

**Analysis of the effects of the plant
growth promoting substances GR24
and smoke water on abiotically-
stressed *Nicotiana benthamiana*
seedlings**

by

Letitia Elizabeth Steenkamp



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degree Master of science at the Institute for Plant
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Supervisor: Dr Paul Hills
Faculty of Natural Sciences
Department of Genetics
Institute for Plant Biotechnology

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Declaration

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Abstract

Almost all processes during the life of a plant are affected by the environment. Changes in phytohormone, metabolite and protein levels follow in response to changes in the environment. Plant growth promoting substances can stimulate changes at these levels to facilitate increased plant growth and yields above what the plant would normally establish. In this study, the effects of two growth promoting substances, smoke water (SW) derived from bubbling smoke from the burning of plant material through water, and a synthetic strigolactone analogue, GR24, on plant growth and architecture, as well as the proteome and metabolome of salt stressed *Nicotiana benthamiana* seedlings were investigated. Physiological studies were conducted to identify the effects of the growth substances on salt stressed seedlings in a tissue culture system. Under non-stress conditions, SW treatment increased seedling fresh mass, root length and leaf area. Under salt stress conditions (100 mM and 150 mM NaCl), SW increased fresh mass, root length, leaf number and lateral root number significantly. Under non-stress conditions, GR24-treated seedlings showed increased fresh mass, leaf number and area and root length. When GR24-treated seedlings were placed under salt stress, the seedlings showed significant increases in fresh mass, leaf number and lateral root number, but only marginal increases in root length and leaf area. Despite these similarities, slight differences were observed in the metabolomes and proteomes of smoke water and GR24-treated seedlings, both with and without the addition of salt stress. Relatively few of the differentially expressed proteins could be identified with the instruments available. Changes in the metabolome indicated that photoassimilation and photosynthesis could be affected in response to smoke water and GR24 treatment. Our results suggest that smoke water and GR24 both promote growth under salt stress conditions in seedlings and we furthermore conclude that, although there are distinct overlaps between treatments, this is accomplished via slightly different mechanisms.

Opsomming

Gedurende 'n plant se lewe word omtrent alle prosesse deur die omgewing geaffekteer. Veranderinge in die omgewing word gevolg deur veranderinge in hormoon, metaboliet en proteïen vlakke. Plant groei stimulant affekteer hierdie vlakke om plant groei en -opbrengs na 'n normale vlakke te verhoog. In hierdie studie word die effek van twee groei stimulant, rook water verkry deur rook van plant materiaal deur water te borrel en 'n sintetiese strigolaktoon, GR24, ondersoek op 'n morfologiese, metaboliese en 'n proteomiese vlak in *Nicotiana benthamiana* saailinge. 'n Studie is onderneem om die veranderinge as gevolg van die onderskeie groei stimulant te ondersoek in 'n weefsel kultuur sisteem. Rook water het onder normale groei omstandighede vars en droë massa, blaar aantal asook wortel en blaar lengte verhoog. Rook water het na sout behandeling (100 en 150 mM NaCl) steeds vars massa, wortel lengte, blaai aantal en laterale wortel aantal beduidend verhoog in vergelyking met die sout stres kontrole. Behandeling met GR24 het ook vars massa, wortel lengte, blaar aantal en grootte verhoog en onder sout stres met GR24 is 'n beduidende vergroting opgemerk in vars massa, blaar grootte en laterale wortel aantal. Ongeag van die veranderinge in groei is klein verskille opgemerk in die metaboliet en proteïen studies. Net 'n paar proteïene kon positief geïdentifiseer word met die apparaat beskikbaar. Verandering in die metabool wys na veranderinge in fotoassimilasie en fotosintese in reaksie tot rook water en GR24. Hierdie resultate lei tot die gevolgtrekking dat rook water en GR24 beide groei verbeter in saailing behandel met sout en ook dat alhoewel daar sekere ooreenkomste is tussen die reaksies as gevolg van die plant groei stimulant, dit wel geskiet deur geringe verskillende meganismes.

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List of abbreviations

Abscisic acid	ABA
Adenosine triphosphate	ATP
Aluminium chloride	AlCl ₃
Arbuscular mycorrhizal	AM
Armadillo repeat	ARM
1-aminocyclopropane-1-carboxylic acid	ACC
AUXIN RESISTANT	AXR
Cadmium chloride	CdCl ₂
Carbon dioxide	CO ₂
Carotenoid cleavage dioxygenase	CCD
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	CHAPS
9- <i>cis</i> -epoxycarotenoid dioxygenase	NCED
DECREASED APICAL DOMINANCE	DAD
DWARF	D
Gas chromatography mass spectrometry	GC-MS
Gibberellin	GA
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Grams per litre	g/L
Gravitational force	<i>xg</i>
Hertz	Hz
Hectare	ha
High performance liquid chromatography	HPLC
Hours	h

Hydrogen peroxide	H ₂ O ₂
Indole-3-acetic acid	IAA
Indole-3-acetic acid acid aspartate	IAA-Asp
Indole-3-butyric acid	IBA
Isoelectric point	pI
Iso-pentenyladenine	iP
Karrikin	KAR
Kilopascal	kPa
Late embryogenesis abundant	LEA
Light-harvesting complex	LHC
Mass per volume	m/v
Matrix-assisted laser desorption/ionization	MALDI
Megapascals	MPa
Meter	m
Microlitre	μL
Millimolar per litre	mmol/L
Minutes	min
Molar	M
MORE AXILLARY BRANCHING	MAX
Murashige and Skoog	MS
Nanograms	ng
Nuclear magnetic resonance	NMR
Ornithine-δ-aminotransferase	δ-OAT
Orthophosphate	P _i
Plant growth promoting substances	PGPS

Photosystem I	PSI
Photosystem II	PSII
Δ^1 -pyrroline-5-carboxylate synthetase	P5CS
RAMOSUS	RMS
Reactive oxygen species	ROS
Ribulose 1,5-bisphosphate carboxylase/oxygenase	RuBisCO
Room temperature	RT
Salt overly sensitive	SOS
Seconds	s
Smoke water	SW
Sodium chloride	NaCl
Sodium nitroprusside	SNP
Time of flight	TOF
Tricarboxylic acid	TCA
TRANSPORT INHIBITOR RESPONSE	TIR
Volt hours	Vh
Volume per volume	v/v
Wildtype	wt

Chapter 1

General introduction

The effective germination and seedling establishment of commercial crops is a major field of study, especially in emerging countries where many of these crops are the staple food without which populations cannot survive. Considerable losses in yearly crop production can be attributed to low germination percentages and poor soil conditions which play an extremely large role in the crop production system, since soil nutrition is expensive and often not accessible, especially because of poor roads in developing countries and the high application costs. In addition, irrigation systems lead to further flushing of essential nutrients to below the rooting zone in the soil, leaving it in a state of high salinity which is often toxic to commercial crops (Badr and Taalab, 2007). Numerous changes occur in the plant under salt stress, placing it under physiological drought conditions which reduce the osmotic potential of the plant. Further effects of salt stress include excessive toxicity of Na⁺ and Cl⁻ ions to cells which disrupt organellar functioning and metabolism and cause nutrient imbalances in the plant, leading to decreases in yields (Allen, 1995).

Since the discovery of the seed germination promoting properties of smoke and the subsequent isolation of a group of compounds responsible for this activity from smoke water, namely karrikins, many likely pathways of activity have been proposed (Keeley and Bond, 1997; Roche *et al.*, 1997a; b; Flematti *et al.*, 2004; Van Staden *et al.*, 2004). The fact that karrikins interact with various hormones and possibly even mimic certain hormonal activities has received considerable attention (Merritt *et al.*, 2005; Jain *et al.*, 2008a; Hayward *et al.*, 2009; Soós *et al.*, 2009). The idea that smoke water could be used as a priming and pre-planting conditioning agent for many commercial crop species has been seen as a cheap and easy means of increasing germination percentage and seedling vigour on a large scale (Jain and Van Staden, 2007).

Strigolactones, a group of plant growth regulators involved in the inhibition of lateral shoot branching, share structural similarities with the karrikins. Strigolactones were first identified from root exudates shown to stimulate the germination of parasitic

weed seeds from the species *Striga* and *Orobanche* (Cook *et al.*, 1966; 1972; Musselman, 1980). Later, strigolactones were found to also increase hyphal branching in arbuscular mycorrhizal (AM) fungi at extremely low concentrations (down to 10^{-13} M) (Bouwmeester *et al.*, 2003; Akiyama *et al.*, 2005) and, most recently, to act as a secondary messenger to auxin in the control of lateral branching (Gomez-Roldan *et al.*, 2008, Umehara *et al.*, 2008). Research on the mode of action of strigolactones has mainly been conducted on shoot branching mutants from *Arabidopsis* (Soferan *et al.*, 2003; Booker *et al.*, 2004), rice (Ishikawa *et al.*, 2005; Zou *et al.*, 2006; Arite *et al.*, 2007), petunia (Napoli 1996; Snowden and Napoli, 2003; Snowden *et al.*, 2005) and pea (Beveridge *et al.*, 1996; Beveridge *et al.*, 1997; Morris *et al.*, 2001). Studies with other growth hormones have also shed some light on the strigolactone activation pathway, especially when looking at auxins and cytokinins, the two main hormones recognised to be controlling of apical dominance (Beveridge *et al.*, 2000; Yang *et al.*, 2004; Bennett *et al.*, 2006).

The effects of smoke water and GR24 on salt stressed plants is still largely unknown, given that most research groups have only looked at hormonal and mutant grafting changes to growth and temperature (Jain *et al.*, 2006) and nutrient (phosphate) fluctuations (López-Ráez *et al.*, 2008). During this study, similarities and differences in the signaling pathways of these two substances were investigated with and without salt stress. Smoke water and GR24 treatment results in increases in biomass in young seedlings and alleviation of salt stress, therefore, the metabolome and proteome were assessed to gain a better understanding of biomass accumulation and increases in salt tolerance in response to PGPS.

Chapter 2

The history and future of plant growth promoting substances

The dynamics of plant growth is a complicated subject. Plants are sessile organisms and due to this limitation, constant adaptation becomes key to their successful development. The precise control and regulation can be seen from embryogenesis, to changes in vegetative and reproductive form and response to stress. Environmental stimuli are the most important factors influencing plant growth and development. A plant has to react to environmental cues in order to survive and this reaction will depend on the plant's genetic make-up and developmental stage. In plants, hormones form a complex network of regulation and control. Responses to environmental cues invoke changes in levels of the various hormones, with constant cross-talk between hormone signaling networks to ensure the survival of the plant from the start of its life to the end.

Agricultural crops and natural plants are always exposed to environmental stresses. Stress arises from environmental variations causing partitioning changes in the plant. Ultimately, stress responses will direct the level of tolerance (Gaspar *et al.*, 2002). The plant's fitness to cope with an unfavorable environment is referred to as its stress tolerance (Gaspar *et al.*, 2002). Adaptation to stress occurs on physiological, genetic and biochemical levels. Oxidative stress, for example, triggers a number of physiological changes in the plant, such as impaired growth rate and leaf wilting (Allen, 1995). Reactive oxygen species (ROS) produced during oxidative stress conditions are highly reactive and toxic and cause oxidative destruction in cells (Allen, 1995). This causes many transcriptional changes, including expression of genes controlling stress hormone regulation (Allen, 1995). On a biochemical level, suppression of photosynthesis and respiration occurs and the production of enzymatic scavengers like catalase, glutathione and superoxide dismutase increases to protect various cellular components and processes against oxidative damage (Allen, 1995; Aono *et al.*, 1991).

Fire, a major environmental factor, forms a natural component of many ecosystems, from forests to shrublands and grasslands (Grumbine, 1994). It affects animals,

plants and the soil in an ecosystem in different ways to provide healthy biodiversity amongst species. Fires differ in severity, from temperature, intensity and frequency, to the amount of land and biomass burnt (Bond and Keeley, 2005). This necessitates different adaptation patterns in different areas. In grasslands like the South African Savanna, older grass is burned away to allow the growth of more palatable and nutritious young grass for animal grazing (Van Wilgen *et al.*, 2000). In forests and scrublands, undergrowth is burned clear to allow for germination of new seeds and fresh growth, since more light and water are available (DeBano *et al.*, 1998)

Fire causes great losses in vegetation, but in many fire-prone areas the plant population is adapted to fire and use it to their advantage, for new growth and breaking seed dormancy. Minerals from ash and charcoal change soil conditions, resulting in an increase in microbial activity and more nutrient-rich soil (Bradstock and Auld 1995; Van Wilgen *et al.*, 2000). In South Africa, a number of fire-prone species, including shrubs, trees, herbaceous perennials, geophytes and annuals, show significant germination responses to smoke, heat, and charred wood treatment. These include *Audouinia capitata* (De Lange and Boucher, 1990), *Leucadendron tinctorum*, *Cyrtanthus ventricosa*, *Heliophila pinnata*, *Castalis nudicaulis* (Keeley and Bond, 1997) and *Albuca canadensis* (Brown, 1993). The effects of fire and powdered charred wood on germination stimulated curiosity towards unique germination cues residing in fire and its products (Keeley *et al.*, 1985). Many fire-annual species in the fire-prone Californian chaparral in North America only germinate after fire and it was found that not only heat but charred wood treatment or heat plus charred wood could promote germination of seeds of these species (Keeley *et al.*, 1985).

After De Lange and Boucher (1990) discovered that smoke from the burning of plant material acts as a cue for breaking seed dormancy in the threatened South African Fynbos species *Audouinia capitata*, as well as 12 other Fynbos species, much research was conducted in all fire-prone areas worldwide, including the South African Fynbos, the American Chaparral, the Australian Kwongan and Mediterranean shrublands (Brown, 1993; Dixon *et al.*, 1995; Keeley and Bond, 1997; Roche *et al.*, 1997a,b). The active ingredients of smoke can be collected by bubbling the smoke

from burning dry and green plant material through distilled water (Baxter *et al.*, 1994) to form a smoke water (SW) solution. Work by Roche *et al.* (1994) showed that high concentrations of SW solutions may have an inhibiting effect on germination. This may be because concentrated solutions of SW are highly acidic and contain numerous organic compounds which could slow germination and growth (Baldwin *et al.*, 1994). This observation could also be explained in the context that smoke may play an important ecological role in managing and controlling new weed species in native and arable communities by posing as an inhibitor to some species (Adkins and Peters, 2001). It was later believed that smoke may act as a germination stimulus only when the seed surrounding conditions are optimum for germination. SW thus has a dual function of promotory and inhibitory compounds and could be very important in seed bank stability and overall plant survival (Light *et al.*, 2002). More recently, it was found that high concentrations of SW may have inhibitory or even include toxic compounds, but that with dilution the inhibitory compounds are reduced while the germination stimulatory effect is still active (Light *et al.*, 2009). The active compound in SW is able to stimulation germination at concentrations as low as 1 nM (Dixon *et al.*, 2009; Light *et al.*, 2009).

When non-fire-prone species, like genera from the family Mesembryanthemaceae, were also found to be stimulated by plant-derived SW, the chemical composition of smoke was suggested to be the most likely germination stimulus (Pierce *et al.*, 1995). This led to further investigations in a wide range of taxonomically-diverse species, from both fire-dependent and non-fire-prone environments, to identify the positive growth effect and whether these effects are independent of seed morphology and plant life structure. The South African fire-climax grass *Themeda triandra* was treated with dry (aerosol) smoke, SW, ash and aqueous ash (Baxter *et al.*, 1994). Dry smoke and SW did stimulate germination but ash and aqueous ash solutions failed to stimulate germination, suggesting that the active compound/s in smoke could be breakdown products of cellulose or hemicelluloses (Baxter *et al.*, 1994).

The effects of dry smoke and SW dilutions were tested on three South African indigenous medicinal plants *Albuca pachyklamys*, *Merwillia natalensis* and *Tulbaghia violacea* and showed that SW increased the vigour of one-week-old

seedlings (Sparg *et al.*, 2005). *Albuca pachyklamys* seedlings germinated in SW of different strengths showed a significant gain in bulb and leaf mass after 75 days, while *A. pachyklamys* and *T. violacea* showed higher seedling survival percentages after treatment with dry smoke (Sparg *et al.*, 2005). The effect of SW was also tested on *Pelargonium hortorum* hypocotyl culture *in vitro* (Senaratna *et al.*, 1999). Smoke water increased germination and also embryo development, which shows that SW could have a growth regulatory effect (Senaratna *et al.*, 1999). Interestingly, the effects of SW could be investigated in tissue culture for an extended period of time, showing that the active compound(s) in SW is stable at high temperatures (during autoclaving) and over a long time period (Senaratna *et al.*, 1999).

The main active biochemical in smoke that stimulates plant growth, 3-methyl-2H-furo[2,3-c]pyran-2-one, a butenolide compound now called karrikinolide (KAR₁), which is active at very low concentrations (10⁻⁹ M), was identified simultaneously by Flematti *et al.* (2004) and Van Staden *et al.* (2004). KAR₁ was identified after germination bioassays in *Lactuca sativa* cv. Grand Rapids and two smoke-responsive Australian species, *Conostylis aculeata* and *Stylidium affine* (Flematti *et al.*, 2004). Van Staden *et al.* (2004) achieved identical results by vacuum-concentrating smoke-saturated water derived from burned *Passerina vulgaris* and *Themeda triandra* and by using bioactivity-guided fractionation. The active compound was analyzed using gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) (Flematti *et al.*, 2004; Van Staden *et al.*, 2004).

Smoke-derived KAR₁, which is stable at high temperatures, effective at a wide range of concentrations and is water-soluble, stimulates germination of a wide range of fire-following species in the same manner as SW (Flematti *et al.*, 2004). Since its discovery, many experiments have been conducted to identify the mechanism by which KAR₁ operates, particularly in terms of its interaction with other growth hormones. KAR₁ enhances the germination of light-sensitive seeds in a similar manner to gibberellic acid (GA) (Merritt *et al.*, 2005), which confirms earlier results on light-sensitive Grand Rapids lettuce seeds where SW and GA₃ substituted for light-mediated germination (Gardner *et al.*, 2001). KAR₁-treated maize kernels showed increased leaf and root numbers, increased shoot height and a higher

survival percentage (Van Staden *et al.*, 2006). The same growth promoting traits were observed in vegetable crop seeds (tomato, okra and bean) that were treated with KAR₁ (Van Staden *et al.*, 2006). A number of further experiments showed that KAR₁ (10⁻¹⁰ M) increased root elongation, seedling mass and the number of lateral roots (10⁻⁸ M) significantly in rice (*Oryza sativa* L.) (Kulkarni *et al.*, 2006a) and that smoke-water (1:500) and KAR₁ (10⁻⁷ M) could substitute for the dark and cold stratification requirements for the germination of the southern African medicinal plant, pineapple lily (*Eucomis autumnalis* subsp. *Autumnalis*) (Kulkarni *et al.*, 2006b).

KAR₁ also has been shown to have cytokinin- and auxin-like activity (Jain *et al.*, 2008). Kinetin and KAR₁ were applied individually or in conjunction to soybean callus at increasing concentrations to determine callus yield and showed similar increases in yield at concentrations of 10⁻¹⁸ to 10⁻¹⁰ M KAR₁ and 2.5 × 10⁻⁸ M kinetin (Jain *et al.*, 2008a). When applied in conjunction, the optimum concentrations for callus growth were 2.5 × 10⁻⁸ M kinetin and 10⁻¹⁶ M KAR₁, these results show that KAR₁ is active at very low concentrations compared with cytokinin (Jain *et al.*, 2008a). Auxin-like activity of KAR₁ (10⁻¹⁶ M) was observed in a mung bean rooting bioassay, where it resulted in the same increase in rooting as when indole-3-butyric acid (IBA) (10⁻⁷ to 10⁻⁶ M) was applied (Jain *et al.*, 2008a). Here it is also evident that KAR₁ is active at extremely low concentrations compared with auxin. The effect of KAR₁ on apical dominance and shoot branching would shed some more light on its hormonal activities and interactions.

Root exudates of several plants have been shown to stimulate germination in the parasitic weed species *Striga* and *Orobanche*. The first germination stimulant to be identified was (+)-strigol (Cook *et al.*, 1966; Cook *et al.*, 1972; Musselman, 1980). A number of structurally and functionally similar germination stimulants have since been isolated and a variety of synthetic analogues, including GR24, prepared (Mangnus *et al.*, 1992; Humphrey *et al.*, 2006) (Fig. 2.1). Similar germination-stimulating compounds have now been identified from root exudates from a wide range of botanical families (Cook *et al.*, 1966; Siame *et al.*, 1993; Yasuda *et al.*, 2003) and are collectively referred to as strigolactones (Cook *et al.*, 1972; Hauck *et*

al., 1992; Siame *et al.*, 1993). The basic skeleton of natural strigolactones is made up of a tricyclic lactone connected to a butyrolactone via an enol-ether bridge.

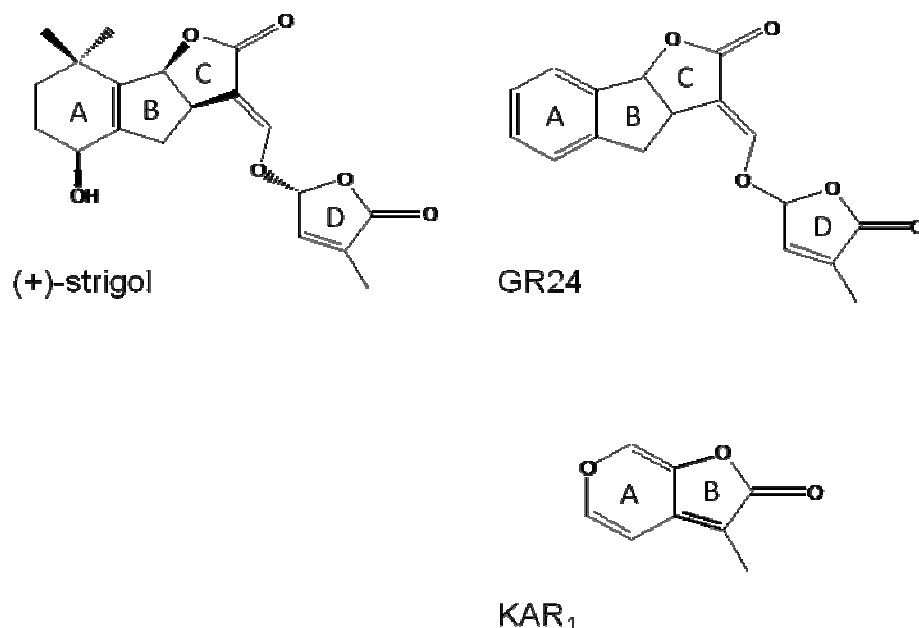


Figure 2.1 Chemical structures of the natural strigolactone (+)-strigol, the synthetic strigolactone GR24 and the main active butenolide compound identified from smoke water, namely 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (karrikinolide, KAR₁). These two groups of compounds share the same furanone ring structure, labelled D in the strigolactones and B in KAR₁.

The methyl-substituted butenolide ring (D-ring) and D- and C-ring connecting enol-ether bridge with α,β -unsaturated ester functionality are essential for the activity of strigolactones, whilst the adjoining structure (A-C-rings) probably modifies the specific activity of the compound (Mangnus and Zwanenburg, 1992). It has been proposed that the molecular mechanism as germination stimulus is achieved by the addition of a nucleophilic species, present in the receptor site, to the enol-ether bridge double carbon bond, followed by the cleaving of the D-ring (Akiyama *et al.*, 2010). This would result in the AB part and, when present, the C-ring being bound to the receptor and active in stimulating germination (Mangnus *et al.*, 1992; Wigchert and Zwanenburg, 1999). For instance, Nijmegen-1, a synthetic strigolactone, has an open C-ring structure but is still active at 1 mg/L in stimulating germination of *S. hermonthica* seeds being in the same range as the activity of GR24 (Zwanenburg *et*

al., 2009). Consequently the D-ring presumably is the essential part as it needs to fit precisely at the receptor site after cleavage (Nefkens *et al.*, 1997; Mangnus and Zwanenburg, 1992). Furthermore, results from experiments on GR24 with nucleophilic compounds support the proposed mechanism. When the D-ring was replaced by other leaving groups, however, GR24 was rendered inactive (Magnus and Zwanenburg, 1992). Interestingly the D-ring of strigolactones is analogous to the B-ring of the KAR₁ molecule (Flematti *et al.*, 2004). KAR₁ also has a methyl substituted butenolide ring (B-ring) and an α,β -unsaturated ester functionality (A-ring) similar to strigolactones but with different conjugations and this could explain why they share similar germination stimulation activity (Zwanenburg *et al.*, 2009)(Fig. 2.1). Strigolactones are only known to trigger germination in parasitic weed species, while KAR₁ has shown to stimulate germination of some parasitic weed species amongst a vast plethora of other genera (Nelson *et al.*, 2009). Because of this it may be possible that different plant species could convert these strigolactones and karrikins into separate bioactive compounds which are metabolically converted into active plant growth regulators that are perceived differently by species because of inherent molecular mechanisms and receptor activations (Nelson *et al.*, 2009).

In addition to their ability to stimulate germination of parasitic weed seeds, strigolactones have also been shown to increase hyphal branching in arbuscular mycorrhizal (AM) fungi at extremely low concentrations (Bouwmeester *et al.*, 2003). Arbuscular mycorrhizal fungi form an obligate symbiosis with more than 80% of land plants. They obtain carbon sources from their host and, in return, supply essential inorganic compounds such as phosphates and nitrates and other soil minerals (Giovannetti *et al.*, 1993). It has been shown that these fungi respond to a signal secreted from the host roots, in order to form these symbiotic associations. Upon recognition of the host-derived signal, the fungus starts branching extensively, tracing the signal concentration gradient towards the host root where the fungus attaches to the roots of hosts to form the symbiosis (Giovannetti *et al.*, 1993; Giovannetti *et al.*, 1994). This signal secreted from the host roots that activate hyphal branching of AM fungi was isolated from the model plant *Lotus japonica* and is a strigolactone, (+)-5-deoxystrigol (Akiyama *et al.*, 2005).

Root extracts from carrot stimulated cell proliferation of the AM fungus, *Gigaspora rosea* at concentrations as low as 10^{-13} M (Besserer *et al.*, 2006). The synthetic strigolactone GR24 caused a rapid increase of mitochondrial density and respiration in two phylogenetically-distant AM fungi *Glomus intraradices* and *Glomus claroideum* (Besserer *et al.*, 2006). This study was extended to include other compounds isolated from root extracts, such as parthenolide, artemisinin and dihydrosorgoleone (Besserer *et al.*, 2006). None of these compounds stimulated germination of *Gigaspora rosea*, which demonstrates that it is only the root derived strigolactones which encourage hyphal branching in the AM fungi (Besserer *et al.*, 2006).

The germination-stimulating activity of strigolactones was recently tested with hydroponically-grown *Arabidopsis thaliana* (Goldwasser *et al.*, 2008). The root exudates were extracted, diluted in methanol and supplied to seeds of the parasitic plant species *Orobanche aegyptiaca*. Results showed that the root exudates promoted germination in this parasitic species. After reverse phase high performance liquid chromatography (LC/MS) analysis on the root exudates, it was shown that *Arabidopsis* produced three major germination stimulants, of which one, Orobanchol, was a strigolactone and the other two, tetrahydro-strigol and didehydro-strigol isomers (Goldwasser *et al.*, 2008). This was the first report which shows strigolactone production in a non-mycotrophic plant (Goldwasser *et al.*, 2008).

As stated earlier, hormonal interaction plays a major role in plant growth and development. A hormone is identified as a small molecule often (but not always) synthesized in one part of the plant and transported to another where it induces a significant change on growth or development at very low concentrations (<http://www.hcs.ohio-state.edu/hcs300/hormone.htm>) Major classes are abscisic acid, auxin, cytokinin, ethylene, brassinosteroids, gibberellins, jasmonates and salicylic acid and now also strigolactones.

Auxin, synthesized in young leaves and in developing fruit and seeds is classically believed to be involved in phototropism, regulation of root system architecture and apical dominance (Phillips, 1964; Blake *et al.*, 1983; Nick *et al.*, 1992). Apical dominance occurs when apically-produced auxin is in abundance and causes shoot apex growth while inhibiting the growth of lateral buds. Auxin (IAA) is transported

basipetally from the apical bud down the plant shoot, affecting the more auxin-sensitive lateral buds. When the apical bud is removed (decapitation) the source of auxin is removed, lowering auxin concentration and initiating lateral bud growth (<http://plantphys.info/apical/apical.html>). Auxin interacts with cytokinin which is synthesized in the root and transported through xylem to the leaves, to signal shoot branching (Sachs and Thimann, 1967; Coenen and Lomax, 1997). Auxin has been shown to down-regulate cytokinin synthesis in the node and in the root in *Arabidopsis* (Tanaka *et al.*, 2006) through an *AXR1*-dependent pathway (Nordström *et al.*, 2004). Consequently, shoot branching is regulated by a network of hormonal interactions throughout the plant.

