STUDIES ON PLANT-APHID INTERACTIONS: A NOVEL ROLE FOR TREHALOSE METABOLISM IN *Arabidopsis* DEFENSE AGAINST GREEN PEACH APHID

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Myzus persicae (Sülzer), commonly known as the green peach aphid (GPA), is a polyphagous insect that can infest over 100 families of economically important plants and is major pest for vegetable crops. This study utilizes the *Arabidopsis*-GPA model system with the aim to elucidate the role of the plant disaccharide trehalose in providing defense against GPA. This study demonstrates a novel role for *TPS11* in providing defense against GPA. *TPS11* expression was found to be transiently induced in *Arabidopsis* plants in response to GPA infestation and the *TPS11* gene was required for curtailing GPA infestation. *TPS11*, which encodes for trehalose phosphate synthase and phosphatase activities, contributes to the transient increase in trehalose in the GPA infested tissues. This work suggests that TPS11-dependent trehalose has a signaling function in plant defense against GPA. In addition, trehalose also has a more direct role in curtailing GPA infestation on *Arabidopsis*.

This work also shows that *TPS11* is able to modulate both carbohydrate metabolism and plant defenses in response to GPA infestation. The expression of *PAD4*, an *Arabidopsis* gene required for phloem-based defenses against GPA, was found to be delayed in GPA infested *tps11* mutant plants along with increased sucrose levels and lower starch levels as compared to the GPA infested wild type plants. This work provides clear evidence that starch metabolism in *Arabidopsis* is altered in response to GPA feeding and that *TPS11*-modulated increase in starch contributes to the curtailment of GPA infestation in *Arabidopsis*.

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LIST OF ABBREVIATIONS

AGpase - ADP-glucose pyrophosphorylase

AKR - *Acyrthosiphon kondoi* Resistance

AMY - Amylase

Avr - Avirulence

Df - Dilution factor

ET - Ethylene

FAC - Fatty acid conjugate

G6P - Glucose-6-phosphate

GBSS - Granule-bound starch synthase

GPA - Green peach aphid

HPI - Hours post infestation

JA - Jasmonic acid

LOX - Lipoxygenease

LRR - Leucine-rich-repeat

M/PAMP - Microbial/pathogen-associated molecular patterns

MAPK - Mitogen-activated protein kinase

MeJA - Methyl jasmonate

MeSA - Methyl salicylate

NBS - Nucleotide-binding site

OPDA - 12-oxo-phytodienoate

PAD4 - Phytoalexin Deficient4

PGM - Phophoglucomutase

R - Resistance

ROS - Reactive oxygen species

SA - Salicylic acid

SS - Starch synthase

T6P - Trehalose-6-phosphate

TPP - Trehalose phosphate phosphatase

TPS - Trehalose phosphate synthase

WT - Wild type

CHAPTER 1

INTRODUCTION

The sessile nature of plants requires the ability to adapt and respond to a variety of threats, including those from other organisms (biotic) and the non-living environment (abiotic). Some important abiotic stressors include exposure to extreme temperatures, water limitation and flooding. Amongst others, biotic stress includes attack by bacterial, fungal and viral pathogens and infestation by insect and nematodes. With the increasing global human population and limited agricultural resources (Oerke and Dehne, 2004), studies concentrating on the plant responses to the biotic and abiotic stresses are more relevant than ever. Insect pests on world crops result in the loss of nearly 10-20% of the annual yield (Oerke and Dehne, 2004; Ferry *et al.,* 2006). The vast number of insect pests of plants, can be categorized into two broad groups based on their feeding strategy: (i) The chewing insects e.g. lepidopteran caterpillars, which use their shear-like mouth parts to cut and chew the foliar tissue and thus cause extensive wounding and tissue loss (Kandoth *et al.,* 2007), and (ii) the piercing-sucking insects in which the mouth parts are modified into slender stylets, which depending on the insect are utilized to either suck cell contents, or consume phloem and/or xylem sap. This feeding behavior of the piercing-sucking insects, as opposed to the chewing insects, causes minimal wounding to the plant (Kaloshian and Walling, 2005). Insect infestation causes enormous loss to plant productivity and quality of plant products. Annually, insect infestation of plants results in the loss of 10-20% of the yield of various economically important crop and vegetable species (Oerke and Dehne, 2004). Plants have evolved multiple mechanisms to counter insect infestations. These include physical and chemical defenses, as well as physiological changes that limit resource availability

to the insect (Walling, 2008). Like any other threat, plants have to perceive the insect pest so that appropriate defenses can be activated.

Plant Perception of Chewing Insects

Plants have evolved highly specialized molecular mechanisms to tightly control the onset of defense responses against chewing insects. Mounting an effective defense response is a costly proposition for the plant and thus there are particular inducible defenses that are distinct from the physical and chemical features of the plant that constitute the constitutive and often the first line of defense (Baldwin, 1998). To avoid unnecessary expenditure of resources, one of the most important distinctions plants make is between the damage caused by insect herbivory and mechanical wounding (Reymond *et al.,* 2000; Reymond *et al.,* 2004). In a manner similar to the recognition of microbial/pathogen-associated molecular patterns (M/PAMPs), plants have evolved to recognize a number of molecular cues from the insect herbivores (Heil, 2009). One of the major insect-derived elicitors that have been characterized is volicitin, which is produced during the interaction of plants with the salivary secretions of *Spodoptera exigua* (beet armyworm). Volicitin and similar elicitors produced during plant-infestation by other lepidopteran species belong to the class of chemicals known as fatty acid conjugates (FACs) that are composed of a fatty acid (either linoleic acid or linolenic acid) that is conjugated to an amino acid. The fatty acid and the amino acid component of volicitins and related compounds are derived from the insect and plant, respectively (Paré *et al.,* 1998). In addition to the lepidopteran larvae, elicitors have been identified in other classes of insects (Yoshinaga *et al.,* 2007). Although little is understood about the perception of FACs it has been shown that salivary secretions from *Manduca sexta* (tobacco hornworm) when applied to *Nicotiana attenuata* leaves are able to induce jasmonic acid (JA) and ethylene (ET) signaling which can contribute to the

induced defenses against grazing insects (Giri *et al.*, 2006; Skibbe *et al.,* 2008). Another example of insect oral secretions resulting in the induction of plant defenses is inceptin (Schmelz *et al.,* 2006). Inceptin is a disulphide-bridged peptide derived from a chloroplastic ATP synthase subunit ingested by the *Spodoptera frugiperda* (fall armyworm) feeding on *Vigna unguiculata* (cowpea) plants. Perception of inceptin by the cowpea plants results in the induction of both hormone and volatile based plant defenses (Schmelz *et al.,* 2006).

Besides the physical act of foliar damage, oviposition by the insect herbivores is known to elicit defense responses from plants. Bruchins, a class of α, ω-diols, identified from the oviposition fluids of *Bruchus pisorum* L. (pea weevil) have been demonstrated to induce the formation of neoplasma or non-differentiated cells on the leaves of pea plants where the eggs are laid. These neoplasmic structures lead to elevation and dropping off of the eggs from the leaf surface (Doss *et al.,* 2000). Production of necrotic lesions on potato leaves is induced by *Leptinotarsa decemlineata* (Colorado potato beetle) ovipostion which ultimately leads to degradation of the lesion area, occluding the growth of the eggs (Balbyshev and Lorenzen, 1997).

Signaling Pathways Triggered in Response to Infestation by Chewing Insects

Following the perception of herbivory, a number of downstream signaling events like Ca^{2+} signaling (Maffei *et al.,* 2004), Mitogen activated protein kinase (MAPK) activation (Wu *et al.,* 2007) are initiated in the plant cells that lead to the induction of defenses. Due to the presence of unpaired valence electrons, reactive oxygen species (ROS) are highly reactive molecules of oxygen which can chemically react to proteins, plasma membranes and other cellular components leading to their degradation. The role of ROS is well established in both

abiotic and biotic plant stresses especially plant-pathogen interactions (Apel and Hirt, 2004). ROS have been suggested to play a role against chewing insects also. For example, feeding by *Helicoverpa zea* (corn earworm) and *Spodoptera littoralis* (African cotton leafworm) on *Glycine max* (soybean) and *Phaseolus lunatus* (lima bean) plants respectively lead to elevated levels of ROS in the infested tissue (Bi and Felton, 1995; Maffei *et al.,* 2006). Another secondary messenger that plays an important role in cellular signaling is Ca^{2+} and changes in cytoplasmic Ca^{2+} concentration are known to induce various signaling cascades under stress conditions in plant cells (Bush, 1995). Differential concentration of Ca^{2+}, across the plasma membrane, is known to influence these responses. It has been demonstrated that in the case of feeding by *S. littoralis*, lima bean tissues undergo membrane depolymerization that is dependent on increased Ca^{2+} flux (Maffei *et al.,* 2004).

Another important signaling cascade that is conserved across eukaryotes is the MAPK signaling. Its role in plant defense responses against pathogens has been demonstrated in a number of plants including *Nicotiana* and *Arabidopsis* (Zhang and Klessig, 2001). With respect to herbivory by *M. sexta* larvae, in *Nicotiana attenuata* plants, it was demonstrated that MAPK activity is highly upregulated in response to larval feeding. In addition, the expression of defense genes induced by herbivory was also found to be dependent on the MAPK pathway (Wu *et al.,* 2007). Silencing of MAPK genes in tomato resulted in reduced JA accumulation and a compromised defense response against *M. sexta* (Kandoth *et al.,* 2007).

Role of Phytohormones in Plant Response to Chewing Insects

Phytohormones like salicylic acid (SA), JA and ET function as important signaling molecules in plant defenses against pathogen and insects (Reymond and Farmer, 1998).

