrast to *N. megalosiphon* which had the highest (20.0 µmol CO_2 mol^{-1} N s^{-1}). This implies that *N. megalosiphon* deployed more N to the chloroplast and thus had greater photosynthetic efficiency. This greater efficiency, which was maintained under heat, encouraged an investigation of RCA homologues of *N. megalosiphon* as heat-tolerant homologues may support increased photosynthetic rates under heat stress. Similarly,

N. megalosiphon had higher mean ITE than *N. tabacum* L. and *N. gossei* under increased temperatures (35 and 40˚C), indicating co-evolution of tolerance to high temperatures and water deficits. This suggests that *N. megalosiphon* tolerates heat above 36° better than other *Nicotiana* species (Fig. 10).

Membrane integrity

The most significant effect of heat on membranes was observed from 40 to 45˚C, suggesting that these temperatures invoke major leakage via membrane damage. *Nicotiana tabacum* L. showed the slowest leakage at 45˚C and *N. simulans* the slowest at 50˚C ($p < 0.05$). Interestingly *N. megalosiphon* showed the greatest leakage at 45˚C, which does not correlate with other physiological responses tested above. Studies show that membrane stability is correlated with heat tolerance, e.g. in cultivars of *Gossypium hirsutum* L. and Syrian landraces of wheat adapted to drought conditions (Ashraf et al., 1994; Bajji et al., 2002; Azhar et al., 2009). Importantly in the wild tobacco comparisons, the differences between cell membrane integrity (biophysical) and biochemical processes (e.g. carbon fixation) suggest that cell membrane integrity is not the determining factor for heat stress tolerance. Rather, our claim is that the efficiency of gas exchange and thus assimilates available for growth and development, which confer heat tolerance on *N. megalosiphon*.

The relative electrical conductivity (REC_t) detected significant differences between *Nicotiana* species between 40 and 50˚C as shown in Figure 12, 13 and Supplementary 1.3 (A). Sigmoidal increases in membrane leakage, such as those shown in this study, have been reported also in sorghum and cowpea (Sullivan and Blum, 1971; Ismail and Hall, 1999). The temperature at which 50% leakage (T_{50}) occurred was highest in in *N. simulans* (48.2˚C) and lowest in *N. megalosiphon* (43.6˚C) as shown in Supplementary 1.3 (B). This again suggests that *N. simulans* has a relatively higher level of heat tolerance. Studies have shown that REC_t increases correlate with increased heat in cotton (Ashraf et al., 1994). However, these studies were not inclusive of T_{50} analysis. Further research would recommend testing leakage with one degree increments from 40 to 50˚C as advised for osmolality. Furthermore, observations have indicated that a substantial amount of leakage can occur over a 24-hour period which does simulate, to some degree, natural situations. This is substantially longer exposure to

abiotic stress then the 2-hour heat treatment used here. This argues for time-course experiments (Whitlow et al., 1992).

Molecular analysis

Targeting RCA from DNA templates derived from cDNA (Experiment 1) identified variation in two species (*N. tabacum* L. and *N. benthamiana*). Amino acid alignment of *N. tabacum* L. RCA and the nucleotide sequences derived from *N. benthamiana* during the first experiment showed two amino acid differences. This suggests that selective pressure on RCAcould be driving a divergence between these two species, as seen in wild rice relatives.

The sequencing of genomic DNA (Experiment 2) using the RCA mRNA template region located 412 bp to 851 bp downstream of the *N. tabacum* L. start codon, the most variable region of the RCA gene, revealed no polymorphisms in nucleotide sequence (Fig. 17). Further, we could not claim that genomic DNA sequences amplified with RCA primers represented transcribed gene sequences due to introns amplifying the size of the RCA gene across species (Fig. 16C). That is, primers designed against RCA transcripts did not produce amplicons that match those amplified when the template was genomic DNA. This requires further investigation.

After no polymorphisms were found among wild Australian *Nicotiana* spp. during Experiment 2 (Fig. 17) it was decided that the *N. tabacum* L. RCA homologue would be evaluated against both *O. sativa* and *O. australiensis*. As *N. tabacum* L. had showed some tolerance to heat it was of interest to see how the RCA gene aligned with the heat tolerant *O. australiensis*. Differences between *Nicotiana* and *Oryza* spp. were pronounced (Fig. 18). Further, the alignment of Solanaceae species *Solanum lycopersicum*, *Solanum tuberosum* L., *Capsicum annuum* and *N. tabacum* L. was carried out to investigate any similarities between species. *Nicotiana tabacum* L. showed the highest variability among these solanaceous species, and yet it has few common characteristics with the heat-tolerant rice, *O. australiensis*. Being so, I propose that the RCA homologues between the two families described in this study (Poaceae and Solanaceae) are highly divergent in evolutionary terms and could have evolved functional tolerance to heat through disparate biochemical mechanisms.

Conclusions

Australian desert plant species are subject to environmental factors that include high temperatures (Laity, 2009). Strategies under these climatic variables include plasticity, acclimation and molecular changes. To escape heat stress a high degree of adaptation may be engaged so that all developmental stages are reached before extreme heat is established (Chaves et al., 2003). Survival strategies in extreme environments also include early vigour in extreme environments, such as those seen in Central Australia, where drought escape is an important adaptation (Bally et al., 2015). *Nicotiana megalosiphon* reaches sexual maturity earlier than *N. tabacum* L. and all other species tested. I propose that selection pressures on *N. megalosiphon* have also increased photosynthetic rates and selected for ecotypes with faster developmental rates. While preliminary data suggest that heat responsive motifs have evolved in the RCA gene, a deeper and more thorough analysis of polymorphisms in key genes that are vulnerable to heat is called for. The discovery and exploitation of abiotic stress tolerance genes using biotechnology and genetically modified organisms (GMOs) may be necessary as environmental conditions become more variable and adverse due to climate change. Not only is the introduction of stress tolerance genes important, an understanding of how these genes interact is required by careful phenotyping. Model plant species are important for understanding how abiotic stress tolerance genes function. Experimental models such as *Arabidopsis*, *N. tabacum* L. and *N. benthamiana* are valuable for the identification of abiotic stress tolerance genes because of the availability of DNA sequence data as well as their efficacy for DNA transformations. Emphasis now has to be on introducing these genes into crop species such as wheat (Sharma et al., 2012) and horticultural species also. However, the complexities involved in creating stable transgenic plants are still being understood, with reliable expression required at both the transcription and translation level (Wang et al., 2003). Until then, the investigation of wild species, particularly those subject to extreme environmental conditions, should continue with the possibility of discovering new genes taking place.

References

Abelenda JA, Navarro C, Prat S (2011) From the model to the crop: genes controlling tuber formation in potato. Curr. Opin. Biotechnol. **22:** 287-292

Ali A, Xu J, Ismail A, Fu B, Vijaykumar C, Gao Y, Domingo J, Maghirang R, Yu S, Gregorio G (2006) Hidden diversity for abiotic and biotic stress tolerances in the primary gene pool of rice revealed by a large backcross breeding program. Field Crops Res. **97:** 66-76

Ashraf M, Harris P (2013) Photosynthesis under stressful environments: an overview. Photosynthetica **51:** 163-190

Ashraf M, Saeed M, Qureshi M (1994) Tolerance to high temperature in cotton (*Gossypium hirsutum* L.) at initial growth stages. Environ. Exp. Bot. **34:** 275-283

Atwell BJ, Wang H, Scafaro AP (2014) Could abiotic stress tolerance in wild relatives of rice be used to improve *Oryza sativa*? Plant Sci. **215:** 48-58

Azhar F, Ali Z, Akhtar M, Khan A, Trethowan R (2009) Genetic variability of heat tolerance, and its effect on yield and fibre quality traits in upland cotton (*Gossypium hirsutum* L.). Plant Breeding **128:** 356-362

Bajji M, Kinet J-M, Lutts S (2002) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regulation **36:** 61-70

Bally J, Nakasugi K, Jia F, Jung H, Ho SY, Wong M, Paul CM, Naim F, Wood CC, Crowhurst RN (2015) The extremophile *Nicotiana benthamiana* has traded viral defence for early vigour. Nature Plants **1:** 15165

Barnabás B, Jäger K, Fehér A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant, Cell Environ. **31:** 11-38

Bernacchi CJ, Portis AR, Nakano H, von Caemmerer S, Long SP (2002) Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. Plant Physiol. **130:** 1992-1998

Bhatnagar-Mathur P, Vadez V, Sharma KK (2008) Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Rep. **27:** 411-424

Blum A (1988) Plant breeding for stress environments. CRC Press, Inc.