When it was observed that a class of shoot branching mutants from different species could all be phenotypically corrected by the addition of strigolactones, a new bioactive strigolactone mechanism was hypothesized (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). Shoot branching is a very important aspect in crop production, especially in commercial flower production, as the number of branches determine the number of flowers a specific plant will bear. Bud outgrowth or shoot branching can be activated through environmental stimuli (mostly when the primary shoot apex is damaged) (Thimann and Skoog, 1933), through hormonal activation (auxin and cytokinin) (Sachs and Thimann, 1967) or through signals coming from the rest of the plant (Tanaka *et al.* 2006).

A variety of mutants with increased shoot branching in *Arabidopsis*, rice (*Oryza sativa*), pea (*Pisum sativum*) and petunia (*Petunia hybrida*) attracted considerable interest and led to extensive studies to understand the mechanism of strigolactone biosynthesis and the importance of strigolactones in regulating shoot branching. It was found that all of these mutants share mutations in genes encoding for carotenoid cleavage dioxygenases (CCDs). In *Arabidopsis*, the *MORE AXILLARY GROWTH* genes *MAX4* (Soferan *et al.*, 2003) and *MAX3* (Booker *et al.*, 2004) were found to encode CCD8 and CCD7 respectively. Later it was found that these genes are involved in strigolactone biosynthesis, since the defective shoot branching mutant phenotype could be corrected by addition of exogenous strigolactones and mutants have lower strigolactone levels compared with the wild-type (wt) (Booker *et al.*, 2005; Bouvier *et al.*, 2005; Alder *et al.*, 2008). *MAX1*, which encodes a

Cytochrome P450 protein that also acts at the strigolactone biosynthetic level, is believed to act downstream from the two CCDs (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008).

A fourth *max* shoot branching mutant, *max2*, was identified which contained wt levels of strigolactones but did not respond to treatment with exogenous strigolactones (Stirnberg *et al.*, 2002, Gomez-Roldan *et al.*, 2008). *MAX2* encodes an F-Box protein and is probably involved in strigolactone signal transduction, localized in the shoot or specific area of action to inhibit branching (Gomez-Roldan *et al.* 2008, Umehara *et al.*, 2008). F-Box proteins, which function as subunits of the multiprotein Skp1p–Cdc53p–F-box protein (SCF)-type E3 ligases, are involved in signal transduction in numerous hormone pathways and other conserved developmental pathways in plants and animals (Patton *et al.*, 1998; Craig and Tyers, 1999). Upon cloning of the *MAX2* gene, it was discovered that it is identical to the previously identified *ORE9* gene (Stirnberg *et al.*, 2002). *ORE9* was isolated as a positive regulator of *Arabidopsis* leaf senescence (Woo *et al.*, 2001) and later as an important mediator for hypocotyl elongation in the light (Stirnberg *et al.*, 2002). Furthermore, it was found that *max2/ore9* mutants display enhanced resistance to oxidative stress (Woo *et al.*, 2004) and suppress the enhanced susceptibility to drought caused by mutations in a gene encoding *CTR1*-like protein kinase (Tang *et al.*, 2005). *MAX2* is expressed universally in the plant and not only at nodes, which explains how it is able to fulfill some of these diverse roles (Stirnberg *et al.*, 2002).

The pathway by which strigolactones stimulate seed germination and hyphal branching is still poorly understood. Currently it is believed that the carotenoid cleavage dioxygenases synthesize a mobile signaling component in the chloroplast, which moves acropetally into the shoot (Beveridge and Kyojuka, 2010). Here, the *MAX1* cytochrome P450 protein catalyses the further biochemical conversion of this bioactive compound into an active strigolactone (Beveridge and Kyojuka, 2010). The strigolactone may possibly then bind to an F-Box protein in the bud or node, although the receptor is not known. The fact that strigolactones are active at very low concentrations could explain why they function through a receptor-mediated signaling mechanism (Bouwmeester *et al.*, 2007). For parasitic plant species, this could be equivalent to hormone perception through receptors (Wigchert and

Zwanenburg, 1999; Matusova *et al.*, 2005), for example, the perception of gibberellins by the *Arabidopsis* F-Box protein SLY1 and the *Arabidopsis* F-Box protein TIR1, which is an auxin receptor (Ueguchi-Tanaka *et al.*, 2005; Kepinski and Leyser, 2005). This is why it is possible that the F-Box protein which is involved in strigolactone signal transduction, acts as a receptor (Beveridge and Kyoizuka, 2010). Table 2.1 represents known genes involved in strigolactone biosynthesis and signaling.

As previously mentioned, orthologues to these *MAX* genes, and some other branching-related genes, have been identified in other species. Pea branching mutants showed defective CCD8 protein function encoded by *RAMOSUS 1 (RMS1)* and in the CCD7 protein encoded by *RMS5* (Beveridge *et al.*, 1996; Beveridge *et al.*, 1997; Morris *et al.*, 2001). Furthermore, root exudates from *rms5* and *rms1* mutants induced less AM fungal hyphae branching and less germination of *Orobanchae* seeds (Gomez-Roldan *et al.*, 2008). *RMS4* in pea encodes an F-Box protein orthologue to *MAX2* and the action of *RMS2* is speculated to be as a feedback inhibition signal of the CCD genes, since it seems to control levels of a mobile substance(s) that interact with exogenous auxin in controlling shoot branching (Beveridge *et al.*, 2000).

In rice the same trend was observed, with *DWARF10 (D10)* that encodes CCD8 and *D17/HTD1* that encodes CCD7 (Ishikawa *et al.*, 2005; Zou *et al.*, 2006; Arite *et al.*, 2007). The rice *D3* gene codes for an F-Box protein, also an orthologue to *Arabidopsis MAX2*, and also does not respond to exogenous strigolactone treatment (Ishikawa *et al.*, 2005). The rice *d10* and *d17/htd1* mutants also showed less signaling towards hyphal branching and therefore, less root infection by *Striga hermonthica* (Umehara *et al.*, 2008)

In petunia, *DECREASED APICAL DOMINANCE 1 (DAD1)* encodes CCD8 (Napoli, 1996; Snowden and Napoli, 2003; Snowden *et al.*, 2005). It has been suggested that *DAD2* may be involved in strigolactone signal transduction, but it is thought not to be an orthologue to *Arabidopsis MAX2*. *DAD3* may possibly be involved in strigolactone biosynthesis (Simons *et al.*, 2007). The *dad3* mutant shows decreased plant height and increased shoot branching, but the defects are not as extreme as the *dad1* and *dad2* mutants (Simons *et al.*, 2007). *DAD1* and *DAD3* may act on the

same step to produce a branching signal, since these mutant phenotypes could not be reverted with grafting to each other (Simons *et al.*, 2007). All three of these petunia branching mutants have reduced strigolactone levels. These findings all show that strigolactones, derived from a carotenoid precursor via a pathway which involves carotenoid cleavage dioxygenases 7 and 8 (CCD7 and CCD8) and a Cytochrome P450 protein, are involved in shoot branching (Matusova *et al.*, 2005). The shoot branching pathway is therefore referred to as the MAX/RMS/D pathway.

Several other genes that are potentially involved in strigolactone biosynthesis or signaling have also been isolated through analyses of branching mutants, although their precise functions have not yet been elucidated. In rice, *D14* and *D27* possibly function in the same pathway as *D3* and *D10*, since the same feedback regulation of *D10* expression was seen in *d14* and *d27* mutants as in the *d3* and *d10* mutants (Arite *et al.*, 2007). The *d14* mutant is phenotypically similar to a strigolactone deficient mutant, however, the mutant phenotype cannot be corrected with strigolactone application (Arite *et al.*, 2009). It was determined that *D14* codes for an α/β -fold hydrolase protein whose function depends on binding with a small molecule and which shows similarities to other proteins with roles in hormone metabolism and signaling (Kaneko *et al.*, 2005; Arite *et al.*, 2009). Similar α/β -fold hydrolase proteins include GID1, a receptor for gibberellins, and SABP2, a salicylic acid binding protein (Forouhar *et al.*, 2005; Kumar and Klessig, 2003). The GID1 protein has no enzymatic activity and allows GA signaling through binding to bioactive GAs and activating an associated F-box protein that degrades negative regulators (Harberd *et al.*, 2009). It is proposed that *D14* might have a similar effect on strigolactone signaling through feedback regulation or, alternatively *D14* may act via an enzymatic conversion similar to SABP2 which converts methyl salicylate to salicylic acid (Kumar and Klessig, 2003; Forouhar *et al.*, 2005; Beveridge and Kyoizuka, 2010). If, however, the *D14* α/β -fold hydrolase does enzymatically convert a strigolactone into a different bioactive product, an F-Box protein would still be necessary for signal transduction (Arite *et al.*, 2009; Beveridge and Kyoizuka, 2010).

Upon characterization of the rice mutant *d27*, which phenotypically has increased branching and reduced plant height, it was observed that the mutant has very low levels of 2'-epi-5-deoxystrigol, the major strigolactone which was identified in root

exudates from rice seedlings (Lin *et al.*, 2009). The mutant branching phenotype could be corrected with GR24. *D27* encodes a novel iron-containing protein and is probably involved in strigolactone biosynthesis, downstream from CCD7 and CCD8 (Lin *et al.*, 2009).

In tomato (*Solanum lycopersicon*), the *sl-ort1* mutant with more lateral shoot branching was previously identified as resistant to the parasitic plant *Orobanche* and was found to have lower levels of AM fungi (*Glomus intraradices*) colonization, probably because of its inability to stimulate AM hyphal branching (Koltai *et al.*, 2010). The mutant also has lower CCD7 levels compared with the wt and biochemical analysis of the root extracts suggests that it produces only minute amounts of two tomato strigolactones (Koltai *et al.*, 2010a). Lateral shoot branching could be phenotypically corrected with GR24 addition, but root exudate strigolactone and CCD7 levels could not be rescued in grafting experiments (Koltai *et al.*, 2010b). It appears that ORT1 could be involved in downstream signaling from the CCDs or even act as a feedback inhibitor of strigolactone biosynthesis.

The fact that the CCD family includes a member of the 9-*cis*-epoxy-carotenoid dioxygenase (NCED) enzyme subfamily which cleaves neoxanthin during ABA biosynthesis suggests that ABA may have an effect on strigolactone biosynthesis or that they may share similar biosynthetic pathways (Matusova *et al.*, 2005; López-Ráez and Bouwmeester, 2008). It has also been proposed that strigolactones might be formed through the action of NCEDs as well as CCDs, since *Arabidopsis nced* mutant root exudates did not induce seed germination in *Striga hermonthica*, whereas wt root exudates do (Matusova *et al.*, 2005).

Strigolactones act as a long-distance messenger for auxin because of their inhibitory effect on bud outgrowth (Dun *et al.*, 2009; Ferguson and Beveridge, 2009). Grafting experiments in pea *rms1* and *rms2* mutants and wt plants suggested that strigolactone acts downstream of auxin (Beveridge *et al.*, 2000). However, further data suggests that strigolactones act upstream from auxin in branch control, since the axillary bud of the *Arabidopsis max4* mutant is resistant to apically-supplied auxin (Bennett *et al.*, 2006). Branching increased in this mutant regardless of auxin supply, suggesting that auxins and strigolactones must interact in branching and that

strigolactone may even control the transport of auxin to some extent (Bennett *et al.*, 2006). Furthermore, when IAA was applied to *max3* and *max4* mutants, MAX3 and MAX4 transcript levels are slightly but significantly up-regulated (Hayward *et al.*, 2009). Auxin up-regulates the *IAA* gene via the AXR1/TIR1 pathway that initiates, for example, apical dominance (Yang *et al.*, 2004). When IAA was applied to the auxin response mutant *axr1-3*, MAX3 and MAX4 transcript levels were not induced and point to the conclusion that MAX3 and MAX4 gene transcription are induced by auxin in an AXR1-dependent manner (Hayward *et al.*, 2009). AXR1 in *Arabidopsis* shoots controls a large proportion of the feedback regulation (Hayward *et al.*, 2009). This phenomenon was also observed in decapitated pea plants, where apically applied IAA (3 and 19 mM) enhanced stem auxin content and also increased RMS1 and RMS5 expression significantly (Hayward *et al.*, 2009; Foo *et al.*, 2005). IAA controls the transcript levels of RMS1 and RMS5, both in intact plants and in decapitated plants, which shows that IAA does not exclusively control shoot branching or strigolactone synthesis but there is also some IAA-independent feedback signal (Foo *et al.*, 2005). Shoot branching can therefore function apart from strigolactone stimulation or interference (Ferguson and Beveridge, 2009). Moreover, these pea grafting experiments showed that auxin, indole-3-acetic acid (IAA), and strigolactone signaling does not necessarily occur in a linear pathway where the one inhibits the other up- or downstream in the meristem (Ferguson and Beveridge, 2009).

Auxin can act as a feedback mechanism from the target area in the plant back to the site of strigolactone synthesis in the MAX/RMS/D pathway through AXR1 and TIR1 in *Arabidopsis* acting as a feedback signal mechanism (Beveridge and Kyosuka, 2010; Hayward *et al.*, 2009). The interaction between auxin and strigolactone in shoot branching is still under debate, whether strigolactones acts up- or downstream of auxin is not clear. Since auxin also inhibits cytokinin biosynthesis which promotes branching, an integrated network is at hand with many unresolved outcomes (Tanaka *et al.*, 2006).

Table 2.1 Representation of all the known genes and their proposed function in strigolactone biosynthesis and signaling in four well-studied species

Function	<i>Arabidopsis thaliana</i>	<i>Oryza sativa</i>	<i>Pisum sativum</i>	<i>Petunia hybrida</i>	Comments
CCD8	<i>MAX4</i>	<i>D10</i>	<i>RMS1</i>	<i>DAD1</i>	
CCD7	<i>MAX3</i>	<i>D17/HTD1</i>	<i>RMS5</i>		
P450	<i>MAX1</i>				
F-Box Protein	<i>MAX2</i>	<i>D3</i>	<i>RMS4</i>		
Fe-containing Protein		<i>D27</i>			Involved in SL biosynthesis, downstream of CCD7 and CCD8 (Lin <i>et al.</i> , 2009)
α/β-hydrolase		<i>D14/D88/HTD2</i>			Similar type of protein to GID1 and SABP2 – could be a SL receptor (Arite <i>et al.</i> , 2007; Gao <i>et al.</i> , 2009; Liu and Van Staden, 2009)
Unknown			<i>RMS2</i>		Feedback inhibition of CCD genes (Beveridge <i>et al.</i> , 2000)
			<i>RMS3</i>		Response gene, localized in the bud, could be similar to D14 (Beveridge <i>et al.</i> , 2008)
				<i>DAD2</i>	Possibly involved in SL signal transduction (Simons <i>et al.</i> , 2007)
				<i>DAD3</i>	SL biosynthesis, possibly a <i>MAX3</i> orthologue (Simons <i>et al.</i> , 2007)

It is now known that strigolactones are involved in shoot branching, stimulate hyphal branching in AM fungi and germination of parasitic weed species, but exactly how they contribute to these developmental changes is still unclear. An investigation on a post-transcriptional level will broaden insight on strigolactone synthesis and mode of action.

Nicotiana benthamiana is widely used as a model plant organism for oxidative and abiotic stress tolerance, the leaves are delicate and can be wounded easily and the plant grows well under controlled conditions and has generally highly homologous expressed sequence tags (ESTs) with agriculturally significant *Solanaceous* crops, such as tomato, potato, pepper, and petunia (Goodin *et al.*, 2008). Therefore, the aim of this study was to investigate the effects of smoke water and GR24 on a physiological and molecular level in salt stressed *Nicotiana benthamiana* seedlings. Given that the active signalling pathways of smoke water and GR24 are relatively unknown, in-depth analysis on the metabolome elucidated some interaction in stress related responses.

Chapter 3

Stress, smoke and strigolactones: Changes in plant growth from the root to the shoot

3.1 Introduction

Plant growth adapts in response to environmental cues, such as extreme temperature changes, drought, salinity and heavy metal toxicity in the soil. Any change in the environment causes some stress effect on the plant, and adjustments at genetic, molecular and physiological levels follow to restore functional growth. Abiotic stresses, especially salt and drought stresses, are the primary cause of crop losses worldwide (Wang *et al.*, 2003). Not only does drought impair plant growth, but in arid and semiarid areas, solutes from irrigation water accumulate in the soil and may reach toxic levels that negatively affect plant growth (Badr and Taalab, 2007). Furthermore, infrequent and little rain in these areas means that the accumulation of solutes is not flushed to below the rooting zone (Badr and Taalab, 2007). Hence, high salt concentrations in the soil destroy soil structure, decreasing porosity and soil permeability.

Osmotic disturbance is one of the main effects of salt stress. The osmotic potential of the roots controls the movement of water from the soil into the roots via osmosis. The passive process of osmosis facilitates the passing of water through a partially-permeable membrane down a water potential gradient. The direction of the water flow depends on solubility properties in the soil and also on the charge, or chemistry, as well as the size of solutes on either side of the membrane (Haynie, 2001). Under saline soil conditions, water may be freely available in the soil, but unavailable to the plant because instead of water moving into the roots, it passively moves out of the roots to the soil. Plants therefore have developed many mechanisms to deal with osmotic disturbances, which include living in symbiosis with Arbuscular Mycorrhizal (AM) fungi. AM fungi are associated with over 80% of all terrestrial plant species and promote plant growth and salt tolerance in many species (Akiyama *et al.*, 2005). The mechanisms by which this is achieved include enhancing nutrient acquisition by increasing root surface area (Al-Karaki and Al-Raddad, 1997). In addition, a higher level of osmolytes such as proline, betaine and polyamines accumulate in plants

living in symbiosis with AM fungi (Jindal *et al.*, 1993). For example, mycorrhizal mung bean plants have a higher proline content than non-mycorrhizal plants at different salt concentrations (Jindal *et al.*, 1993). A similar observation was made in soybean AM and non-AM plants treated with increasing salt concentrations (0, 50, 100, 150 and 200 mM NaCl) (Sharifi *et al.*, 2007). In the presence of AM fungi, salt-stressed plants photosynthesise more efficiently and antioxidant production increases. More salt-tolerant plants also have a lower transport rate of Na⁺ and Cl⁻ to leaves, which prolongs normal photosynthesis compared with salt-sensitive species (Munns, 2002). These adjustments extend normal plant life, but slow growth (Munns, 2002).

High salt concentrations in the soil cause an accumulation of ions in the cell, leading to toxicity. These ions include Na⁺, Cl⁻ and SO₄²⁻, which displace plasma membrane Ca²⁺ and lower membrane transport activity (Cramer and Läuchli, 1986). A change in plasma membrane permeability causes a leakage of K⁺ from the cell and decreases control of plasma membrane function (Cramer *et al.*, 1985). Na⁺ also disrupts ion homeostasis by competing with K⁺ for transport proteins. This negatively disturbs plant nutrient uptake and other vital processes such as cell elongation, leaf and stomatal movements and germination (Kochian and Lucas, 1988). Ion toxicity also inactivates enzymes by binding to sulphhydryl groups and subsequently inhibits protein synthesis (Kneera and Zenk, 1992). Furthermore, high concentrations of Na⁺ and Cl⁻ in the chloroplast inhibit photosynthesis through affecting either carbon metabolism or photophosphorylation. Other secondary effects include accumulation of toxic molecules such as reactive oxygen species (ROS) that eventually lead to cell death. A basic way to combat these stress conditions is for plants to develop extensive root systems, reaching deeper into the soil to accumulate nutrients and water, and to reduce leaf size or lose leaves via leaf abscission (Hasegawa *et al.*, 2000).

3.1.1 Plant growth promoting substances

Genetic variation within plant species and evolutionary change has resulted in the development of a range of mechanisms for protection and tolerance against salt and heavy metal stress. Plants can productively use cues from the environment to their own advantage, such as heat and chemical stress caused by wild-fires. After fire,

chemicals leach into the soil from organic matter left behind, causing changes in soil nutrient supply and bacterial activity which facilitate new growth and stimulate the germination of dormant seeds.

Smoke from fire is known to stimulate the germination of approximately 1200 species from more than 80 genera worldwide (Dixon *et al.*, 2009). The use of fire and smoke in agriculture is not a new practice. Maize (*Zea mays*) farmers have been storing their seeds above fireplaces for centuries. Studies by Modi (2002; 2004) have shown that seeds stored in this way have substantially higher germination percentages than untreated seeds and produce heavier and taller seedlings. The effects of fire and smoke on germination have been thoroughly studied, but the molecular effects of smoke water on plants are still being elucidated.

Smoke water (SW) application has shown beneficial effects in temperature-, osmotic- and salt-stressed plants. For example, tomato seeds treated with SW and exposed to temperatures altering from 40°C to 25°C and from 10°C to 25°C showed a higher vigour index compared with control seeds (Jain *et al.*, 2006). Smoke water-treated tomato seeds showed significant increases in germination compared with the untreated control upon exposure to increasing temperature conditions, but even though germination was stimulated by SW, not all treated seeds developed into normal seedlings, especially at higher temperatures (Jain *et al.*, 2006).

The bioactive compound in smoke, KAR₁, was used as pre-treatment on tomato seeds exposed to increasing temperatures (10, 15, 20, 25, 30 and 35°C). All treated seeds had increased germination percentages compared with the controls (Jain *et al.*, 2006). Furthermore, KAR₁-primed seeds treated with NaCl (0, 100, 125, 150 mM) as a salt stress treatment, and polyethylene glycol 6000 (0, -0.05, -0.15, -0.30 and -0.49 MPa) as an osmotic stress, had higher germination percentages, seedling vigour and seedling weight at all concentrations for all treatments compared with the control plants (Jain *et al.*, 2006).

When seeds of the cereal crop *Eragrostis tef* were treated with SW and exposed to various temperature and osmotic stress conditions, SW increased the germination percentage at all temperatures and also increased seedling length and overall vigour at 25, 30, 35 and 40°C and alternating 30/15°C (Ghebrehiwot *et al.*, 2008). In

addition, SW-treated seeds had higher germination percentages at a lower osmotic potential compared with control seeds and resulted in taller and more vigorous seedlings at an osmotic potential of 0 and -0.30 MPa (Ghebrehiwot *et al.*, 2008).

Smoke water and KAR₁ enhance germination and relieve stress, but the molecular mechanism via which this occurs remains largely unknown. A microarray study on SW-treated maize seeds showed high expression levels of stress-responsive genes (particularly abscisic acid [ABA]-related genes) and provided insight into the interaction of SW and phytohormones to reduce stress (Soós *et al.*, 2008). It was suggested that SW mimics stress conditions, which leads to better adaptation later during development (Soós *et al.*, 2008). KAR₁ enhances expression of GA biosynthetic genes during seed imbibition (Nelson *et al.*, 2009). GA typically initiates germination by suppressing the germination inhibiting activities of ABA (Olszewski *et al.*, 2002). However, ABA suppresses the germination response to KAR₁, which suggests that KAR₁ interacts with these growth-regulating hormones on a biochemical level (Nelson *et al.*, 2009).

Strigolactones, a newly discovered class of plant hormones, are secreted from roots of host plants to stimulate growth of AM fungi by acting as a chemical cue to facilitate identification of the host roots (Bouwmeester *et al.*, 2003). Certain species of parasitic plants, such as those from the genera *Striga* and *Orobancha* (Cook *et al.*, 1966; 1972; Wigchert and Zwanenburg, 1999), have evolved to take advantage of this chemical signaling between plants and their mycorrhizal symbionts. Seeds from these species are extremely small and contain minimal storage reserves; they therefore need to infect their host plants within a very short time following germination before these stores are exhausted. The seeds remain dormant in the soil until germination is stimulated by strigolactones exuded from nearby roots of their host plants (Press, 1995; Butler 1995). Strigolactones share structural similarities with KAR₁ and it has been proposed that karrikins and strigolactones may act on the same signalling pathway (Flemmati *et al.*, 2004). Smoke water has also been shown to stimulate germination in a number of parasitic weed species like *Orobancha aegyptiaca* and *Chenopodium album* (Nun and Mayer, 2005; Daws *et al.*, 2007). Other studies have found contradicting results regarding the stimulation of germination of parasitic weed species by KAR₁. To investigate the similarities between karrikins and strigolactones, *Arabidopsis* seeds were treated with four

synthetic karrikins and the synthetic strigolactone analog GR24 (Nelson *et al.*, 2009). KAR₂ was the most effective in stimulating germination, KAR₁ and KAR₃ were less effective and KAR₄ had either no effect or an inhibitory effect on germination (Nelson *et al.*, 2009). GR24 stimulated germination, but only at concentrations a hundred-fold greater than that of the weakest active karrikin, KAR₃ (Nelson *et al.*, 2009). In addition, GR24 could not stimulate germination in *Brassica tournefortii*, a strongly karrikin-responsive species and KAR₁ could not stimulate germination in the strigolactone-responsive parasitic weed *Orobanche minor* (Nelson *et al.*, 2009). Chiwocha *et al.* (2009) found that synthesized KAR₁ could not stimulate *O. aegyptiaca* germination (Chiwocha *et al.*, 2009). Since it is very difficult to purify KAR₁ from SW, it is possible that purified KAR₁ may in some instances not be completely pure, which might explain the differences between KAR₁ treatments in different experiments (Chiwocha *et al.*, 2009). In the bioassay-guided fractionation purification of KAR₁, KAR₁ and pyrone are isolated in the same fraction even after several separation steps, and also have similar UV absorbance and mass spectrum readings (Flematti *et al.*, 2008). Pyrone shares structural similarities with coumarin, which stimulates germination in *O. aegyptiaca* (Num and Meyer, 2005). Consequently it was hypothesized that karrikins and strigolactones are not interchangeable and may act via distinct mechanisms, because although both stimulate germination, it appears to be species-specific with strigolactones being more selective (Chiwocha *et al.*, 2009; Nelson *et al.*, 2009). It is also possible that different plant species convert strigolactones and karrikins into separate bioactive compounds and that these could metabolically be converted into an active plant growth regulator that is perceived differently by species because of inherent molecular mechanisms and receptor activations (Chiwocha *et al.*, 2009).

The post-germination effects of strigolactones on salt-stressed plants have, as far as can be ascertained, never been studied. There is, however, some evidence which suggests that strigolactones may play a role in various stress conditions. Production of orobanchol, a natural strigolactone first isolated from red clover root extracts, was significantly stimulated upon limited-phosphate (Pi) conditions, suggesting that Pi availability regulates strigolactone production to some extent (Yoneyama *et al.*, 2007). Similarly, strigolactone biosynthesis was also promoted in tomato plants exposed to Pi starvation conditions (López-Ráez *et al.*, 2008). Phosphate-deprived tomato was grown with and without a carotenoid biosynthesis inhibitor, fluridone,

together with the tomato ABA mutant *notabilis* (López-Ráez *et al.*, 2008). Root exudates were collected for all three treatments and their corresponding controls and applied to different weed seeds and AM fungi (López-Ráez *et al.*, 2008). Phosphate starvation increased the germination percentage of *Orobancha ramosa* and hyphal branching in the AM fungi. The fluridone-treated root exudates decreased the germination percentage of the weed seeds. Liquid chromatography-mass spectrometry/mass spectrometry (LC/MS/MS) analysis of the tomato root exudates showed that the biological action and changes were due to changes in the occurrence of a number of strigolactones (López-Ráez *et al.*, 2008). Root exudates from the tomato ABA mutant *notabilis* decreased germination by 40% compared with the wild-type. The *notabilis* mutant has a null mutation in the gene *LeNCED1*. This could suggest that the enzyme NCED1 plays a role in strigolactone synthesis and/or regulating strigolactone biosynthesis on a post-transcriptional level (López-Ráez *et al.*, 2008). Subsequently, NCEDs play a role in ABA biosynthesis and reduced induction of ABA may trigger reduced strigolactone biosynthesis (López-Ráez and Bouwmeester, 2008). The ABA biosynthetic pathway involves cleavage of carotenoid precursors similar to those used in the strigolactone biosynthetic pathway (López-Ráez and Bouwmeester, 2008). Furthermore, in three *Arabidopsis* ABA-deficient mutants decreased levels of strigolactone biosynthetic genes, *LeCCD7* and *LeCCD8* were observed as well as reductions in root exudate strigolactone levels, specifically, solanacol and the two didehydro-orobanchol isomers, compared with the wild-type (López-Ráez *et al.*, 2010). Thus, a definite correlation can be observed between ABA and strigolactone biosynthesis. All of these results suggest the presence of an interactive cascade between strigolactones, and the growth hormones, ABA and GA, on a germination level and even post-germination. In this study, the physiological effects of the synthetic strigolactone analog, GR24 and SW on salt-stressed *Nicotiana benthamiana* seedlings were analyzed and possible reasons for growth changes are discussed.