Furthermore, cross-talk (synergy and antagonism) between SA, JA, and ET signaling is involved in fine-tuning plant defenses (Reymond and Farmer, 1998). JA is one of the best studied plant hormones in defense responses against herbivores (Stintzi *et al.,* 2001; Cipollini *et al.,* 2004; Kessler *et al.,* 2004). Impairment of the JA biosynthesis or signaling process in plants renders them highly susceptible to insect herbivory (Walling, 2000; Kessler *et al.,* 2004). In *Arabidopsis*, the role of JA in defense against insect herbivory was demonstrated in experiments with the *fad3 fad7 fad8* triple mutant plant that is deficient in linolenic acid, the fatty acid precursor to JA. The *fad3 fad7 fad8* triple mutant is highly susceptible to herbivory by *Bradysia impatiens* (McConn *et al.,* 1997). Stinzi *et al*. (2001) further showed that the JA precursor, 12-oxo-phytodienoate (OPDA) can act as a potent signal for turning on plant defense against insect herbivory. JA also forms conjugates with a number of molecules, especially amino acids. The role of JA-Ile (JA conjugated to isoleucine) in defense against chewing insects is evident from the impaired resistance of *Nicotiana attenuata* plants lacking JA-Ile, against *M. sexta* (Wang *et al.,* 2008).

The role of SA in providing defense against chewing insects remains unclear. Induction in the expression of JA and ET responsive genes was observed in *Arabidopsis* plants under pathogen attack, and in plants infested with chewing- and piercing-sucking insects. However, the chewing insect (*Pieris rapae*) did not elicit any SA based defenses (De Vos *et al.,* 2005). In the case of feeding by a specialist herbivore, *M. sexta*, *N. attenuata* plants displayed bursts in the levels of JA and ET but not SA. In contrast, feeding by a generalist feeder *Spodoptera exigua*, high levels of SA were accumulated along with lower JA and ET bursts (Diezel *et al.,* 2009). It has been suggested that SA, which is known to antagonize the activation of JA signaling (Reymond and Farmer, 1998), can be manipulated by insect herbivores to colonize a host

(Walling, 2008). SA and JA have been shown to act antagonistically in their defense role against *S. exigua* feeding on *Arabidopsis* plants (Cipollini *et al.,* 2004).

In conjunction with JA, the role of ET has also been established in providing defense against chewing insects. Bursts in ET levels are observed in response to insect herbivory and these levels were found to be accentuated by application of *M. sexta* oral secretions to wounded *N. attenuata* plants (Von Dahl *et al.,* 2007). Similar alterations in ET levels were reported in response to the application of *Leptinotarsa decemlineata* oral secretions to potato and bean plants (Kruzmane *et al.,* 2002)

Plant Responses Against Piercing-Sucking Type of Insects

Infestation by piercing –sucking type of insects results in minimal wounding damage to the plant but the plant productivity and health are negatively affected due to the loss of nutrients via the cell contents or phloem sap (Walling, 2000). Insects like aphids, whiteflies and leafhoppers feed on the plant sap whereas other piercing-sucking insects like spider mites and thrips feed on the epidermal or mesophyll cell contents of the leaves (Walling, 2000). Aphids belong to the family Aphidoidea and are further classified into the order Hemiptera and suborder Sternorrhyncha. Within this classification the aphids have evolved a very unique pattern of plant herbivory that is in vast contrast to other insect herbivores. The modified mouth parts of the aphids constitute a slender stylet bundle that punctures the leaf surface and then penetrates predominantly in an intercellular manner to reach the sieve element where the insect can the feed on the nutrient-rich phloem sap (Kaloshian and Walling, 2005). Due to the presence of a large concentration of carbohydrates in the phloem sap, an osmotic imbalance is created in the aphid gut which can result in dehydration of the insect (Walling, 2008). One of the mechanisms by

which aphids maintain their water balance is by occasionally feeding through the xylem which conducts water and mineral nutrients in plants (Spiller *et al.,* 1990).

Host Selection by Aphids

Selection of a suitable host by the aphid involves perception by the aphid of both visual and olfactory cues from the host plant (Powell *et al.,* 2006). In the case of *Myzus persicae*, the role of color in host plant selection has been demonstrated by the presence of three different spectral photoreceptors (Kirchner *et al.,* 2005). The waxy cuticle of plant leaves act as a rich source of volatile compounds that are perceived by aphids. *Aphis fabae* (black bean aphid) uses the volatile cocktail emitted by *Vicia faba* plants as an odor source for host location and settling (Webster *et al.,* 2008).

Unique Composition of the Aphid Saliva

As opposed to the grazing insects, aphid feeding results in minimal wounding damage to the plants leaves. One of the reasons that allows for this is the unique salivary constitution of the aphids. Aphids produce two types of saliva. The gelling saliva is viscous and contains complex carbohydrates, phospholipids and protein species that appear to be consistently represented in the sheath saliva of a number of aphid species (Miles, 1999; Cherqui and Tjallingii, 2000). The gelling saliva is secreted when the stylet is penetrating host tissue and forms a tight sheath around the stylet as it traverses the plant tissue. The sealing effect of the sheath minimizes any counter reactions from the plant cells as it effectively isolates the stylet from the adjoining host cells (Tjallingii, 2006).

A second type of saliva, the watery saliva, produced by aphids contains a myriad of hydrolytic enzymes like pectinases and cellulases, and others like polyphenoloxidases, glucose

oxidase and peroxidases (Miles, 1999). En route to the sieve element the aphid stylet occasionally punctures a few cells, and in the process delivers a small amount of the watery saliva into the cells and concomitantly the insect ingests a mixture of the saliva and host plant cytoplasmic contents (Mart *et al.,* 1997). It has been suggested that uptake of this cytoplasm and saliva mixture allows the aphid to sample plant contents and make a decision on whether to continue feeding on the host. Occasional puncturing of cells has also been suggested to help the aphid assess the location of the stylet within the leaf anatomy (Tjallingii and Esch, 1993; Powell *et al.,* 2006). The watery saliva is also delivered into the sieve element when the insect is feeding from the sieve element. The enzymes in the watery saliva collectively help the aphid repress plant defense responses (Ma *et al.,* 1990; Cherqui and Tjallingii, 2000; Harmel *et al.,* 2008). Another aspect where the water saliva appears to play an important role is the prevention of sieve element occlusion. To prevent the loss of phloem sap in response to injury and damage, plants deposit callose which can occlude the porous ends of a sieve element (Knoblauch and van Bel, 1998). A similar function is performed by proteinaceous structures called forisomes in legume cells and phloem proteins in cucurbits (Knoblauch *et al.,* 2001). Influx of Ca^{2+} ions from the sieve element via the activation of both voltage gated and mechano-sensitive channels seems to be important for phloem occlusion in response to aphid infestation (Knoblauch *et al.,* 2001; Furch *et al.,* 2009). Artificial induction of forisome formation leading to the occlusion of sieve elements in *Vicia faba* altered the feeding behavior of *Megoura viciae* from active ingestion to watery salivation. In the same study at least two proteins with Ca^{2+} binding properties were identified from the saliva of *M. viciae* (Will *et al.,* 2007). A protein with high homology to regucalcin, a calcium binding protein, was identified from the salivary secretions of greenbug (*Schizaphis graminum*) (Carolan *et al.,* 2009).The watery saliva composition varies depending on

the diet. Furthermore, the watery saliva composition varies among different aphid species (Carolan *et al.,* 2009; Will *et al.,* 2009). The essential role of salivary proteins is evident from the fact that the RNAi-mediated down-regulation of expression of the *Acyrthosiphon pisum* (pea aphid) C002 protein-encoding gene resulted in lethality of the insects on its host plant, *Vicia faba* (Mutti *et al.,* 2006). A glucose oxidase has been identified from the saliva of *Myzus persicae* (Harmel *et al.,* 2008) which is similar to glucose oxidase from the oral secretions of the lepidopteran herbivores. This enzyme can generate H_2O_2, by oxidizing glucose, which can potentially stimulate SA production in plants (Vandenabeele *et al.,* 2003). Peptides in the 3-10 kDa fraction of *Myzus persicae* saliva were found to be responsible for eliciting local defenses in *Arabidopsis* (De Vos and Jander, 2009).

Physical Defenses Against Piercing-Sucking Type of Insects

Plant resistance to piercing-sucking type of insects broadly falls into three categories: antibiosis, antixenosis and tolerance (Smith, 2005). Antibiosis and antixenosis are resistance categories that result from the direct interaction of plant and insects and influences the insects' fecundity or behavior. Antibiosis impacts the insects' growth and development due to the presence of a chemical or physical feature on the plant (Smith, 2005). Antixenosis results from the insects' inability to select the plant as a suitable host which may result from both physical and chemical cues emanating from the plant (Smith, 2005)

Trichomes are perhaps the most important physical feature of the plant surface that influence aphid settling and host selection. Presence of trichomes may be considered the first line of direct defense, which may be both antibiotic and antixenotic in nature. Both the type and density of glandular aphids influenced host selection and mortality of the potato aphid on wild tomato plants (Musetti and Neal, 1997). The presence of trichomes is a desirable wild type

characteristic as demonstrated in the case of another wild tomato species *Solanum pennellii* (synonym: *Lycopersicon pennellii*) which was shown to be resistant to *Macrosiphum euphorbiae* (potato aphid) settling and feeding due to the presence of both simple and glandular trichomes and also the toxic exudations by the latter (Goffreda *et al.,* 1989). Trichomes also provide effective defense against whiteflies, another piercing-sucking type of insect (Neal and Bentz, 1999).