Boyle RK, McAinsh M, Dodd IC (2015) Stomatal closure of *Pelargonium*× *hortorum* in response to soil water deficit is associated with decreased leaf water potential only under rapid soil drying. Physiol. Plant. **156(1)**: 84-96.

Bray EA, Bailey-Serres J, Weretilnyk E (2000) Responses to abiotic stresses. Biochemistry and molecular biology of plants**:** 1158-1203

Byrne M, Yeates D, Joseph L, Kearney M, Bowler J, Williams M, Cooper S, Donnellan S, Keogh J, Leys R (2008) Birth of a biome: insights into the assembly and maintenance of the Australian arid zone biota. Mol. Ecol. **17:** 4398-4417

Cao H, Wang Y, Xie Z, Huang L, Xu H, Zhang L, Bian M, Sun G, Han S, Yang L (2013) TGB: the tobacco genetics and breeding database. Mol. Breed. **31:** 655-663

Cavallini E, Zenoni S, Finezzo L, Guzzo F, Zamboni A, Avesani L, Tornielli GB (2013) Functional diversification of grapevine MYB5a and MYB5b in the control of flavonoid biosynthesis in a petunia anthocyanin regulatory mutant. Plant and Cell Physiology**:** pct190

Change IPOC (2007) Climate change 2007: The physical science basis. Agenda **6:** 333

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought—from genes to the whole plant. Funct. Plant Biol. **30:** 239-264

Chen J-R, Lü J-J, Liu R, Xiong X-Y, Wang T-x, Chen S-Y, Guo L-B, Wang H-F (2010) DREB1C from *Medicago truncatula* enhances freezing tolerance in transgenic *M. truncatula* and China Rose (*Rosa chinensis* Jacq.). Plant growth regulation **60:** 199-211

Clay J (2013) World agriculture and the environment: a commodity-by-commodity guide to impacts and practices. Island Press

De Souza J, Da Silva JV (1987) Partitioning of carbohydrates in annual and perennial cotton (*Gossypium hirsutum* L.). J. Exp. Bot. **38:** 1211-1218

Demirevska-Kepova K, Holzer R, Simova-Stoilova L, Feller U (2005) Heat stress effects on ribulose-1, 5-bisphosphate carboxylase/oxygenase, Rubisco binding protein and Rubisco activase in wheat leaves. Biol. Plant. **49:** 521-525

Dexter ST, Tottingham W, Graber L (1932) Investigations of the hardiness of plants by measurement of electrical conductivity. Plant Physiol. **7:** 63

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32:** 1792-1797

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra S (2009) Plant drought stress: effects, mechanisms and management. *In* Sustainable Agriculture. Springer, pp 153-188

Fischer R, Byerlee D, Edmeades G (2012) Crop yields and global food security. *In*. Canberra: Australian Center for International Agricultural Research

Flexas J, Barbour MM, Brendel O, Cabrera HM, Carriquí M, Díaz-Espejo A, Douthe C, Dreyer E, Ferrio JP, Gago J (2012) Mesophyll diffusion conductance to CO 2: an unappreciated central player in photosynthesis. Plant Sci. **193:** 70-84

Flexas J, Ribas-Carbó M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R (2006) Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO2in vivo. The Plant Journal **48:** 427-439

Fukayama H, Ueguchi C, Nishikawa K, Katoh N, Ishikawa C, Masumoto C, Hatanaka T, Misoo S (2012) Overexpression of Rubisco activase decreases the photosynthetic CO2 assimilation rate by reducing Rubisco content in rice leaves. Plant and Cell Physiology **53:** 976-986

Gillings MR (2014) Rapid extraction of PCR-competent DNA from recalcitrant environmental samples. *In* Environmental Microbiology. Springer, pp 17-23

Golldack D, Lüking I, Yang O (2011) Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep. **30:** 1383-1391

Goodin MM, Zaitlin D, Naidu RA, Lommel SA (2008) *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. Mol. Plant-Microbe Interact. **21:** 1015-1026

Greer DH, Weedon MM (2012) Modelling photosynthetic responses to temperature of grapevine (*Vitis vinifera* cv. Semillon) leaves on vines grown in a hot climate. Plant, Cell Environ. **35:** 1050-1064

Gupta US (2007) Physiology of Stressed Crops: Membrane System. Taylor & Francis

Hamrick JL, Godt M (1996) Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society B: Biological Sciences **351:** 1291-1298

Hanba YT, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K, Terashima I, Katsuhara M (2004) Overexpression of the barley aquaporin HvPIP2; 1 increases internal CO2 conductance and CO2 assimilation in the leaves of transgenic rice plants. Plant and Cell Physiology **45:** 521-529

Hasanuzzaman M, Nahar K, Alam MM, Roychowdhury R, Fujita M (2013) Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. International journal of molecular sciences **14:** 9643-9684

Havaux M, Tardy F (1999) Loss of chlorophyll with limited reduction of photosynthesis as an adaptive response of Syrian barley landraces to high-light and heat stress. Funct. Plant Biol. **26:** 569-578

Hennessy F, Nicoll WS, Zimmermann R, Cheetham ME, Blatch GL (2005) Not all J domains are created equal: implications for the specificity of Hsp40–Hsp70 interactions. Protein Sci. **14:** 1697-1709

Hiriart J-B, Aro E-M, Lehto K (2003) Dynamics of the VIGS-mediated chimeric silencing of the *Nicotiana benthamiana* ChlH gene and of the tobacco mosaic virus vector. Mol. Plant-Microbe Interact. **16:** 99-106

Hoekema A, Hirsch P, Hooykaas P, Schilperoort R (1983) A binary plant vector strategy based on separation of vir-and T-region of the Agrobacterium tumefaciens Ti-plasmid. **30**: 179-180

Hossain M, Takeda H, Senboku T (1995) Improved method of determination of membrane thermostability for screening heat-tolerant and sensitive varieties in Brassica. JIRCAS Journal for Scientific Papers (Japan)

Huang B, Rachmilevitch S, Xu J (2012) Root carbon and protein metabolism associated with heat tolerance. J. Exp. Bot. **63:** 3455-3465

Ismail AM, Hall AE (1999) Reproductive-stage heat tolerance, leaf membrane thermostability and plant morphology in cowpea. Crop Sci. **39:** 1762-1768

Jada B, Soitamo AJ, Siddiqui SA, Murukesan G, Aro E-M, Salakoski T, Lehto K (2014) Multiple Different Defense Mechanisms Are Activated in the Young Transgenic Tobacco Plants Which Express the Full Length Genome of the Tobacco Mosaic Virus, and Are Resistant against this Virus. **9(9)**: e107778.

Jan AT, Singhal P, Haq QMR (2013) Plant abiotic stress: Deciphering remedial strategies for emerging problem. Journal of Plant Interactions **8:** 97-108

Jin T, Chang Q, Li W, Yin D, Li Z, Wang D, Liu B, Liu L (2010) Stress-inducible expression of GmDREB1 conferred salt tolerance in transgenic alfalfa. Plant Cell, Tissue and Organ Culture (PCTOC) **100:** 219-227

Julie Gray SP, Shabbeer J, Schuch W, Grierson D (2012) Molecular biology of fruit ripening and its manipulation with antisense. 10 Years Plant Molecular Biology **19:** 69-87

Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002) Chloroplast avoidance movement reduces photodamage in plants. Nature **420:** 829-832

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A combination of the Arabidopsis DREB1A gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant and Cell Physiology **45:** 346-350

Kong F, Deng Y, Wang G, Wang J, Liang X, Meng Q (2014) LeCDJ1, a chloroplast DnaJ protein, facilitates heat tolerance in transgenic tomatoes. Journal of integrative plant biology **56:** 63-74

Kotak S, Larkindale J, Lee U, von Koskull-Döring P, Vierling E, Scharf K-D (2007) Complexity of the heat stress response in plants. Curr. Opin. Plant Biol. **10:** 310-316

Kurek I, Chang TK, Bertain SM, Madrigal A, Liu L, Lassner MW, Zhu G (2007) Enhanced thermostability of Arabidopsis Rubisco activase improves photosynthesis and growth rates under moderate heat stress. The Plant Cell **19:** 3230-3241