3.2 Materials and Methods

3.2.1 Growth substances

The smoke water used in all experiments was generously donated by Prof J van Staden (Research Centre for Plant Growth and Development, School of Biological

and Conservations Sciences, University of KwaZulu-Natal, Pietermaritzburg, South-Africa). The solution was prepared as described in Baxter and Van Staden (1994). GR24 was purchased from Prof B Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands).

3.2.2 Plant materials and growth conditions

Nicotiana benthamiana seeds were surface decontaminated for 5 min in 70% ethanol, followed by 5 min in 1.75% sodium hypochlorite containing Tween 20 (two drops in 20 mL). The seeds were then rinsed 5 times in sterile dH₂O. Thereafter the surface decontaminated seeds were germinated for two days on filter paper with sterile dH₂O in a 16h:8h light:dark photoperiod, under cool, white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 $\mu\text{moles photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at $25\pm 2^\circ\text{C}$. After the germination period, the seeds were plated out in Cellstar[®] 100x20mm cell culture dishes (Greiner Bio-One) onto half-strength Murashige and Skoog (MS) salts (Highveld Manufacturing) as control and with the addition of 1:1000 smoke water solution in the smoke control and 10^{-7} M GR24 in the strigolactone control. The stress treatments included varying concentrations of NaCl. The pH of all media was adjusted to 5.9 using KOH prior to adding 8 g/L bacteriological agar (Biolab) and autoclaving at 121°C, 100 kPa for 20 minutes. Each treatment consisted of five replicates of five seeds per plate (n=25). Following germination, the seedlings were grown for a further 21 days under the same light and temperature conditions. The plates were held in a vertical position (75°) using white-painted steel racks designed for this purpose. After the growth period the plants were removed from the plates and gently blotted dry. Fresh mass was determined, followed by measurements of root and shoot length, root number and lateral root formation as well as leaf number and leaf area. Leaf area was digitally acquired and calculated by comparison with a 100 mm² reference marker processed within each image. The entire plant was oven-dried at 60°C for at least 2 days, followed by measurement of dry mass. Each experiment was independently repeated three times.

3.2.3 Statistical analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) using the GraphPad Prism 5 (GraphPad Software, Inc., USA). The Fisher's least

significant difference (LSD) at the 5% level was used to analyze the differences between the means of growth parameters of seedlings.

3.3 Results

3.3.1 Identification of appropriate NaCl concentrations

Increasing concentrations of 50 mM, 100 mM, 150 mM and 200 mM NaCl were applied to impose salt stress on *N. benthamiana* seedlings. A concentration of 200 mM NaCl resulted in minimal or no growth of the seedlings, whilst 50 mM NaCl (Fig. 3.1) induced no visible stress effect after 21 days of growth. Significant decreases in growth could be observed following 100 and 150 mM NaCl treatment and these concentrations were used to establish the growth effects of SW and GR24.

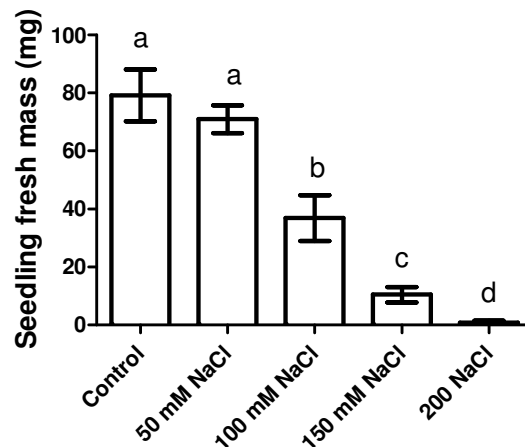


Figure 3.1 Changes in seedling fresh mass (mg) in response to increasing NaCl concentrations. Seedlings were grown for 21 days in half-strength MS medium containing 0, 50, 100, 150 and 200 mM NaCl to establish salt tolerance and adaptation of *N. benthamiana* seedlings.

3.3.2 Growth effects of SW and GR24 treatment

To identify the most effective concentration at which the SW solution was active in this tissue culture system, different dilutions (1:500, 1:1000, 1:2000 and 1:5000) were applied to the media. A dilution of 1:1000 SW showed the most significant increase in growth compared with the untreated controls. A concentration of 10^{-7} M GR24 was used in all experiments, as it resulted in significant increases in growth compared with untreated controls at this concentration. The SW and GR24 treatments

increased seedling fresh mass by 64.8% and 69.7% respectively, compared with the untreated control seedlings (Fig. 3.2a). This trend was also observed for dry mass measurements (Fig. 3.2b). The SW and GR24 treatments increased root length and treated seedlings had much thicker roots. Although lateral root number did not increase, all lateral roots of the treated seedlings were much longer (Fig. 3.2c and Fig. 3.3). The SW and GR24 treatments increased leaf area (Fig. 3.2g) but decreased shoot length (Fig. 3.2d) compared with the controls. Furthermore, GR24 treatment also increased leaf number (Fig. 3.2f).

3.3.3 SW and GR24 ameliorate salt stress in *N. benthamiana* seedlings

Salt stress decreased fresh mass in a concentration-dependent manner but SW reduced this effect at 100 mM NaCl. The GR24-treated seedlings were also larger (although this was not statistically significant) than the control seedlings at 100 mM NaCl and showed a significant increase in fresh mass at 150 mM NaCl (Fig. 3.2a). Both SW and GR24 treatment resulted in a significant increase in dry biomass accumulation at 100 mM NaCl compared with the untreated seedlings (Fig. 3.2b). No differences in dry mass were observed between the control and treated seedlings at 150 mM NaCl. Salt stress impaired root growth but not to the same extent as shoot length and overall plant mass were affected (Fig. 3.2a, c, d and Fig. 3.3). Lateral root number increased significantly in 100 mM NaCl with SW and GR24 compared with the untreated stressed seedlings. Salt treatment caused significant decreases in leaf number at 100 and 150 mM NaCl, at both salt concentrations, however, SW and GR24 treatment mitigated this effect significantly (Fig. 3.2f). A major decrease in leaf area was observed when seedlings were under salt stress, correlating with the decreases in fresh mass under stress (Fig. 3.2a and g). Without salt treatment, SW and GR24 caused significant increases in leaf growth, but under stress these treatments caused little change in leaf size compared with the control seedlings (Fig. 3.1g). Only at 100 mM NaCl did GR24 increase leaf area significantly in comparison to the untreated stressed seedlings. Both salt stress treatments resulted in significant increases in root:shoot fresh mass ratios. Upon SW and GR24 treatment this trend increased even more, although not always significantly compared with the untreated stressed seedlings (Fig. 3.2i). Treatment with a light stress of 50 mM NaCl, which was seen as a very low stress for *N. benthamiana* seedlings resulted in increases in lateral root formation (Fig. 3.3). Roots appeared to

be much thicker and sturdier with lateral roots being much longer. At a concentration of 100 mM NaCl, however, root growth and overall growth was extremely impaired, although SW and GR24 treatment reduced the extreme effects of salt stress and SW and GR24 treatment resulted in increases in root number and length compared with the control (Fig. 3.3).

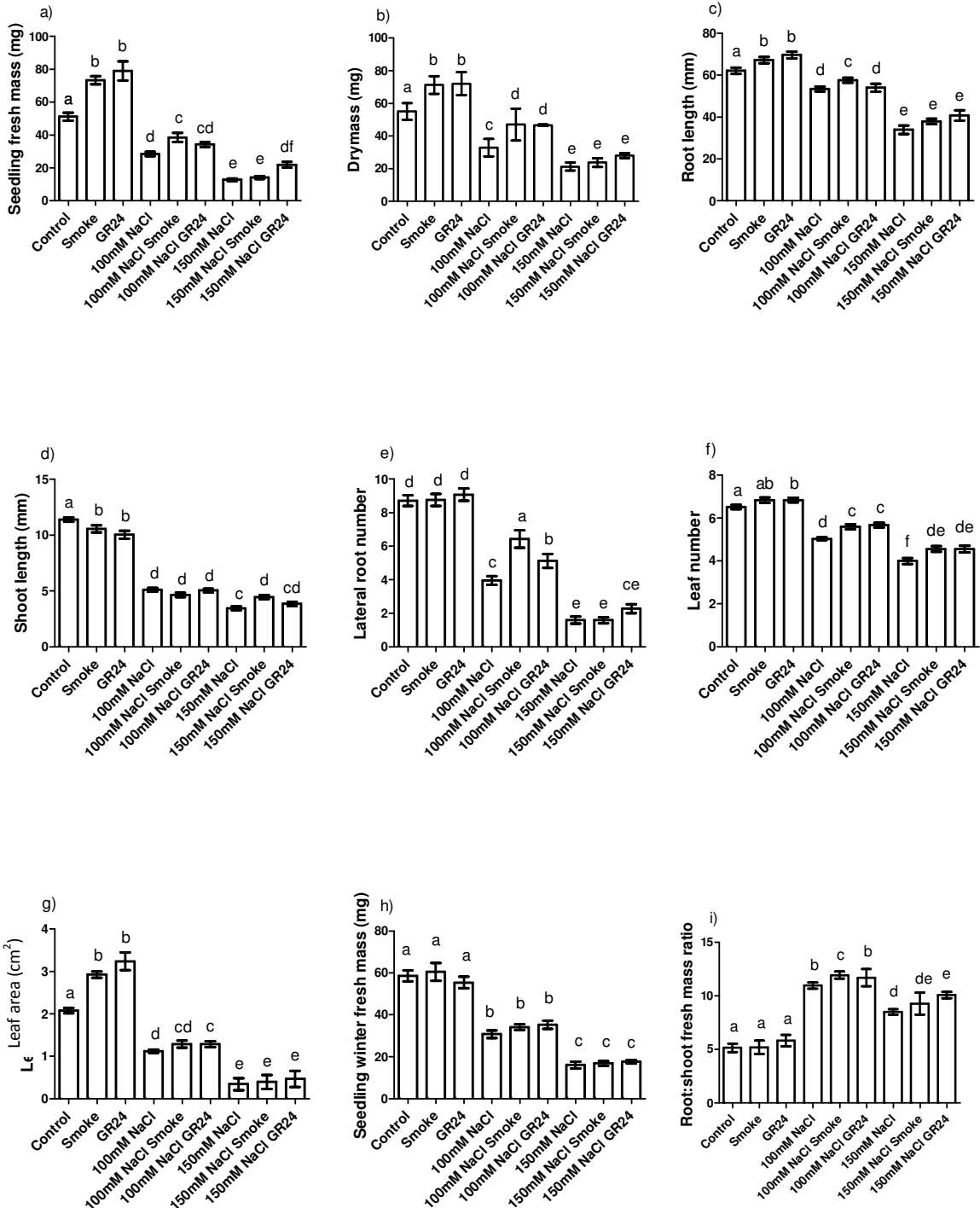


Figure 3.2: The effects of smoke water and GR24 on 100 and 150 mM NaCl-treated and non-salt treated, 21-day old *N. benthamiana* seedlings. The seeds

were pre-germinated in sterile dH₂O before being subjected to the various treatments. a, seedling fresh mass (mg); b, dry mass (mg) measured as a batch of 25 seedlings from three independent experiments; c, Root length (mm); d, shoot length (mm); e, lateral root number; f, leaf number; g, leaf area (cm²); h, winter seedling fresh mass; i, root:shoot mass ratio. Different letters indicate values that were determined by ANOVA (Fisher LSD test) to be significantly different ($P < 0.05$) from each other.

3.3.4 Changes in growth in response to increasing salt treatments with SW and GR24

Increases in salt stress with the growth promoting substances resulted in varying physiological changes. For instance, SW treatment increased fresh mass, dry mass, root length and lateral root number and leaf number significantly at 100 mM NaCl stress but at 150 mM NaCl stress, SW only ameliorated the stress effect for fresh mass, shoot length and leaf area. GR24 treatment showed the same trend, at 100 mM NaCl stress GR24 treatment resulted in significant increases in dry mass, lateral root number and leaf number compared with the untreated stressed seedlings. However, at 150 mM NaCl stress treatment with GR24, increases were observed only for fresh mass and leaf number.

An interesting observation that was made was that at different seasons of the year, different growth vigour was observed in *N. benthamiana*, even in the temperature controlled growth room. In summer months, significant differences in growth were consistently observed between treated and untreated seedlings (Fig 3.2 a-g). In winter months, however, the seedlings from all treatments were significantly smaller than during the summer months, as a result the differences in growth as a result of the growth stimulatory effects from SW and GR24 became statistically non-significant or became inconsistent (Fig. 3.2 h).

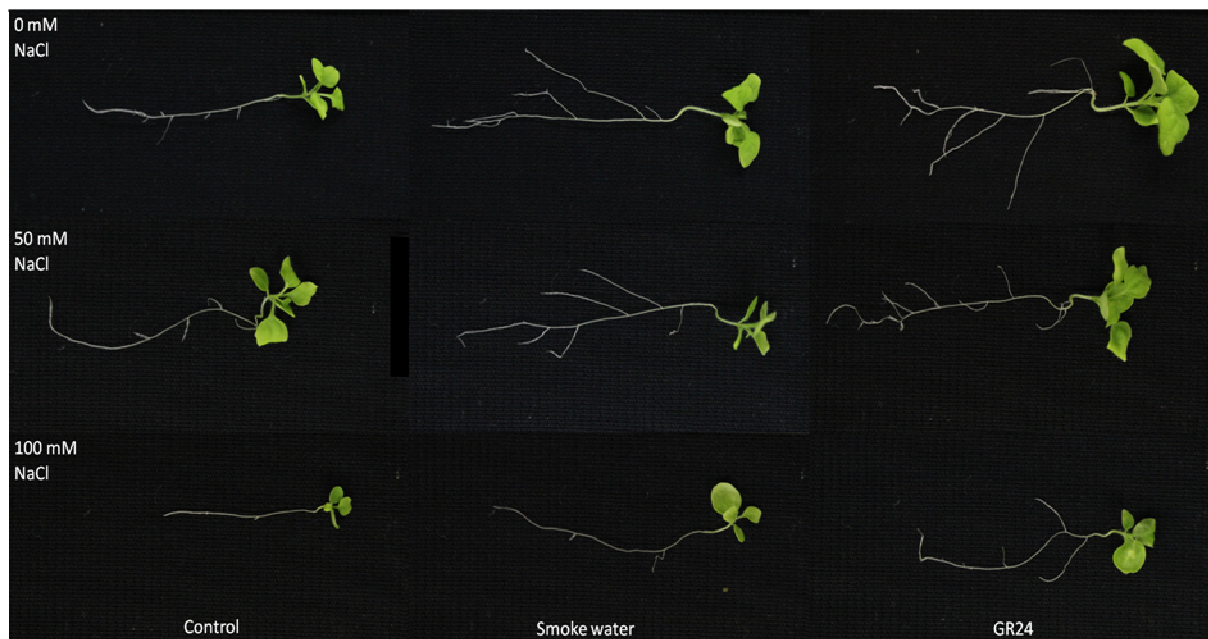


Figure 3.3: Lateral roots formation and root length in 21-day-old *Nicotiana benthamiana* seedlings, under smoke water, GR24 and control treatments with the addition of 0, 50 and 100 mM NaCl.

3.4 Discussion

Seed germination is greatly affected by very low concentrations of the active compound in SW (Kulkarni *et al.*, 2006b), KAR₁ (Flematti *et al.*, 2004; Van Staden *et al.*, 2004) in a large variety of plants. Strigolactones, including synthetic examples such as GR24, are also important germination cues for a number of species (Besserer *et al.*, 2006). In *N. benthamiana*, however, none of these treatments has any effect on either percentage germination or the germination rate (Kotze, 2010). Nevertheless, all seeds used in this study were germinated on water before being transferred onto the different media containing NaCl, SW or GR24. Low concentrations of SW (1:1000) and GR24 (10^{-7} M) applied to the growth medium significantly improved the vigour of 21-day old seedlings compared with the control seedlings. The effects of SW and GR24 on seedling vigour therefore took place post-germination and are not due to increased rates of germination. Heat from fire inhibits germination in the genus *Centaurea* but the chemicals released from burning stimulated seedling vigour showing a SW-post-germination response similar to this study (Riba *et al.*, 2002) and this phenomenon has also been observed in numerous other studies (Keeley *et al.*, 1985; 1997; Jain *et al.*, 2007).

SW and GR24 increased leaf area significantly compared with control seedlings, which could facilitate increased photosynthesis but photosynthetic performance needs to be assessed in future in order to validate this. Shoot length decreased with the addition of the growth stimulators, SW and GR24, compared with the control seedlings. This is comparable to triadimefon treatment which showed decreased shoot length in soybean seedlings (Panneerselvam *et al.*, 1998), as well as hypocotyl length in cucumber (Fletcher and Arnold, 1986) and radish (Panneerselvam *et al.*, 1997). Triadimefon belongs to a group of highly active fungicides which inhibit sterol biosynthesis and also protect the plant from injury due to drought, ozone and chilling (Fletcher and Hofstra, 1985). Furthermore, GR24-treated rice seedlings grown in darkness had decreased mesocotyl elongation compared with control seedlings (Hu *et al.*, 2010). The mesocotyl is present in most monocot seedlings and can be described as partly hypocotyl and partly cotyledon and thus forms part of the primary extension of the seedling that develops into the stem (<http://www.knowledgerush.com>). Strigolactone insensitive or deficient mutants had enhanced mesocotyl elongation compared with wild-type plants (Hu *et al.*, 2010). Mesocotyl elongation could be corrected with GR24 in strigolactone-deficient (*d10-1*) mutants, but not in strigolactone-insensitive mutants (*d3-1* and *d14-1*) (Hu *et al.*, 2010). Similarly, GR24-treated *N. benthamiana* seedlings had decreased shoot length compared with the untreated control seedlings and GR24, therefore negatively regulates shoot elongation. Although the shoot formation mechanisms between dicotyledonous and monocotyledonous apical meristems are different, orthologs of the *Arabidopsis* MAX genes were also isolated in pea and rice suggest that dicots and monocots share a conserved MAX/RMS/D-involved carotenoid-derived branching signal pathway (Wang and Li, 2008). Therefore, *N. benthamiana* may also share a conserved region for strigolactone signaling to control shoot formation.

In this study, exogenously applied strigolactone resulted in considerably longer and more developed lateral roots. However, other studies have found the opposite. Strigolactones have been suggested to affect root growth in the presence of exogenously-applied auxin, especially by restraining lateral root formation (Koltai *et al.*, 2010b). A study by Kapulnik *et al.* (2010) indicated that the lack of strigolactones in strigolactone-deficient *Arabidopsis thaliana* mutants improved lateral root formation after 12 days of growth. Furthermore they found that exogenously applied GR24 decreased lateral root density in a concentration-dependent manner (Kapulnik *et al.*,

2010). However, root-hair length was increased with GR24 (1×10^{-6}) addition to wild-type *Arabidopsis* (Kapulnik *et al.*, 2010). Lateral root formation was repressed by GR24 and when strigolactone sensing was suppressed, lateral root growth was enhanced (Kapulnik *et al.*, 2010). Therefore, GR24 at higher concentrations of 2.7×10^{-6} and 8.1×10^{-6} M affects lateral root development rather than elongation thereof (Kapulnik *et al.* (2010) and, furthermore, treatment with 10^{-6} and 5×10^{-6} M GR24 significantly decreased lateral rooting (and general growth) of *N. benthamiana* seedlings (P. Hills, *pers. comm.*). However, treatment of 10^{-7} M GR24 for 21 days resulted in lateral root formation in *N. benthamiana* similar to those of control seedlings which is similar results to a previous study on *N. benthamiana* seedlings (Kotze, 2010). Therefore, the effects of strigolactones on lateral root development is concentration dependent, with concentrations higher than 10^{-7} M inhibiting lateral rooting. This effect may also be species-specific, but future research will be required to fully elucidate this.

3.4.1 Salt stress impairs growth

Sodium chloride, at concentrations of 100 mM and 150 mM, was applied to the medium to induce salt stress conditions. Exposure of plants to high salt concentrations enforces constraints on growth and can markedly affect development and reproductive success (Gaspar *et al.*, 2000). Salinity affects growth by changing water relations and by affecting the nutrient balance in plant tissue (Pessaraki *et al.*, 1991). Physiological mechanisms by which plants respond to salt stress include, decreases in fresh mass, reductions in leaf growth and increases in root growth and length (Pessaraki *et al.*, 1991; Evers *et al.*, 1998; Khodary, 2004). During this physiological study, all parameters measured decreased upon salt stress treatment. An increase in root:shoot fresh mass ratio was observed under salt stress conditions, which correlates with many other species responding to salt stress, such as peanut seedlings exposed to 30 mM NaCl (Muthukumarasamy and Panneerselvam, 1997) and the legume *Phaseolus aconitifolius* exposed to 50 to 100 mM NaCl (Hema and Karadge, 1991). A study by Meloni *et al.* (2004) showed similar results, where root growth was less affected than shoot growth in *Prosopis alba* at NaCl concentrations as high as 300 mmol.L^{-1} . An increased root:shoot mass ratio thus appears to be a common adaptation to salinity, resulting in longer roots with increased lateral root development to facilitate more efficient water and nutrient uptake under salt stress

(Gorham *et al.*, 1985). With the addition of the two growth promoting substances, the root:shoot mass ratio of salt-stressed seedlings increased even more although not always significantly. Plant growth is controlled by a well-regulated balance between root and shoot growth. In general, roots can only supply as much water and nutrients as are available from the soil, and in relation to the amounts of photosynthates received from the shoot, and *vice versa*. If the plant is under salt or water stress, the below-ground structures expand to facilitate growth and supply nutrients and water to the above-ground structures for normal growth (Saab *et al.*, 1990). Seedlings grown with GR24 and 100 mM NaCl showed a significant increase in lateral root formation. As mentioned before, under salt stress conditions, root development is enhanced in search of nutrients and water, and as suggested by Kapulnik *et al.* (2010) strigolactones play a hormonal role in the plant to control root responses to growth conditions such as responding to low nutrient status. Treatment with SW at 100 mM NaCl had a similar effect on lateral root number, but did not only cause increases in lateral root formation but also significant increases in root length which could enhance the uptake of nutrients from the root system under osmotic disturbances.

Leaf number and area decreased significantly in response to salt stress in this study similar to many other studies (Higbie *et al.*, 2006; Al-Maskri *et al.*, 2010). However, although leaf area was not affected by the growth promoting substances under either moderate or severe salt stress conditions, both SW and GR24 treatment resulted in a small but significant increase in leaf number at 100 and 150 mM NaCl compared with untreated seedlings.

It was evident that under stress conditions, the growth stimulators SW and GR24 enhance seedling vigour and general development. Under moderate salinity stress (100 mM NaCl), SW and GR24 enhanced the stress tolerance/adaptation of *N. benthamiana* seedlings significantly more than at a higher stresses (150 mM NaCl) (Fig. 3.1 a-g). Throughout these growth experiments, SW-treatment only facilitated tolerance to salt stress at 100 mM NaCl and failed to facilitate growth (fresh mass) at 150 mM NaCl. GR24, however, did facilitate fresh mass growth at both 100 and 150 mM NaCl. This could be seen in almost all parameters and suggests that different mechanisms are active at different stress intensities in the plant, with SW and GR24 contributing or maybe interacting in these stress pathways to relieve the stress effect to some extent, especially in root growth (Fig. 3.1c and e).

3.5 Conclusion

Research focused on enhancing seedling vigour and stress tolerance in commercial crops is of immense significance for the expansion and advance of agricultural production, since saline areas are increasing yearly (Wang *et al.*, 2003). After exposure to an environmental stress, plants respond with reduced growth rates and impaired acquisition of nutrients (Chapin, 1991).

In summary, these results demonstrated a significant positive effect when salt-stressed *N. benthamiana* seedlings were supplied with SW and GR24 as growth stimulators. These results showed that SW and GR24 enhanced growth under ideal conditions, improving overall plant development and growth, resulting in significant increases in biomass. SW and GR24 treatment also enhanced the ability of plants to respond to salt stress. SW had an enhancing effect on the innate salt stress tolerating mechanisms by increasing root length, leaf area and number and by decreases in shoot length. GR24 similarly affected overall plant growth by facilitating lateral root formation, increasing root length as well as increasing leaf area and number and thereby increasing growth of above ground structures to compensate for increases in root growth. Strigolactones (e.g. GR24) have not previously been shown to facilitate lateral root development similar to control treatment or even enhance lateral root formation under salt stress condition as noted in this study.

These experiments were conducted over a period of 21 days and there is a strong possibility that sustained SW and GR24 treatment could have an even greater effect on growth in a later developmental stage of *N. benthamiana*. Also, much of the increase in fresh mass of the SW and especially GR24-treated seedlings could be attributed to the increase in leaf area and leaf number of the seedlings suggesting that these growth promoters might be useful in increasing vegetable crop biomass. This would clearly be a valuable point of further investigation. Further field and greenhouse experiments should be conducted using additional intermediate stress conditions to determine more effectively whether SW and GR24 have any benefit in the long term and whether these growth stimulators can be used as preconditioning agents.

Chapter 4

Comparative analysis of protein changes in response to growth promoting substances and salt stress in *Nicotiana benthamiana*

4.1 Introduction

The effects of salt stress on plant growth have been well documented on a physiological level (Munns, 2002; Yokoi *et al.*, 2002). Salt stress causes physiological drought to plants by reducing osmotic potential and resulting in a high uptake of Na⁺ and Cl⁻ ions that leads to toxicity to cells (Gaspar *et al.*, 2000). This leads to disruption of cell organelles and their metabolism, causing nutrient imbalances and affecting plant growth, which ultimately decreases plant yield (Evelin *et al.*, 2009). On a molecular level, changes in transcription, translation and hormonal levels facilitate salt stress tolerance and the mechanisms involved therein are very important to identify and understand adaptation to environmental changes. Research for understanding salt stress responses on a molecular level is therefore essential in order to develop salt-stress tolerant plant varieties.

The conversion of solar energy to chemical energy by plants is a complex process that includes electron transport and photosynthetic carbon metabolism. Numerous proteins, genes and metabolites are involved in this system of energy supply. The main protein complex regulating photosynthesis and, more precisely the Calvin cycle which facilitates carbon metabolism, is ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO). Increases in RuBisCO levels generally result in increased photosynthesis leading to enhanced growth. However, the converse is not always true, since decreases in RuBisCO levels do not necessarily result in decreases in growth (Quick *et al.*, 1991a; Teramura *et al.*, 1994). It is a well-known fact that plant growth, including photosynthetic activity, is influenced negatively by abiotic stress (Caruso *et al.*, 2009). RuBisCO content changes in response to stress or to forced decreases in expression but without significantly altering the photosynthetic rate (Caruso *et al.*, 2009). For example, decreases in photosynthetic rate occurred only after a 60% reduction in RuBisCO levels in tobacco mutant plants (Quick *et al.*, 1991a). It is generally believed, therefore, that some plant species such as tobacco carry excess levels of RuBisCO to avoid decreases in photosynthesis

(Quick *et al.*, 1991b). However, changes in growth rate in response to changes in RuBisCO activity are dependent on environmental as well as species specific responses (Kawaguchi *et al.*, 2003; Kubien *et al.*, 2003; Bota *et al.*, 2004; Flexas *et al.*, 2006). For example, water stress down-regulates soybean RuBisCO activity but has no adverse affect on tobacco RuBisCO activity (Flexas *et al.*, 2006).

Another essential part of plant growth; shoot branching, was believed to be mainly controlled by auxin and cytokinin, but recent knowledge of another shoot branching regulator, strigolactones, has suggested a new protein-degradative pathway to control shoot branching (Gomez-Roldan *et al.*, 2008, Umehara *et al.*, 2008). A number of proteins are involved in the biosynthesis and signalling pathway of strigolactones, primarily carotenoid cleavage dioxygenase (CCD) enzymes and some F-Box proteins, and it has also been suggested that an α,β -hydrolase receptor may also be involved (Gomez-Roldan *et al.*, 2008, Umehara *et al.*, 2008; Arite *et al.*, 2007; Gao *et al.*, 2009; Liu and Van Staden, 2009). The complete biosynthetic route for strigolactones is still largely unknown, but studies on shoot branching mutants have answered some questions regarding the biosynthetic and signalling mechanisms and interactions with other growth hormones. A list of the genes known to be involved in strigolactone synthesis and signaling and their proposed functions is presented in Chapter 2 (Table 2.1).