Gene-For-Gene Interaction in Defense Against Aphids

The compatible and incompatible nature of plant-pathogen interactions depends on the interaction of *R* (resistance) gene product from plants and *avr* (avirulence) gene product from the pathogen (Martin *et al.,* 2003). A similar example of gene for gene interaction based defense mechanism against insects is the *Mi-1* gene from tomato (Rossi *et al.,* 1998). *Mi-1*, structurally, belongs to the Nucleotide Binding Site (NBS)-Leucine Rich Repeat (LRR), the largest *R* gene family identified in plants and was originally identified as conferring resistance in tomato against the root-knot nematode (Milligan *et al.,* 1998). Tomato plants carrying the *Mi-1* gene have been shown to be resistant against the potato aphid as compared to the plants lacking the gene (de Ilarduya *et al.,* 2003). Similarly the *Vat* gene from melon *Cucumis melo* (melon), which is also a member of the NBS-LRR-*R* gene family, confers resistance against *Aphis gossypii* (melon aphid) (Dogimont *et al.,* 2008). Interestingly another member of the NBS-LRR, *R* gene family, *AKR* (*Acyrthosiphon kondoi* resistance) has been shown to be responsible for resistance against bluegreen aphid (synonym: blue alfalfa aphid) in *Medicago truncatula* (Klingler *et al.,* 2005)

Involvement of Reactive Oxygen Species in Defense Against Aphids

Production of H_2O_2 and other ROS in response to pathogen attack is a well known plant defense mechanism (Wojtaszek, 1997). In fact, H_2O_2 production is observed under a number of

plant stress conditions (Bolwell and Wojtaszek, 1997). It was suggested early on that the generation of H_2O_2 in response to aphid feeding enhances plant defenses as it leads to the production of antioxidants in the plant cells (Miles and Oertli, 1993). The presence of oxidizing enzymes within the aphid saliva was considered a counter mechanism against the same (Miles and Oertli, 1993). Microarray analysis revealed that greenbug infestation on *Sorghum bicolor* (sorghum) plants leads to the induction of genes encoding for ROS accumulation in the infested leaves (Park *et al.,* 2006). Alteration in the redox status of aphid infested plants is suggested by the gene expression changes induced by cabbage aphids in *Arabidopsis* leaves (Kusnierczyk *et al.,* 2008). The MAPK cascade induced by H_2O_2 and several ROS detoxification enzyme encoding genes were found to be upregulated in response to feeding by cabbage aphid on *Arabidopsis* (Kusnierczyk *et al.,* 2008). One hypothesis to explain the accumulation of H_2O_2 in response to aphid feeding is that both the H_2O_2 burst and in response the accumulation of soluble peroxidases may lead to cell wall strengthening which may render the plant more resistant to aphid attack (Moloi and van der Westhuizen, 2006).

Role of Jasmonates in Defense Against Aphids

Lipoxygenase (LOX) is one of the first enzymes involved in the biosynthesis of JA. Aphid feeding induces the expression of *LOX* genes as demonstrated in the case of *Macrosiphum euphorbiae* feeding on tomato plants (Fidantsef *et al.,* 1999) and *M. persicae* on *Arabidopsis* (Moran and Thompson, 2001). Wheat and sorghum plants, resistant to Russian wheat aphid and greenbug respectively were found to have a higher expression of *LOX* genes in response to aphid infestation (Boyko *et al.,* 2006; Park *et al.,* 2006). A similar response, with respect to LOX expression, was demonstrated in wild tobacco plants infested with *Myzus nicotianae* (tobacco aphid) (Voelckel *et al.,* 2004). Genes encoding for the octadecanoid pathway, responsible for JA

biosynthesis, were also induced in response to bluegreen aphid infestation in *M. truncatula* (Gao *et al.,* 2007). Transcript profiling in sorghum plants infested with greenbug similarly demonstrated a higher expression of JA biosynthesis genes and JA response genes, for example the *VEGETATIVE STORAGE PROTEIN* (*VSP*) (Zhu-Salzman *et al.,* 2004).

Application of MeJA, the methyl ester of JA, on plants reduces aphid fecundity. MeJA treatment of alfalfa and tomato plants reduced the infestation of blugreen aphid and potato aphid respectively (Cooper and Goggin, 2005; Gao *et al.,* 2007). Zhu-Salzman and co-workers (2004) demonstrated a similar function of MeJA application in reducing aphid infestation on sorghum. As a corollary, JA hyper-accumulating *cev1* mutants of *Arabidopsis* displayed increased resistance to GPA as compared to the wild type plants (Ellis *et al.,* 2002). These results suggest that both JA and MeJA have an important role in providing plant defense against aphids.

Role of Salicylic Acid in Plant-Aphid Interaction

Extensive field studies with MeSA, the volatile SA derivative, showed that its application to cereal fields reduced infestation by at least three different aphid species (Pettersson *et al.,* 1994). The role of MeSA and in conjunction that of SA signaling as defense against aphids is observed in the release of MeSA, as a repellant for black bean aphids, by *Vicia faba* plants (Hardie *et al.,* 1994). As discussed above, the *Mi-1* gene confers resistance to potato aphid in tomato plants. SA accumulation, in response to potato aphid feeding, was found to be more rapid in tomato plants harboring *Mi-1* as compared to *mi-1* plants (de Ilarduya *et al.,* 2003). The role of SA in mediating defense via *Mi-1* was confirmed by the loss of potato aphid resistance in *Mi-1* tomato plants carrying the *NahG* transgene, which encodes a salicylate hydroxylase that blocks SA accumulation due to conversion of SA to catechol (Li *et al.,* 2006). Global expression analysis of genes in *Arabidopsis* plants infested with cabbage aphids displayed upregulated

expression of SA biosynthesis and SA responsive genes as compared to uninfested control plants (Kusnierczyk *et al.,* 2008). The participation of SA in plant-aphid interaction is also implicated from the observation that increased levels of SA were observed in barley plants under attack from *S. graminum* (Chaman *et al.,* 2003). Another crop species belonging to the Poaceae family, sorghum, accumulated elevated levels of SA responsive defense proteins (Zhu-Salzman *et al.,* 2004). Similar increase in the expression of SA biosynthesis and responsive genes was observed in bluegreen aphid infested *M. truncatula* plants (Gao *et al.,* 2007). Green peach aphid infestation also led to increased expression of SA responsive genes like *PR-1*, *BGL2* and *PDF1.2* in *Arabidopsis* plants indicating the involvement of SA in the defense response against aphids (Moran and Thompson, 2001). However, genetic studies conducted in *Arabidopsis* suggest that SA per se does not contribute to host defense. Instead, SA increase in this case and in case of squash interacting with whitefly might be due to insect manipulating host physiology. Increase in SA, prevents the timely activation of JA signaling and thus facilitates insect infestation (DeVos *et al.,* 2007)

Role of Ethylene in Plant-Aphid Interaction

Expression of both ethylene biosynthesis and responsive genes was found to be upregulated in response to bluegreen aphid feeding in *M. truncatula* plants (Gao *et al.,* 2007). In barley plants infested with two aphid species, *Schizaphis graminum* and *Rhopalophum padi*, increased ET production was observed. In addition, barley varieties resistant to aphids displayed higher ethylene levels, in response to aphid feeding, as compared to susceptible varieties (Argandona *et al.,* 2001). Melon plants carrying the *Vat* gene display resistance against *Aphis gossypii* infestation and demonstrate an increased induction in the expression of ethylene signaling and responsive genes. This indicates a role for ethylene in contributing *Vat* mediated

resistance against *A. gossypii* (Anstead *et al.,* 2010). Similar response has been observed in *Arabidopsis* where *M. persicae* infestation resulted in the upregulation of genes involved in ethylene biosynthesis (Moran *et al.,* 2002).

Cross-Talk Between Plant Defense Signaling Mechanisms in Aphid-Infested Plants

Aphid infestation leads to the simultaneous upregulation of both SA and JA based defenses as demonstrated in the case of cabbage aphid (Moran and Thompson, 2001) and green peach aphid (Fidantsef *et al.,* 1999) infestations on *Arabidopsis*. The antagonistic nature of SA and JA during signal crosstalk has been well documented. One of the proposed models for the coordinated action of SA and JA based defense mechanisms against aphid attack is the suppression of JA based defenses by induction of SA signaling which may help the aphids propagate on a plant (de Vos *et al.,* 2007). GPA populations have been observed to be similar between wild type *Arabidopsis* plants and mutant plants deficient in both SA biosynthesis and signaling (Moran and Thompson, 2001; Pegadaraju *et al.,* 2005). However, Mewis and co-workers (2005) found that lower aphid numbers were supported by SA deficient plants as compared to the wild type. This contrary result was explained by suggesting that low SA levels lead to the activation of JA signaling which results in lower aphid numbers on SA deficient plants (Mewis *et al.,* 2005). Evidence on similar lines has been obtained in the case of another piercing-sucking kind of insect, the silverleaf whitefly (Zarate *et al.,* 2007). Lower growth of whitefly larvae was supported by SA deficient *NahG* plants whereas a higher larval growth was supported by SA overexpressing *cim10* plants as compared to the wild type plants (Zarate *et al.,* 2007). The crosstalk between JA and ethylene is clear from the convergence of both pathways into the *Ethylene Response Factor 1* (*ERF1*), a transcription factor required for the expression of a number of pathogen defense genes (Lorenzo *et al.,* 2003). However, in case of the piercing

sucking kind of insects, both synergistic and antagonistic interactions have been observed. Both MeJA and ethylene were found to induce the expression of defense genes in local and systemic leaves of squash in response to feeding by whitefly (van de Ven *et al.,* 2000). However, increased accumulation of ethylene in response to green peach aphid feeding on *Arabidopsis* was found to have a negative impact on JA signaling (Dong *et al.,* 2004) .

Aphid Infestation Impacts Source-Sink Patterns In the Host Plant

Aphids feeding on the phloem sap, the nutritional link between the source (photosynthesizing mature tissue) and sink (growing young tissue) tissues of the plant, cause major changes in the physiology and gene expression of the infested plant to alter source-sink relationship (Larson and Whitham, 1991; Voelckel *et al.,* 2004; Douglas, 2006). The galling aphid *Pemphigus betae* feeding on *Populus angustifolia* (narrowleaf cottonwood) form galls that act as strong sinks which compete with the existing plant sink tissue for nutritional resources (Larson and Whitham, 1991). GPA infestation on *Arabidopsis* results in the upregulation of a monosaccharide symporter in the infested tissue indicating an increase in the sink strength of the aphid infested tissue (Moran and Thompson, 2001). In a similar vein, it was observed that *M. nicotianae* prefers to feed on the young leaves, which act as strong sink tissues, of native tobacco plants and result in the alteration of expression of genes involved in maintaining the source-sink balance (Voelckel *et al.,* 2004). Hemipterans have evolved a very unique feeding behavior wherein they feed from a nutrient source that does not provide them with all the necessary nutrition (Douglas, 2006). The phloem sap, from which aphids acquire nutrition, is rich in carbohydrates but deficient in amino acids. Thus the host plant does not satisfy the nitrogen needs of aphids, which have evolved to harbor an endosymbiont protobacterium *Buchnera aphidicola*, which synthesizes essential amino acids required by the insect (Douglas, 2006).