Laity JJ (2009) Deserts and desert environments, Vol 3. John Wiley & Sons

Latchman DS (1997) Transcription factors: an overview. The international journal of biochemistry & cell biology **29:** 1305-1312
Latz PK & GREEN J (1995). Bushfires & bushtucker: Aboriginal plant use in Central Australia, Iad Press, Alice Springs.
Lauriano J, Lidon F, Carvalho C, Campos P, do Céu Matos M (2000) Drought effects on membrane lipids and photosynthetic activity in different peanut cultivars. Photosynthetica **38:** 7-12
Leport L, Turner N, French R, Barr M, Duda R, Davies S, Tennant D, Siddique K (1999) Physiological responses of chickpea genotypes to terminal drought in a Mediterranean-type environment. European Journal of Agronomy **11:** 279-291
Lewis RS (2011) *Nicotiana*. *In* Wild Crop Relatives: Genomic and Breeding Resources. Springer, pp 185-208
Li G-L, Chang H, Li B, Zhou W, Sun D-Y, Zhou R-G (2007) The roles of the atDjA2 and atDjA3 molecular chaperone proteins in improving thermotolerance of Arabidopsis thaliana seedlings. Plant Sci. **173:** 408-416
Lin MT, Occhialini A, Andralojc PJ, Parry MA, Hanson MR (2014) A faster Rubisco with potential to increase photosynthesis in crops. Nature **513:** 547-550
Lim, KY, Matyasek R, Kovarik A, & Leitch AR (2004) Genome evolution in allotetraploid *Nicotiana*. Biological Journal of the Linnean Society **82(4):** 599-606
Marchand FL, Mertens S, Kockelbergh F, Beyens L, Nijs I (2005) Performance of high arctic tundra plants improved during but deteriorated after exposure to a simulated extreme temperature event. Global Change Biol. **11:** 2078-2089
Marks CE, Ladiges PY (2011) Comparative morphology and phylogeny of *Nicotiana* section *Suaveolentes* (Solanaceae) in Australia and the South Pacific. Aust. Syst. Bot. **24:** 61-86
Matsukura S, Mizoi J, Yoshida T, Todaka D, Ito Y, Maruyama K, Shinozaki K, Yamaguchi-Shinozaki K (2010) Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes. Mol. Genet. Genomics **283:** 185-196
McCouch SR, McNally KL, Wang W, Hamilton RS (2012) Genomics of gene banks: A case study in rice. Am. J. Bot. **99:** 407-423
McDowell JM, Woffenden BJ (2003) Plant disease resistance genes: recent insights and potential applications. Trends Biotechnol. **21:** 178-183
Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in plant abiotic stress responses. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms **1819:** 86-96
Moon J-C, Ham DJ, Hwang S-G, Park YC, Lee C, Jang CS (2014) Molecular characterization of a heat inducible rice gene, OsHSP1, and implications for rice thermotolerance. Genes & genomics **36:** 151-161
Morales D, Rodríguez P, Dell'Amico J, Nicolas E, Torrecillas A, Sánchez-Blanco MJ (2003) High-temperature preconditioning and thermal shock imposition affects water relations, gas exchange and root hydraulic conductivity in tomato. Biol. Plant. **47:** 203-208
Mounet F, Moing A, Kowalczyk M, Rohrmann J, Petit J, Garcia V, Maucourt M, Yano K, Deborde C, Aoki K (2012) Down-regulation of a single auxin efflux transport protein in tomato induces precocious fruit development. J. Exp. Bot. **63:** 4901-4917
Mueller LA, Solow TH, Taylor N, Skwarecki B, Buels R, Binns J, Lin C, Wright MH, Ahrens R, Wang Y (2005) The SOL Genomics Network. A comparative resource for Solanaceae biology and beyond. Plant Physiol. **138:** 1310-1317

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant Physiol. **149:** 88-95

Parry MA, Andralojc PJ, Scales JC, Salvucci ME, Carmo-Silva AE, Alonso H, Whitney SM (2013) Rubisco activity and regulation as targets for crop improvement. J. Exp. Bot. **64:** 717-730

Piramila B, Prabha A, Nandagopalan V, Stanley A (2012) Effect of heat treatment on germination, seedling growth and some biochemical parameters of dry seeds of black gram. Int. J. Pharm. Phytopharmacol. Res **1:** 194-202

Pons TL, Welschen RA (2003) Midday depression of net photosynthesis in the tropical rainforest tree Eperua grandiflora: contributions of stomatal and internal conductances, respiration and Rubisco functioning. Tree Physiology **23:** 937-947

Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species differing in relative growth rate. Oecologia **83:** 553-559

Portis Jr AR (2003) Rubisco activase–Rubisco's catalytic chaperone. Photosynthesis Res. **75:** 11-27

Raven PH, Evert RF, Eichhorn SE (2005) Biology of Plants. W. H. Freeman

Rodríguez M, Canales E, Borrás-Hidalgo O (2005) Molecular aspects of abiotic stress in plants. Biotecnol. Apl. **22:** 1-10

Rosenzweig C, Elliott J, Deryng D, Ruane AC, Müller C, Arneth A, Boote KJ, Folberth C, Glotter M, Khabarov N (2014) Assessing agricultural risks of climate change in the 21st century in a global gridded crop model intercomparison. Proceedings of the National Academy of Sciences **111:** 3268-3273

Rosenzweig C, Hillel D (2008) Climate variability and the global harvest: impacts of El Niño and other oscillations on agroecosystems. Oxford University Press New York. **48(5)**: 2049-2050

Saelim S, Zwiazek JJ (2000) Preservation of thermal stability of cell membranes and gas exchange in high temperature acclimated *Xylia xylocarpa* seedlings. J. Plant Physiol. **156:** 380-385

Sakuma S, Salomon B, Komatsuda T (2011) The domestication syndrome genes responsible for the major changes in plant form in the triticeae crops. Plant and Cell Physiology **52:** 738-749

Salvucci ME, Crafts-Brandner SJ (2004) Relationship between the heat tolerance of photosynthesis and the thermal stability of Rubisco activase in plants from contrasting thermal environments. Plant Physiol. **134:** 1460-1470

Sambrook J, Russell D (2001) Molecular cloning: a laboratory manual-New York, NY: Cold Spring Laboratory Press. *In*. Cold Spring Harbor. 2-43

Scafaro AP, Haynes PA, Atwell BJ (2009) Physiological and molecular changes in *Oryza meridionalis* Ng., a heat-tolerant species of wild rice. J. Exp. Bot.**:** erp294

Scafaro AP, Von Caemmerer S, Evans JR, Atwell BJ (2011) Temperature response of mesophyll conductance in cultivated and wild *Oryza species* with contrasting mesophyll cell wall thickness. Plant, Cell Environ. **34:** 1999-2008

Scafaro AP, Yamori W, Carmo-Silva AE, Salvucci ME, Von Caemmerer S, Atwell BJ (2012) Rubisco activity is associated with photosynthetic thermotolerance in a wild rice (*Oryza meridionalis*). Physiol. Plant. **146:** 99-109

Schaff D, Clayberg C, Milliken G (1987) Comparison of TTC and electrical conductivity heat tolerance screening techniques in *Phaseolus*. HortScience **22:** 642-645

Sharma AD, Rakhra G, Singh J (2012) Expression analysis of BsAPase14 acid phosphatase, a stress responsive boiling-stable protein from *Triticum aestivum*. Journal of Crop Science and Biotechnology **15:** 41-45