Another growth stimulating substance that has received considerable attention is smoke water, obtained by bubbling smoke from the burning of plant material through water. The active compounds in smoke water (SW), karrikins (especially KAR₁), stimulate germination and seedling vigour in a number of species, particularly from dormant seeds in fire-prone areas (Keeley *et al.*, 1985; De Lange and Boucher, 1990; Sparg *et al.*, 2005). The effect of KAR₁ on cotyledons and embryos of germinated tomato seed (*Lycopersicon esculentum* Mill.) was analysed at different time-points using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and showed little variation in the proteome compared with control seeds (Jain *et al.*, 2008c). Changes in recorded band intensities at different developmental stages in germination suggested that KAR₁ might be playing a role in the rate of protein synthesis and hydrolysis of stored material in the seed (Jain *et al.*, 2008c).

GR24 and SW treatment facilitates changes in *N. benthamiana* seedlings to overcome salt stress on a physiological level (Chapter 3), but the response to salt stress with these growth promoting substances on a molecular, and more specifically a protein level, remains unknown. Therefore, the aim of the following work was to perform a comparative study using a proteomics approach, based on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS), in order to identify the effects of SW and GR24 on the proteome of *N. benthamiana* seedlings, the salt-stress responsive proteins in *N. benthamiana* and the proteomic effects which SW and GR24 exerts on these seedlings which enhances their tolerance to the salt stress. *Nicotiana benthamiana* seedlings were grown under moderate salt stress conditions with or without GR24 and SW. Proteome changes were recorded and compared and mechanisms via which SW and GR24 may potentially increase growth and stress tolerance are discussed.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

Nicotiana benthamiana seeds were surface decontaminated and germinated as described in Chapter 3, section 3.2.2. After the germination period the seeds were plated out on half-strength Murashige and Skoog (MS) salts (Highveld Manufacturing) as control and with the addition of 1:1000 smoke water solution in the smoke control and 10^{-7} M GR24 in the strigolactone control. The seedlings were placed under salt stress by the addition of 100 mM NaCl. The pH of all media was adjusted to 5.9 using KOH, prior to adding 8g/L bacteriological agar (Biolab) and autoclaving at 121 °C at 100 kPa for 20 minutes. The treatments that were compared are listed in Table 4.1. Each treatment consisted of 10 replicates of five seeds per plate. Following germination, seedlings were left to grow for 21 days in a 16h:8h light:dark photoperiod, under cool, white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 $\mu\text{moles photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at $25 \pm 2^\circ\text{C}$. After 21 days, the plants were removed from five of the plates, chosen at random, and gently blotted dry. The fresh mass was determined, followed by measurements of root and shoot length. The seedlings from the second set of plates (five plates with five seedlings each)

were then flash frozen in liquid nitrogen and stored at -80°C for later protein extraction.

Table 4.1 Comparisons made between different treatments in order to investigate changes in the proteome brought about by smoke water or GR24 treatment, under both salt stress and non-salt stress conditions.

Comparison	Physiological effect under investigation
Control vs GR24	Plant growth promotion
Control vs smoke water	Plant growth promotion
Control vs 100mM NaCl	Salt stress
100mM NaCl vs 100mM NaCl + GR24	Alleviation of salt stress
100mM NaCl vs 100mM NaCl + smoke water	Alleviation of salt stress

4.2.2 Protein extraction

Phenol extraction of proteins was performed according to the modified protocol of Hurkman and Tanaka (1986). Approximately 2 mL of extraction buffer (0.1 M Tris-HCl [pH 8.8]; 10mM EDTA; 0.4% [v/v] β -mercaptoethanol; 0.9 M sucrose) was added to 600 mg of ground tissue. After vortexing for 30 sec, 2 ml of phenol (Tris-buffered, pH 8.0) was added and samples were vortexed for an additional 30 sec and agitated by shaking for 30 min at 4°C. The phenol phase was separated by centrifugation at 5000 xg for 10 min at 4°C. The aqueous phase was removed and placed in a new tube. An equal volume of fresh Tris-buffered phenol was added to the aqueous phase, vortexed for 30 s and agitated for 30 min at 4°C. Again, the phenol phase was separated at 5000 xg for 10 min at 4°C and added to the previous fraction. This fraction was back extracted with an equal volume of extraction buffer and agitated for 15 min at 4°C, the phenol phase then separated by centrifugation and the final phenol fraction transferred to a new tube. Phenol extracted proteins were precipitated by adding 6 volumes of 0.1 M ammonium acetate in 100% (v/v) methanol (pre-chilled at -20°C), vortexing

thoroughly and incubating overnight at -20°C. The precipitate was collected by centrifugation at 4000 $\times g$ for 30 min at 4°C and the pellet washed twice with ice-cold 0.1 M ammonium acetate in methanol containing 10 mM dithiothreitol (DTT). The pellet was resuspended in approximately 100 μ l of buffer containing 7 M urea and 5 mM DTT. The protein concentration was determined according to Bradford (1976) using BSA as a standard.

4.2.3 Two-dimensional gel electrophoresis

All equipment, chemicals and kits were purchased from Bio-Rad. In preparation for the first dimension, samples were cleaned with the ReadyPrep 2D Cleanup Kit, according to the manufacturer's instructions. The resulting pellet was resuspended in rehydration/sample buffer, composed of 8 M urea, 2% (m/v) CHAPS, 10 mM DTT and 0.2% (m/v) Bio-Lyte™ (Bio-Rad) for isoelectric focusing (IEF) and the protein concentration determined with the *RC DC* protein assay before applying 400 μ g protein to an immobilized pH gradient (IPG) strip (pH 5 – 8). Three technical replicates were run for each biological replicate. Isoelectric focusing was carried out overnight by applying a linear increase in voltage from 0 – 250 V over 20 minutes, 250 V to 8000 V over 2.5 h, and holding at 8 000 V until a total of 20 000 Vh was obtained. Following IEF, each strip was equilibrated for 20 minutes in 4 ml of equilibration buffer I (6 M urea; 0.375 M Tris-HCl [pH 8.8]; 2% [m/v] sodium dodecyl sulphate [SDS]; 20% [v/v] glycerol and 2% [m/v] DTT), followed by 20 min in equilibration buffer II (6 M urea; 0.375 M Tris-HCl [pH 8.8]; 2% [m/v] SDS; 20% [v/v] glycerol and 2.5% [m/v] iodoacetamide). For the second dimension, the equilibrated strip was applied to a 12% Bis-Tris Criterion XT precast gel (11 cm; 1.0 mm) for separation according to the molecular weight and run for approximately 50 min at 200 V. Gels were silver-stained with Silver Stain Plus Kit™ according to the manufacturer's instructions.

4.2.4 Image acquisition and data analysis

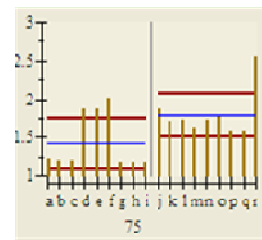
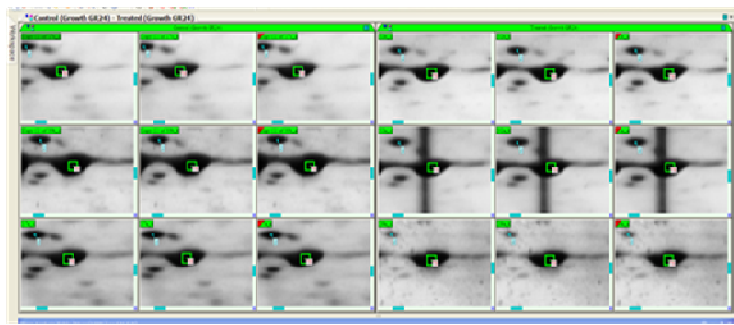
The silver-stained gels were scanned with the Pharos FX scanner from Bio-Rad (Hercules, CA, USA) and analysed for differential protein expression using the Melanie 7.0 2D software (Swiss Institute of Bioinformatics, Switzerland). A spot detection algorithm was applied; manual selection of landmarks made and all images

were matched. To validate the suggestions made by the software based on a Student's t-test at the 95% significance level, the spots were checked for (a) spot detection quality, (b) matching quality, (c) spot position and (d) spot intensity. Criteria (a) and (b) determine the quality of spot quantification i.e. the reliability of the statistics and criteria (c) and (d) determine the likelihood of successful protein identification (Fig. 4.1).

A total of 323 spots were chosen from across the five treatments according to the criteria mentioned above. These spots were excised using an EXQuest spot cutter (Bio-Rad) and subjected to tryptic in-gel digestion.

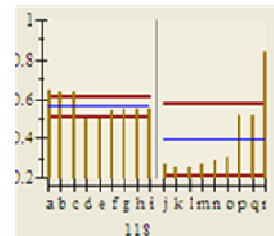
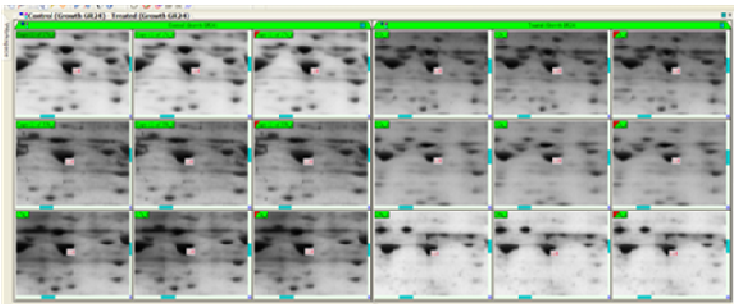
4.2.5 In-gel digestion

In-gel tryptic digestion followed by peptide extraction was performed according to Shevchenko *et al.* (1996). The individual spots were transferred to PCR tubes for digestion. The digestion buffer was added after dehydration for 10 min at RT with acetonitrile, reduction at 56°C with 10 mM DTT in 100 mM NH₄HCO₃ for 1h and 45 min alkylation in the dark with 55 mM iodoacetamide in 100 mM NH₄HCO₃. After alkylation the gel pieces were washed with 100 µl of 100 mM NH₄HCO₃ for 10 min, dehydrated by addition of acetonitrile, swelled by rehydration in 100 mM NH₄HCO₃, and shrunk again by addition of the same volume of acetonitrile. The digestion buffer consisted of 12.5 ng/µl trypsin (Promega sequencing-grade modified porcine trypsin, Cat. #V511A) in 50 mM NH₄HCO₃ without 5 mM CaCl₂. The samples were digested overnight and after several wash steps with 5% formic acid and 50% acetonitrile solution to collect the cleaved peptides, the samples were vacuum-dried and stored at -20°C until analysis.



Match ID 75

<0.033



Match ID 118

<0.022

Figure 4.1 Enlarged images and expression profiles of 2 of the 7 differentially expressed protein spots which could be positively identified. Expression profiles were calculated as the averages of three biological replicates using the Melanie 7.0 2D software. Match ID 75 was identified as Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (PsbO) (*Nicotiana benthamiana*) and Match ID 118 was identified as Ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) (*Nicotiana tabacum*) - (fragment). Both of these spots were isolated upon comparison of the proteome of GR24-treated seedlings compared with the proteome of untreated seedlings. (Additional identified spots are represented in Appendix A)

4.2.6 Mass spectrometry

Mass spectra of the resulting tryptic digests were acquired by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) on a Voyager-DE STR mass spectrometer (Applied Biosystems). The peptide digests were dissolved in 20 μ l 50% (v/v) acetonitrile and 0.1% (v/v) formic acid solution, containing approximately 10 mg α -cyano-4 hydroxy-cinnamic acid as matrix and 4 μ g/ μ l Glu-1-Fibrinopeptide B (m/z 1 570.6887) as an internal analytical standard to monitor mass accuracy stability and overall performance (by peak height) of the MALDI-TOF MS. The instrument was externally calibrated using a calibration mixture 2 (Applied Biosystems) containing Angiotensin (m/z 1 296.6853), ACTH clip 1-17 (m/z 2 039.0867) and ACTH clip 18-39 (m/z 2 465.1989). One μ l of the peptide mix

was spotted onto the MALDI plate and air-dried before measurement. The MALDI-TOF MS was operated in reflector mode with delayed ion extraction, using a 20 kV accelerating voltage, a 64.7% grid voltage, and a 50 ns delay time. Spectra, acquired from seven different regions of each spot, were the result of averaging 300 separate laser shots. The laser power was modulated in order to obtain the best signal to noise ratio. All spectra were processed by applying a noise filter with a correlation factor of 0.7, baseline correction, and peak deisotoping. Peak lists generated from the digest spectra were submitted for peptide mass fingerprinting (PMF) with the MASCOT search engine (www.matrixscience.com; Matrix Science, London, UK) using online protein databases (SwissProt, NCBI non-redundant protein database, MSDB). Parameters for the MASCOT search were as follows: monoisotopic mass accuracy, 100 ppm tolerance, missed cleavages 1, allowed variable modifications: oxidation (methionine), taxonomy *Viridiplantae* (Green Plants). Predicted proteins with the highest MOWSE (molecular weight search) score were checked for sequence coverage and pI. All the proteins with corresponding pI values (5 – 8) were reported. These predicted proteins were further characterised using NCBI BLAST (blastp) and TIGR (http://blast.jcvi.org/euk-blast/plantta_blast.cgi) to search for related proteins in the Solanaceae family and are referred to by their function according to their identification by either of these databases.

4.3 Results and Discussion

In this study, 21-day-old *Nicotiana benthamiana* seedlings were treated with the growth promoting substances SW and GR24, with and without the addition of salt stress (100 mM NaCl). Smoke water treatment increased seedling fresh mass, root length and leaf area significantly, whilst GR24 treatment had a similar effect to SW but also showed a significant increase in leaf number compared with the untreated control (Chapter 3). To gain insight into the mode(s) of action for these growth promoting substances, proteomic changes induced by these different treatments were studied.

Firstly, the effects of the growth promoting substances SW and GR24 were compared with untreated controls and yielded 74 and 48 differentially-expressed spots, respectively. The proteomes of 0 and 100 mM NaCl-treated seedlings were also compared and 92 statistically significant different spots were isolated. Lastly the

alleviating affects of SW and GR24 on salt-stressed seedlings was investigated and 69 and 40 differentially expressed spots could be isolated from each of these treatments respectively. These spots were analysed using MALDI-TOF MS. Of these 323 spots, only 7 could be positively identified (Table 4.2, Fig. 4.2 and Fig. 4.3). It is, however, important to note that many other recent studies also showed little success in identifying proteins with MALDI-TOF MS (Caruso *et al.*, 2009; Witzel *et al.*, 2009; Wen *et al.*, 2010). Caruso *et al.* (2009) were able to identify only 36 of 300 spots from drought responsive wheat proteins and Wen *et al.* (2010) could positively identify only 11 of at least 200 observed spots from rice shoots. MALDI-TOF MS was carried out because of easy access, relatively short analysis time and having a straightforward protocol.

Of the seven proteins identified using MASCOT, two were involved in growth changes as a result of GR24 treatment. Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (PsbO) (Fig. 4.2 C, spot 75) was 1.3-fold up-regulated compared with the untreated control seedlings. The PsbO protein is part of photosystem II (PSII) which is present in all photosynthetic organisms and forms part of the oxygen-evolving protein group (Ifuku *et al.*, 2010). This protein forms part of the first membrane-bound protein complex in the light-dependent reactions involved in splitting H₂O, O₂, protons and electrons (Kok *et al.*, 1970; Gibney and Tommos, 2005). This complex, composed of 20 subunits as well as other accessory light-harvesting proteins, ultimately provides electrons for the rest of photosynthesis to occur. *Arabidopsis*, for example, has two isoforms of PsbO and it has been suggested that one (PsbO2) might be involved in PSII repair (Lundin *et al.*, 2007). Since this protein forms part of a complex which is essential in metabolism and energy production, it is logical that PsbO was up-regulated upon GR24 treatment, correlating with increases in seedling vigour and leaf area compared with the controls. Interestingly, this protein was also up-regulated in salt stressed seedlings in response to SW treatment (Fig. 4.3 B, spot 119). This increase could be to counteract decreases in photosynthetic activity as a result of stress. This complex protein is specifically salt-stress inducible (Abbasi *et al.*, 2004) and since high NaCl concentrations affect photosynthesis by disturbing the K:Na ratio, changes in membrane-bound photosynthetic proteins are only to be expected (Sudhir and Murthy, 2004). The same result was found in a proteomic study on salt-stressed rice, particularly after 24 h of 50 and 100 mM NaCl treatment (Abbasi *et al.*, 2004).

Therefore, the fact that treatment of salt-stressed seedlings with SW caused increases in this protein compared with 100 mM NaCl-treated control seedlings suggests that SW caused a further increase in this salt-stress inducible protein, over and above the induction normally caused by salt stress. This suggests that SW treatment may enhance the plant's natural responses to stress and thus enhance salt stress adaptations such as increases in leaf area and fresh mass and consequently may also induce genes involved in salt stress tolerance. For example, drought stress affects photosynthesis and leaf physiology, especially concerning water and proton transport in wheat which resulted in increased photosynthetic oxygen-evolving protein content (Caruso *et al.*, 2009). Salt stress may have the same effect on water and proton and electron transport, by interfering with membrane permeability resulting in an increase in oxygen-evolving protein expression in 100 mM NaCl-treated seedlings. The addition of SW could repair damage from NaCl to the photosynthetic transport system by stimulating further increases in photosynthetic oxygen-evolving protein content. The fact that GR24 treatment also resulted in an increase in this protein subunit could indicate strigolactone involvement in inducing stress responsive processes to prepare the seedling for such circumstances, especially since after an initial increase in levels this protein is known to decline to control levels after prolonged exposure (longer than 48 h) of 50 and 100 mM NaCl in rice (Abbasi *et al.*, 2004). This protein also functions independently of ABA signal transduction under salt stress conditions (Abbasi *et al.*, 2004). However, it was recently shown that the expression of genes putatively involved in light-harvesting in PSI and PSII were significantly induced by GR24 in the tomato WT and *SI-ORT1* strigolactone deficient mutant (Mayzlish-Gati *et al.*, 2010). The mutant also had reduced expression levels in a number of light-harvesting associated genes as well as reduced chlorophyll levels (Mayzlish-Gati *et al.*, 2010). Furthermore, GR24 application to tomato WT and *SI-ORT1* leaves also resulted in an induction of most of the light-harvesting associated genes (Mayzlish-Gati *et al.*, 2010). This effect of increased levels in light-harvesting expression was observed in both the presence and absence of exogenously applied IAA, although IAA treatment alone could not induce these genes.

Ribulose-bisphosphate carboxylase activase (Fig. 4.2 C, spot 118) expression was down-regulated by GR24 treatment. This protein is vital in facilitating the rapid formation of the critical carbamate in the active site of ribulose-1.5-biphosphate

carboxylase/oxygenase (RuBisCO) (Portis, 2003). RuBisCO is a key enzyme in the Calvin cycle, is essential in photosynthesis and overall plant growth and represents up to half of the leaf nitrogen (Woodrow and Berry, 1988). A similar down-regulation of this protein was also observed in rice seedlings treated with GA₃ (Wen *et al.*, 2010) and an overall decrease in Calvin cycle proteins was reported in 150 mM NaCl *Arabidopsis* (Pang *et al.*, 2010). Although it would be assumed that increases in growth are accompanied by increases in photosynthetic rate, growth does not always correlate with photosynthetic rate but rather depends on the efficiency of the photosynthate available for use by the cell (Chapin *et al.*, 1990). The mechanism through which photosynthetic activity decreases is not well characterised, but it is evident that decreases in RuBisCO activity do not impair growth to an extreme extent (Quick *et al.*, 1991a; Stitt *et al.*, 1991; Stitt and Schulze, 1994). Furthermore, only after a 60% reduction in RuBisCO expression in tobacco transgenic plants could photosynthetic inhibition be observed, which further decreased proportionally with decreases in RuBisCO expression (Quick *et al.*, 1991a; Stitt *et al.*, 1991). For plant biomass the same trend was observed, growth only decreased when about half of RuBisCO expression was impaired (Quick *et al.*, 1991b). As for other growth parameters, a decrease in RuBisCO content caused an increase in the shoot:root ratio, leaf composition changed with water content rising in transgenic plants and the leaf area doubled (Quick *et al.*, 1991b). The same trend was observed in this study, a 1.4-fold decrease in RuBisCO activase expression levels accompanied an significant increase in leaf area upon GR24 treatment.

Table 4.2 Differentially-expressed proteins identified by peptide mass fingerprinting following treatment with either 0 or 100 mM NaCl, with and without smoke water or GR24. MS – MASCOT score, PM – peptides matched, % - percentage coverage. Accession numbers obtained from www.ncbi.nlm.nih.gov and www.uniprot.org.

Spot no.	Accession number	Mr/pI	MS	PM	%	Fold change	Protein name and species	Molecular function
Control vs GR24								
75	AAX53163	35206/5.89	59/57	6	23	1.3	Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (PsbO) (<i>Nicotiana benthamiana</i>)	Calcium ion binding
118	CAA78702	25913/5.01	88/66	8	32	-1.4	Ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) (<i>Nicotiana tabacum</i>) - (fragment)	Monooxygenase and ribulose-biphosphate carboxylase activity
100 mM NaCl vs 100 mM NaCl smoke water								
119	AAX53163	34202/5.4	65/57	8	23	1.4	Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (<i>Nicotiana benthamiana</i>)	Calcium ion binding
4	P69249	20379/6.82	73/72	5	28	-1.6	Cristal-Glass1 protein (<i>Capsicum annuum</i>)/Ribulose biphosphate carboxylase small chain	Monooxygenase and ribulose-biphosphate carboxylase activity
3	P69250	10175/5.3	69/72	5	47	-1.4	Ribulose-1,5-biphosphate carboxylase (<i>Nicotiana sylvestris</i>)	[Ribulose-biphosphate carboxylase]-lysine N-methyltransferase activity
9	ADI46214	114392/5.88	76/72	1 5	12	-3.4	Armadillo repeat-containing protein (<i>Nicotiana tabacum</i>)/CMPG1b (<i>Nicotiana benthamiana</i>)	Binding, ubiquitin-protein ligase activity
100 mM NaCl vs 100 mM NaCl GR24								
278	YP_358683	35491/5.54	60/57	6	24	1.5	ATP synthase CF1 beta subunit (<i>Nicotiana sylvestris</i>)	ATP binding

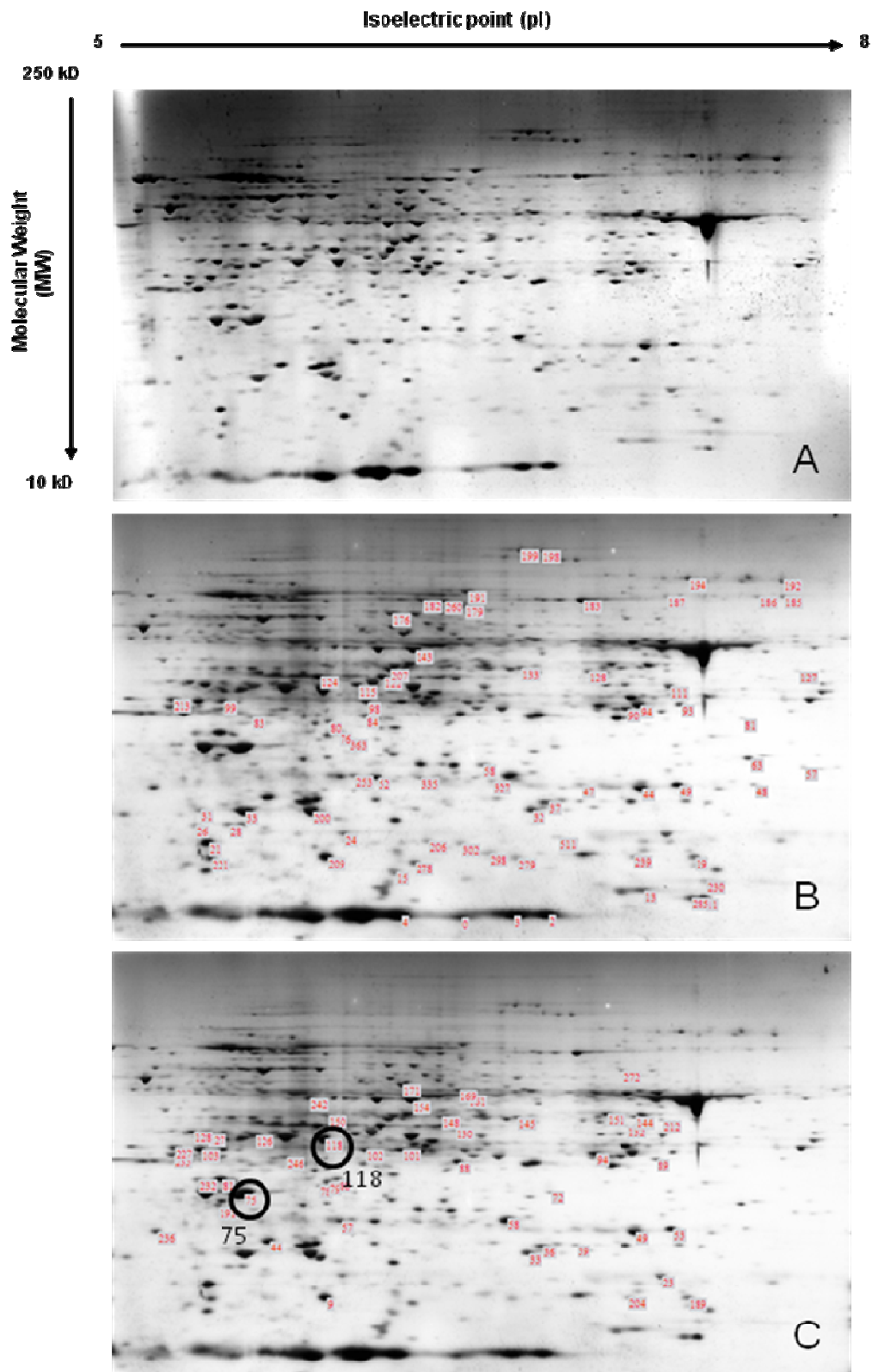


Figure 4.2 2D-PAGE profiles of proteins extracted from whole 21-day-old *N. benthamiana* seedlings. Proteins marked with a circle were differentially expressed and positively identified using PMF. Seedlings were grown on $\frac{1}{2}$ -strength MS medium (A), or the same medium supplemented with 1:1000 SW (B) and 10^{-7} M GR24 (C).