However, aphid feeding does alter nitrogen availability of the infested tissue as observed in the case of russian wheat aphid (*Diruaphis noxia*) feeding on wheat plants (Telang *et al.,* 1999). Similarly, reduction in stem elongation in alfalfa plants infested with the pea aphid was explained by the conversion of nitrogen source tissues into nitrogen sinks (Girousse *et al.,* 2005). This shift in source to sink nature has also been observed in celery plants under attack from GPA. Feeding by *Myzus persicae* led to the induction of a number of nitrate and carbohydrate transport genes in the infested tissue as compared to the control tissues (Divol *et al.,* 2005). Feeding by two genotypes of GPA has been shown to induce nitrate reductase activity in cabbage seedlings (Wilson *et al.,* 2011). Increased nitrogen metabolism activity in the infested plants has been observed in the case of *M. nicotianae* feeding on wild tobacco plants (Voelckel *et al.,* 2004).

Plant Carbohydrates: Involvement in Plant-Aphid Interaction

Sugars play an important role in the energy metabolism of plants and act as the major resource of nutrition and storage (Rolland *et al.,* 2006). In case of lower organisms, especially the fungus *Saccharomyces cerevisiae* (Baker's yeast) the role of sugars as controllers of metabolism is well established (Rolland *et al.,* 2001). Besides their well established role it has been shown that sugar molecules can act as signaling factors that regulate gene expression with respect to growth and development (Koch, 1996) as well as environmental stress (Roitsch, 1999) and changes in phyto-hormone levels (Finkelstein and Gibson, 2002). The disaccharide sucrose is the main photosynthate that acts as a signaling link between the source and the sink tissues. The amount of sugars flowing into these tissues is coordinated by both the demand and the circadian clock of the plant. It's the involvement and perception of sugars as signaling molecules which controls the flux that optimizes the utilization of photosynthetic resources (Coruzzi and

Zhou, 2001; Osuna *et al.,* 2007). The signaling role of sucrose (Rolland *et al.,* 2006) and more recently trehalose (Paul *et al.,* 2008) has been documented with regards to plant growth and development. Despite being present in trace amounts in most plants, trehalose and its metabolic intermediate trehalose 6 phosphate (T6P) are emerging as key players, in control of various physiological functions in plants(Paul *et al.,* 2008). The structures of sucrose, trehalose and T6P are depicted in Figure 1.1.

The Disaccharide Trehalose

Trehalose is present in a wide spectrum of living organisms, as varying as bacteria and fungi to insects and angiosperms. Trehalose is a water soluble disaccharide composed of two molecules of glucose and is non-reducing in nature. The disaccharide molecule is similar in its molecular weight to sucrose (Figure 1.1). The two glucose molecules are linked in an α, α-1,1-linkage (Figure 1.1). There are two more isomers of trehalose α, α- and α, β- trehalose, but only α, α-1, 1-trehalose is present in biological systems (Elbein, 1974).

Trehalose accumulates in spores of microorganisms and in response to a number of abiotic stresses such as heat and cold shock and dehydration in bacteria and fungi (Elbein *et al.,* 2003). In lower organisms, trehalose can be easily hydrolyzed to glucose and thus acts as a ready source of glucose for glycolysis during adverse growth conditions (Galinski, 1993). Trehalose acts as a protectant for cellular membranes and proteins during events of environmental stresses largely due to its high solubility and ability to form hydrogen bonds with sugar and phosphate molecules (Peterbauer *et al.,* 2002). Also it acts as a recalcitrant storage form for the much reactive glucose molecules. Over the years trehalose has been used as a commercial food and enzyme protectant (Colaco *et al.,* 1992).

There are a total of five known pathways for trehalose synthesis and metabolism. The pathway that exists in angiosperms has been termed the OtsA-OtsB pathway due to its initial elucidation in bacteria. This pathway is found in a number of other organisms including yeast and is the only one that employs trehalose-6-phosphate (T6P) as an intermediate (Paul *et al.,* 2008). UDP-Glucose (UDPG) and glucose 6-phosohate (G6P) are converted to T6P and uridine diphosphate by the enzyme trehalose phosphate synthase (TPS) (Figure 1.2). Subsequently, T6P is de-phosphorylated by trehalose phosphate phosphatase (TPP) resulting in the formation of trehalose. Glucose is regenerated from trehalose by the action of trehalase (Paul *et al.,* 2008).

Trehalose Metabolism in Plants

Little more than a decade ago the occurrence of trehalose in plants was believed to be limited to a few resurrection plants like *Myrothamnus flabellifolia* (Bianchi *et al.,* 1993). With the aim to create drought and desiccation tolerant plants, *otsA* and *otsB* genes (encoding TPS and TPP respectively) from *E. coli* were transformed into tobacco (Goddijn *et al.,* 1997). Also these plants were treated with trehalase inhibitor, validamycin A to enhance the accumulation of trehalose. The transgenic plants showed a stunted phenotype but what was more surprising was the accumulation of trehalose in wild type plants treated with validamycin A which indicated, for the first time, the presence of a trehalose biosynthetic pathway in angiosperms (Goddijn *et al.,* 1997). In 1998, two studies independently identified the functional genes encoding for TPS and TPP from the crucifer *Arabidopsis thaliana* (Blazquez *et al.,* 1998; Vogel *et al.,* 1998). The whole genome sequence of *Arabidopsis* revealed a surprising total of 21 genes involved in trehalose metabolism. These genes have been classified in to three different classes based on their sequence similarity to yeast *TPS1* (TPS) and *TPS2* (TPP) genes along with a single gene encoding for trehalase (Leyman *et al.,* 2001).

The eleven genes encoding for TPSs in *Arabidopsis* are classified into two separate classes (Figure 1.3). Till date only *TPS1*, amongst class I TPSs (*AtTPS1-4*), has been shown to be functional by its complementation of mutant yeast strains lacking a functional TPS (Blazquez *et al.,* 1998). Sequence analysis of these genes reveals that class I TPS have a eukaryotic origin and are closely related to yeast TPS. On the other hand the class II TPSs (*AtTPS5-11*), which encode for both synthase and phosphatase domains, may have evolved in response to the evolution of TPPs which are close to bacterial TPP genes. The basis for this classification is that class I TPS do not contain the typical phosphatase sequences within their C-terminal phosphatase domains whereas the class II genes have retained these phosphatase boxes (Leyman *et al.,* 2001). It was believed that the class II TPSs are not functional (Vogel *et al.,* 1998) but recently it was shown that *AtTPS6* can complement both *tps1* and *tps2* mutant strains of yeast, which lack TPS and TPP respectively, and has a role in determining cell shape of the leaf epidermis (Chary *et al.,* 2008). Also the sequence analysis of class II TPS genes shows that codon substitutions at the nucleotide level are the same at the level of proteins indicating towards conserved protein functions (Avonce *et al.,* 2006). AtTPS1 has a unique N-terminal sequence, not found in all the other TPSs, which is involved in regulating the activity of the enzyme. This sequence is similar to the one found in TPS1 from *Selaginella lepidophylla*, a resurrection plant with high levels of trehalose (Van Dijck *et al.,* 2002). This N-terminal sequence can potentially bind to a molecular motor protein, KCA (Geelen *et al.,* 2007). Function of the remaining class I genes is not known except that *TPS2* and *4* are expressed in developing seeds (Lunn, 2007). Amongst the class II genes, *AtTPS5* is required for thermo-tolerance in *Arabidopsis* plants and has been demonstrated to interact with the transcriptional co-activator MBF1c (Suzuki *et al.,* 2008).AtTPS8-11 have conserved sites for SnRK1 mediated phosphorylation and can be phosphorylated by crude

Arabidopsis leaf extracts (Glinski and Weckwerth, 2005). In a similar manner, AtTPS5, 6 and 7 are expected to be phosphorylated by SnRK1 and have been sown to bind to 14-3-3 proteins (Harthill *et al.,* 2006).

With the exception of the recent reports showing a biological function for *AtTPS6* and *AtTPS11* the role of other class II TPS genes is not clear. Gene expression studies have shown that expression of class II TPS genes is impacted by a number of biotic and abiotic factors and plant hormones. For example, *TPS8* was expressed at high levels in *Arabidopsis* plants treated with cytokinin (Brenner *et al.,* 2005). Expression of a number of these genes is also influenced by both light and sugar (Harmer *et al.,* 2000; Thimm *et al.,* 2004). The diurnal control of gene expression coupled with changing sugar levels has lead to the hypothesis that these genes are involved in various processes via sugar sensing (Thimm *et al.,* 2004). Class II TPS proteins have been shown to associate with protein kinases. *TPS5-7* are targeted by the protein kinase SnRK1 in *Arabidopsis* (Harthill *et al.,* 2006) and all the class II TPS proteins have defined amino acid sites for CDPK binding and phosphorylation (Glinski and Weckwerth, 2005). Recently, (Rolland *et al.,* 2006) have published a detailed account of their functional and *in planta* expression analysis of the class II TPS genes.

Stress Protection by Trehalose

Trehalose application or accumulation can result in plants tolerant to a number of biotic and abiotic stresses. For example, spraying tomato plants with validamycin A, a trehalase inhibitor, that promotes trehalose accumulation, protected the plants against tomato wilt disease caused by *Fusarium oxysporum* (Ishikawa *et al.,* 2005). Similarly, exogenous application of trehalose has been shown to be quite effective against fungal growth and penetration. Trehalose application stimulated resistance against powdery mildew in wheat (Reignault *et al.,* 2001).

Resistance against *Hyaloperonospora parasitica* has been observed in trehalose fed *Arabidopsis* plants and also those that overexpress *otsA* (Schluepmann *et al.,* 2002 European patent application No. 1375669). Work presented in this dissertation has demonstrated that trehalose injected *Arabidopsis* plants are also more resistant to green peach aphid as compared to water injected control plants (Singh *et al.,* 2011).