Smith NG, Dukes JS (2013) Plant respiration and photosynthesis in global-scale models: incorporating acclimation to temperature and CO2. Global Change Biol. **19:** 45-63
Sreenivasulu N, Grimm B, Wobus U, Weschke W (2000) Differential response of antioxidant compounds to salinity stress in salt-tolerant and salt-sensitive seedlings of foxtail millet (*Setaria italica*). Physiol. Plant. **109:** 435-442
Stone P (2001) The effects of heat stress on cereal yield and quality. Crop responses and adaptations to temperature stress**:** 243-291
Sullivan CY, Blum A (1971) Drought and heat resistance of sorghum and corn. University of Nebraska. Departments of horticulture, forestry and agronomy
Sumesh K, Sharma-Natu P, Ghildiyal M (2008) Starch synthase activity and heat shock protein in relation to thermal tolerance of developing wheat grains. Biol. Plant. **52:** 749-753
Sunkar R, Chinnusamy V, Zhu J, Zhu J-K (2007) Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. Trends Plant Sci. **12:** 301-309
Symon D (2005) Native tobaccos (Solanaceae: *Nicotiana* spp.) in Australia and their use by Aboriginal peoples. Beagle: Records of the Museums and Art Galleries of the Northern Territory. **21:** 1
Tester M, Bacic A (2005) Abiotic stress tolerance in grasses. From model plants to crop plants. Plant Physiol. **137:** 791-793
Tholen D, Zhu X-G (2011) The mechanistic basis of internal conductance: a theoretical analysis of mesophyll cell photosynthesis and CO2 diffusion. Plant Physiol. **156:** 90-105
Todaka D, Nakashima K, Shinozaki K, Yamaguchi-Shinozaki K (2012) Toward understanding transcriptional regulatory networks in abiotic stress responses and tolerance in rice. Rice **5:** 1-9
Väinölä A, Repo T (2000) Impedance spectroscopy in frost hardiness evaluation of Rhododendron leaves. Ann. Bot. **86:** 799-805
Van der Hoorn RA, Laurent F, Roth R, De Wit PJ (2000) Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr 9/Cf-9-induced and Avr 4/Cf-4-induced necrosis. Mol. Plant-Microbe Interact. **13:** 439-446
Wahid A, Rasul E, Rao R, Iqbal R (2005) Photosynthesis in leaf, stem, flower and fruit. Handbook of Photosynthesis, 2nd ed., CRC Press, Florida**:** 479-497
Wang G, Kong F, Zhang S, Meng X, Wang Y, Meng Q (2015) A tomato chloroplast-targeted DnaJ protein protects Rubisco activity under heat stress. J. Exp. Bot.**:** erv102
Wang J, Cui L, Wang Y, Li J (2009) Growth, lipid peroxidation and photosynthesis in two tall fescue cultivars differing in heat tolerance. Biol. Plant. **53:** 237-242
Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1-14
Wang W, Vinocur B, Shoseyov O, Altman A (2000) Biotechnology of plant osmotic stress tolerance physiological and molecular considerations. *In* IV International Symposium on In Vitro Culture and Horticultural Breeding 560, pp 285-292
Wang X, Jiang J, Wang Y, Luo W, Song C, Chen J (2006) Responses of ephemeral plant germination and growth to water and heat conditions in the southern part of Gurbantunggut Desert. Chin. Sci. Bull. **51:** 110-116
Waterhouse PM, Helliwell CA (2003) Exploring plant genomes by RNA-induced gene silencing. Nature Reviews Genetics **4:** 29-38
Whitlow TH, Bassuk NL, Ranney TG, Reichert DL (1992) An improved method for using electrolyte leakage to assess membrane competence in plant tissues. Plant Physiol. **98:** 198-205

Whitney SM, Houtz RL, Alonso H (2011) Advancing our understanding and capacity to engineer nature's CO2-sequestering enzyme, Rubisco. Plant Physiol. **155:** 27-35

Wiech H, Buchner J, Zimmermann R, Jakob U (1992) Hsp90 chaperones protein folding in vitro. Nature **358:** 169-170

Wright NT (2003) On a relationship between the Arrhenius parameters from thermal damage studies. Journal of biomechanical engineering **125:** 300-304

Xu Q, Huang B (2001) Morphological and physiological characteristics associated with heat tolerance in creeping bentgrass. Crop Sci. **41:** 127-133

Yamori W, Noguchi K, Hanba YT, Terashima I (2006) Effects of internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. Plant and Cell Physiology **47:** 1069-1080

Yang KZ, Xia C, Liu XL, Dou XY, Wang W, Chen LQ, Zhang XQ, Xie LF, He L, Ma X (2009) A mutation in Thermosensitive Male Sterile 1, encoding a heat shock protein with DnaJ and PDI domains, leads to thermosensitive gametophytic male sterility in Arabidopsis. The Plant Journal **57:** 870-882

Yeates C, Gillings M (1998) Rapid purification of DNA from soil for molecular biodiversity analysis. Lett. Appl. Microbiol. **27:** 49-53

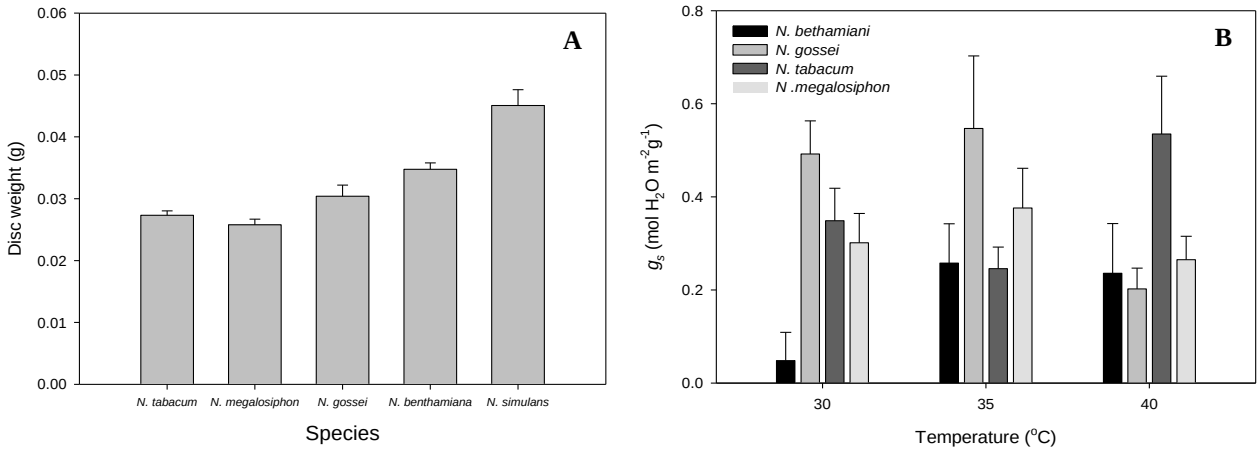
Yun-Ying C, Hua D, Li-Nian Y, Zhi-Qing W, Shao-Chuan Z, Jian-Chang Y (2008) Effect of heat stress during meiosis on grain yield of rice cultivars differing in heat tolerance and its physiological mechanism. Acta Agronomica Sinica **34:** 2134-2142

Zamir D (2001) Improving plant breeding with exotic genetic libraries. Nature reviews genetics **2:** 983-989

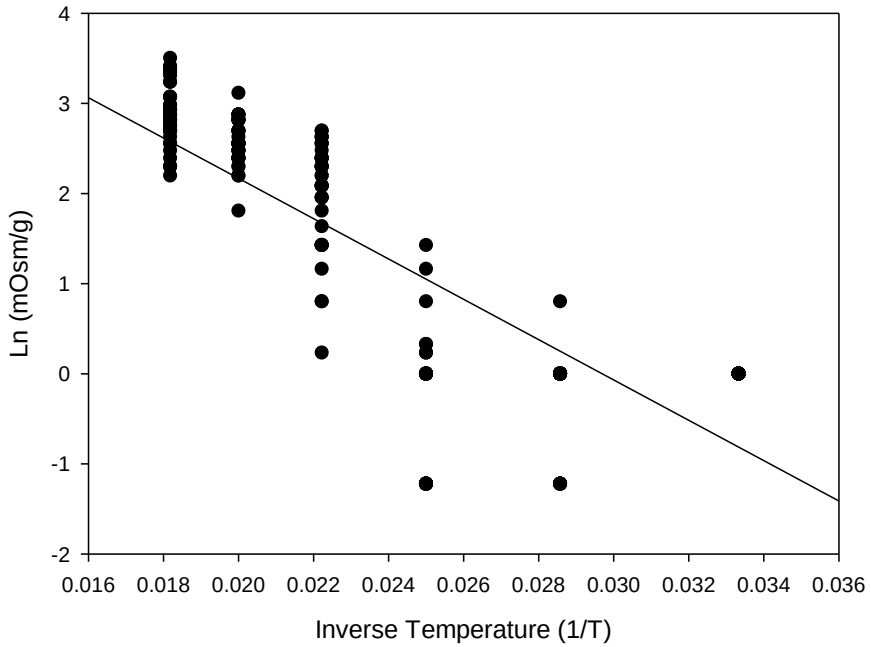
Zhu X-G, Long SP, Ort DR (2010) Improving photosynthetic efficiency for greater yield. Annu. Rev. Plant Biol. **61:** 235-261