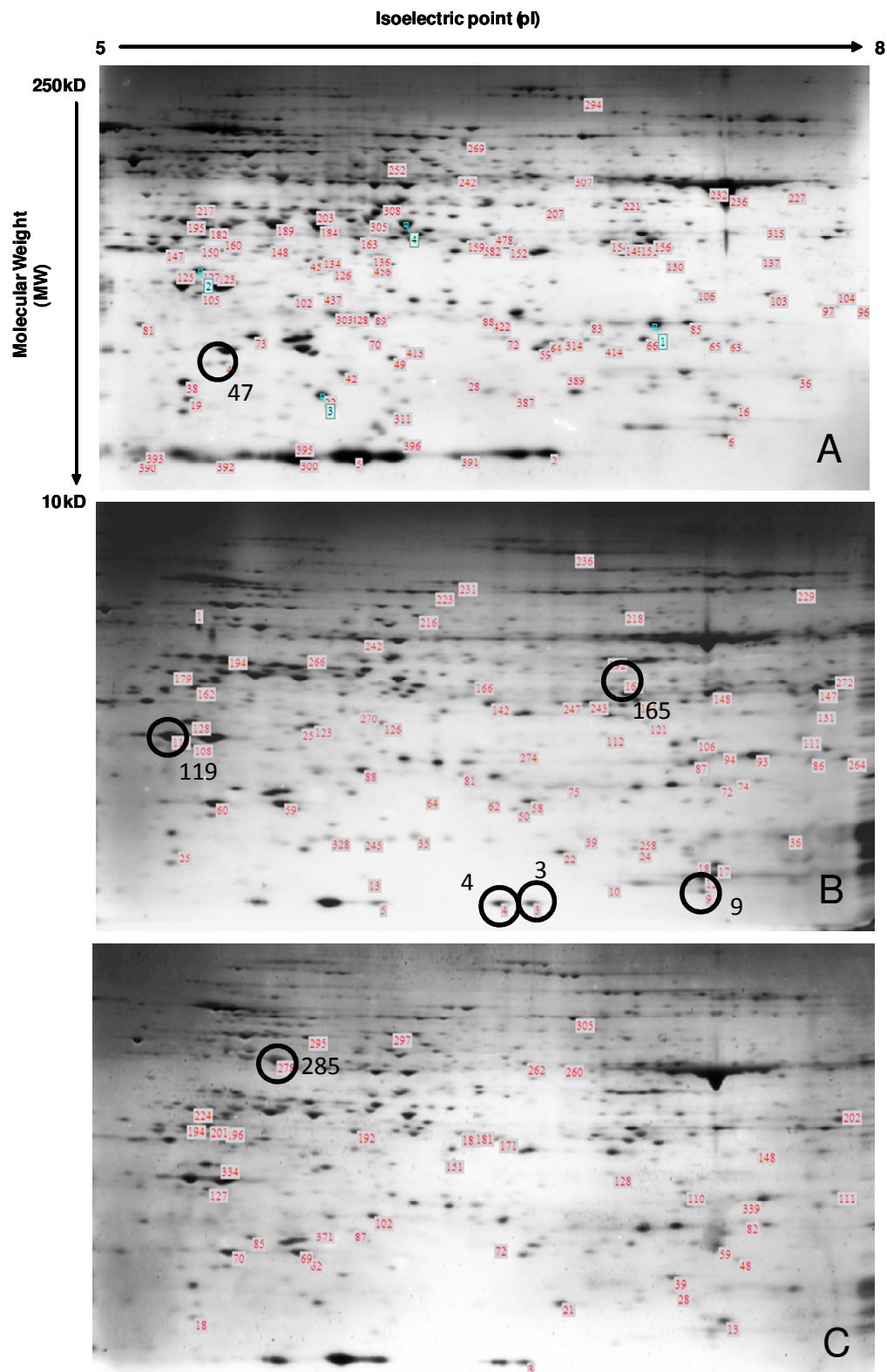


Figure 4.3 2D-PAGE whole plant protein profiles of 21-day-old *N. benthamiana* seedlings exposed to 100 mM NaCl (A), 100 mM NaCl with SW (B) and 100 mM NaCl with GR24 (C). Proteins marked with a circle were differentially expressed and positively identified using PMF, except for spot numbers 47 and 165 which could only be identified with near significance.

Two proteins, forming part of the RuBisCO complex were altered in response to SW and salt stress treatment, the ribulose biphosphate carboxylase small chain (Fig. 4.3 B, spot 4) and ribulose-1,5-biphosphate carboxylase (Fig. 4.3 B, spot 3). Both of

these enzymes were down-regulated in response to SW treatment of salt-stressed seedlings. Under drought stress conditions in wheat, RuBisCO was down-regulated, and it was suggested that this could be because of inhibitor compounds increasing which are normally synthesized at night, inhibiting RuBisCO catalytic activity (Caruso *et al.*, 2009). However, other studies have shown the opposite effect of increases in RuBisCO content or no change in RuBisCO content between stressed and control plants (Delfine *et al.*, 1999; Chen *et al.*, 2003). For example, in a study on salt-stressed (1% [m/v] NaCl) spinach leaves, RuBisCO content and activity were similar between stressed and control seedlings for the first 22 days of treatment (Delfine *et al.*, 1999). Another study on rice RuBisCO content revealed that increases in RuBisCO led to increases in growth via increases in photosynthetic activity indicators compared with the control plants (Chen *et al.*, 2003). Treatment with SW and GR24 caused increases in growth in salt-stressed seedlings and decreases in RuBisCO-related enzymes, resulting in a common trend which suggests that seedling vigour under salt stress conditions is not dependent on changes in RuBisCO levels.

Expression of an armadillo repeat-containing protein (*Nicotiana tabacum*)/CMPG1b (*Nicotiana benthamiana*) (Fig. 4.3 B, spot 9) decreased significantly in SW-treated salt-stressed seedlings. This family of proteins is involved in protein-protein interactions (Huber *et al.*, 1997), the ABA response and other abiotic stresses, however, there are over 90 armadillo repeat proteins in *Arabidopsis* and their functions are still largely unknown (Kim *et al.*, 2004). A well characterised potato armadillo (ARM) protein, PHOR1, is involved in GA signalling and over-expression of this gene increases GA sensitivity and internode length (Amador *et al.*, 2001). In rice, an armadillo repeat-containing protein was up-regulated by chilling stress (Yan *et al.*, 2006) and six of these ARM-repeat motifs are conserved in SPOTTED LEAF II (SPLII) in rice (*Oryza sativa*). SPLII plays a role in the ubiquitination system in the control of plant cell death and defence (Zeng *et al.*, 2004). The rice (*Oryza sativa*) *spIII* mutant causes a cell death phenotype with a clear association to being defence activated, especially to plant-pathogen responses (Zeng *et al.*, 2004). CMPG1b, a U-box protein, is essential for plant defence and disease resistance, especially inducing a stronger hypersensitive response (HR) (Zeng *et al.*, 2004). The proteolytic activities involving U-Box proteins are more important in disease resistance and HR than at an abiotic stress adaptation level. Up-regulation of this motif was observed in a study on the proteome of cold-stressed rice but in

response to SW treatment with NaCl this protein had decreased levels which demonstrate the complexity of the stress signal transduction pathway (Yan *et al.*, 2006). However, since neither the precise ARM protein affected by SW and salt treatment, nor its precise function are known, it is impossible to say what the actual significance is to this study.

One polypeptide (Fig. 4.3 B, spot 278) gave a significant match to an ATP synthase CF1 beta subunit, with a 1.5-fold increase in 100 mM NaCl + GR24-treated seedlings compared with 100 mM NaCl control seedlings. ATP synthase synthesises adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate in the chloroplast. This protein, located specifically in the chloroplast (Boyer, 1989), is usually down-expressed in response to drought, chilling and salt stress, affecting the Calvin cycle (Caruso *et al.*, 2009; Chen *et al.*, 2009; Yan *et al.*, 2006). With addition of GR24, expression of this enzyme increased, possibly ameliorating the salt stress effect and facilitating increased growth.

A few other proteins warranted investigation since previous studies have found significant changes in the levels of their expression after salt stress treatment and these spots also had MASCOT scores and pI values close to theoretical values, although these were not statistically significant. A small molecular heat shock protein (HSP) increased 1.5-fold in response to salt stress (Fig. 3.3A, spot 47). This protein is part of a class of functionally-related proteins which show increased expression when cells are exposed to environmental stress and plays an important role in folding and translocation of proteins across membranes (Downs and Heckathorn, 1998). It is also known that small HSPs can be systemically induced in plants and may be important in re-adaptation or recovery after an environmental stress has passed (Hamilton and Coleman, 2001).

Another identified protein that showed very high similarity to the experimental pI value acquired was a chloroplastic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) B (Fig. 4.3 B, spot 165), which is involved in phosphorylation in the Calvin cycle. This protein was 1.4-fold up-regulated in response to 100 mM NaCl with SW treatment and was one of the most abundant proteins in a recent proteome study on *N. tabacum* BY2 cell lines (Duby *et al.*, 2010). It is important in cell energy supply as

it facilitates the breakdown of glucose during glycolysis, but it has also been found to be involved in apoptosis (Tarze *et al.*, 2007). The expression of GAPDH commonly increases under salt and drought stress conditions and in response to ABA treatment (Ingram and Bartels, 1996). This increase could reflect an increase in energy demand brought about by addition of SW which ultimately increases growth under stress conditions.

The remaining spots that were excised, all of which fall within a pI range of 5-8 but with non-significant MOWSE scores, are listed in Appendix B (www.matrixscience.com) and will be further characterised when more peptide information on *Nicotiana benthamiana* is available. A number of these identified proteins did, however, display theoretical pI values very close to the experimental pI values. Although the *N. benthamiana* genome has not been sequenced, most of the proteins were identified using sequence data from various *Nicotiana* species, and other *Solanaceae* species such as potato and tomato. Some proteins were categorised based on sequences from the known genomes for *Oryza sativa* and *Arabidopsis*.

It has been suggested that increases in reactive oxygen species (ROS) associated with oxidative stress degrades RuBisCO via proteases in barley (Desimone *et al.*, 1996). Increases in ROS from salt stress may therefore also be responsible for the decreases in proteins involved in photosynthesis and defence such as the ARM protein and RuBisCO subunits and isoforms. When stressed seedlings were treated with SW, changes in the proteome mostly correlated with proteins involved in photosynthetic activity. However, the observed decrease in photosynthetic proteins was not related to growth rate in this study, as has been previously observed (Quick *et al.*, 1991a; Stitt *et al.*, 1991). Furthermore, there was a decrease in levels of an ARM protein, commonly involved in defence and HR, in response to SW treatment, which could be an energy-saving response by the plant in reaction to SW-treatment. However, large numbers of ARM proteins have been identified with different functions which have not all been confirmed, therefore the precise significance of the identified ARM protein in this study is not presently clear and will need further detailed analysis to elucidate the exact function.

The changes in proteome levels demonstrated that GR24 increased growth but not through increases in photosynthetic enzymes. Decreases of Calvin cycle protein levels (RuBisCO activase and RuBisCO subunits) could lead to an accumulation of ATP and ATP synthases in the leaves, to protect the seedling against photo-oxidative damage during salt stress conditions as have also been hypothesised in other studies (Zhu, 2001; Bohler *et al.*, 2007; Gao *et al.*, 2011). Energy metabolism is very important in plants under stress, root growth increase significantly under moderate salt stress conditions in search for nutrients and water and ATP production is essential in this process. From these results it seems that SW and GR24 enhance normal responses to stress, which could also be observed in the physiological study where SW and GR24-treated plants showed growth patterns that mirror the responses of salt tolerance in plants (Chapter 3).

4.4 Conclusion

For a more complete look at the proteome when salt-stressed seedlings are treated with SW and GR24, quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) should be used, to form a clearer picture of the predicted polypeptides in the sample. Financial limitations prevented further studies to gain more results and information on stress-associated proteins as well as identification of proteins involved in SW and GR24 related responses. The fact that there are only a limited number of databases available which are PMF-search compatible also limits success in this regard.

The growth promoting substances SW and GR24 increase seedling vigour, even under salt stress conditions. However, the mechanisms by which this is achieved are still poorly understood. The *Nicotiana benthamiana* genome has not been sequenced and proteins expressed in response to salt stress in this species are not well characterised (Goulet *et al.*, 2010). Furthermore, the response of SW treatment on the proteome is still a poorly understood field. Only a few transcription factors have been explored as possible regulators in the pathway and no definite conclusions could be drawn as to where or how SW or karrikins facilitate growth (Nelson *et al.*, 2010). Similarly, the strigolactone synthesis and signalling mechanism is also still poorly understood. From the results presented in this chapter, it may be concluded that GR24 and SW treatment, with and without stress treatment, caused

decreases in enzymes involved in the Calvin cycle but resulted in increased levels of light reaction related proteins (Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit) which could possibly ensure adequate energy for other processes important during salt stress.

Chapter 5

The effects of smoke water and GR24 on the metabolome of salt-stressed *Nicotiana benthamiana* seedlings[†]

[†]Experimental disclaimer: All experimental work, with the exception of metabolite identity assignment and annotation, was done by LE Steenkamp.

5.1 Introduction

Salt-rich soils, caused by several biotic and abiotic factors, result in major crop losses yearly through inhibition of growth and disruption of ion homeostasis in the plant (Wang *et al.*, 2003). Many plants have evolved several strategies to adapt to these stress conditions in order to facilitate growth under unfavourable conditions. For example, salt stress triggers alterations in root formation and elongation, ion transport and compartmentation, and the adjustment of osmotic balance by producing solutes to aid the cell in rescuing turgor and facilitating water uptake (Munns and Termaat, 1986). Apart from assisting in cellular osmotic balance, many solutes buffer cellular redox potential and protect cell integrity against toxic ions under stress conditions and are referred to as osmoprotectants (Pattanagul and Thitisaksakul, 2008, Widodo *et al.*, 2009). Osmoprotectants have polyhydroxylic properties and include sugars such as sucrose, glucose and fructose, various oligosaccharides, and polyols such as *myo*-inositol and trehalose. Other solutes important in conferring stress tolerance or adaptation include nitrogen-containing compounds such as proline and other amino acids as well as polyamines which are synthesised via an arginine/ornithine pathway with methionine donating the aminopropyl groups via a decarboxylated step (Bohnert *et al.*, 1995; O'Hare *et al.*, 1998; Kaur-Sawhney *et al.*, 2003). Furthermore, phytohormones such as auxins, ethylene and abscisic acid (ABA) have also been shown to be involved in salinity stress responses although the exact molecular mechanism remains unclear (Mehlhorn and Wellburn, 1987; He *et al.*, 2005; Kempa *et al.*, 2008).

As signalling molecules, phytohormones play an important role in adaptation to environmental changes and form an intriguing complex network with substances inside and outside the plant. The study of phytohormones and other plant growth promoting substances (PGPS) has enjoyed much attention, either as an economic alternative to facilitate plant growth under environmentally-constrained conditions,

such as salt and drought stress, or a means to study the fundamental molecular mechanism(s) underlying the alleviation of these stresses (Van Staden *et al.*, 1995; Jain *et al.*, 2006; Egamberdieva and Kucharova, 2009; Soos *et al.*, 2009). Smoke water (SW), derived from the bubbling of smoke from the burning of plant material through water, have been shown to exhibit hormone-like activities in various studies (Van Staden *et al.*, 1995; Merrit *et al.*, 2005; Jain *et al.*, 2008a). Although the exact mode of action remains unresolved, it is believed that the principle active compound in smoke, namely karrikin 1 (KAR₁), could interact with specific hormones within plant metabolism. In a similar manner to gibberellin A3 (GA₃), KAR₁ has been shown to be able to substitute for red-light stimulation of germination of light-sensitive lettuce seeds (Van Staden *et al.*, 1995; Gardner *et al.*, 2001). This was achieved by overcoming the inhibitory effect of far-red light and was thought to be achieved by decreasing the sensitivity of lettuce seeds to ABA (Van Staden *et al.*, 1995). However, while KAR₁ has similar effects on stimulation of germination to GA₃ in Australian *Asteraceae* spp. (Merrit *et al.*, 2005), the KAR₁ germination response could only be partially recovered in *Arabidopsis* gibberellin signalling mutants (Nelson *et al.*, 2009). In addition, KAR₁ treatments do not significantly enhance sensitivity to GA in GA biosynthetic mutants nor do they decrease sensitivity to ABA in ABA biosynthetic mutants (Nelson *et al.*, 2009). These results suggest that while KAR₁ cannot substitute for GA or ABA in germination responses, a degree of cross-talk between these compounds is apparent. One plausible explanation is that KAR₁ might interact with other phytohormones *in vivo*. In this regard, Jain *et al.* (2008a) have shown that KAR₁ also possesses cytokinin- and auxin-like activity, by increasing cell division rates in soybean callus and rooting in mung bean, respectively. However, phytohormones are involved in many processes during growth and development and although KAR₁ may manipulate some growth processes it does not necessarily possess the same activities as any one specific hormone.

High levels of auxin have also been observed in *Arabidopsis*, pea, rice and petunia strigolactone mutants (Beveridge *et al.*, 2000; Foo *et al.*, 2005; Beveridge *et al.*, 2009; Dun *et al.*, 2009). Elevated auxin levels have been found to enhance endogenous strigolactone levels via an auxin AXR1/TIR1-dependent (AUXIN RESISTANT 1/TRANSPORT INHIBITOR RESPONSE 1) pathway leading to enhanced carotenoid cleavage dioxygenase (CCD) 7 and 8 expression in

Arabidopsis (Hayward *et al.*, 2009) as well as their orthologs in other species (Sorefan *et al.*, 2003; Bainbridge *et al.*, 2005; Foo *et al.*, 2005; Johnson *et al.*, 2006; Arite *et al.*, 2007; Liang *et al.*, 2010). It is believed that strigolactones influence shoot branching by acting as auxin-promoted secondary messengers that move up into buds inhibiting outgrowth (Ferguson and Beveridge, 2009; reviewed by Dun *et al.*, 2009). Strigolactone biosynthesis might also be affected by ABA metabolism. ABA levels have been shown to correlate with strigolactone production in a CCD-dependent manner in tomato (López-Ráez *et al.*, 2010). ABA biosynthesis involves utilization of the same plastidial carotenoid genes, namely 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), to produce precursors shared by the strigolactone biosynthetic pathway (Zeevaart and Creelman, 1988; López-Ráez and Bouwmeester, 2008; López-Ráez *et al.*, 2010). Furthermore, ABA is known as the plant stress hormone and plays a vital role in mediating stress responses in the plant, including salinity stress (Kempa *et al.*, 2008). At low water potentials, ABA promotes root growth and inhibits shoot growth (Creelman *et al.*, 1990) and also plays an integral role in stomatal closure and transpiration efficiencies under these conditions (Zimmermann and Sentenac, 1999). Curiously, the interaction of strigolactones, ABA and salinity stress have not received any attention to date despite the involvement of these chemicals in growth and stress tolerance. An integrated signalling pathway may exist which could lead to a better understanding of strigolactone metabolism.

In context with previously-described observations that both SW and GR24 increase biomass accumulation of *N. benthamiana* seedlings under salt stress conditions (Chapter 3), and the mounting evidence from the literature that biochemical constituents are either involved in salinity stress, or karrikin- and strigolactone-like responses, primary metabolite and phytohormone levels of salt-stressed seedlings were determined in this study in order to identify molecular mechanisms that may aid in this plant stress adaptation response.

5.2 Materials and Methods

5.2.1 Chemicals

All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich (www.sigma-aldrich.co.za). The smoke water used in these experiments was

obtained from Kirstenbosch National Gardens (GPS coordinates: 33°59'22" S 18°25'49" E; Cape Town, South Africa), and optimum concentrations to increase biomass accumulation was established using serial dilutions of the solution (D. Dempers, unpublished data). GR24 was purchased from Prof B Zwanenburg (Department of Organic Chemistry, Radboud University, Nijmegen, The Netherlands), and prepared as described in Kapulnik *et al.* (2011).

5.2.2 Plant material and growth conditions

Nicotiana benthamiana seeds were surface decontaminated and germinated as described in Chapter 3, Section 3.2.2. After the germination period the seeds were plated out on half-strength Murashige and Skoog (MS) salts (Highveld Biological, Johannesburg, South Africa) as control and with the addition of 1:1000 smoke water solution and 10^{-7} M GR24, respectively. The stress treatments included 50 mM and 100 mM concentrations of NaCl. The pH of all media was adjusted to 5.9 using KOH prior to adding 8 g/L bacteriological agar (Biolab) and autoclaving at 121 °C, 100 kPa for 20 minutes. Plant growth promoting substances were filter-sterilised and added after autoclaving the media. Each treatment consisted of five replicates of five seeds per plate. The seedlings were grown for 21 days in a 16h:8h light:dark photoperiod, under cool, white fluorescent tubes (Osram L 58V/740) with a light intensity of 50 $\mu\text{moles photons.m}^{-2}\text{s}^{-1}$ at $25 \pm 2^\circ\text{C}$. The plates were held in a vertical position (75°) using white painted steel racks designed for this purpose. After the growth period the plants were removed from the plates and gently blotted dry. Fresh mass was determined, followed by measurements of root and shoot length from two plates containing 5 seedlings each. The seedlings from the three remaining plates, containing five replicates each were flash frozen with liquid nitrogen and stored at -80°C until further analyses.

5.2.3 Primary metabolite profiling

Primary metabolites were extracted and analysed as described by Roessner *et al.* (2000). Whole frozen seedlings of four individual plants per treatment were used for primary metabolite extractions. The seedlings were homogenised in a tissue-lyser (Qiagen, www.qiagen.com) by shaking at 30 Hz for 30 s while maintaining a frozen temperature, and the polar metabolite fraction, obtained from approximately 10 mg

homogenised tissue was extracted in 350 μL of 100% methanol (containing 23.5 ng ribitol as internal standard for quantification). Samples were incubated at 70°C, with rapid shaking for 15 min. After centrifugation (10 min at 4000 xg) the supernatant was transferred to a fresh tube, and 400 μL CHCl_3 and 750 μL dH_2O added and vortexed. The sample was centrifuged at 2 200 xg for 10 min, and 150 μL from the polar phase placed into a fresh Eppendorf tube. The samples were then reduced in volume under vacuum without heating.

The dried samples were subsequently derivatised in 60 μL of 15 mg/mL methoxyamine-HCl in pyridine. The samples were vortexed and incubated at 37°C for 2 h. All drops were spun down and 70 μL methylsilyl trifluoroacetamide (MSTFA) added to all samples, followed by addition of 10 μL alkane mix to the polar phase and shaking for 30 min at 37°C. The samples were then transferred to glass inserts and analysed by GC-QUAD MS technology. The GC MS system was composed of an Agilent technologies 6890N network GC system coupled to an Agilent 5975 MS (www.agilent.com). Chromatographic separation was performed on a 10 m guard column and a 30 m Restek column (www.restek.com) with a 2.5 μm film thickness. Running conditions were as described in Roessner *et al.* (2000). Helium was used as a carrier gas at a flow rate of 1 $\text{ml}\cdot\text{min}^{-1}$. The injection temperature was set at 230°C, the interface at 250°C and the ion source at 200°C. The oven temperature was set at 70°C for 5 min followed by 5°C/min increase to 310°C with 1 min of heating at 310°C. The system was equilibrated for 6 min at 70°C before the injection of the next sample.

Instrument control and data acquisition was performed by the MSD Chemstation software (v 02.00.237, www.agilent.com). Data pre-processing for baseline correction, scaling and alignment was conducted with MetAlign software, with parameters as specified in “Platform for Riken Metabolomics” (<http://prime.psc.riken.jp/lcms/>) (MetAlign v 200410, www.metalign.wur.nl/UK/), and metabolites annotated in comparison with authentic standards and cross-comparison with the Golm metabolome database (www.csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd).

5.2.4 Phytohormone profiling

For phytohormone analyses, homogenised seedling material was extracted in 0.05 M sodium phosphate buffer (pH 7.0) in a 1:3 ratio according to Edlund *et al.* (1995). The samples were subsequently vortexed and incubated for 1 h in the dark with continuous shaking at 4 °C. The pH was adjusted to 2.6 using HCl, and approximately 35 mg Amberlite XAD-7 (Serva, Heidelberg, Germany) was added for solid phase extraction for the phytohormones of interest. The sample was further incubated for 1 h in the dark with continuous shaking at 4 °C. The sample was centrifuged at 5000 xg for 5 min, and the XAD-7 washed twice with 500 μ L 1% (v/v) acetic acid before elution with 500 μ L dichloromethane for 30 min. The elution step was repeated once more. The combined dichloromethane fractions were reduced to dryness under vacuum.

Derivatisation of the dried samples was achieved by adding 15 μ L 2.0 M trimethylsilyl diazomethane in hexane and 10 μ L methanol (modified from Schmelz *et al.*, 2003). The sample was incubated at RT for 90 min. Following this period, the excess diazomethane was destroyed by addition of 15 μ L 1% (v/v) acetic acid, and the samples again dried to completion under vacuum without heating. The dried samples were resuspended in 50 μ l heptane and injected splitless into a GCT Premier TM benchtop orthogonal acceleration time-of-flight (oa-TOF) MS (Waters). Running conditions were as previously described (Edlund *et al.*, 1995), and phytohormone identification and quantification was done by means of linear calibration curves for authentic standards.

5.2.5 Statistical analysis

Analysis of variance (ANOVA) was used to assess the significance of treatment means. Differences between treatments were compared using the Fisher's least-significant difference test (LSD) at the 0.05 probability level using R Statistical Software (Institute for Statistics and Mathematics, Vienna University of Economics and Business, Germany).

5.3 Results

5.3.1 Phenotypic assesment

For the biochemical analyses, plant material to be used was firstly assessed for the phenotypic attributes of smoke water (SW) and strigolactone (GR24) treatment upon salinity stress. In agreement with the previous results (Chapter 3; Fig. 3.1), both SW and GR24 alleviated the inhibition of biomass production upon salt stress. However, in contrast to previous results, 50 mM salt treatment did result in a significant decrease in biomass which could be alleviated by SW and GR24 treatments (Table 5.1). It was thus decided to include this lower concentration of salt stress, namely 50 mM, as well as 100 mM NaCl for metabolite analyses since the severe inhibition of 150 mM salt (Fig. 3.2) probably will more likely resemble programmed cell death responses (Keyster, 2011).

Table 5.1 Biomass accumulation of *N. benthamiana* seedlings treated with smoke water (SW) and the synthetic strigolactone, GR24, under two salinity treatments. Seedling fresh mass (mg) were taken after 21 days, and salt treatments included 50 and 100 mM NaCl with and without SW and GR24. Values are presented as means \pm SE of 10 individual seedlings and significant differences ($P < 0.05$) were determined by ANOVA followed by Fishers least significant difference (LSD) test. Values that do not share the same letter in parentheses are significantly different from each other.

Treatment	Salt Concentration		
	0 mM	50 mM	100 mM
Control	17.8 \pm 1.08 (a)	11 \pm 1.72 (c)	9.13 \pm 0.91 (c)
Smoke water	25.81 \pm 1.37 (b)	16.9 \pm 0.67 (ad)	13.5 \pm 1.09 (d)
GR24	29.02 \pm 1.42 (b)	19.26 \pm 2.67 (ad)	14.67 \pm 1.15 (d)

5.3.2 Primary metabolite profiles of seedlings treated with smoke water or GR24

In order to evaluate the biochemical effect in response to these growth observations, primary metabolites were profiled via GC QUAD MS technology. Upon salt stress, several metabolic perturbations were evident in the metabolome of the plant (Fig. 5.1A). Proline and galactose levels decreased significantly upon both 50 and 100 mM NaCl treatment compared with the untreated controls, while the levels of alanine, arginine, histidine and lysine increased significantly at these salt stress concentrations (Fig. 5.1A). Furthermore, malate and fumarate levels also increased at 50 mM NaCl treatment. Whilst trehalose levels increased, maltose and mannose

levels decreased at 100 mM NaCl treatment compared with the unstressed control (Fig. 5.1A).

Figure 5.1 Relative metabolite content of *N. benthamiana* seedlings subjected to 50 and 100 mM salt treatments (A), and the metabolite content subjected to the same conditions with the addition of the plant growth promoting substances (PGPS), namely smoke water (SW) (B) and the synthetic strigolactone, GR24 (C). Data are normalised with respect to mean response calculated for the 0 mM salt treatment without any addition of PGPS (to allow statistical assessment in the same way). Values are presented as mean \pm SE of four individual plants per treatment and values marked with a black dot were determined by Student's t-test to be significantly different ($P < 0.05$) from the untreated, unstressed control.