Ectopic expression of yeast TPS1 in tobacco plants resulted in drought tolerant plants (Holmström *et al.,* 1996; Romero *et al.,* 1997; Karim *et al.,* 2007). These plants were found to retain higher water levels as compared to wild type plants suggesting a basis for improved drought tolerance. A similar phenotype of improved water retention has been observed in drought tolerant transgenic potato plants expressing yeast TPS1 (Stiller *et al.,* 2008). However, constitutive expression of *TPS1* resulted in smaller leaves (Holmström *et al.,* 1996; Romero *et al.,* 1997). This phenotype was overcome by targeting yeast *TPS1* to the chloroplast without affecting improved drought tolerance (Karim *et al.,* 2007). Protection against abiotic stress can be mediated via increased accumulation of trehalose is evident from transgenic rice plants expressing gene fusion of *E. coli otsA/otsB*. These plants were found to be less damaged by photo-oxidative damage during drought conditions as compared to control plants (Garg *et al.,* 2002). Overexpression of rice TPP1 gene resulted in trehalose hyper-accumulating transgenic rice plants that were demonstrated, in comparison to control plants, to be tolerant to cold and salt stress (Ge *et al.,* 2008).

Transgenic *Arabidopsis* plants expressing yeast *TPS1* and *TPS2* genes were found to contain 20-30% less stomata than wild type control plants suggesting that the drought tolerant phenotypes may be a result of improved water retention and photosynthesis due to an impact of trehalose hyper-accumulation on guard cell development (Karim *et al.,* 2007).

Impact of Trehalose on Carbon Allocation and Storage in Plants

The low concentration at which trehalose is present in most of the studied angiosperms excludes the possibility that it can be effectively used as a nutrition source. However, trehalose application to growing *Arabidopsis* seedlings has profound impact on plant carbon allocation and storage (Wingler *et al.,* 2000). *Arabidopsis* seedlings treated with 25 mM trehalose displayed large accumulation of starch in cotyledons with a concomitant lack of starch storage in the roots (Wingler *et al.,* 2000). Addition of trehalose at higher concentrations (100 mM) arrests *Arabidopsis* seedling root growth and can be rescued by the addition of other metabolizable sugars (Schluepmann *et al.,* 2004). These observations suggest that trehalose may be required for carbon transport and allocation in higher plants and is toxic at a higher concentration which explains the low amounts at which this sugar is present in higher plants. The toxic nature of trehalose, when applied at higher concentrations, is evident from the observation that *Arabidopsis* seedlings grown in the presence of 30 mM trehalose displayed the accumulation of stress detoxification enzymes (Bae *et al.,* 2005).

The *Arabidopsis*-Green Peach Aphid Model System

The model plant *Arabidopsis thaliana*, in the last two and a half decades has become an indispensible tool for studying plant molecular biology. With a small size, short life span, ease of transformation and availability of mutants has made this crucifer a cornerstone of plant research (Meinke *et al.,* 1998).*Arabidopsis* has been utilized as a model plant to study plant defense against insects. Both chewing and piercing-sucking type of insects have been studied on *Arabidopsis* e.g. Lepidopteran larvae like fall armyworm (Cipollini *et al.,* 2004), corn earworm (Cardoza, 2011) and piercing sucking insects like silverleaf whitefly (*Bemisia tabaci*) (Zarate *et al.,* 2007), cabbage aphid (Kusnierczyk *et al.,* 2008) and GPA (Pegadaraju *et al.,* 2007; Louis *et*

al., 2010; Singh *et al.,* 2011) *Myzus persicae* (Sülzer) or the Green peach aphid (GPA) is a prominent member of the family Aphididae. Depending on the availability of the nutrient source, GPA demonstrates an alteration between two forms. A suitable host is colonized by the apterous or the "wingless" form. These yellowish green 1-2 mm long apterous insects reproduce asexually, via parthenogenesis, with the subsequent generations developing within the mother and the embryos. This "telescopic" generation allows the GPA to effectively colonize a compatible host. Depleted nutrient source lead to the appearance of the alate or "winged" forms. These dark colored alate GPA are able to migrate to newer hosts and can reproduce sexually. However, the majority of reproduction is completed asexually (Blackman and Eastop, 2000). As the name suggests, peach trees are the primary host of the GPA. The aphid displays a world-wide distribution and is particularly successful in the temperate regions of the world. GPA is considered a polyphagous aphid and is known to infest more than 100 species of plants including *Arabidopsis*. Besides the obvious loss of nutrition due to its feeding, GPA can vector over 100 economically important plant viruses (Blackman and Eastop, 2000). To study the nature of plant-aphid interaction we have successfully employed the *Arabidopsis*-GPA model system in our laboratory (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007; Louis *et al.,* 2010a; Louis *et al.,* 2010b; Singh *et al.,* 2011).

PHYTOALEXIN DEFICIENT4 (*PAD4*) was identified as a gene involved in defense against pathogens and was found to be involved in SA signaling and camalexin metabolism (Zhou *et al.,* 1998; Jirage *et al.,* 1999). Pegadaraju and co-workers (2005) demonstrated that PAD 4 is involved in both antibiotic and antixenotic defenses against GPA, in an SA independent manner. Knockout of PAD4 function resulted in mutant plants that displayed delayed senescence in response to aphid feeding (Pegadaraju *et al.,* 2005), suggesting that PAD4

mediated premature senescence may be employed by the plants as defense mechanism against GPA to control its colonization of the host plant (Pegadaraju *et al.,* 2005). In case of pathogen infection it had been observed that PAD4 function was dependent upon a physical interaction with ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) protein (Feys *et al.,* 2005). However, in case of defense against GPA, PAD4 function was found to be independent of EDS1 (Pegadaraju *et al.,* 2007).Use of the extremely powerful electrical penetration graph (EPG) technique (Tjallingii, 1990) demonstrated that *PAD4* has an essential role in providing a phloem based defense mechanism against GPA (Pegadaraju *et al.,* 2007). As the aphid and the plant are made parts of a continuous circuit, EPG allows deciphering the various feeding patterns of the phloem feeder as a series of distinctive waveforms that can be recorded and analyzed (Tjallingii, 1990; Tjallingii and Esch, 1993; Pegadaraju *et al.,* 2007).

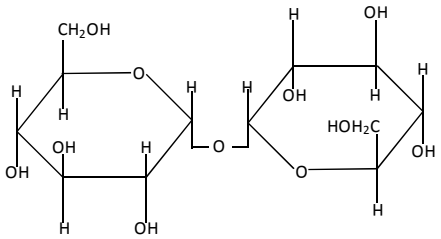
In a manner similar to *PAD4*, the expression of another potential lipase encoding gene *MYZUSPERSICAE INDUCED LIPASE1* (*MPL1*) was found to be upregulated in response to GPA feeding with *mpl1* mutant plants susceptible to GPA infestation as compared to wild type plants (Louis *et al.,* 2010b). Together these studies (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007; Louis *et al.,* 2010b) suggest an important role for lipids and lipid metabolism in *Arabidopsis* defense against GPA.

Objectives of This Study

The presented dissertation provides unique insights into the unique role of *AtTPS11* in *Arabidopsis* defense against GPA and also its impact on primary carbon metabolism in response to GPA feeding. The work presented here suggests a novel role for a class II trehalose metabolism gene. In chapter 2, the characterization of *tps11* mutant plants and their role in providing both antibiotic and antixenotic defenses against GPA is discussed. Chapter 3 delves

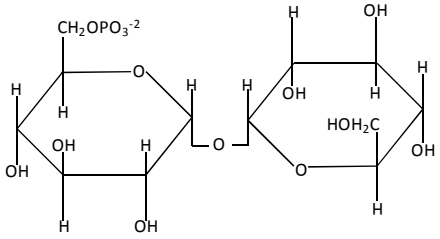
into the impact of *AtTPS11* on trehalose metabolism. The *tps11* mutant plants lack the transient accumulation of trehalose in response to aphid feeding, displayed by wild type plants. In addition, the role of trehalose, as a supplement to the artificial aphid diet and *in planta* application, is discussed. It was observed that trehalose can directly act as an antibiotic agent against GPA. Trehalose application and accumulation by *Arabidopsis* plants also led to the induction of *PAD4*, a gene involved in providing defense against GPA (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007). Chapter 4 describes the impact of trehalose metabolism in general and the role of AtTPS11 in modulating carbohydrate metabolism. Both sucrose and starch were found to hyper-accumulate in response to aphid feeding. However, it was observed that *TPS11* promoted the accumulation of starch at the expense of sucrose in GPA infested leaves and that this has an important role in curtailing insect infestation on *Arabidopsis*. The same chapter also discusses the role of starch metabolism in *Arabidopsis* defense against GPA.

(a)



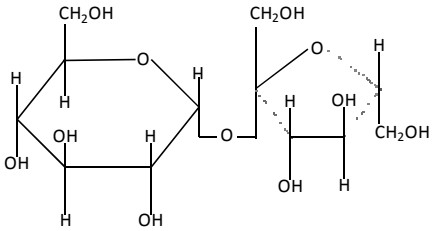
Trehalose

(b)



Trehalose-6-Phosphate

(c)



Sucrose

Figure 1.1. Molecular structures of trehalose, trehalose-6-phosphate and sucrose.