Supplementary Material

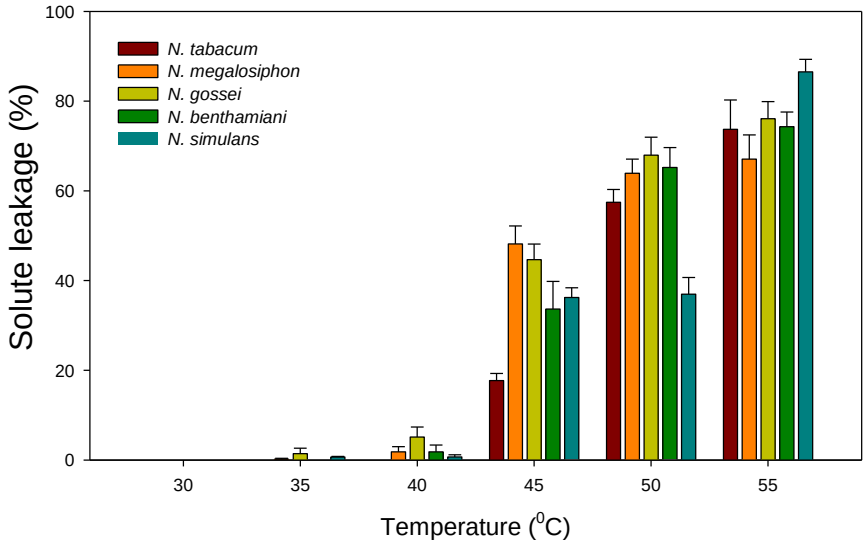
Supplementary I:



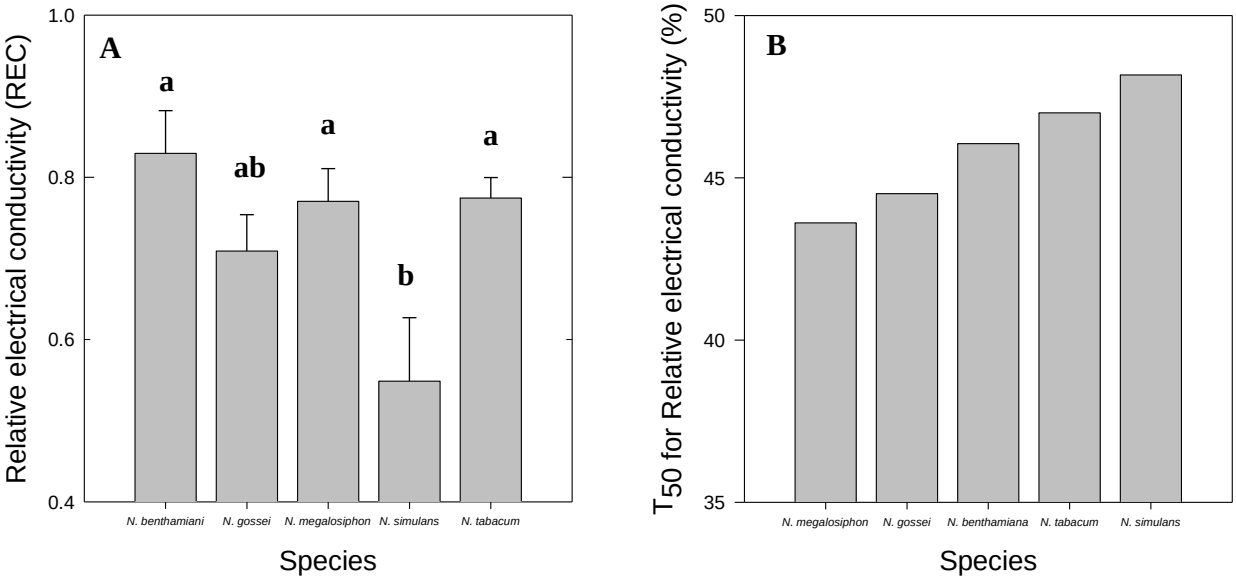
Supplementary 1.1 (A) Disc weights used to calculate mOsmoles g^{-1} (*n* = 10). Supplementary 1.1 (B) Stomatal conductance (g_s) of *N. benthamiana, N. gossei, N. tabacum L.* and *N. megalosiphon*. No statistical analysis was carried out on gs due to large SE variation. Bars show SE (mean).



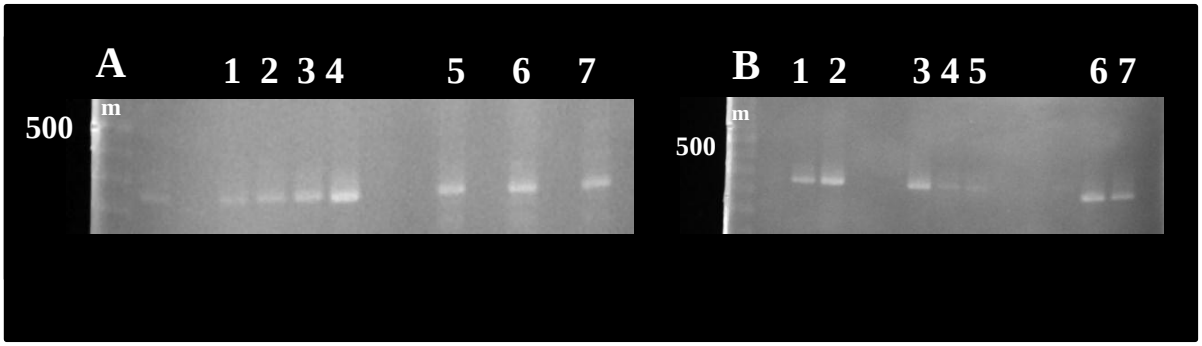
Supplementary 1.2 Arrhenius plot of pooled *Nicotiana* species solute leakage (mOsmoles) used to determine membrane transition temperature.



Supplementary 1.3 Proportion of leaked electrolytes after a two-hour incubation at 30, 35, 40, 45, 50 and 55˚C in five species (*N. tabacum* L., *N. megalosiphon, N. gossei, N. benthamiana* and *N. simulans*; *n* = 8).



Supplementary 1.4 (A) Relative electrical conductivity (%) at 50˚C. Data compiled in this figure have been derived from Figure 13. Columns represent REC mean averages +/- SE (*n* = 8). Means that do not share a lower case letter are significantly different (Tukey's HSD). Supplementary 1.4 (B) Temperature (˚C) at which 50% relative electrical conductivity occurred for tobacco species (*N. megalosiphon, N. gossei, N. benthamiana, N. tabacum* L. and *N. simulans*).



Supplementary 1.5 (A) Representative gel showing amplicons of RCA. Lane labels are as follows: *m* = 100 bp ladder. Gene fragments are noted as independent lines where lanes 1, 2, 3 and 4 represent the region Nf412 to Nr851 for species *N. tabacum, N. benthamiana, N. simulans,* and *N. tabacum.* Lanes 5 to 7 are replicates of *N. megalosiphon.* Supplementary 1.5 (B) lanes 1, 2, 3, 4 and 5 represent amplicons from region Nf412 to Nr851 for species *N. benthamiana, N. simulans, N. tabacum* and *N. megalosiphon* (twice) respective. Lanes 6 and 7 show amplicons specific to regions defined by Nf1036 to Nr1217 for *N. megalosiphon* (twice).

Supplementary II: Instructions to authors

ORGANIZATION

Submit manuscript with elements arranged in the following order, numbering all pages consecutively. All material should be 1.5-spaced. Font: Times New Roman, Courier New, Arial. (Other fonts may cause conversion problems).

1. Page 1: Running head not to exceed 50 characters and spaces; name, address, telephone number, and e-mail address of author(s) to whom all correspondence should be sent (*Plant Physiology* now permits more than one Corresponding Author in articles when such recognition can be justified and is approved by the Monitoring Editor); and Research Area most appropriate for the paper (a secondary Research Area should be indicated also, if relevant, and be identified as such). Research Areas are listed above.

2. Page 2: Title of article, not to exceed 150 characters and spaces; all authors' full names (necessary for accurate indexing and abstracting); institution address (es); and a summary of the most important findings and/or advance set out in the article. This summary will follow

the title in the Table of Contents and should be written for the non-expert. It must not exceed 200 characters including spaces. Please see "One-sentence Summary" below for guidance.

3. Page 3: Footnotes in the following order: financial source (if any) and the experiment station or institution paper number (if any); present address(es) of authors if different from heading; corresponding author with e-mail address.