A. Salinity

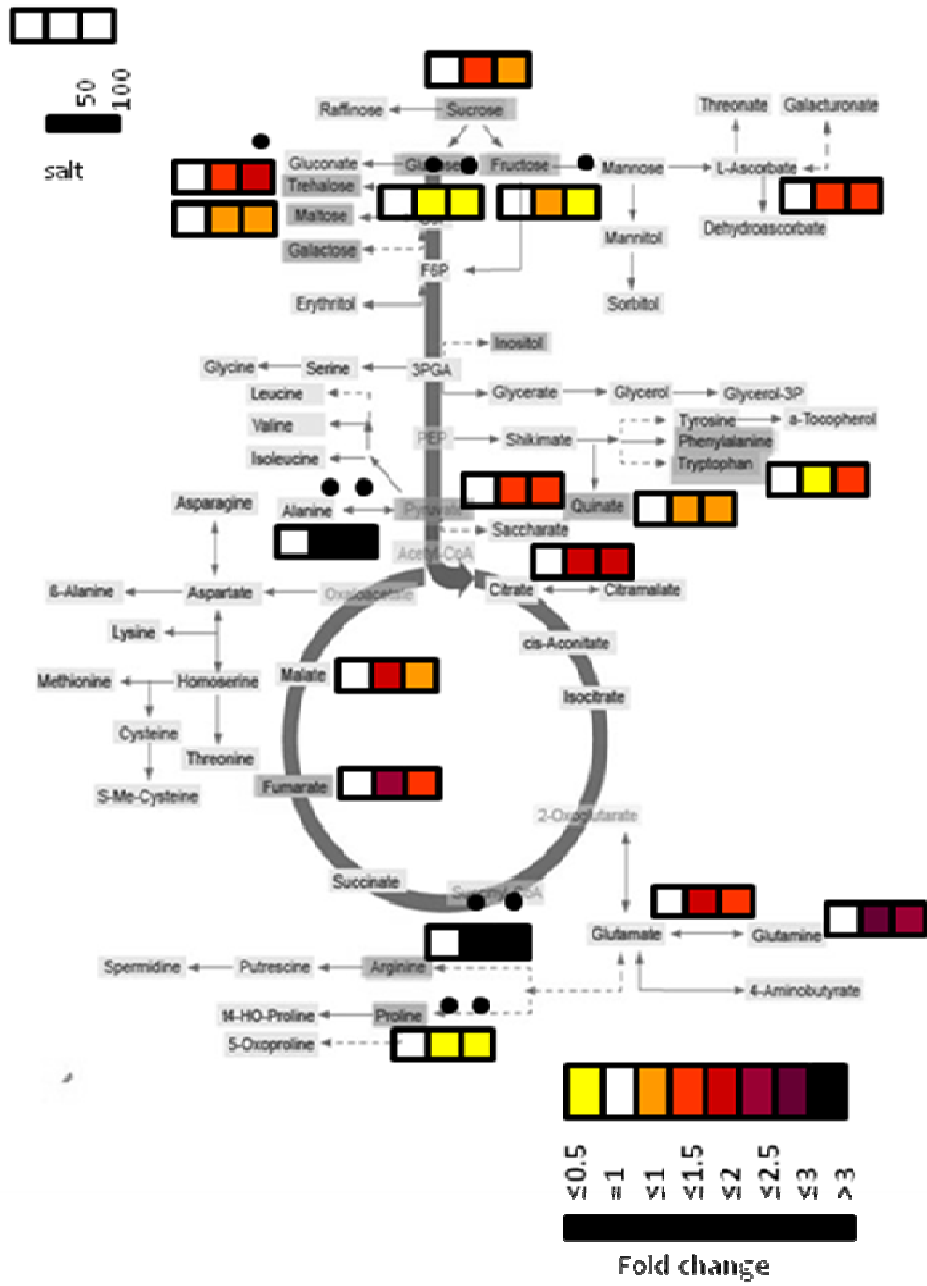


Figure 5.1 (Legend on previous page)

B. Smoke

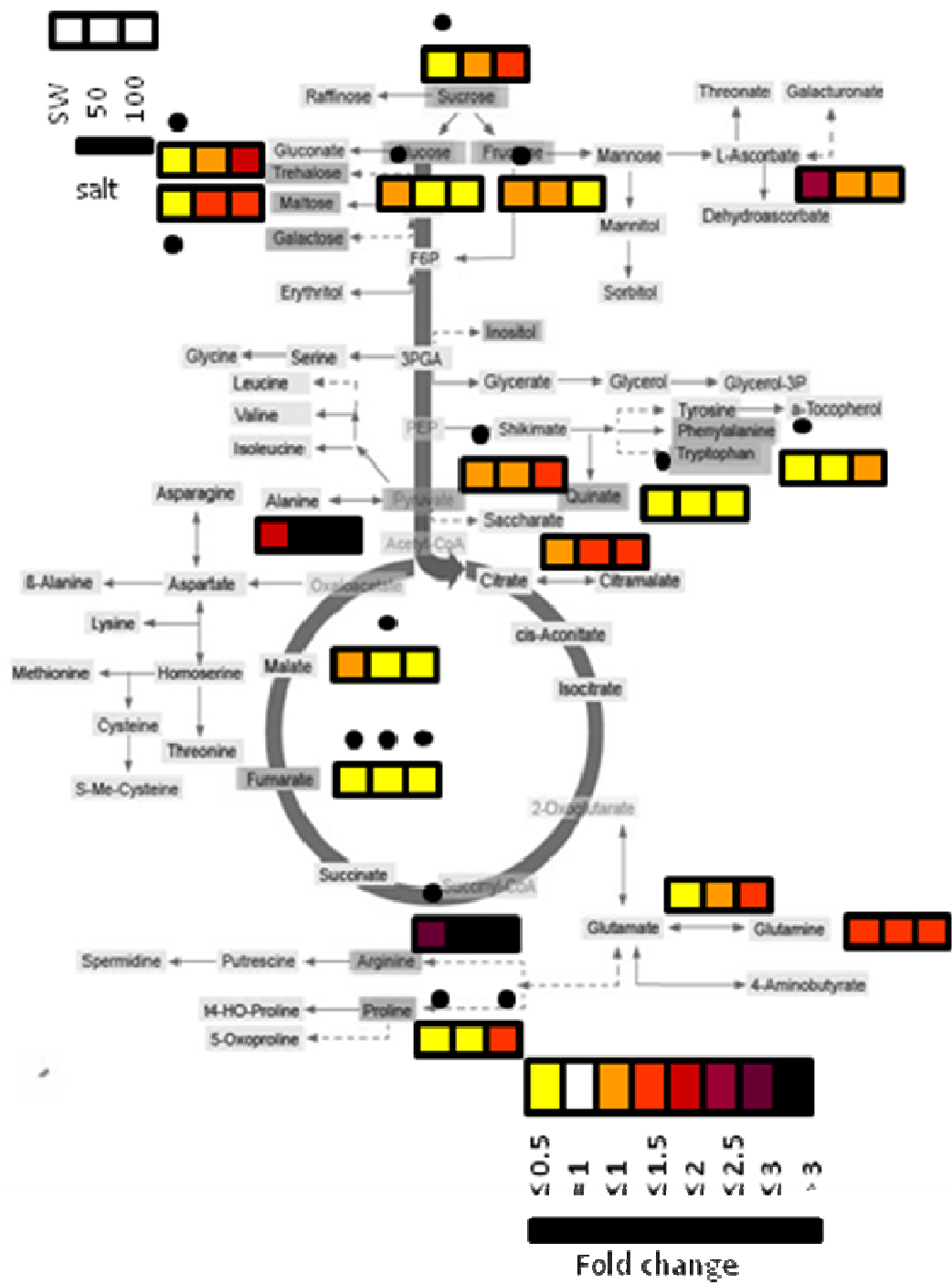


Figure 5.1 (Legend on previous page)

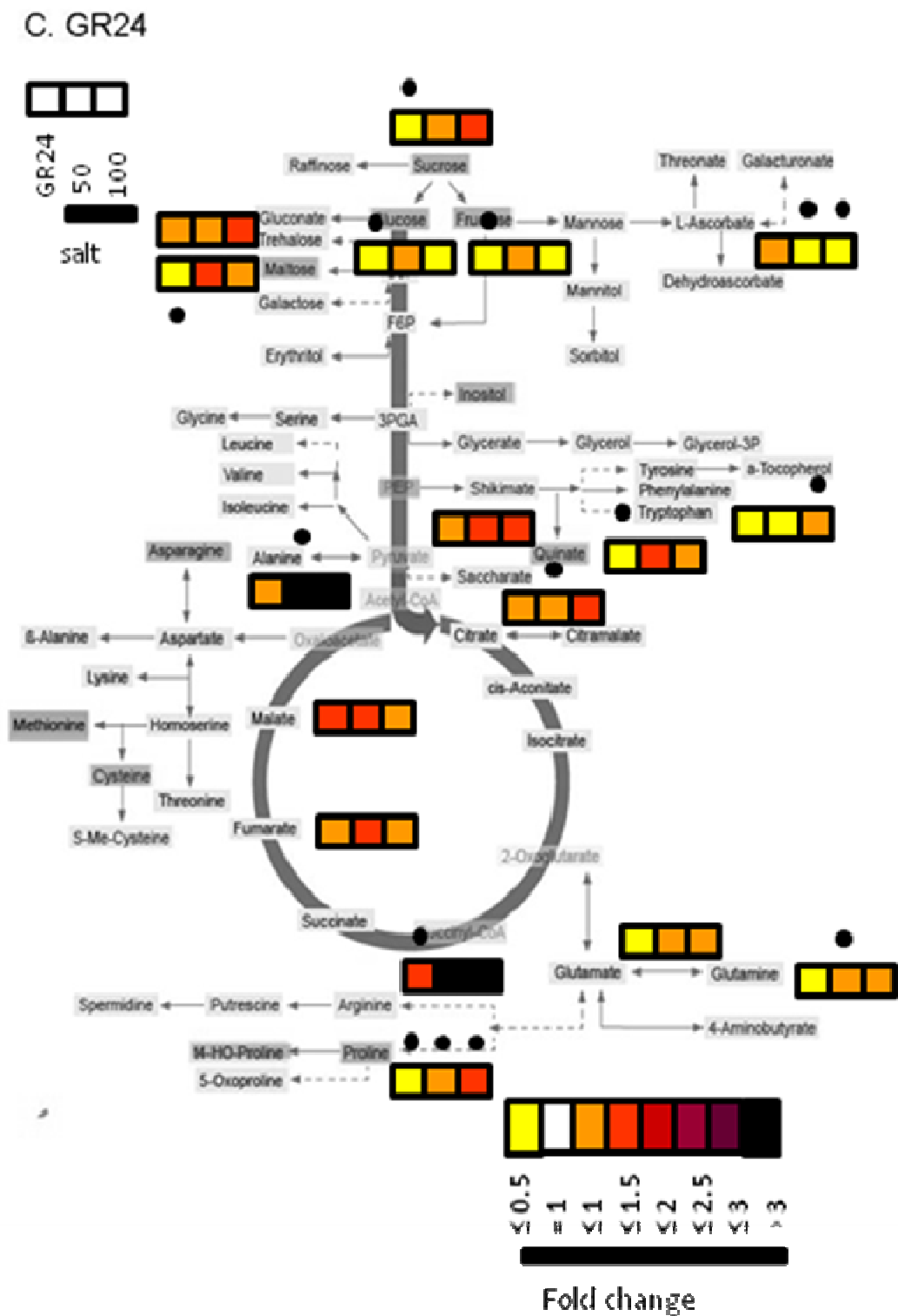


Figure 5.1 (Legend on previous page)

With the addition of the growth promoting substances, significant changes in several organic acids, sugars and amino acids were observed (Fig. 5.1B, C). The addition of SW led to significant decreases in the organic acids, pyruvate, fumarate and quinate

levels (Fig. 5.1B). Furthermore, metabolite changes as a result of SW treatment caused significant decreases in fructose, glucose, galactose, rhamnose, *myo*-inositol, sucrose, trehalose and maltose (Fig. 5.1B). In contrast, SW treatment resulted in significant increased ribose levels (Supplemental Table S1). Amino acid changes in response to SW were mainly observed with regard to decreased proline, phenylalanine and tryptophan and greatly enhanced arginine levels (Fig. 5.1B).

On the other hand, GR24 treatment resulted in significant decreases in quinate, as well as decreased levels for fructose, glucose, rhamnose, *myo*-inositol, sucrose and maltose (Fig. 5.1C). Like SW, GR24 treatment also resulted in decreased levels of proline, with further additional reductions in the amino acids of trans-4-hydroxy-proline, asparagines, and cystine (Fig. 5.1C).

Under salinity stress, a significant decrease could be observed in fumarate levels upon SW treatment (Fig. 5.1B). Furthermore, the combination of salt and SW treatment further led to a significant decrease in malate upon 50 mM salt application (Fig. 5.1B). Strikingly, with the addition of Na⁺ ions (in combination with the PGPS treatments), both SW (except at 50 mM NaCl with SW) and GR24 treatment significantly increased proline levels to levels approaching those of the untreated unstressed control (Fig. 5.1B, C). In contrast, under the same treatments, the control salinity treatments had a significant decrease in proline levels (Fig. 5.1A). Furthermore, it was also evident from the GR24 treatment that, under increasing salt concentrations, several organic acids were significantly affected; both ascorbate and citrate levels decreased under 50 mM salt, while isocitrate and ascorbate decreased under 100 mM salt treatment (Fig. 5.1C). Valine, leucine, isoleucine, proline, serine, methionine, tryptophan, phenylalanine and arginine levels increased significantly upon 50 and 100 mM NaCl with SW compared with the unstressed SW-treated seedling levels. In addition, serine, threonine, trans-4-hydroxy-proline, asparagine and lysine levels also increased significantly from 50 and 100 mM NaCl with GR24 compared with the unstressed GR24-treated seedlings.

5.3.3 Phytohormone levels of smoke water and GR24 with and without salt stress

In light of the involvement of SW and GR24 in phytohormonal responses, the levels of these were determined next in *N. benthamiana* seedlings, with and without salt

stress (Fig. 5.2 and 5.3). Increasing salt concentrations resulted in increased levels of auxin-conjugates (IAA-Asp and IAA-Ile) at 50 and 100 mM NaCl determined in *N. benthamiana* (Fig. 5.2). The same result was observed for most cytokinin levels with significant increases at 50 and 100 mM NaCl treatment, including *trans*-zeatin (tZ), isopentenyl adenosine (iPR) and *trans*-zeatin riboside (tZR). ABA and JA levels also increased upon 50 and 100 mM NaCl treatment compared with the unstressed seedling levels (Fig. 5.3).

Smoke water treatment to unstressed seedlings caused increased ABA, GA₃, tZR and IAA-Ile levels and decreased salicylic acid (SA) levels (Fig. 5.2 and 5.3). Also, SW treatment resulted in significant decreases in SA and JA levels under salt stress compared with untreated stressed seedlings (Fig. 5.2 and 5.3). Similarly, GR24 treatment also resulted in decreased SA levels compared to the controls and significantly increased ABA and tZR levels (Fig. 5.2 and 5.3). As previously observed, high auxin (IAA) levels were also evident in the GR24 treatments (Fig. 5.2; Kotze, 2010). However, upon SW and GR24 salinity treatments, IAA levels were below those of the respective salt controls (Fig. 5.2). Although GA₄ levels increased significantly after 50 and 100 mM NaCl treatment (as well as for GA₃ at 100mM salt), the addition of SW or GR24 resulted in significant decreased GA levels compared with the respective salt controls under these conditions (Fig. 5.2).

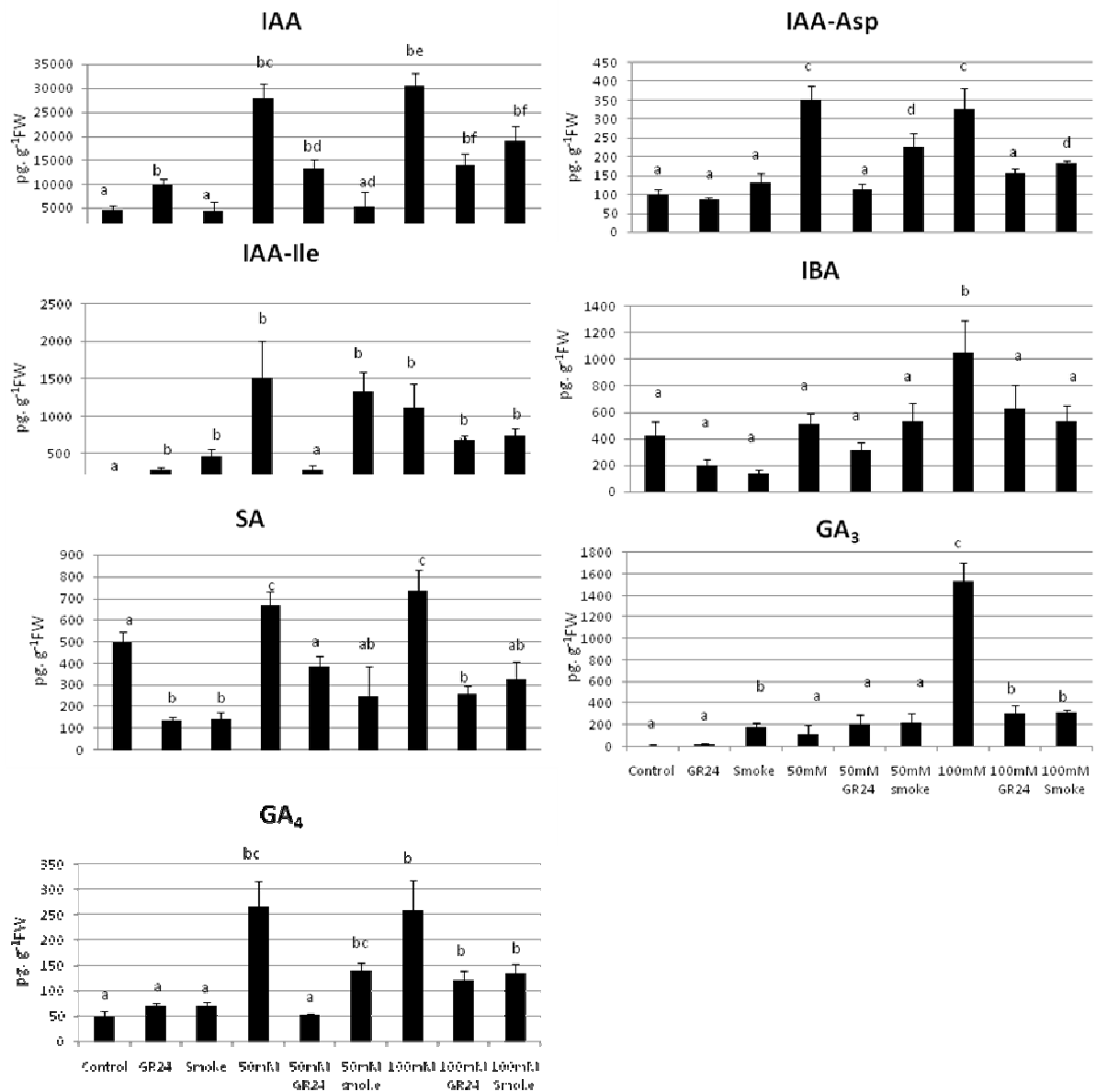


Figure 5.2 Phytohormone levels in response to smoke water and GR24 in salt stressed (50 mM and 100 mM) *Nicotiana benthamiana* seedlings (pg/g FW). Abbreviations: IAA, indole-3-acetic acid, IAA-Asp, indole-3-acetic acid-aspartate, IAA-Ile, indole-3-acetic acid-isoleucine, IBA, indole-3-butyric acid, SA, salicylic acid, GA₄ and GA₃, gibberellin A₄ and A₃, respectively. Values are presented as means ± SE of six individual seedlings and statistical significance was determined by analysis of variance (ANOVA) ($P < 0.05$) followed by Fishers least significant difference (LSD) test. Bars that share the same letter are not significantly different from each other.

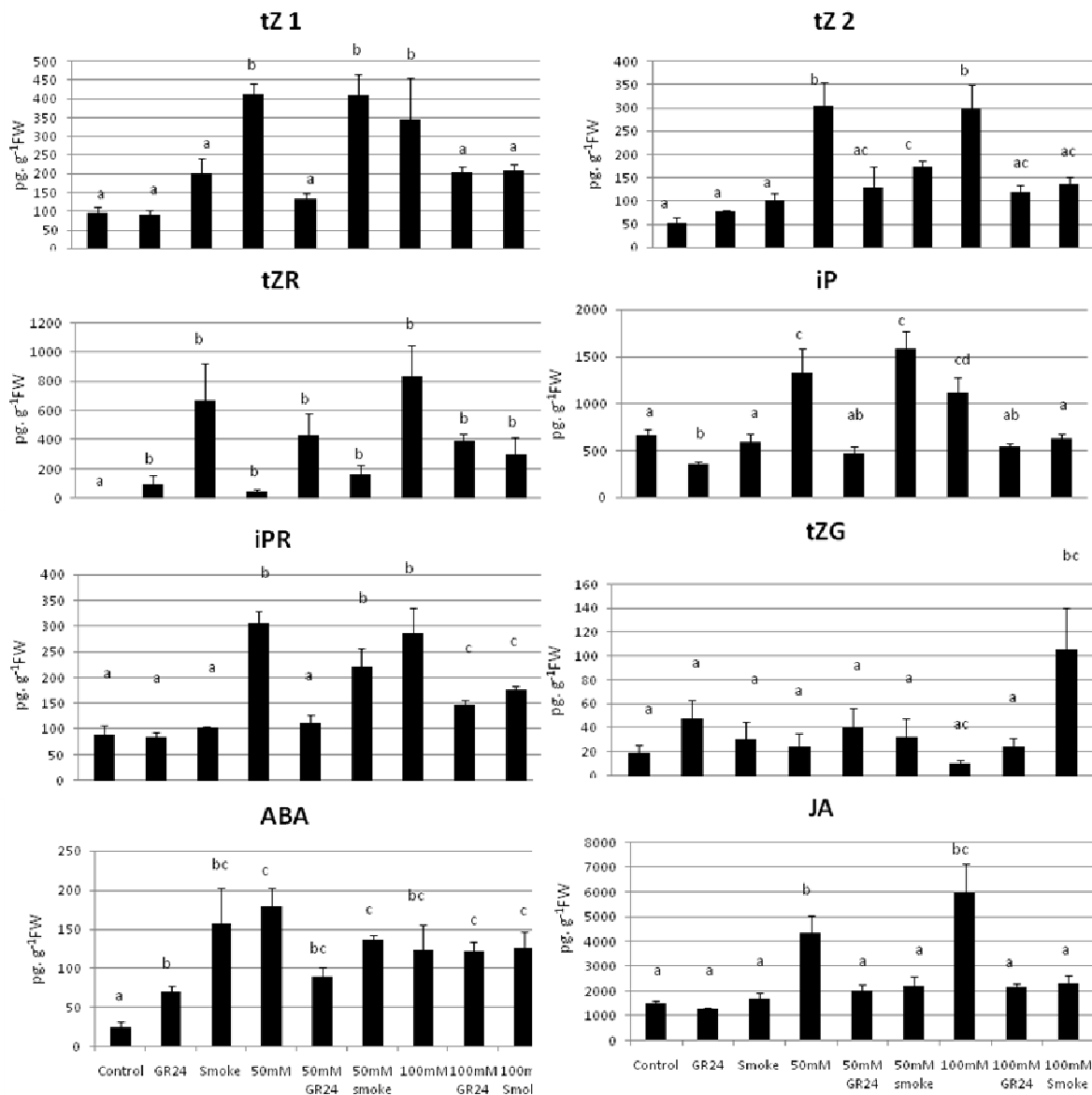


Figure 5.3 Phytohormone levels in response to smoke water and GR24 in salt stressed (50 mM and 100 mM) *Nicotiana benthamiana* seedlings (pg. g⁻¹FW). Abbreviations: tZ, *trans*-Zeatin, tZR, *trans*-zeatin 9-riboside, iPR, isopentenyl adenosine, iP, isopentenyl adenine, tZG, *trans*-zeatin-9-glucoside, ABA, abscisic acid, JA, jasmonic acid. Values are presented as means \pm SE of six individual seedlings and statistical significance was determined by analysis of variance (ANOVA) ($P < 0.05$) followed by Fishers least significant difference (LSD) test. Bars that share the same letter are not significantly different from each other.

5.4 Discussion

Salt stress is a major constraint on yield and plant performance, with an approximate 831 million ha land worldwide negatively affected by salinity (Martinez-Beltran and

Manzur, 2005). Poor quality irrigation water containing high salt levels with insufficient means of leaching is one of the main causes for increases in global soil salt toxicity. Also, irrigation followed by quick evaporation of water results in salt-rich upper soil levels (Rengasamy, 2006). Salinity stress retards plant growth, especially root and shoot growth, followed by leaf damage as a result of salt accumulation in leaves. Most crop species are moderately salt tolerant during germination but as seed emergence and seedling development progress, seedlings become exceptionally susceptible to salinity stress (Lauchli and Epstein, 1990; Maas and Grattan, 1999). In this study, *Nicotiana benthamiana* seedlings showed severe growth retardations upon salt stress. The response could be significantly alleviated by SW and GR24 treatments (Chapter 3, Fig. 3.2, Table 5.1) and were subsequently evaluated on a biochemical level in order to identify putative molecular targets or biosynthetic pathways that may aid in this salt stress perception and adaptation. Metabolomics (the study of a range of metabolites present in a cell) has become a popular choice to study these underlying mechanisms associated with a particular phenotype, as the presence and levels of these molecules are a reflection of transcriptional, translational and post-translation regulation in the cell (Fiehn 2002; Weckwerth 2003). Furthermore, understanding the relationships between SW, GR24 and metabolite levels may provide a rational basis for improving salt stress tolerance and biomass production in food and energy crops.

5.4.1 Metabolites associated with photosynthetic performance and respiration are significantly affected by salinity stress and plant growth promoting substance treatments

Environmental stress has a significant effect on photosynthesis, respiration, and general mitochondrial metabolism and could be compensated for by, amongst others, redundancy in organic acid levels and subcellular localization, facilitating these processes to maintain function and homeostasis (reviewed in Nunes-Nesi *et al.*, 2011). Strikingly, levels of several organic acids increased significantly in response to salt stress, especially at 50 mM NaCl, namely malate (between 1.5- and 6-fold), citrate (between 1.7- and 2-fold) and fumarate (between 1.5- and 3-fold) (Fig. 5.1A). These metabolites are constituents in the tricarboxylic acid (TCA) cycle and are mainly involved in mitochondrial respiration and the generation of redox equivalents involved in ATP synthesis. Furthermore, malate is also a redox regulator of

photosynthesis (Backhausen *et al.*, 1998) and plays an integral role in stomatal movement in the vacuole and apoplast, leading to the control of CO₂ assimilation (reviewed in Nunes-Nesi *et al.*, 2011). Recently it has been shown that a minor isoform of fumarase (responsible for fumarate biosynthesis) leads to increased carbon accumulation into fumarate during the day, acting as a temporary storage molecule in photosynthesis when starch accumulation is prevented (Pracharoenwattana *et al.*, 2010). Furthermore, tomato plants with reduced mitochondrial fumarase and malate dehydrogenase activity, the two sequential steps in generating fumarate and then hydrolysing fumarate to malate, improves both carbon assimilation and above ground growth (Nunes-Nesi *et al.*, 2005; 2007). Decreased fumarate levels upon SW treatment (Fig. 5.1B), along with decreases in major and minor sugars (including sucrose, glucose, fructose, maltose, trehalose and *myo*-inositol; Fig. 5.1B) suggest that photoassimilation and photosynthate supply could be significantly affected by this treatment.

Furthermore, upon SW treatment with salt, maltose levels increased significantly to reach similar levels to the unstressed control seedlings (Fig. 5.1B). Maltose is the main degradation product of starch. Increased maltose levels were previously observed in salt stressed *Arabidopsis* and lotus plants (Sanchez *et al.*, 2008; Kempa *et al.*, 2008). It has also been demonstrated that maltose acts as a solute stabilizing factor which alleviates abiotic stress in *Arabidopsis* (Kalpan and Guy, 2004); yet in this study this was only evident upon addition of the PGPS treatment. A similar observation was made with trehalose levels that increased in salt stressed SW seedlings compared with the SW unstressed controls (Fig. 5.1B). Widodo *et al.* (2009) showed the same response to salt stress with trehalose levels increasing under salinity stress in barley leaves but, again, this response in the *Nicotiana* seedlings was only evident under the SW conditions (Fig. 5.1B). Trehalose acts as an osmoprotectant in resurrection plants; but in other higher plants a deficiency in the pathway leads to an arrest in embryo development, while partial complementation of trehalose mutants indicates its essential role in vegetative development stages (Lunn *et al.*, 2006). One plausible pathway that trehalose interacts with is starch metabolism (Kolbe *et al.*, 2005). Starch acts as a temporary storage molecule during photosynthetic carbon assimilation. Previous work has, however, shown that, upon SW or GR24 treatments, starch levels were unaltered despite significant increases in transcript levels associated with trehalose metabolism (Kotze, 2010). The same

assessment under saline conditions might provide a more direct link between starch, trehalose and PGPS treatment, and should be determined in future.