Depiction of the molecular structures of (a) trehalose and its metabolic intermediate (b) trehalose-6-phosphate. (c) Molecular structure of sucrose

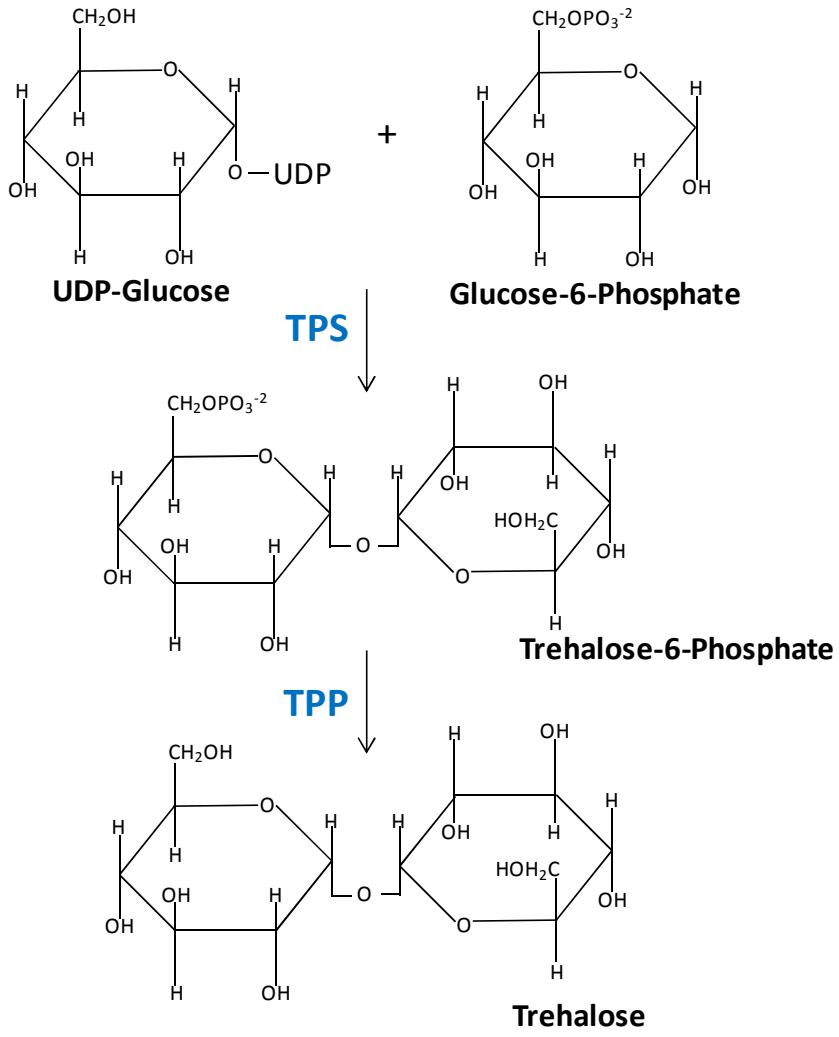


Figure 1.2. Trehalose biosynthetic pathway in plants

UDP-glucose and glucose -6-phosphate are converted to trehalose-6-phohsphate, the metabolic intermediate by the activity of trehalose phosphate synthase (TPS) which is further converted to trehalose by the removal of phosphate by trehalose phosphate phosphatase (TPP). Plants also contain the trehalose degrading enzyme trehalase which can catalyze the formation of glucose molecules from trehalose.

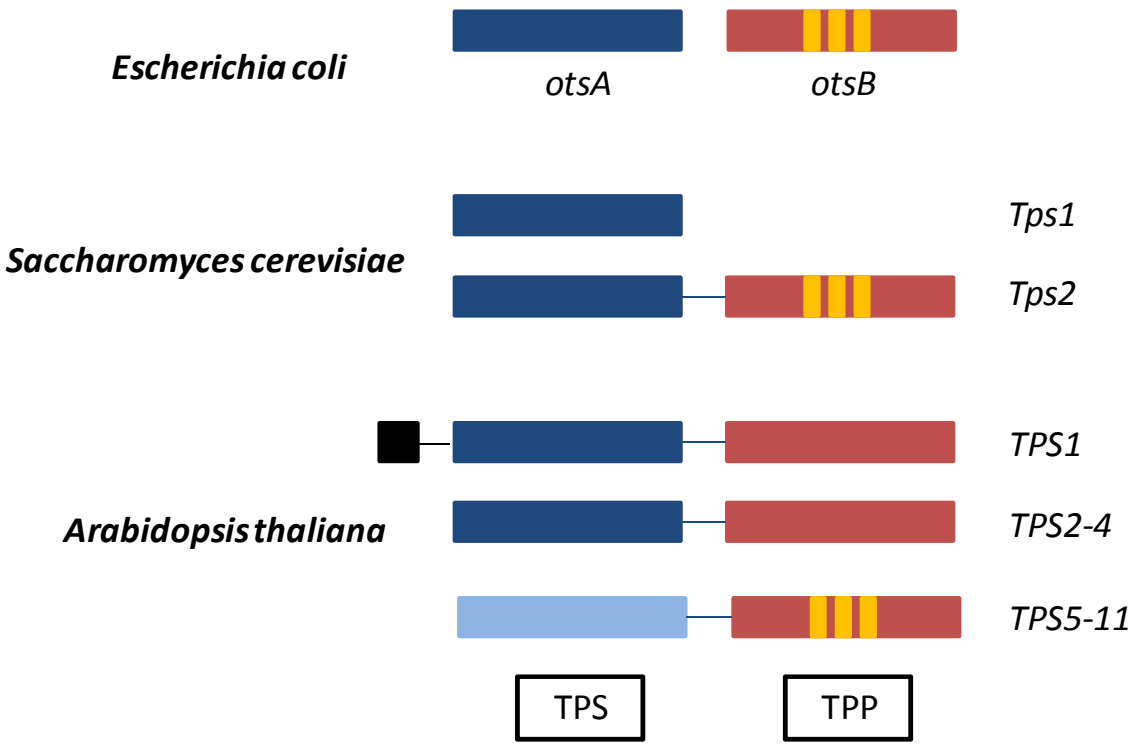


Figure 1.3. Diagram depicting various domains of the trehalose metabolism proteins in bacteria, yeast and plants.

E. coli genes *otsA* and *otsB* encode trehalose phosphate synthase (TPS) and trehalose phosphate phosphatase (TPP), respectively. *Arabidopsis TPS1*-encoded protein is similar to both bacterial and yeast TPS except for the presence of an N-terminal domain. Class II trehalose metabolism genes *TPS2-4* encode proteins that have a TPS domain similar to TPS1 but the TPP domains are inactive. *TPS5-11*-encoded proteins display low similarity to the TPS domain but appear to have an active TPP domain. Figure adapted from Leyman *et al.*, 2001. Blue boxes represent the TPS domain whereas the red boxes represent the TPP domain. Yellow boxes within the TPP domain represent the phosphatase domains.

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CHAPTER 2

CHARACTERIZATION OF *ARABIDOPSIS* CLASS II TREHALOSE METABOLISM GENE *TREHALOSE PHOSPHATE SYNTHASE11* (*TPS11*) AND ITS ROLE IN DEFENSE AGAINST GREEN PEACH APHID

Abstract

Agricultural productivity is limited by the removal of sap, alterations in source–sink patterns, and viral diseases vectored by aphids, which are phloem-feeding pests. Here I show that *TREHALOSE PHOSPHATE SYNTHASE11* (*TPS11*) gene-dependent trehalose metabolism regulates *Arabidopsis thaliana* defense against *Myzus persicae* (Sülzer), commonly known as the green peach aphid (GPA). GPA infestation of *Arabidopsis* resulted in a transient increase in expression of the *TPS11* gene, which encodes a trehalose-6- phosphate synthase/phosphatase. Knockout of TPS11 function in *tps11* mutant plants attenuated both antibiotic defenses that curtail GPA fecundity and antixenotic defenses that impact GPA behavior. Complementation of yeast *tps1* and *tps2* mutants by *TPS11* cDNA suggests that *TPS11* can encode both trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase activities. Evidence presented here demonstrates that GPA induced expression of the *PHYTOALEXIN DEFICIENT4* gene, which is a key modulator of defenses against GPA, is modulated by *TPS11*. These results uncover an erstwhile unknown role for *TPS11*, a class II trehalose metabolism gene from *Arabidopsis*.

[1]Results presented in this chapter have been published in The Plant Journal, Singh *et al.,* 2011. 67 (1), 94-104. Used with permission from John Wiley and Sons

Introduction

Aphids are an important group of phloem-feeding insects that limit plant productivity by removing nutrients and altering source-sink patterns (Dixon, 1998; Blackman and Eastop, 2000; Goggin, 2007). In addition, several aphids also vector viral diseases, thus causing further damage to the host plant (Kennedy *et al.,* 1962; Matthews, 1991). Aphids use their slender stylets to penetrate plant tissue intercellularly to access the sieve elements for feeding (Pollard, 1973; Pollard, 1973; Walling, 2000). However, occasionally the stylets also puncture cells, and minute amounts of cell contents are ingested (Tjallingii, 1990). Plants utilize a variety of mechanisms to control aphid infestation. These include anti-xenotic factors that impact insect behavior and thus deter aphids from settling on the plant and limit feeding, and antibiotic factors that adversely affect insect reproduction, survival, or growth and development (Smith, 2005; Smith, 2005; Powell *et al.,* 2006; Powell *et al.,* 2006; Powell *et al.,* 2006; Goggin, 2007; Goggin, 2007; Walling, 2008; Walling, 2008). However, the molecular mechanisms underlying plant defense against aphids and the regulation of these defenses are poorly understood. In recent years, study of the interaction between *Arabidopsis thaliana* and *Myzus persicae* (Sülzer), commonly known as the green peach aphid (GPA), a polyphagous insect with a wide host range (Blackman and Eastop, 2000), has provided insights into the regulation of plant defense against aphids. These studies have revealed a role for jasmonate signaling in plant defense against aphids (Ellis *et al.,* 2002; Mewis *et al.,* 2005). Salicylic acid (SA) signaling is also activated in GPA-infested *Arabidopsis*. However, studies with *Arabidopsis* genotypes that are defective in SA synthesis and signaling confirmed that SA signaling does not promote defense against GPA (Pegadaraju *et al.,* 2005). On the contrary, Mewis et al. (2005) reported that insect numbers were lower on plants defective in SA accumulation and signaling than in wild-type (WT) plants. It has been suggested

that phloem-feeding insects may trick the host plant into activating SA signaling as a means of suppressing activation of JA signaling (Walling, 2008). The *PHYTOALEXIN DEFICIENT4* (*PAD4*) gene is an important component of *Arabidopsis* defense against GPA (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007; Louis *et al.,* 2010a; Louis *et al.,* 2010b). *PAD4* expression is rapidly induced in GPA-infested leaves. Insect numbers were higher on the *pad4* mutant than the WT plant (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007). In contrast, resistance against GPA was enhanced in transgenic plants that over-express *PAD4* (Pegadaraju *et al.,* 2007). *PAD4* was required for the accumulation of an antibiosis activity that could be recovered in vascular sap-enriched petiole exudates collected from WT leaves (Pegadaraju *et al.,* 2007). In addition, the presence of *PAD4* had a deterrent effect on settling of GPA on WT *Arabidopsis* (Louis *et al.,* 2010a). Electrical monitoring of aphid behavior on *Arabidopsis* further revealed that PAD4 was also required to limit insect feeding from the sieve elements (Louis *et al.,* 2010a). Although *PAD4* also regulates synthesis of camalexin and SA, and SA signaling, genetic studies have indicated that the role of *PAD4* in *Arabidopsis* defense against GPA is independent of its involvement in these mechanisms (Pegadaraju *et al.,* 2005). Furthermore, although the participation of PAD4 protein in plant defense against pathogens is dependent on its physical interaction with the EDS1 protein (ENHANCED DISEASE SUSCEPTIBILITY1), *EDS1* is not required for the involvement of *PAD4* in *Arabidopsis* defense against GPA (Pegadaraju *et al.,* 2007), suggesting that *PAD4* has a unique role in defense against GPA that is distinct from its involvement in plant defense against pathogens.