4. Page 4: Abstract (include genus and species). Abstracts cannot exceed 250 words (Research Article, Breakthrough Technologies) or 150 words (Research Report, Update, Topical Review) or the processing of the paper will be delayed. An Abstract must not be included in Scientific Correspondence.

5. Page 5 and subsequent pages: The text, comprising Introduction (not to exceed 10,000 words for a Research Article), Results, Discussion, and Conclusions, Materials and Methods, and Acknowledgments (do not use the space to supply financial source). Results, Discussion, and Conclusions may be combined in shorter submissions. Please note that Research Reports are strictly limited to six Journal pages and Scientific Correspondence to three Journal pages, including tables, figures, and references.

6. Literature Cited. (Authors are responsible for accuracy in citations. Citations will be copyedited for format only.)

7. Figure legends (must also be included below the figure in each individual figure file).

8. Tables with brief and concise titles and legends (one table per page).

9. Original figure files (with corresponding figure legends contained in each file). See FIGURE PREPARATION, DIGITAL ART, COVER SUBMISSIONS, and SUPPLEMENTAL DATA.

10. Supplemental data files. See SUPPLEMENTAL DATA.

One–Sentence Summaries.

When preparing the summary, please:

1. Keep to short, simple sentences (200 characters, maximum)

2. Avoid abbreviations

3. Use the active voice and avoid infinitives when possible

4. Avoid using specific gene/protein names and specialist terminologies

5. Do not use 'we' and phrasing such as 'this paper shows'

6. Do draw the readers' attention to the most important concept to come from the research

7. Use wording that can be easily understood by the non-expert

8. Avoid using multiple adjectives, adverbs and long, dependent clauses

The following examples may be helpful:

Overexpression of a BAHD Acyltransferase, OsAt10, Alters Rice Cell Wall Hydroxycinnamic Acid Content and Saccharification
Laura E. Bartley, Matthew L. Peck, Sung-Ryul Kim, Berit Ebert, Chithra Manisseri, Dawn M. Chiniquy, Robert Sykes, Lingfang Gao, Carsten Rautengarten, Miguel E. Vega-Sánchez, Peter I. Benke, Patrick E. Canlas, Peijian Cao, Susan Brewer, Fan Lin, Whitney L. Smith, Xiaohan Zhang, Jay D. Keasling, Rolf E. Jentoff, Steven B. Foster, Jizhong Zhou, Angela Ziebell, Gynheung An, Henrik V. Scheller, and Pamela C. Ronald
Plant Physiol. 2013 161: 1615-1633.
An acyltransferase reduces cross linking in grass cell walls, yielding grass leaves and stems that can be more easily broken down to make biofuels.

The Epiphytic Fungus Pseudozyma aphidis Induces Jasmonic Acid- and Salicylic Acid/Nonexpressor of PR1-Independent Local and Systemic Resistance
Kobi Buxdorf, Ido Rahat, Aviva Gafni, and Maggie Levy
Plant Physiol. 2013 161: 2014-2022.
An epiphytic fungus induces plant resistance against pathogens.

The Anticipation of Danger: Microbe-Associated Molecular Pattern Perception Enhances AtPep-Triggered Oxidative Burst
Pascale Flury, Dominik Klauser, Birgit Schulze, Thomas Boller, and Sebastian Bartels
Plant Physiol. 2013 161: 2023-2035.
Microbial elicitors and the plant defense hormone jasmonic acid differentially modulates the plant's innate immune response.

Understanding the Role of Defective Invertases in Plants: Tobacco Nin88 Fails to Degrade Sucrose

Katrien Le Roy, Rudy Vergauwen, Tom Struyf, Shuguang Yuan, Willem Lammens, Janka Mátrai, Marc De Maeyer, and Wim Van den Ende

Plant Physiol. 2013 161: 1670-1681.

An inactive invertase may indirectly stimulate the activity of active cell wall invertases.

TEXT REQUIREMENTS

Style and format. Manuscripts should be written in simple declarative sentences and must conform to accepted standards of English style and usage. Consult recent issues for style and placement of main headings, subheadings, and paragraph headings and for other details of format. Authors may wish to consult Scientific Style and Format, 6th Ed., 1994, Council of Biology Editors, Bethesda, MD, for current scientific and editorial conventions.

Nomenclature. In the abstract, at first mention in the text, and in "Materials and Methods," include complete botanical names (genus, species, and, when appropriate, cultivar) for all experimental plants. Do NOT use the genus name alone, unless that is the accepted common name. Identify algae and microorganisms by a collection number or that of a comparable listing. Following first mentions, generic names should be abbreviated to the initial, except when confusion could arise by reference to genera with the same initial. Common names can be used after first mention. When the genus name is the accepted common name, the name should be in lowercase, roman type. Arabidopsis (no italics) is an accepted common name for *A. thaliana*.

Genes and Proteins. For genes and proteins, use the following format: the ABC (ATP-binding cassette) gene or the ABC (ATP-binding cassette) protein (when informal description is appropriate). If the gene/protein definition contains italic/underlined initial letters, remove italics or underline (and follow instructions below). Define whichever one appears first; there is no need to define it twice. If the gene abbreviation is adequately defined by the text, there is no need to also define it parenthetically (e.g. in the STA11 gene, which results in significantly reduced granular starch deposition and major modifications in amylopectin structure). Gene names are italic; protein names are not.

Abbreviations in Gene and Protein names are acceptable when used two or more times. Numbers should be closed up on gene names that are spelled out (i.e. *APETALA2*). Avoid

using an abbreviation first, unless spelling out the name of a gene would make the sentence indecipherable. If this is the case, use the abbreviation first, followed by the spelled-out name in parentheses with the word "for" (i.e. *APT2* (for *APETALA2*)). If *APT2* is given as the gene name of *APETALA2*, then it is unnecessary to define any of the other *APETALA* names (e.g. *APT1*). *APT1* may be used without first referring to*APETALA1*. *VERNALIZATION1*, *2*, and *3* (*VRN1*, *2*, and *3*) should read: *VERNALIZATION1* (*VRN1*), *VRN2*, and*VRN3*. Taxonomic prefixes (*At*, *Os*, *Nt*, etc.) should be closed up to the gene/protein names and match the style (italic or normal type) of the name.

Abbreviations. Do not abbreviate words or measures in the title other than those standard for international usage. Chemical symbols can be used in the title, but spell out chemical elements. Units of measure can be abbreviated in the abstract. Please click on the following link for a list of abbreviations that can be used in the remainder of the text and the running head:List of Abbreviations. Introduce all other abbreviations parenthetically following the term both in the abstract (if used three times) and at first use of the term in the text. Abbreviations must be used three times in the text (this includes table and figure legends) or the term must be spelled out.

Units of measure. The metric system is standard, and SI units must be used as much as possible. Use negative exponents to indicate units in the denominator when three or more units are used (e.g., μmol m^{-2} s^{-1} rather than μmol/m^2/s).

Numbers and fractions. Write out numerals one through nine, except when used with units of measure. Write out all numbers or fractions that begin a sentence, or rephrase the sentence to avoid beginning with a numeral. Use the preposition "to" between numerals (do not use a dash): e.g., "13 to 22 min" and "3°C to 10°C." Exceptions: in tables, figures, graphs, legends, and within parentheses in the text, dashes are used. Decimals are preferred over fractions; however, when simple fractions are used, write them out as a hyphenated unit: "two-thirds."

Statistical treatment. When appropriate, include statistical analysis. Define all statistical measures and models clearly and include relevant analysis of statistical probabilities and the software or statistical packages used for analysis. Identify the number of independent replications of experimental treatments and the number of times individual experiments were duplicated.

Ratios. In describing mixtures, use "to" if a ratio is stated in words: "the chloroform to methanol ratio"; use a colon if a numerical ratio is provided: "chloroform:methanol (2:1, v/v)"; use a hyphen if a numerical value is not given: "used in chloroform-methanol."

Solutions. Describe solutions of common acids and bases in terms of normality (N), e.g., 1 N NaOH, and those of salts in terms of molarity (M). Express fractional concentrations by decimals: 0.1 N acetic acid (not N/10 acetic acid). Define % as (w/w), (w/v), or (v/v); 10% (w/v) signifies 10 g/100 mL. Express concentrations as micrograms per gram ($\mu g\ g^{-1}$) or micrograms per milliliter ($\mu g\ mL^{-1}$) rather than as parts per million (ppm).