GR24 treatment also resulted in significant changes in major and minor sugar metabolism (Fig. 5.1C). However, in contrast to the SW treatment, levels of several organic acids, including citrate (50 mM salt), isocitrate (100 mM salt) and ascorbate (both salt concentrations), decreased upon salt and GR24 treatment (Fig. 5.1C). Ascorbate is an important antioxidant that protects plants from oxidative damage resulting from osmotic disturbances, photosynthetic breakdown products and contributes to photosynthetic electron transport regulation (Smirnoff, 1996). Enhanced ascorbate levels increase salt stress resistance, reduce lipid peroxidation and is associated with redox regulation of photosynthesis (Wang *et al.*, 1999; Shalata and Neumann *et al.*, 2001; Nunes-Nesi *et al.*, 2005). One way to interpret these results is that photosynthesis could also be negatively affected in GR24 salt-stressed treatments. In support of this, in Chapter 4, GR24 treatment has led to decreased RuBisCO activase protein amounts (Table 4.2). However, exogenous GR24 application to *Arabidopsis* lead to enhanced chlorophyll content and improved photosynthetic capacity (Nelson *et al.*, 2010), suggesting that this hypothesis appears unlikely. Another plausible explanation is that mitochondrial respiration efficiency might be significantly affected. Tomato roots with down-regulated fumarase and malate dehydrogenase activity had significant reduced biomass, along with decreased respiration rates (Van Der Merwe *et al.*, 2009). From the decreased citrate, isocitrate and ascorbate levels upon GR24 treatment, it appears likely that enhanced adenylate generation could contribute to plant fitness and performance when GR24 are applied, especially since ATP synthase CF1 beta subunit also increased in response to GR24 treatment (see Chapter 4, Table 4.2). Therefore, both photosynthesis and respiration measurements needs to be performed in future in order to determine how these two fundamental processes contribute to plant growth and stress alleviation upon the different PGPS treatments.

5.4.2 Primary metabolite and phytohormone interactions induced by smoke water and GR24

A striking observation from the PGPS primary metabolite profiles were the significant decreases observed in specific organic- and amino acids that serve as precursors for

phytohormone biosynthesis (Fig. 5.2 and 5.3). One of the routes of auxin biosynthesis is via L-tryptophan (Bialek *et al.*, 1992), while ethylene is commonly formed from L-methionine (reviewed by Bleecker and Kende, 2000). In addition, ABA and strigolactone are synthesised from carotenoid precursors driven by Calvin cycle intermediates in the plastid. Additionally, phytohormone regulation is finely coordinated with substrates from primary carbon metabolism, with N-conjugation of glucose and alanine, and isoleucine and aspartate inactivating cytokinin and auxin activity, respectively (Bandurski *et al.*, 1995; McGaw and Burch, 1995; Kende and Zeevaart, 1997). Furthermore, the biosynthesis of many gibberellins requires 2-oxoglutarate as cosubstrate and ascorbate as a cofactor (Kende and Zeevaart, 1997). In addition, the induction of hexose transporters by cytokinins is well-documented (Ehneß and Roitsch, 1997). Profiling of these messengers from secondary metabolism revealed that ABA, IAA, tZR and GA₃ (SW only), increased significantly upon PGPS treatment (Fig. 5.2 and 5.3).

ABA is involved in many plant responses, including stress, seed dormancy, root and shoot growth and has been shown to interact with sugar metabolism (Creelman *et al.*, 1990; Arenas-Huertero *et al.*, 2000; Cheng *et al.*, 2002). In emerging seedlings a positive relationship exists where by exogenous glucose increased ABA levels (Arenas-Huertero *et al.*, 2000; Cheng *et al.*, 2002) and exogenously applied glucose and ABA increased sucrose levels (Kashem *et al.*, 1998). Starch is also closely correlated with ABA levels under salinity stress (Kempa *et al.*, 2008). In salt stressed *Arabidopsis*, ABA marker genes are positively correlated with levels of the starch breakdown product, maltose, suggesting that ABA may be involved in this metabolic reaction (Kempa *et al.*, 2008); yet the exact mechanisms and role players remain to be elucidated, especially since strigolactones also interact with ABA and alleviated salt stress.

It has previously been shown that *myo*-inositol content increases upon salt stress treatment, and is involved in salt stress tolerance in *Mesembryanthemum crystallinum* (Nelson *et al.*, 1998) and osmotic stress response in *Actinidia* species (Klages *et al.*, 1999). However, in *Nicotiana tabacum*, *myo*-inositol levels increased under salt stress but did not result in salt tolerance (Klages *et al.*, 1999). *Myo*-inositol is also important in many biochemical pathways, including signalling and phosphate storage via inositol phosphates (Hubel and Beck, 1996) and forms part of

membranes via inositol-containing lipids (Mathews and Van Holde, 1990). *Myo*-inositol may also be involved in long-distance transport of auxin in the plant (Cohen and Bandurski, 1982). GR24 and SW treatment caused decreased *myo*-inositol and enhanced IAA levels (Fig. 5.1B, C, Fig. 5.2). In addition, upon salt stress, these treatments did not induce *myo*-inositol levels significantly above untreated control levels (Fig. 5.1A-C). GR24 is known to alter PIN protein efflux carriers, which are responsible for auxin transport and root development (Benkova *et al.*, 2003; Bennett *et al.*, 2006; Crawford *et al.*, 2010; Ruyter-Spira *et al.*, 2010; Kapulnik *et al.*, 2011). In addition, null *Arabidopsis* mutants for *myo*-inositol-1-phosphate synthase (MIPS) activity, the initial step in *myo*-inositol biosynthesis, have slower PIN2 kinetics and are characterized by reduced polar auxin transport (Chen and Xiong, 2010). This suggests that *myo*-inositol and strigolactone metabolism might interact to facilitate auxin distribution in the plant; the exact nature of this relationship remains an exciting topic for future research.

IAA levels decreased in response to SW and GR24 treatment in salt stressed seedlings (Fig. 5.2). In other words, auxin levels did not reach the enhanced levels as those observed under 50 and 100 mM salt without SW and GR24. This decrease in IAA levels can be correlated with the effects of salt stress on biomass accumulation upon SW and GR24 (Table 5.1). Other hormones that showed the same response to salt stress after treatment with SW and GR24 include several cytokinins, GAs, SA and JA. SA and JA are the main signalling molecules involved in defence against diseases and herbivore resistance, respectively (Clarke *et al.*, 2000). Salicylic acid has also been documented as an important molecule in physiological processes such as flowering, thermogenesis and stomatal closure, as well as responses to abiotic stress (Catinot *et al.*, 2008). Production of SA is dependent on isochorismate in *Nicotiana benthamiana* (Catinot *et al.*, 2008). When wild type and a SA biosynthesis mutant were exposed to 100 mM NaCl, the wild type plants showed higher levels of oxidative damage, suggesting that SA is involved in the generation of reactive oxygen species (ROS) upon salt stress (Chen *et al.*, 1993; Borsani *et al.*, 2001). Similarly, IAA biosynthesis is dependent on hydrogen peroxide (H₂O₂) generation, which would need to be efficiently dissipated in order to avoid oxidative stress responses (Gazarian and Lagrimini 1996a; b; Gazaryan *et al.* 1998; Savitsky *et al.* 1999). Thus, the protection of seedlings against high ROS production upon SW and GR24 treatment could be by decreasing or limiting hormone levels.

Influencing some of these hormonal reactions provide one mechanisms to protect seedlings against the detrimental effects of salinity and ROS production.

5.4.3 Amino acid metabolism is increased upon salt stress and plant growth promoting substances treatments

Of all the amino acids measured, proline showed the most striking changes after the various treatments (Fig. 5.1). Proline is one of the most commonly known osmoprotectants and increases in response to salt, drought and heavy metal stress in many plant species (Sarvesh *et al.*, 1966; Kohl *et al.*, 1991; Muthukumarasamy and Panneerselvam, 1997; Schat *et al.*, 1997; Kavi Kishor *et al.*, 2005; Kempa *et al.*, 2008). Proline application to stressed plants causes a reduction in levels of free radicals (Singh *et al.*, 1996, Jain *et al.*, 2001) associated with oxidative damage. Proline is also involved in stabilization of proteins, membranes and subcellular structures and plays a crucial role in protecting photosynthetic activity under stress (Vanrensburg *et al.*, 1993; Kavi Kishor *et al.*, 2005). Proline may be synthesised either from glutamate or arginine/ornithine (Adams and Frank, 1980; Kavi Kishor *et al.*, 2005) via the rate-limiting steps of Δ^1 -pyrroline-5-carboxylate synthase (P5CS) or ornithine- δ -aminotransferase (δ -OAT), respectively. After salt treatment, both these enzyme activities are significantly reduced; however, P5CS activity is affected by exogenous ROS addition and is active regardless of ROS levels, while δ -OAT seems to be regulated by ROS changes under salt stress (Carrion *et al.*, 2008). Transgenic plants engineered for enhanced proline levels have increased tolerance to abiotic stress and improved growth (Hong *et al.*, 2000; Maggio *et al.*, 2002; Roosens *et al.*, 2002); however, enhanced levels, from over-expression of *tomPRO2* (a Δ^1 -pyrroline-5-carboxylate synthetase, P5CS, from tomato) in yeast resulted in an inverse correlation to cell growth, suggesting that plant growth could be impaired under unstressed conditions (Maggio *et al.*, 2002). Furthermore, proline levels are also stimulated by ABA via *P5CS1* regulation (Strizhov *et al.*, 1997; Abraham *et al.*, 2003) and ABA levels increased significantly upon SW and GR24 treatment compared with the controls. ABA seems to respond differently to stress compared with the other phytohormone levels, suggesting a response of this hormone to the PGPS, both with and without salt stress. In addition, proline levels decreased significantly in SW and GR24 treated seedlings under replete conditions and, with salt stress treatment (in parallel with the growth substances), increased significantly in both salt treatments

with GR24, while proline levels increased significantly at higher salt concentration (100mM) after SW treatment (Fig. 5.1 B, C). In contrast, control seedlings had decreased proline levels upon salinity stress, while arginine/ornithine levels increased significantly (Fig. 5.1 A), suggesting an important switch in this particular part of metabolism concerned with the biosynthesis of these osmoprotectants. One speculation is that, upon SW and GR24 treatment, under salinity increased ABA levels could enhance P5CS activity driving proline biosynthesis through this route. Alternatively, the proposed regulated ROS generation upon PGPS application could maintain δ -OAT activity under salinity stress, as was observed in *in vitro* experiments performed on Chinese cabbage (*Brassica rapa* L. cv. Onekilo) treated with NaCl and increasing concentrations of the nitric oxide donor SNP (Carrion *et al.* 2008). It would be interesting to determine whether one or both of these possibilities are plausible in this situation.

It was further observed that GR24 treatment led to decreased isoleucine, trans-4-hydroxy-proline, asparagine and cystine levels (Fig. 5.1 C). Apart from amino acid metabolism being directly altered, this could also imply that protein translation might be affected following these treatments. Similarly, salinity stress led to increased glycine, valine, leucine, isoleucine, proline, serine methionine, tryptophan, phenylalanine and arginine levels following the addition of both SW or GR24 (Fig. 5.1 B, C). Protein translation has been previously correlated with biomass accumulation (Usadel *et al.*, 2008) and, in combination with alterations in amounts of highly abundant proteins (Chapter 4), could suggest an alteration in protein turnover or re-allocation of nitrogen caused by the PGPS and/or stress treatment (Kempa *et al.*, 2008).

5.5 Conclusion

In conclusion, metabolite profiling reveals a re-configuration of primary carbon metabolism involving decreased sugar and organic acids, and altered amino acid metabolism under SW, GR24 and salinity treatments. While some of the effects appear to be related to altered carbon assimilation and respiration, it is also likely that some of these substrates form either vital biosynthetic precursors or regulate the distribution of classical phytohormones associated with growth and stress responses. Treatment with SW and the strigolactone analogue GR24 further revealed that, under

stress conditions, phytohormone levels are more effectively controlled than in the respective salt controls, providing a plausible mechanism to limit the generation of harmful reactive oxygen species. Lastly, it appears that salt stress tolerance mediated by SW or GR24 is mainly regulated by proline and ABA levels. The novel regulatory role(s) these compounds may plausibly play in proline or ABA metabolism provide an interesting strategy to explore in increasing salt tolerance without the detrimental effects caused by direct interference in the proline/ABA biosynthetic pathway under unstressed growing conditions. However, the exact mechanism how this is achieved remains to be elucidated, and this should be a key research endeavour in future.

Chapter 6

General conclusion and future prospects

In this study, *Nicotiana benthamiana* seedlings were exposed to different concentrations of salt stress, with and without the plant growth promoting substances, SW and GR24. Changes in physiological growth, the proteome and the metabolome were analysed. Both of the PGPS invoked similar physiological changes of enhanced salt tolerance and increased biomass accumulation (Chapter 3). Fresh and dry mass, root length, leaf area and number increased significantly in response to SW and GR24 treatment. Under salt stress conditions, decreases in fresh mass and all other growth parameters were observed in a concentration-dependent manner. Interestingly, in response to SW treatment, different growth responses were observed at different salt concentrations. It is therefore obvious that different mechanisms are activated in response to the severity of the salt stress. Smoke water could significantly increase fresh mass, root length and lateral root number at 100 mM NaCl, but not at 150 mM NaCl. There was also a definite increase in root development in salt stressed seedlings in response to SW and GR24 treatment, resulting in increases in lateral root number and length at both 100 and 150 mM NaCl. Although SW has previously been shown to have beneficial effects on stressed (but not salt stressed) seedlings, this represents a completely novel finding for strigolactones. Interestingly, seedlings responded differently to SW and GR24 treatment in winter months compared with summer months. This shift in growth and metabolism could be due to an inbuilt circadian rhythm in the seedling and future studies on this phenomenon may give some clues into the mechanism involved in salt tolerance in response to the PGPS.

Proteome analysis using 2D-PAGE and PMF via MALDI-TOF mass spectrometry showed that SW and GR24 treatment resulted in decreases in RuBisCO-associated proteins in both stressed and unstressed seedlings (Chapter 4). However, decreases in RuBisCO levels do not necessarily result in decreases in photosynthetic rate of growth (Quick *et al.*, 1991a; Stitt *et al.*, 1991). Interestingly, both growth promoting substances resulted in increases in levels of a chloroplast photosynthetic oxygen-evolving protein (33 kDa subunit). This complex protein is specifically salt-stress inducible (Abbasi *et al.*, 2004) and SW and GR24 may, therefore, mimic a

stress response which could facilitate the seedling towards adaptation against salt stress. Unfortunately, little knowledge could be gained from the proteome study because of the fact that only a small number of proteins could be positively identified. This is, however, not uncommon in MALDI-TOF MS studies. When more information on the *Nicotiana benthamiana* proteome becomes available, further analysis of the peptide mass fingerprints should be conducted, which could facilitate a better understanding into the response mechanism active during treatment with PGPSs.

Despite the similarities observed in basic growth, metabolomic studies suggested slight differences in the response mechanism (Chapter 5). Both SW and GR24 treatment resulted in significant changes in major and minor sugar metabolism, but in contrast to SW treatment, GR24 with salt treatment resulted in decreases in a number of organic acids such as ascorbate, which is an important antioxidant that protects plants from oxidative damage.

Of the amino acids, proline showed the most changes in response to different treatments. Proline is an important cellular osmoprotectant and decreased in response to salt stress and in response to SW and GR24. However, under salt stress conditions in untreated seedlings, levels of arginine, which is a precursor to many other osmoprotectants (Bohnert *et al.*, 1995; Kaur-Sawhney *et al.*, 2003), increased significantly. Also, in seedlings treated with the growth promoting substances during salt stress, proline levels increased significantly. Therefore a definite shift in osmoprotectant metabolism was observed upon treatment with the PGPS. The SW and GR24 treatments, both of which resulted in increases in biomass, also resulted in increased proline levels under salt stress. This is a novel finding and demonstrates the positive effects the PGPS have on abiotically-stressed seedlings. Proline accumulation is stimulated in many plants by ABA and salt stress, but increasing proline levels in unstressed plants have detrimental effects on growth (Strizhov *et al.*, 1997; Abraham *et al.*, 2003; Maggio *et al.*, 2002). Because of this undesirable response, little advance has been made in increasing salt tolerance in crop species via this route. Therefore manipulation of the strigolactone biosynthetic pathway through genetic engineering strategies could both increase biomass under replete conditions, as well as aid in salt tolerance through increased proline levels in young seedlings.

Furthermore, SW and GR24 treatment did not alter hormone levels under salt stress with the exception of ABA (Chapter 5). Salicylic acid (SA) biosynthesis generates harmful ROS (Chen *et al.*, 1993; Borsani *et al.*, 2001) and IAA biosynthesis is dependent on ROS (Gazarian and Lagrimini 1996a; b; Gazaryan *et al.* 1998; Savitsky *et al.* 1999), therefore part of the mechanism via which SW and GR24 alleviate salt stress may involve protecting the seedlings from the generation of harmful oxidative molecules by decreasing hormonal biosynthesis under salt stress. The changes in ABA levels in response to the PGPS followed a different pattern from the other phytohormone levels, in that ABA levels increased compared with the unstressed controls for all treatments. Increases in ABA levels are only to be expected, since ABA is intimately involved in the response to drought and salt stress. Future in-depth studies on the exact effect the PGPS have on ROS generation and ABA biosynthesis could facilitate a better understanding into the mechanism(s) via which they aid seedling salt tolerance.

Although the exact mechanisms by which SW and GR24 promote biomass accumulation and enhance salt tolerance could not be identified, some valuable points for future study have been identified. Firstly, changes in the metabolome, proteome and growth suggest that photoassimilation and photosynthesis supply could be significantly affected by the PGPS. This was not determined during this study because the seedlings were grown in a tissue culture system and, upon removal from the plates, seedlings started to wilt quickly, preventing the measurement of the photosynthetic rate. It would be extremely valuable in future studies to find a means to measure photosynthesis in these seedlings and determine whether the changes in the photosynthesis-related protein levels and metabolites correlate with actual changes in photosynthetic performance. A second interesting point of future study would be to look into changes in ROS generation and metabolism in response to the PGPS with and without abiotic stress. Finally, all of the observed increases in biomass and changes in the proline and other metabolite levels were established in a tissue culture system. In the future, these physiological and metabolomic observations need to be validated in pot trial experiments.

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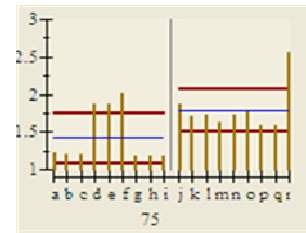
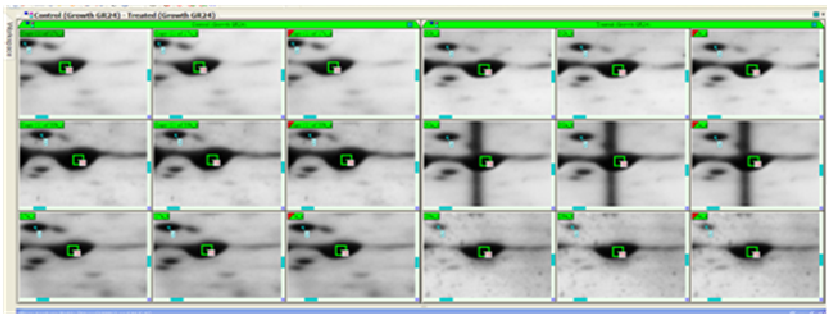
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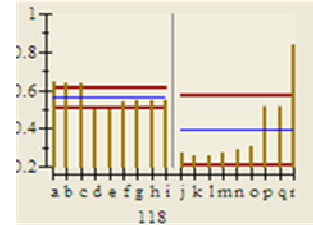
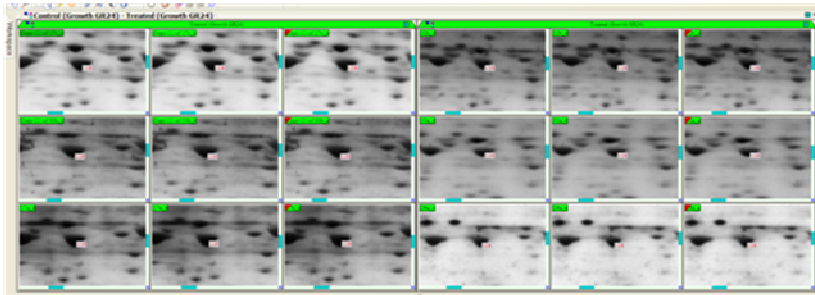
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Appendices



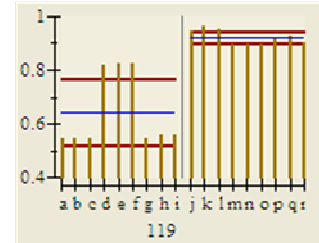
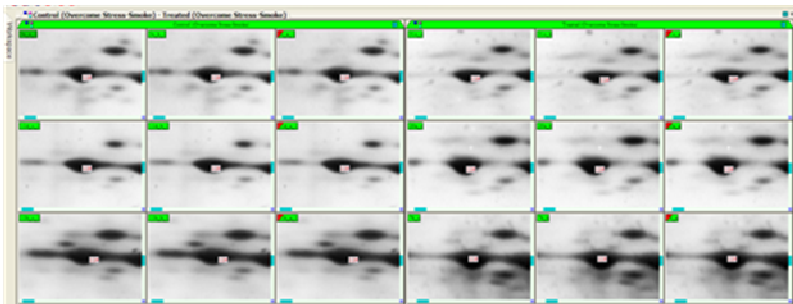
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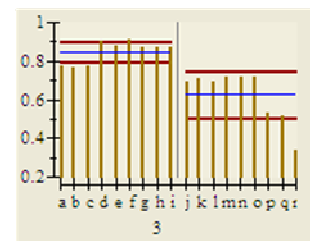
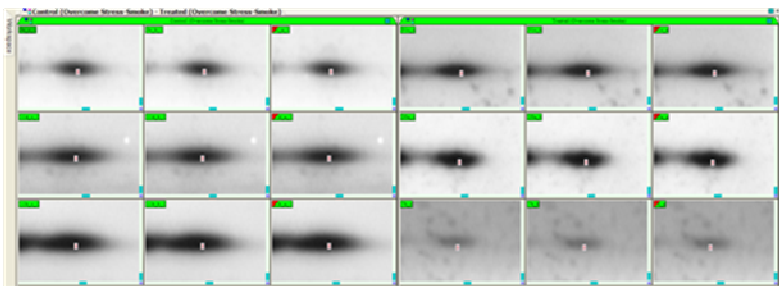
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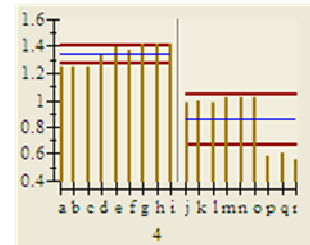
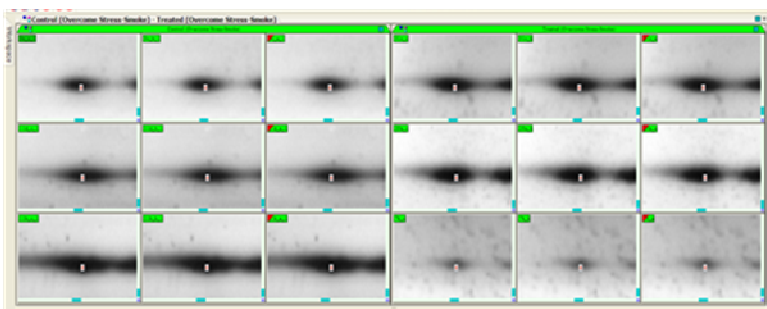
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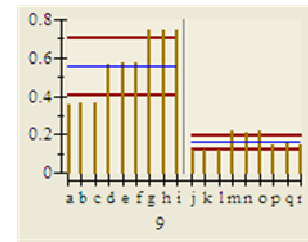
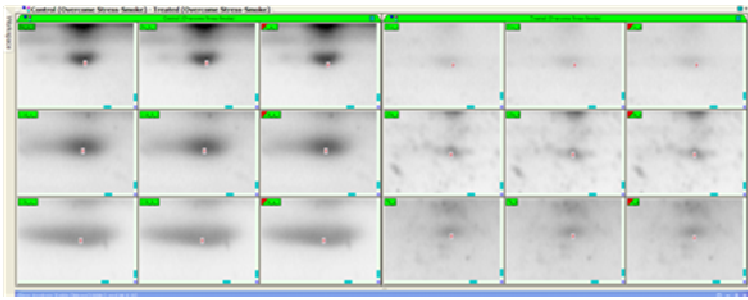
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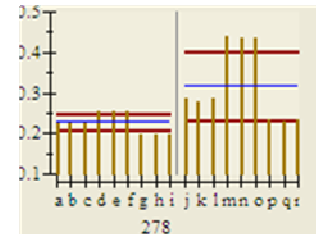
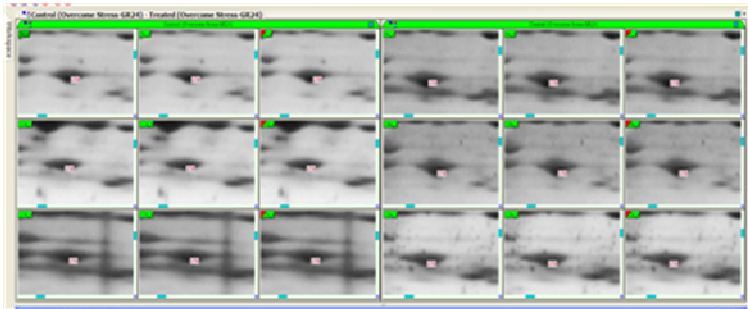
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Match ID 9

< 3.49E-06



Match ID 278

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Appendix A: Magnified screenshots and expression profiles of the 7 differentially expressed protein spots which could be positively identified.

Expression profiles were calculated as the averages of three biological replicates using the Melanie 7.0 2D software. Match ID 75 was identified as Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (PsbO) (*Nicotiana benthamiana*) and Match ID 118 was identified as Ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) (*Nicotiana tabacum*) - (fragment). Both of these spots were isolated upon comparison of the proteome of GR24-treated seedlings compared with the proteome of untreated seedlings. Match ID 119 was identified as Chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (*Nicotiana benthamiana*) and Match ID 3 as Ribulose-1,5-bisphosphate carboxylase (*Nicotiana sylvestris*), Match ID 4 represents the positively identified Cristal-Glass1 protein (*Capsicum annuum*)/Ribulose bisphosphate carboxylase small chain and Match ID 9 was identified as Armadillo repeat-containing protein (*Nicotiana tabacum*)/CMPG1b (*Nicotiana benthamiana*). Match IDs 119, 3, 4 and 9 were all isolated upon comparison of 100 mM NaCl treatment with 100 mM NaCl with smoke water treatment. Match ID 278, identified from the comparison of the proteomes of 100 mM NaCl treated compared with 100 mM NaCl with GR24 treated seedlings, was identified as ATP synthase CF1 beta subunit (*Nicotiana sylvestris*).

Appendix B: Salt stress, SW and GR24 responsive proteins which fall within a pI range of 5-8 but with non-significant MOWSE scores. MS – MASCOT score, PM – peptides matched, % - percentage coverage.