Trehalose is a non-reducing α, α-1, 1-linked glucose disaccharide that functions as an energy source and a storage form of more reactive glucose in lower organisms (Galinski,

1993). The ability of trehalose to establish hydrogen bonds with membranes and macromolecules under conditions of dehydration makes it a suitable osmoprotectant as well (Peterbauer *et al.,* 2002; Fernandez *et al.,* 2010). Plants also synthesize trehalose. However, except for a few desiccation tolerant species, plants contain only trace amounts of trehalose, arguing against its involvement as an osmoprotectant and energy source. The general consensus is that trehalose and trehalose-6-phosphate (T6P) have a signaling function in plants (Paul *et al.,* 2008; Fernandez *et al.,* 2010). However, this remains to be proven. The morphological aberrations of the maize (*Zea mays*) *rasmosa3* and *Arabidopsis tps1* and *tps6* mutants, which have defects in trehalose metabolism, suggest that trehalose metabolism has a role in plant growth and development (Eastmond *et al.,* 2002; Gomez *et al.,* 2006; Satoh-Nagasawa *et al.,* 2006; Chary *et al.,* 2008). In addition, stress tolerance/resistance was higher in mutant and transgenic plants that constitutively accumulated high levels of trehalose, and in plants treated with trehalose (Garg *et al.,* 2002; Cortina and Culiáñez- Maciá , 2005; Renard-Merlier *et al.,* 2007; Fernandez *et al.,* 2010), indicating that elevated trehalose content can promote stress resistance in plants. However, whether plants engage trehalose metabolism to regulate stress responses has not been demonstrated. In plants, trehalose is synthesized in two steps by a pathway that has been termed the OtsA–OtsB pathway (also referred to as the TPS–TPP pathway) (Paul *et al.,* 2008). This pathway is found in a number of other organisms, including bacteria and yeast, and is the only one that uses T6P as an intermediate (Paul *et al.,* 2008). The first step in this pathway, which is catalyzed by T6P synthase (TPS), results in synthesis of T6P. Subsequently, T6P is dephosphorylated by T6P phosphatase (TPP) to yield trehalose. Trehalose in turn can be degraded by trehalase to yield glucose. *Arabidopsis* contains a single trehalase-encoding gene (*TRE1*). The 11 genes encoding TPSs in *Arabidopsis* are classified into two separate classes

(Leyman *et al.,* 2001; Paul *et al.,* 2008). The class I genes, which are closely related to the yeast *TPS1* gene, include *TPS1–TPS4*. Of these four class I TPS-encoded proteins, synthase activity has only been demonstrated for *TPS1* (Blazquez *et al.,* 1998). The seven predicted proteins encoded by the class II genes *TPS5–TPS11* contain both synthase and phosphatase domains. Indeed, *Arabidopsis* TPS6 complemented the phenotypes of the *Saccharomyces cerevisiae tps1* and *tps2* mutants, which are deficient in TPS and TPP activities, respectively (Chary *et al.,* 2008), confirming the dual activity of this class II TPS. However, the biochemical and biological functions of the other class II TPSs remain to be determined. Here we show that the *Arabidopsis TPS11* gene encodes a trehalose-synthesizing enzyme with TPS and TPP activities that is required for anti-xenosis and antibiosis against GPA. The evidence provided here reveals a previously unrecognized regulatory function for *TPS11*-dependent trehalose in plant defense against aphids. *TPS11* contributes to defense by regulating expression of *PAD4*.

Results

TPS11 is Required to Limit GPA Infestation

TPS11 was identified in a microarray experiment as a gene that was down-regulated 48 h post-infestation (hpi) with GPA (Pegadaraju *et al.,* 2005). However, as shown in Figure 2.1, time-course experiments indicated that *TPS11* expression is in fact transiently induced in *Arabidopsis* leaves infested with GPA. Like *PAD4*, *TPS11* expression was induced within 3 hpi. However, unlike the *PAD4* transcript, which remained at elevated levels during the course of the experiment, *TPS11* expression peaked by 12 hpi and declined thereafter (Figure 2.1) (Pegadaraju *et al.,* 2005).

To evaluate the contribution of *TPS11* in *Arabidopsis* interaction with GPA, a no-choice bioassay was performed to compare insect performance on WT plants and the *tps11-1* mutant, in which the *TPS11* gene contains a T-DNA insertion within the coding region (Figure 2.2a) that disrupts *TPS11* expression (Figures 2.1 and 2.2b). Insect numbers (adults + nymphs) were significantly higher on *tps11-1* than the WT plant (Figure 2.2c). Similarly, insect numbers were higher on the *tps11-2* and *tps11-3* mutants (Figure 2.2c), both of which contain T-DNA insertions within the *TPS11* promoter region (Figure 2.2a) that affect *TPS11* expression (Figure 2b). Resistance was restored in the *tps11-1* background by expression of *TPS11* from the *CaMV* 35S gene promoter (Figure 2.2d). These results confirm that *TPS11* is important for *Arabidopsis* defense against GPA.

TPS11 Contributes to Antixenosis and Antibiosis

To determine whether *TPS11* contributes to antixenosis and thus impacts the ability of the insect to settle on *Arabidopsis*, the insect was provided with the choice of settling on either a WT plant or the *tps11-1* mutant. As shown in Figure 2.3a, the insects preferred to settle on the *tps11-1* mutant, indicating that *TPS11* is required for deterring insects from settling on *Arabidopsis*. *Arabidopsis* petiole exudates, which are enriched in vascular sap, contain a factor(s) that, when added to a synthetic diet, curtails growth of the insect population (Figure 2.3b) (Louis *et al.,* 2010a; Louis *et al.,* 2010b). To examine whether *TPS11* is required for this antibiosis activity, the insect population size was monitored on a synthetic diet supplemented with petiole exudates collected from *tps11-1* leaves. In comparison to petiole exudates collected from the WT plant, *tps11-1* petiole exudates did not limit the insect population (Figure 2.3b), indicating that *TPS11* is required for accumulation of an antibiosis activity in the petiole exudates of

Arabidopsis leaves. Singh et al. (2011) also showed that *TPS11* impacts insect feeding behavior and was required for curtailing insect feeding from sieve elements. Taken together, the above results indicate that *TPS11* is required for antixenotic and antibiotic defenses against GPA.

TPS11 Encodes for Trehalose-6-Phosphate Synthase and (TPS) Trehalose-6-Phosphate Phosphatase (TPP) Activities

The TPS11 protein exhibits homology to *Saccharomyces cerevisiae* TPS1- and TPS2-encoded TPS and TPP, respectively (Figure 2.4 a, b). In *S. cerevisiae*, TPS1 deficiency results in an inability to grow in the presence of glucose due to accumulation of high levels of sugar phosphates and the corresponding depletion of Pi and ATP, and TPS2 deficiency results in poor growth at an elevated temperature due to the accumulation of high levels of T6P (Hohmann *et al.,* 1996).

To determine whether the TPS11 protein possesses TPS and TPP activities, the ability of TPS11 to complement the glucose sensitivity of the yeast *tps1* mutant and the temperature sensitivity of the *tps2* mutant was tested. As shown in Figure 2.5, TPS11 complemented the *tps1* mutant phenotype, resulting in enhanced growth of the *TPS11* transformed *S. cerevisiae tps1* strain, compared to the non-transformed *tps1* strain on minimal medium containing glucose. Similarly, *TPS11* also complemented the *tps2* mutant phenotype as evident by the improved growth of the *TPS11* transformed *tps2* strain of *S. cerevisiae* at the 37°C, compared to the non-transformed *tps2* mutant strain (Figure 2.5). These results confirm that the TPS11 protein possesses both TPS and TPP activities.

PAD4 does not regulate *TPS11* expression in GPA-infested *Arabidopsis*

Like *TPS11*, the *PAD4* gene is also required for antixenosis and antibiosis against GPA (Pegadaraju et al. 2005, 2007; Louis et al. 2010). Like *TPS11*, expression of *PAD4* is upregulated in response to GPA infestation. *PAD4* was previously shown to be a key regulator of gene expression in aphid-infested *Arabidopsis* (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007; Louis *et al.,* 2010a; Louis *et al.,* 2010a; Louis *et al.,* 2010b). It is plausible that the transient upregulation of *TPS11* in GPA-infested plants is modulated by *PAD4*. To determine this hypothesis, *TPS11* expression was monitored in the leaves of uninfested and GPA-infested WT and *pad4-1* mutant plants. Previous studies have shown that the *PAD4* transcript is undetectable in the *pad4-1* mutant, thus suggesting that it is a likely null allele (Louis et al. 2010). As shown in Figure 2.6, the aphid infestation-induced expression of *TPS11* was unaffected in the *pad4* mutant, indicating that *PAD4* does not regulate *TPS11* expression and thus does not function upstream of *TPS11*.

TPS11 modulates expression of *PAD4* in GPA-infested plants

The evidence presented above indicates that *PAD4* does not regulate *TPS11* activity. To determine if *TPS11* functions upstream of *PAD4* and thus regulates *PAD4* expression in GPA infested plants, we monitored *PAD4* expression over a 24 h period in the GPA-infested leaves of WT and *tps11-1* mutant plants. As shown in Figure 2.1, the GPA infestation-induced expression of PAD4 was weaker in the *tps11-1* mutant than in the WT. In contrast, basal expression of *PAD4* was constitutively higher in the 35S:*TPS11* plants (Figure 2.7). As summarized in Figure 2.8, these results indicate that *TPS11* functions upstream of *PAD4* to modulate the upregulation of *PAD4* expression in GPA-infested leaves of *Arabidopsis*.