Gases. To indicate volume of gases, use microliters per liter ($\mu L\ L^{-1}$) or nanoliters per liter ($nL\ L^{-1}$) rather than ppm or ppb.

Ions. Represent ions as follows: Na^{+}, Mn^{3+}, Br^{-}, PO_4^{3-}.

Isotopically labeled compounds. For simple molecules, indicate the labeling by writing the chemical formulae, for example: $^{14}CO_2$, $H_2{}^{18}O$, $^{2}H_2O$ (or D_2O), $H_2{}^{35}SO_4$. For other molecules, place the isotopic symbol in square brackets attached to the name or the formula without a hyphen or space: [^{14}C]glucose, [^{32}P]ATP, [^{2}H]C_2H_2, sodium [^{14}C]lactate. In the case of generic names, write the isotope without brackets and follow with a hyphen: ^{131}I-albumin, ^{14}C-amino acids, ^{14}C-photosynthate. Place letter and symbols indicating configuration and the like before the square bracket: D-[^{14}C]glucose, L-[^{14}C]alanine, alpha[^{14}C]naphthaleneacetic acid. Indicate the positions of isotopic labeling by Arabic numerals, Greek letters, or prefixes placed in the square bracket and before the symbol of the element to which they are attached by hyphen: D-[3-^{14}C]lactate, L-[2-^{14}C]leucine, L-[2,3-^{14}C]malate, [γ-^{32}P]ATP. Use the term U to indicate that the isotope is uniformly distributed among all six carbons: [U-^{14}C]glucose.

Molecular weight and mass. Two equivalent expressions should be distinguished: "molecular weight" (M_r) is the ratio of the mass of a molecule to 1/12 of the mass of carbon 12 and is, therefore, dimensionless. "Molecular mass" (the mass of one molecule of a substance) is not a ratio and can be expressed in daltons (D). Say "the molecular mass of X is 20,000 daltons" (20 kD) or "the molecular weight (M_r) is 20,000," but do not express M_r in daltons. Expressions such as "the 20-kD peptide" and "the mass of a band on a gel is 240 kD" are acceptable for an entity that is not a definable molecule.

Trade names and suppliers. Whenever possible, use the generic name of equipment, chemicals, or other things used in research, followed by the trade name (capitalized) in parentheses with the name and location of the manufacturer. Avoid the use of trade names and code numbers of experimental chemical compounds used in research; rather, identify such compounds by common name (American Standards Association) if such a name exists, or by chemical name and structural formula.

Materials and methods. This section should reference all standard procedures but must be complete enough so that results can be verified by other laboratories.

For reports of experiments in which growth rooms were used to simulate the natural environment, growth room conditions must be described according to the guidelines in Scientific Style and Format, Council of Biology Editors, 1994, 6th Ed., pp.434–436. The following information must be provided:

For plants grown under controlled or semi-controlled growth chamber conditions, the primary parameters needed are the timing and levels of illumination (e.g., 16/8 hr photoperiod at 200 μmol m^{-2} s^{-1}) and the temperature(s) at which the plants were grown.

For plants grown in greenhouses, the major items needed are (1) the time of year that the plants were grown, (2) whether any supplemental lighting was provided and, if so, its nature and (3) details of any temperature control.

For both chamber and greenhouse conditions, the application of any fertilization regime, if present, should also be given.

Accession numbers should be provided at the end of the Material and Methods for any data or materials available in a public repository. Novel DNA sequences must be deposited in GenBank (http://www.ncbi.nlm.nih.gov) and accession numbers provided. If a number has not yet been assigned at the time of submission, use x's as place holders to be updated later. In the case of data sets too large for inclusion in the manuscript (such as EST sequences, gene or genome annotations, genetic maps, microarray data, metabolic profiles, etc.), complete data sets must be uploaded as electronic supplementary material, to be peer reviewed and posted with the online article. See also sections on Large-Scale Data Sets and Other Supplemental Data, Distribution of Materials and Gene Expression Profiling Data.

Literature cited. Cite all references in text by last names and year of publication. Grouped text citations should be arranged from the earliest to most recent year, alphabetized by name within the same year. For entries in "Literature Cited," alphabetize by first author's last name and follow the styles below exactly for capitalization, punctuation and order of elements. **The accuracy in the "Literature Cited" section is the responsibility of the authors. The Journal will only proofread references for format. Any mistakes in reference formats may affect the conversion of html references.**

Journal articles: **Author AB,Author BB** (1977) Title of article. Plant Physiol **59**: 45–59

Book articles: **Author AB,Author BB,Author CC** (1974) Title of article. In A Smith, B Jones, eds, Title of Book, Ed 2 Vol 3. Publisher, City, pp 14–19

Theses: **Author BC** (1974) Title of thesis. PhD thesis. University, City

Online: **Author A** (year of publication) Title. Source Title, http://www.utopia.com/talent/lpb/muddex/essay

Patent: **Author B,Author BC** inventors. January 1, 1997. Endogenous nonstarch polysaccharide hydrolyzing enzymes. European Patent Application No. XXX

No Authors or Editors: Title of Booklet, Pamphlet, etc. (1975) Publisher (or Company), City

If you are citing an article that only exists as an early online version, including the PubMed ID (PMID) number will allow reviewers to link directly to the article. At the bottom of the PubMed record for the article, you will find the PMID number which you should include in the reference as follows:

Author AB,Author BB (2006) Title of article. PMID: 16723506

Write out in full all one-word journal titles. Use the BIOSIS List of Serials for abbreviations of multiple-word journal titles; write out in full the names of journals not listed there. Unpublished data (submitted articles and articles in preparation) and personal communications are not acceptable as literature citations, so they must be referred to parenthetically in the text. Please include initials and last names of all authors. Articles that are "in press" may be so designated in "Literature Cited." Note: An article may be referred to as "in press" only if it has been accepted for publication; cite the journal in which the article

will appear. For personal communications, it is the corresponding author's responsibility to ensure that those cited are aware of the citation and have approved the content of the personal communication.

TABLES

1. Number tables consecutively with Roman numerals.

2. First mention of tables in the text must be in sequential order.

3. Provide each table with a short, concise title followed by a legend that will make the general meaning of the table comprehensible without reference to the text.

4. Provide a descriptive heading for each column.

5. Do not separate data within the body of the table with new column headings or data. Do not arrange tables in sections labeled as, e.g., A or B. Instead, create another table to express data unconnected to or separate from that already presented. Authors will be contacted and asked to supply a new table if submitted in this form.

6. Use superscript lowercase letters to indicate footnotes. Asterisks or other symbols should not be used in place of the letters and will be changed accordingly.

7. For all submitted tables, please use Word's "create table" feature, with no tabbed text or tables created with spaces and drawn lines.

8. Do not use color, shading, or graphics in tables.

Numerals. Check both tabular data and numerical values reported in the text for the proper number of significant figures. For decimals smaller than one, insert a zero before the decimal point: 0.349.

Powers. To avoid numbers with many digits, express such numbers as powers of 10. The unit may be changed by the use of prefixes such as "m" or "μ." For example: enter "5" to express a g value of 0.005 under the heading g × 10^{-3} or a g value of 5,000 under the heading g × 103; conversely, express a concentration of 0.0015 M as 1.5 under the heading "concn (mM)."

FIGURE PREPARATION, DIGITAL ART, COVER SUBMISSIONS and SUPPLEMENTAL DATA

Figure preparation. Number figures consecutively according to the order in which they are called out in the text. Manuscripts submitted with substandard figures will be delayed. Format your figures according to our "Help With Digital Art" document. To assist the editors and reviewers, please also include the figure number in the figure image itself. Each figure should have its corresponding legend contained within the figure file below the figure. Figures should be unambiguous and as conceptual as possible and provide enough information so that the reader can understand them without significant input from the text. For those figures that contain more than one panel, designate the panels with capital letters, no parentheses and no periods following letters, in the upper left-hand corner. Whenever possible, position panels vertically for one-column reproduction in the Journal. For the best possible reproduction of gel blots, submit combination figures in which the labels and photographs or autoradiographs are composite images. Format the width of sequence data in the paper to one column.