Plant growth promotion

spot nr	Accession number	Mr/pI	MS	PM	%	Fold change	Homologous Protein
<u>Control vs GR24</u>							
44	Q6ATW0	9745/5.5	23/72	2	17	-1.5	retrovirus-related reverse transcriptase homolog - rice retrotransposon copia-like (fragment)
9	O05214	43840/5.33	20/57	3	6	-1.5	actin [<i>Nicotiana tabacum</i>]
57	Q2A9I2_BRAOL	19669/7.52	41/66	4	23	-1.6	GRF zinc finger containing protein.- <i>Solanum tuberosum</i> <i>Nicotiana benthamiana</i> chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (psbO) mRNA, complete cds; nuclear gene for chloroplast product.
75	AAX53163	35206/5.89	59/57	6	23	1.3	ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) (clone TA1.1) - common tobacco (fragment)
118	P69250	25913/5.01	88/66	8	32	-1.4	NADH-enoyl ACP reductase [<i>Brassica napus</i> , Peptide Partial, 15 aa]
145	P80030	1630/5.69	34/66	2	73	3.4	predicted protein [<i>Populus trichocarpa</i>]/ <i>Solanum tuberosum</i>]
58	B9I035	20333/5.94	55/72	5	20	-1.7	<i>Nicotiana tabacum</i> Cluster: Double-strand break repair protein
49	P94102	73134/6.76	55/72	6	13	-1.5	MRE11
53	P23322	3416/6.34	27/57	2	61	-1.4	33kDa precursor protein of oxygen-evolving complex [<i>Solanum lycopersicum</i>]
126	S25484	25913/5.01	57/66	6	15	-1.6	ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) (clone TA1.1) - common tobacco (fragment)
94	Q6L3S2	10756/5.69	35/72	3	21	-1.8	<i>Solanum lycopersicum</i> Cluster: Retrotransposon protein
33	O24131	18662/5.97	37/72	4	23	-1.2	fiber annexin [<i>Nicotiana tabacum</i>]
36	PSB5A_ARATH	29648/6.00	20/57	2	6	-1.3	<i>Nicotiana tabacum</i> Cluster: Peptidase T1A, proteasome beta-subunit
127	C7EC57	23111/5.76	38/66	4	20	-1.8	Ankyrin.- <i>Medicago truncatula</i> (Barrel medic)/ankyrin repeat-rich protein [<i>Nicotiana benthamiana</i>]
103	Q8RVS9	17974/5.32	29/66	3	18	-1.8	putative transposase-like protein [<i>Oryza sativa</i>]
227	Q6NPL1ARATH	33154/5.26	33/66	4	11	-1.6	<i>Solanum tuberosum</i> Ubiquitin
102	TA17482_4097	44141/5.22	41/57	5	15	-1.5	<i>Nicotiana tabacum</i> Cluster: Putative serine protease inhibitor
148	CV477853	4090/6.55	40/66	3	81	1.7	<i>Solanum tuberosum</i> BEL1-related homeotic protein 11

89	EB683546	19924/6.69	17/57	2	11	1.4	<i>Nicotiana tabacum</i> Cluster: Zinc finger, RING-type; n=1
189	EB451068	15650/5.52	46/72	4	31	-1.8	<i>Nicotiana tabacum</i> Cluster: AUX/IAA protein
232	TA16561_4097	18243/5.11	42/72	4	13	-1.9	<i>Nicotiana tabacum</i> Cluster: 101 kDa heat shock protein
128	CV019872	10825/5.88	21/66	2	21	-1.6	<i>Nicotiana tabacum</i> Cluster: Proteasome alpha subunit-like protein
242	BP529501	21001/6.16	17/66	2	9	-1.5	<i>Nicotiana tabacum</i> Cluster: Retrotransposon gag protein
151	BT014438	7188/5.34	25/57	2	40	-1.4	<i>Solanum lycopersicum</i> Cluster: L-asparaginase precursor <i>Solanum lycopersicum</i> Cluster: Ribosomal protein L11
144	TA55063_4081	8617/5.77	38/66	3	40	1.7	methyltransferase-like protein <i>Nicotiana tabacum</i> Cluster: Phospholipid hydroperoxide glutathione
132	TA15814_4097	18919/6.37	28/66	3	17	3.0	PEROXIDASE
191	Q6T7E8	50886/5.93	25/57	4	7	-2.0	Adenylosuccinate synthetase, chloroplastic [<i>Nicotiana tabacum</i>]
246	Q9SXX8	79555/5.59	34/66	6	5	-1.9	heat shock factor [<i>Nicotiana tabacum</i>]
150	P82856	4142/7.82	42/72	3	58	-1.5	unknown protein <i>Nicotiana tabacum</i>
169	B3F8F4	13602/5.39	19/57	2	14	-2.0	glutaredoxin [<i>Solanum tuberosum</i>] class II small heat shock protein Le-HSP17.6 [<i>Solanum</i>
272	Q96489	17843/7.71	18/57	2	7	1.5	<i>lycopersicum</i>] photosystem I light-harvesting chlorophyll a/b-binding protein
78	P27492	26522/6.97	23/72	3	13	1.8	[<i>Nicotiana tabacum</i>]
161	P29130	7232/6.06	38/72	3	40	-2.0	<i>Nicotiana tabacum</i> Cluster: Alpha-soluble NSF attachment protein

Control vs smoke water

33	Q33BV4	2244/6.51	23/57	2	73	1.4	Ycf15 protein [<i>Nicotiana tomentosiformis</i>]
200	Q9FSF8	30218/5.3	33/66	6	20	1.5	protein phosphatase 2C [<i>Nicotiana tabacum</i>] Pentatricopeptide repeat domain containing protein, putative
98	Q60D18	77823/5.66	37/57	12	10	2.7	[<i>Solanum demissum</i>]
4	T14642	3722/6.51	24/66	2	61	-1.2	Alpha-galactosidase [<i>Solanum lycopersicum</i>]
0	P54214	31393/5.41	22/57	5	19	-5.4	SF-assemblin [<i>Dunaliella bioculata</i>] pathogenesis-related protein (PR-5 protein) [<i>Solanum</i>
3	P04284	3589/6.12	22/57	2	34	-1.6	<i>lycopersicum</i>]
2	Q9ATE7	16773/5.58	35/66	5	31	-1.5	MADS-box transcription factor FBP22 [Petunia x hybrida]
94	Q2PYY3	38837/6.95	37/72	7	22	1.3	transporter-like protein [<i>Solanum tuberosum</i>]

44	D4P3S0	39112/6.46	24/57	5	16	1.9	sinapyl alcohol dehydrogenase 2 [<i>Nicotiana tabacum</i>]
31	CAC07357	2023/6.48	14/66	1	94	1.9	Sequence 8 from Patent WO0004176 (Fragment)[<i>Calluna vulgaris</i>] Cluster: ATP synthase subunit beta mitochondrial precursor
209	TA12596_4097	53128/5.06	9/57	3	8	-1.5	[<i>Nicotiana tabacum</i>] Cluster: ADP-glucose pyrophosphorylase small subunit-like [
24	TA13341_4097	52114/5.54	24/57	6	11	1.8	<i>Nicotiana tabacum</i>] Cluster: Myb-like DNA-binding domain SHAQKYF class family
143	TA50786_4081	35033/6.98	26/66	6	12	1.4	protein [<i>Solanum lycopersicum</i>]
206	TA15591_4097	11804/5.41	23/72	3	25	-9.1	Cluster: Tumor-related protein [<i>Nicotiana tabacum</i>] transferase, transferring glycosyl groups / transferase [<i>Solanum</i>
47	TA46749_4113	10032/6.88	37/72	4	25	1.4	<i>tuberosum</i>]
13	TA42226_4113	18650/6.9	20/57	3	20	-2.1	cytochrome c biogenesis orf452 [<i>Solanum tuberosum</i>]
49	DB692289	27704/5.11	21/57	5	17	1.3	Cluster: Syntaxin/epimorphin family [<i>Solanum lycopersicum</i>]
192	TA31068_4113	50063/5.44	27/57	6	13	2.4	Flavonoid 3-glucosyl transferase [<i>Solanum tuberosum</i>]
99	Q53J26	2640/6.12	24/72	2	68	1.5	hypothetical protein LES1_20t00006 [<i>Solanum lycopersicum</i>] ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
182	Q33C26	45694/6.42	30/66	7	15	3.6	[<i>Nicotiana tomentosiformis</i>]
207	Q1W375	28175/5.54	21/57	4	10	1.4	phosphomannomutase [<i>Nicotiana tabacum</i>]
122	Q6XX15	16983/5.04	26/66	4	21	1.5	glutathione S-transferase U3 [<i>Nicotiana benthamiana</i>]
32	Q2LFC4	9981/5.28	15/57	2	15	1.4	AGO1-1 [<i>Nicotiana benthamiana</i>] Cluster: H ⁺ -transporting two-sector ATPase alpha/beta subunit
93	TA14672_4097	14629/6.59	21/57	3	27	1.9	[<i>Nicotiana tabacum</i>]
63	BQ116994	5753/6.81	23/72	3	38	1.9	<i>Solanum tuberosum</i> Hypothetical protein At5g20635
213	Q52QJ6	14268/6.58	32/72	4	44	1.2	methionine rich arabinogalactan [<i>Solanum lycopersicum</i>]
221	Q93YF0	10375/7.18	27/66	4	51	-1.2	hypothetical protein NitaMp031 [<i>Nicotiana tabacum</i>]
58	P00826	19657/7.77	27/57	4	17	-1.7	ATP synthase CF0 B subunit [<i>Nicotiana tabacum</i>]
302	P00826	19657/7.77	19/57	3	17	-4.1	ATP synthase CF0 B subunit [<i>Nicotiana tabacum</i>]
298	Q9FS86	16139/5.81	39/57	5	31	-4.3	Ga20 oxidase [<i>Solanum tuberosum</i> subsp. <i>andigena</i>]
37	Q9SE09	9357/5.81	27/57	3	43	-1.7	cystatin [<i>Solanum lycopersicum</i>]
90	TA8565_4100	15275/7.44	31/72	4	33	1.3	<i>Nicotiana benthamiana</i> Cluster: F-box/LRR-repeat protein 20
115	AF479624	13320/5.97	30/66	5	27	1.6	<i>Nicotiana benthamiana</i> Cluster: Prf-like protein
179	TA1177_164110	11735/5.5	29/57	4	42	2.2	Vacuolar ATP synthase subunit G 1 [<i>Nicotiana tabacum</i> (Common tobacco)]

335	TA23170_4113	23407/6.1	36/72	5	18	-4.8	Serine acetyltransferase [<i>Nicotiana plumbaginifolia</i> (Leadwort-leaved tobacco)]
15	TA20825_4097	33903/7.6	17/57	4	16	-1.3	<i>Nicotiana tabacum</i> Cluster: Clp protease
278	TA5239_4072	7294/5.22	29/72	3	276	-5.0	<i>Capsicum annuum</i> Alcohol dehydrogenase [<i>Lycopersicon esculentum</i>]
187	TA11484_4100	12791/5.6	34/57	4	30	1.8	<i>Nicotiana benthamiana</i> Cluster: Putative glutaredoxin-like protein
285	CK294135	6104/6.07	31/72	4	50	-5.2	<i>Nicotiana benthamiana</i> Cluster: Aldo/keto reductase
83	EB451223	9165/6.26	32/72	4	53	2.1	<i>Nicotiana tabacum</i> Cluster: Hydrolase-like protein
128	TA15172_4097	32011/6.62	32/72	5	27	1.7	<i>Nicotiana tabacum</i> Cluster: Auxin-responsive protein IAA26
176	TA11484_4100	12791/5.6	22/57	3	16	1.7	<i>Nicotiana benthamiana</i> Cluster: Putative glutaredoxin-like protein
511	EH365931	46941/6.54	24/57	5	13	-6.8	<i>Nicotiana benthamiana</i> Cluster: F-box/Kelch-repeat protein At3g61590
239	TA13162_4097	12412/5.91	36/66	5	29	-1.5	<i>Nicotiana tabacum</i> Cluster: Vignain precursor
186	TA42226_4113	18657/6.04	21/66	3	22	1.5	<i>Solanum tuberosum</i> cytochrome c biogenesis orf452
185	TA36032_4081	57208/6.77	20/57	5	11	1.7	<i>Solanum lycopersicum</i> Cluster: Beta-mannosidase
363	TA16154_4097	14428/6.11	36/72	4	45	-15.2	<i>Nicotiana tabacum</i> Cluster: Serine palmitoyltransferase subunit2
253	TA49244_4081	24970/7.78	32/66	5	26	-3.6	<i>Solanum lycopersicum</i> Cluster: Putative phospholipase

Salt stress
Control vs 100 mM NaCl

391	Q84RC1	15030/6.78	22/57	3	15	-1.2	ent-kaurene synthase [<i>Nicotiana sylvestris</i>]
2	Q40399	24275/5.66	24/57	4	12	-1.2	ACC oxidase [<i>Nicotiana glutinosa</i>]
232	Q93YF2	16727/5.71	28/72	4	26	-2.0	hypothetical protein [<i>Nicotiana tabacum</i>]
236	DV160864	7221/7.82	26/66	3	40	-2.4	<i>Nicotiana tabacum</i> Cluster: Biotin carboxyl carrier protein subunit chloroplastic
103	O65149	10903/6.89	28/66	4	31	3.6	late embryogenis abundant protein 5 [<i>Nicotiana tabacum</i>]
184	Q40399	24275/5.66	24/57	4	12	-1.2	ACC oxidase [<i>Nicotiana glutinosa</i>]
163	Q40399	24275/5.66	24/57	4	12	1.9	ACC oxidase [<i>Nicotiana glutinosa</i>]
22	Q40399	24275/5.66	24/57	4	12	-1.3	ACC oxidase [<i>Nicotiana glutinosa</i>]
59	Q40399	24275/5.66	24/57	4	12	1.9	ACC oxidase [<i>Nicotiana glutinosa</i>]
156	P25856	37652/7	26/66	6	16	-2.2	glyceraldehyde 3-phosphate dehydrogenase A subunit [<i>Arabidopsis thaliana</i>]

393	Q657Y9_ORYSA	8577/6.54	28/66	3	37	-2.8	Hypothetical protein P0005A05.15. [<i>Oryza sativa</i>]
73	Q9FYP8	7221/7.82	38/66	4	46	-1.1	hypothetical protein [<i>Oryza sativa</i> Japonica Group]
303	E1U7Y5	13656/5.72	27/72	4	25	-1.2	microtubule-associated protein [<i>Nicotiana benthamiana</i>]
28	P81161	23595/6.45	24/57	5	14	1.7	mitochondrial small heat shock protein [<i>Solanum lycopersicum</i>]
38	Q76MX6	2818/5.96	35/72	3	65	-1.4	QB protein [<i>Nicotiana tabacum</i>] RNA-directed DNA polymerase homolog; AltName: Full=Reverse transcriptase homolog
203	Q9ZRM0	16789/6.9	28/72	4	20	1.4	
305	Q8GSC4	51400/6.33	29/72	6	12	-1.7	DNA topoisomerase II [<i>Nicotiana tabacum</i>]
70	P09094	22413/6.59	26/66	6	21	2.0	glyceraldehyde-3-phosphate dehydrogenase [<i>Nicotiana tabacum</i>]
159	ABA94318	5832/6.54	30/66	3	56	2.0	DP000010 NID: - <i>Oryza sativa</i> (japonica cultivar-group) 10 kda C-II-like Bowman-Birk proteinase inhibitor {N-terminal}
72	Q9S8K0	13040/6.84	25/66	3	25	3.9	[<i>Solanum tuberosum</i>]
104	Q002B3	34535/7.85	19/57	4	13	2.1	cytokinesis negative regulator RCP1 [<i>Nicotiana tabacum</i>]
151	Q9AVMS	16789/6.9	19/57	3	15	1.5	reverse transcriptase [<i>Nicotiana tabacum</i>]
390	A9XAN0	12475/6.56	30/72	5	21	-3.8	TMV resistance protein N [<i>Nicotiana tabacum</i>]
195	Q6L3K4	67189/6.99	18/57	5	6	-1.9	Myosin heavy chain-like protein, putative [<i>Solanum demissum</i>]
47	E1AWP1	10250/5.34	21/72	3	22	1.5	small molecular heat shock protein [<i>Nicotiana tabacum</i>]
148	ABA94318	5832/6.54	31/66	3	56	1.8	DP000010 NID: - <i>Oryza sativa</i> (japonica cultivar-group)
395	Q9AVP4	12083/5.82	16/57	2	22	-3.9	BY-2 kinesin-like protein 10 [<i>Nicotiana tabacum</i>]
126	P07920	31643/5.12	31/72	4	12	1.5	1-aminocyclopropane-1-carboxylate oxidase [<i>Solanum lycopersicum</i>]
308	P82816	35652/6.33	21/57	4	12	-1.7	malate dehydrogenase [<i>Nicotiana tabacum</i>]
49	P090946	22413/6.59	33/72	5	20	-1.7	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast precursor [<i>Nicotiana tabacum</i>]
311	Q8H0G9	41913/5.98	34/72	6	12	-5.9	MCM protein-like protein [<i>Nicotiana tabacum</i>]
221	A9XAN0	12475/6.56	33/72	5	21	1.4	TMV resistance protein N [<i>Nicotiana tabacum</i>]
182	Q9XEZ1	13040/6.84	24/66	3	25	-3.0	NT4 [<i>Nicotiana tabacum</i>]
19	Q75WVO	13124/5.33	20/72	3	19	-1.4	ALG2-interacting protein X [<i>Nicotiana tabacum</i>]
307	Q9S8K0	13040/6.84	24/66	3	25	1.8	10 kda C-II-like Bowman-Birk proteinase inhibitor {N-terminal}
150	CK283772	5832/6.54	19/66	2	43	-1.8	[<i>Solanum tuberosum</i>] Cluster: Mitochondrial prohibitin 1 [<i>Nicotiana benthamiana</i>]

134	Q4W6T8	8831/5.49	27/66	3	33	1.6	polypeptide with a gag-like domain [Petunia x hybrida]
413	Q40399	24275/5.66	25/57	4	12	-5.7	ACC oxidase [<i>Nicotiana glutinosa</i>]
582	Q8RXH5	10956/5.84	31/66	4	27	-5.4	osmotic stress-activated protein kinase [<i>Nicotiana tabacum</i>]
478	Q05214	43824/5.33	24/57	5	11	-5.1	actin [<i>Nicotiana tabacum</i>]
207	Q84VS3	108005/5.67	32/57	10	7	2.4	potyviral helper component protease-interacting protein 1 [<i>Solanum tuberosum</i> subsp. andigena]
387	Q40399	24275/5.66	25/57	4	12	-5.7	ACC oxidase [<i>Nicotiana glutinosa</i>]
217	Q6TF27	11577/6.36	23/72	3	13	1.4	rapid alkalization factor 3 [<i>Solanum chacoense</i>]
125	EB448653	6468/6.23	19/66	2	32	-4.2	Cluster: ADP/ATP carrier protein [<i>Nicotiana tabacum</i>]
123	Q40399	24275/5.66	25/57	4	12	-1.4	ACC oxidase [<i>Nicotiana glutinosa</i>]
294	C4T7Z2	37652/7.48	34/72	6	16	2.2	DnaJ-like protein [<i>Nicotiana tabacum</i>]
314	TA12226_4097	35652/6.33	20/57	4	9	-3.3	Cluster: Malate dehydrogenase [<i>Nicotiana tabacum</i>]
130	B7UDM1	2818/5.96	20/72	2	60	1.6	photosystem II protein D1 [<i>Nicotiana tabacum</i>]
437	A9XI04	13890/6.23	32/72	4	22	-7.3	class S F-box protein [<i>Nicotiana alata</i>]
456	Q40399	24275/5.66	25/57	4	15	-7.8	ACC oxidase [<i>Nicotiana glutinosa</i>]
428	Q8LP11	17292/5.29	37/72	6	19	-9.0	L-galactono-1,4-lactone dehydrogenase [<i>Solanum lycopersicum</i>]
269	Q43497	4133/6.34	21/66	2	56	-1.9	ascorbate free radical reductase [<i>Solanum lycopersicum</i>]

Alleviation of salt stress
100 mM NaCl vs 100 mM NaCl GR24

70	Q6IVK8	28142/6.64	49/66	5	20	-1.4	ADH-like UDP-glucose dehydrogenase (<i>Nicotiana tabacum</i>).
69	Q9M2X6	17820/5.62	41/66	4	27	-1.7	hypothetical protein T16K5.170 - <i>Arabidopsis thaliana</i> AY128278 NID: - <i>Arabidopsis thaliana</i> /RNA helicase [<i>Arabidopsis thaliana</i>]
62	Q8L7S8	81120/7.66	53/66	7	9	-1.4	Metacaspase-9 OS= <i>Arabidopsis thaliana</i> GN=AMC9 PE=1 SV=1/metacaspase type II [<i>Nicotiana tabacum</i>]
102	Q9FYE1	35483/5.81	26/57	3	18	-1.2	Actin-depolymerizing factor 12 OS= <i>Arabidopsis thaliana</i> GN=ADF12 PE=2 SV=2/pollen specific actin-depolymerizing factor 2 [<i>Nicotiana tabacum</i>]
192	Q9ZNT3	15874/5.57	19/57	2	18	-1.9	<i>Solanum tuberosum</i> Pollen coat-like protein
171	CV498319	5319/5.48	45/72	3	77	-1.4	DP000009 NID: - <i>Oryza sativa</i> (japonica cultivar-group)/vacuoler processing enzyme 1 [<i>Solanum tuberosum</i>].
21	P93231	1826/5.96	25/66	2	93	-1.6	

278	YP_358683	35491/5.54	60/57	6	24	1.5	ATP synthase subunit beta, chloroplastic OS= <i>Pisum sativum</i> GN=atpB PE=3 SV=1/ATP synthase CF1 beta subunit [<i>Nicotiana sylvestris</i>].
260	TA14923_4097	39244/5.56	21/57	3	10	-2.3	<i>Nicotiana tabacum</i> Cluster: Dimethylallyltransferase
59	BM060303	15172/5.24	42/66	4	35	1.9	<i>Capsicum annuum</i> Transposase family tnp2, putative
13	TA17466_4097	21902/5.95	40/57	4	22	1.2	<i>Nicotiana tabacum</i> Cluster: NAD(P)H:quinone oxidoreductase
28	TA13368_4097	25293/6.04	27/57	3	24	1.8	<i>Nicotiana tabacum</i> Cluster: Nt-iaa28 deduced protein
371	BI929472	32816/6.75	44/66	5	13	1.3	<i>Solanum lycopersicum</i> Cluster: F14J9.26 protein
87	TA778_4096	27218/5.09	46/66	5	16	-1.4	<i>Nicotiana sylvestris</i> Elongation factor TuB, chloroplast precursor
151	TA56770_4081	26477/5.23	36/66	4	14	-1.3	<i>Solanum lycopersicum</i> Cluster: Probable glutathione-S-transferase
262	CK287453	30081/5.8	56/72	6	23	-1.4	<i>Nicotiana benthamiana</i> Cluster: Protein At4g15417
127	TA14853_4097	27252/5.67	24/66	3	12	-1.3	<i>Nicotiana tabacum</i> Cluster: Stearoyl-ACP desaturase
196	CN743199	14170/6.12	29/66	3	33	-1.5	<i>Nicotiana benthamiana</i> Cluster: Auxin-induced protein-like
295	TA14851_4097	13016/5.09	31/72	4	26	-1.2	<i>Nicotiana tabacum</i> Cluster: Small heat shock protein
297	TA26910_4113	41003/5.67	21/57	3	10	1.2	<i>Solanum tuberosum</i> Putative RING-H2 zinc finger protein
82	DN848998	32267/6.17	34/72	4	17	1.2	<i>Solanum tuberosum</i> Dihydrodipicolinate reductase-like protein
48	TA11701_4097	7584/7.98	38/66	3	44	1.3	<i>Nicotiana tabacum</i> Cluster: Ribulose bisphosphate carboxylase small chains, chloroplast precursor

100 mM NaCl vs 100 mM NaCl smoke water

119	AAX53163	34202/5.4	65/57	8	23	1.4	chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit (<i>Nicotiana benthamiana</i>)
*60	Q40458	21958/5.28	48/72	6	23	1.3	23 kDa polypeptide of water-oxidizing complex of photosystem II [<i>Nicotiana tabacum</i>]/photosystem II 23 kDa polypeptide [<i>Nicotiana tabacum</i>].
59	Q3S1N4	57246/6.34	46/72	6	13	1.3	LOC100282820 [<i>Zea mays</i>]/cytosolic pyruvate kinase [<i>Solanum tuberosum</i>].
4	P69249	20379/6.82	73/72	5	28	-1.6	Cristal-Glass1 protein [<i>Capsicum annuum</i>]/Ribulose bisphosphate carboxylase small chain
3	P69250	10175/5.3	74/72	5	47	-1.4	ribulose-1,5-bisphosphate carboxylase [<i>Nicotiana sylvestris</i>]

142	TA12442_4097	55172/6.06	48/72	8	12	-1.2	<i>Nicotiana tabacum</i> Cluster: Glutamate decarboxylase isozyme 1; conserved hypothetical protein [<i>Ricinus communis</i>]/cytokinin oxidase/dehydrogenase [<i>Solanum tuberosum</i>]
148	C3VPM6	15645/5.02	49/72	5	34	-1.4	
36	Q9ZTY8	11697/6.56	46/72	3	56	-2.7	predicted protein [<i>Physcomitrella patens</i> subsp. <i>patens</i>]/glucose acyltransferase [<i>Solanum pennellii</i>]/ <i>Solanum tuberosum</i> NADH:ubiquinone oxidoreductase-like protein
35	TA38778_4113	15250/5.03	45/72	7	24	-1.9	hypothetical protein [<i>Oryza sativa</i> Japonica Group]/anthocyanin 2 [<i>Petunia integrifolia</i>].
88	Q8VXD2	120337/6.35	49/72	6	11	1.1	predicted protein [<i>Physcomitrella patens</i> subsp. <i>patens</i>]/P70 protein [<i>Nicotiana tabacum</i>]
242	C5J0G6	27275/5.87	40/72	7	23	-1.4	enolase [<i>Nicotiana tabacum</i>]
50	Q589Y2	19686/6.15	43/72	5	34	-1.4	glycosyltransferase [<i>Nicotiana tabacum</i>]
22	Q9FYW3	87207/6.46	50/72	12	10	-1.6	synaptonemal complex protein, putative [<i>Ricinus communis</i>]/BAC19.13 [<i>Solanum lycopersicum</i>]
75	H84470	13898/7.93	27/66	4	24	-1.5	probable microtubule-associated protein [imported] - <i>Arabidopsis thaliana</i>
141	CK283637	33307/7.79	32/66	5	14	1.6	Tuber-specific and sucrose-responsive element binding factor related cluster
9	AF383149.1	114392/5.88	76/72	15	12	-3.4	armadillo repeat-containing protein [<i>Nicotiana tabacum</i>]/CMPG1b [<i>Nicotiana benthamiana</i>]
162	Q8S950	8492/6.56	38/83	4	47	1.3	guanine nucleotide binding protein gamma 4 [synthetic construct]/kinesin-like protein NACK1 [<i>Nicotiana tabacum</i>]
123	Q7XAE0	11246/5.09	40/72	4	28	-1.9	S2 self-incompatibility locus-linked G221 protein [<i>Petunia integrifolia</i> subsp. <i>inflata</i>]
10	Q1S798_MEDTR	14381/5.91	34/66	4	24	-1.4	Histidine triad (HIT) protein.- <i>Medicago truncatula</i> (Barrel medic)./SLT1 protein [<i>Nicotiana tabacum</i>]
179	Q40492	31649/5.39	24/72	5	13	2.2	cyclin A-like protein [<i>Nicotiana tabacum</i>]
328	T06261	21611/6.29	28/66	4	11	-3.5	caffeoyl-CoA 3-O-methyltransferase 5 [<i>Nicotiana tabacum</i>]
62	Q6SSJ2	2015/5.16	32/66	3	93	-1.9	phytochalpain [<i>Nicotiana benthamiana</i>]
243	Q307X6	25754/6.22	30/72	5	17	1.4	hydroxyacylglutathione hydrolase cytoplasmic-like [<i>Solanum tuberosum</i>]
192	Q1KTH5	11603/5.02	50/72	5	44	-1.4	mitochondrial ATP synthesis coupled proton transport protein [<i>Physalis</i> sp. TA1367]

18	A9RZ32	23646/8.45	25/72	4	16	-1.3	predicted protein [<i>Physcomitrella patens</i> subsp. <i>patens</i>]
194	Q8H9C1	14678/6.71	43/75	5	33	-2.0	citrate binding protein [<i>Solanum tuberosum</i>]
252	BP887488	5676/7.98	20/72	3	51	-1.2	Hypothetical protein OSJNBa0038P01.2 related cluster
270	TA2340_62890	5212/8.54	20/72	2	48	-5.6	Amidase [<i>Medicago truncatula</i> (Barrel Medic)] Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic
165	Q38IX0_SOYBN	48199/7.1	37/66	7	14	1.3	<i>Nicotiana tabacum</i>
86	Q6K1T7_ORYSA	5676/7.98	20/66	2	51	-1.8	Self-incompatibility RNase [<i>Physalis cinerascens</i>] Hypothetical protein OSJNBa0038P01.36.- <i>Oryza sativa</i> (japonica cultivar-group).
108	BP887488	5676/7.98	20/66	2	51	1.7	zinc finger (C3HC4-type RING finger) family protein [<i>Arabidopsis thaliana</i>]
*223	Q7X843	24948/6.89	28/66	5	16	-1.7	Putative zinc finger CCCH domain-containing protein 58 OS= <i>Oryza sativa</i> subsp. japonica GN=Os09g0305900 PE=4 SV=1/Cys-3-His
*166	C3H58_ORYSJ	26145/7.05	12/57	3	16	-1.4	zinc finger protein [<i>Capsicum annuum</i>]
258	A7UF45	15829/8.63	27/72	4	31	-1.8	granule-bound starch synthase [<i>Nolana ramosissima</i>]
229	O23680	48891/7.1	22/66	6	20	2.8	Hypothetical protein.- <i>Arabidopsis thaliana</i> (Mouse-ear cress).
272	Q7X9W1_PINMO	17976/5.56	36/66	5	25	-1.4	PR10 protein.- <i>Pinus monticola</i> (Western white pine).
264	Q6TKR0	11706/5.97	39/66	4	38	-1.6	ribosomal protein L3A [<i>Nicotiana tabacum</i>]
274	Q43187	24144/5.56	30/57	5	13	-2.4	soluble inorganic pyrophosphatase [<i>Solanum tuberosum</i>]