Figure manipulation. *PLANT PHYSIOLOGY* does not allow certain electronic enhancements or manipulations of micrographs, gels, or other digital images using Photoshop or any other software. If multiple images are collected into a single figure, be sure to separate them clearly with lines. If a digital tool is used to adjust contrast, brightness, or color, it must be applied uniformly to an entire image; targeted alteration of only part of an image is prohibited. *PLANT PHYSIOLOGY*reserves the right to ask authors to provide supporting data on which figures were based. Please refer to J Cell Biol (2002) 158: 1151 (http://www.jcb.org/cgi/content/full/158/7/1151) for guidance on acceptable and unacceptable digital image manipulation.

Free online color is available for all accepted papers. Authors may request to have figures presented in color online and in black and white in print free of charge. The use of online-only color will be subject to editorial review to ensure that color is not critical for data interpretation but aids in presentation. The online submission system will prompt you to select whether each figure is intended to be printed in color online only; please upload only one file in RGB color mode for that figure.

NOTE ON PREPARING FIGURES FOR COLOR VISION-DEFICIENT READERS: Many readers of the journal (1 in 12 males, on average) have some form of color-deficient vision; therefore, when preparing your figures, please observe the following guidelines to ensure that all readers will be able to comprehend your data.

1: In fluorescent double-staining micrographs and DNA chips, do not use the combination of red and green; use magenta and green instead.

2: For micrographs with triple or more channels, additionally show either grayscale image of each channel or the combination of the two most important channels in magenta and green.

3: For graphs and line drawings, label elements on the graph itself rather than making a separate color-coded key. Do not try to convey information in color only, but use BOTH color and shape (solid and dotted lines, different symbols, various hatchings, etc.).

4. For more information, see the following web site: http://jfly.iam.u-tokyo.ac.jp/color/

Digital art. ***Authors must submit correctly formatted digital art files.***Please visit the following URL for a complete guide on formatting figure files. http://submit.plantphysiol.org/journals/plantphysiol/forms/digital_art.pdf

Figure labels must match text for capitalization, italics and use of symbols. If labels contain typographic errors or inconsistencies, the author will be asked to upload a corrected figure(s). Each figure file must contain its corresponding legend, included below the figure as part of each individual figure file. If you have any questions regarding the preparation of quality digital art files, please contact Ashton Wolf at the *PLANT PHYSIOLOGY* editorial office (301-296-0916;awolf@aspb.org).

Cover figure submissions. Each cover of *PLANT PHYSIOLOGY* will have an illustration selected from one of the articles published in that issue. Authors who would like to have a color photograph considered should submit an image online via BenchPress when submitting a manuscript, or email submissions to the manuscript tracking office at awolf@aspb.org. The image will then be forwarded by the manuscript office to the Editor-in-Chief for consideration. If your photograph is selected, you will be contacted by the Production Manager (Jon Munn jmunn@aspb.org , who will provide additional instructions.

Expression profiling data, large-scale data sets and other supplemental data. Supplemental data (those too large to be submitted comfortably for print publication and videos) must be submitted for inclusion in the online version of *PLANT PHYSIOLOGY* via BenchPress at the time of submission. Note that Supplemental Material normally will not be accepted as part of a Research Report, except for instances in which submissions include

videos or similar material that cannot be presented otherwise. Where possible, all supplemental figures, materials, methods and tables in Word and other Word-based supplemental data, should be combined into one pdf file with legends included for easy reader access. Supplemental materials presented at *PLANT PHYSIOLOGY* Online are subject to the same copyright restrictions as published manuscripts and cannot be presented elsewhere without proper citation. Expression profiling data and large-scale data sets, including data sets from next-generation sequencing, must be made available for review at time of submission. PLEASE NOTE: All supplemental data will be peer reviewed.

Supplemental materials must be briefly described in the manuscript (in the Results or Materials and Methods section, as appropriate) with direct reference to each item, such as Figure S1, Table S1, Protocol S1 and Video S1 (numbering should always start at 1, since these elements will be numbered independently from those that will appear in the printed version of the article) and the location of the data sets must be noted. Each supplemental item that is posted online should be listed after the Materials and Methods section under the heading "Supplemental Material" along with a brief descriptive title. Protocols for experiments presented as supplemental material must be described in the print article, at least in general terms; a more detailed methods description may be provided as a Supplemental Protocol. Text of supplemental materials such as in legends, footnotes and protocols must follow Journal style. Preferred file formats for uploading are Microsoft Excel (.xls) for large-scale data sets, PDF for figures and QuickTime format (.mov) for videos (please keep video files to 6 MB or less to allow for easy downloading).

Supplemental materials that cannot be submitted via BenchPress are occasionally hosted by authors, or for authors, on a third-party hosting site. For supplemental data of this nature, it is the responsibility of the authors to both provide instruction for access to this material within the manuscript under the heading "Supplemental Material" and to remove or disable access to this material upon completion of the peer-review process. Authors providing this sort of supplemental data are responsible for the security of the data and accept that third-party access to the confidential account may be a risk. We encourage reviewers to use an anonymous web-browsing site, such as http://anonymouse.org to further promote confidentiality.

Image data and micrography must provide information about the acquisition and processing of the images, including:

Make and model of microscope
Type, magnification and numerical apertures of the objective lenses
Imaging medium
Fluorochromes and related material
Make and model of any camera systems
Acquisition software and version
Software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

All micrographs must include a bar to indicate the scale. In general, images representing experimental data should be supported by statistical analyses based on multiple data sets. Statistical analyses of image data must be carried out on all available data, not just on data from a reprensentative experiment. Statistics and error bars should only be shown for independent experiments and not for replicates within a single experiment. For a detailed discussion of error bars in experimental biology, see Cumming et al., J. Cell Biol. 177: 7-11.

In general, large-scale data sets must be complete and must include the experimental design and details of the number of biological and analytical replicates and relevant statistical analyses (means, standard deviations, etc.) as well as methods of data normalization, transformation, missing value handling, statistical tests used, the degrees of freedom and the statistical package or program used. Manuscripts that contain gene expression profiling data are required to describe the experiments according to MIAME guidelines (Brazma et al. 2001 Nature Genetics 29: 365-371; (http://www.mged.org). Papers with unreplicated gene expression profiling experiments will not be accepted for publication in *PLANT PHYSIOLOGY*. Supporting experiments, such as real-time PCR for selected genes, generally are not a substitute for replication within the profiling experiment. Transcript profiling must include the complete set of genome sequences analyzed, ESTs identified, genes queried in transcript profiling and data supporting transcript profiling experiments must include complete sequence information (accession numbers, relevant annotation data, locus identifiers (e.g. for Arabidopsis as specified by TAIR,http://www.arabidopsis.org).

For electrophoresis or other intact seperatory approaches, proteomic and MS-based experiments, where biologically important differences in protein (gene) expression are reported confirmatory data (e.g. from validated immunoassays) are desirable. In validation studies, the sensitivity and specificity of any biomarker(s) should be provided wherever possible. The method(s) used to generate the mass spectrometry data must be described, as should the methods used to create peak lists from raw MS or MS/MS data. The names and versions of programs used for database searching must be included, the values of critical search parameters (e.g. parent ion and fragment mass tolerance, cleavage rules used, allowance for number of missed cleavages) and the name and version of the database(s) searched must be provided. With large data sets, estimates of the false positive rates are expected (e.g. through searching randomized or reversed sequence databases). Where individual proteins are identified, statistical measures of certainty must be provided. For MS/MS, included number of peptides used to identify the protein as the sequence and charge state of each peptide. For peptide mass fingerprinting, the number of peptides that match the sequence and the total percent of sequence coverage should be quoted. Reports of post-translational modifications should include the methods used to discover the modification(s). When mapped to amino acid(s) by fragmentation analysis, data should be reported as ambiguous if mapping to a single amino acid is not possible. Provision of information according to the more detailed "Minimum Information About a Proteomics Experiment" recommendations (http://www.psidev.info/MIAPE) is encouraged.

At the time of publication, supplemental data must be placed in a permanent public repository if one is available, or if none is available, in *PLANT PHYSIOLOGY* Online. Examples of accepted public gene expression repositories are SRA (http://www.ncbi.nlm.nih.gov/sra,) GEO (http://www.ncbi.nlm.nih.gov/geo) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress). As with microarray data, complete datasets from next-generation sequencing must be deposited in an accepted format in a permanent public repository with free access (e.g., GEO or NCBI's Short Read Archive sequence database). This includes data from small RNA, mRNA, specialized RNA libraries, ChIP-seq, whole-genome re-sequencing or genotyping, whole-genome bisulfite sequencing, etc. Links to web sites other than a permanent public repository are not an acceptable alternative because they are not permanent archives.