Discussion

The *Arabidopsis* genome has 21 genes that are annotated as genes involved in trehalose metabolism. Prior to this study only *TPS1* and *TPS6* were shown to encode a bonafide trehalose biosynthesis enzyme (Leyman *et al.*, 2001). *TPS1* was shown to have a role in embryo development. Considering that *Arabidopsis* contains several more trehalose biosynthesis genes, including those containing both TPS and TPP activities (Class II genes), it has been speculated that the class II trehalose metabolism genes (*TPS5-11*) may serve secondary biological functions in addition to their speculated function in the synthesis of T6P and trehalose (Leyman *et al.*, 2001). Indeed, *TPS6* was shown to have a role in controlling epidermal cell shape and trichome structure in *Arabidopsis* leaves (Chary *et al.*, 2008). *TPS11*is also annotated as a class II trehalose metabolism gene that encodes a putative protein with both TPS and TPP domains (Leyman *et al.*, 2001). The evidence provided here confirms that *TPS11* indeed encodes a protein with both TPS and TPP activities. Expression of *TPS11* complemented the growth deficiencies of the *S. cerevisiae tps1* and *tps2* mutants. This study also uncovered an important biological function of *TPS11*. Genetic studies with *Arabidopsis tps11* mutant plants confirmed an important role for *TPS11* in *Arabidopsis* defense against GPA. Choice and no-choice assays confirmed that *TPS11* is required for both antibiosis that curtails insect fecundity and antixenosis that has a detrimental effect on insect behavior thus limiting its full potential on *Arabidopsis*. Vascular sap-enriched petiole exudates from *tps11* lacked an antibiosis activity that was present in petiole exudates from WT plants. Loss of this antibiosis activity likely contributes to the improved performance of GPA on the *tps11* mutant compared to the wild type plants.

TPS11 expression was transiently induced in response to GPA infestation. Upregulation of *TPS11* expression was detectable as early as 3h post infestation and continued to rise until

12hpi, after which it declined. Whether the depression in *TPS11* expression later in infection is also critical in *Arabidopsis* defense is not clear and will require additional experimentation. *TPS11* may also be involved in the plant response to pathogen infection since its expression was down regulated in *Arabidopsis* challenged with *Pseudomonas* sp. (Verhagen *et al.,* 2004) and upregulated in plants exposed to *Tobacco mosaic virus* (Golem and Culver, 2003). *TPS11* expression is also impacted by plant hormones. For example, *TPS11* was upregulated in young *Arabidopsis* seedlings exposed to the auxin IAA (Goda *et al.,* 2004). *TPS11* expression is also tightly regulated by both the light and the carbon status of the plant. *TPS11* expression decreased upon first light and reintroduction of sucrose in the growth media, whereas its expression was high during darkness (Osuna *et al.,* 2007; Usadel *et al.,* 2008). *TPS11* expression was also induced in plants that exhibited enhanced salt tolerance due to over-expression of a bZIP transcription factor (Fujita *et al.,* 2007), and in plants that are rendered heat tolerant due to the over-expression of transcription factor MBF1c (Suzuki *et al.,* 2008). These results suggest that *TPS11* may also have a role in plant response to abiotic stress. The above mentioned studies clearly indicate that *TPS11* expression is dependent upon a number of environmental factors and presumably TPS11 has a broader function in plant stress tolerance.

Besides *TPS11*, *PAD4* gene is another gene which was previously shown to play an important role in regulating both antibiotic and antixenotic defenses against GPA (Pegadaraju *et al.,* 2005, 2007). *PAD4* was demonstrated to serve a phloem based defense function against GPA in *Arabidopsis* (Pegadaraju *et al.,* 2007).

Although the biochemical function of PAD4 remains unknown the protein contains the conserved triad of amino acids that confer a lipase/acylhydrolase like activity and has previously been shown to regulate expression of genes associated with plant defense against stress

(Pegadaraju et al. 2005: Wiermer et al. 2005). However, the transient upregulation of *TPS11* expression in GPA infested plants was not influenced by *PAD4* (Figure 2.6). By contrast, *PAD4* upregulation in response to GPA infestation was slower and weaker in the *tps11* mutant compared to the wild type, thus suggesting that *TPS11* modulates *PAD4* expression and thus functions upstream of *PAD4*. Thus the impact of *TPS11* on plant defense against GPA is in part likely mediated through *PAD4*. These results also suggest that *TPS11* might have a regulatory role in plant defense, presumably a signaling function upstream of *PAD4*. In summary, evidence presented here clearly demonstrates that *AtTPS11* plays an important role in the *Arabidopsis* defense against GPA. Cogent proof has been provided that *TPS11* has both TPS and TPP activities and that presence of *TPS11* function is essential for the timely expression of *PAD4*, an important regulator of both antibiotic and antixenotic defenses against GPA.

Materials and Methods

Plant and insect materials

Green peach aphid (GPA; *Myzus persicae* Sülzer) (Kansas State University, Museum of Entomological and Prairie Arthropod Research, voucher specimen #194) was reared as previously described (Pegadaraju *et al.,* 2005) on a 50:50 mixture of commercially available radish (*Raphanus sativus*) and mustard (*Brassica juncea*) plants. Approximately 4-week-old *Arabidopsis* plants cultivated at 22_C under a 14 h light (100 μE m^{-2} sec^{-1})/10 h dark regime were used for all experiments. The *pad4-1* (CS3806), *tps11-1* (SALK_082979), *tps11-2* (SALK_072451), *tps11-3* (SALK_072450) mutant plants and are all in the accession Columbia.

No-choice and choice tests

In no-choice assays (Pegadaraju *et al.,* 2005), a total of 20 adult apterous (wingless) aphids were released on each plant, and the total numbers of insects counted 2 days later. For choice tests, one plant each of two genotypes was cultivated in each pot (Pegadaraju *et al.,* 2007). Twenty adult GPA were released equidistant from the two plant genotypes being tested. The numbers of adult aphids recovered on each plant were counted over a 24 h period. For caging experiments, five insects were released on each leaf at the center of a cage constructed using double-sided foam tape. Leaf tissue from the caged region and the upper insect-free leaves were analyzed for trehalose, sucrose and starch content.

Petiole exudate collection and feeding trials

Petiole exudates were collected from *Arabidopsis* leaves using a previously described method (Chaturvedi *et al.,* 2008). A synthetic diet containing sucrose as the major carbon source (Mittler *et al.,* 1970) was used for the feeding trial bioassays (Louis *et al.,* 2010b). The total number of GPA in each feeding chamber was determined 4 days after release of three adult insects.

PCR analysis

All primers used in this study are listed in Table 2.1. A set of PCR reactions involving primers tps11-1-F plus tps11-1-R and tps11-1-R plus the T-DNA left border primer, which amplify the wild-type TPS11 and tps11-1 alleles, respectively, were used to identify plants that were homozygous for the tps11-1 allele. To identify plants that were homozygous for the tps11-2 and tps11-3 alleles, the primer TPS11-G-R plus the T-DNA left border primer were used to

follow the mutant alleles, and the primers tps11-G-F and tps11-G-R were used to follow the presence or absence of the *TPS11* allele. PCR using gene-specific primers was performed under the following conditions: 95ºC for 5 min, followed by 35 cycles of 95 ºC for 30 sec, 50 ºC for 1 min and 68 ºC for 2.5 min, with a final extension of 68 ºC for 7 min. PCR using the T-DNA left border primer and one gene specific primer was performed under the following conditions: 95 ºC for 5 min, followed by 30 cycles of 95 ºC for 30 sec, 50 ºC for 1 min and 72 ºC for 2.5 min, with a final extension of 72 ºC for 7 min.

Northern blot and RT-PCR analysis

RNA extraction from leaves was performed as previously described (Pegadaraju *et al.,* 2005). DIG labeling of probes and hybridization were performed using kits from Roche Applied Sciences (http:// www.roche.com). Gene-specific primers used for RT-PCR are listed in Table S1. The PCR conditions were as follows: 95 ºC for 5 min, followed by 25 cycles of 95 ºC for 30 sec, 60 ºC for 30 sec and 72 ºC for 30 sec, followed by a final extension step of 72 ºC for 7 min.

Cloning of the *TPS11* coding region and yeast complementation analysis

RNA extracted from GPA-infested WT Columbia plants at 12 hpi was reverse-transcribed, and the resulting cDNA was amplified using the forward primer TPS11-start (Table S1), which contains the start codon, and the reverse primer TPS11-stop, which contains the stop codon. The KpnI/XhoI-digested TPS11 amplicon was ligated into the KpnI/XhoI-restricted pYES2 vector (Chary *et al.,* 2008) to yield the pYES2-TPS11 plasmid, which was transformed into *Escherichia coli* BL21. pYES2-TPS11 was transformed into the *Saccharomyces cerevisiae* tps1 and tps2 deletion strains (Invitrogen Corporation, http://www.invitrogen.com/), and the

ability to complement Δtps1 and Δtps2 mutants was determined using previously described protocols (Gietz and Woods, 2002; Chary *et al.,* 2008).

35S:*TPS11* transgenic plants

TPS11-C-F and TPS11-C-R primers were used to amplify the TPS11 coding region from pYES2-TPS11, which was cloned into the pCR_8/GW/TOPO_ vector® (Invitrogen), which was subsequently used in an LR recombination reaction with the destination vector pMDC32 (Curtis and Grossniklaus, 2003) to yield pMDC32-TPS11. TPS11 expression in pMDC32-TPS11 is driven by the *CaMV* 35S gene promoter. *Agrobacterium tumefaciens* strain GV3101 was used to mobilize the pMDC32-TPS11 into *tps11-1* plants by the floral- dip method (Clough and Bent, 1998)to yield hygromycin-resistant 35S:TPS11 plants.

Statistical analysis

For the no-choice assays, choice assays and artificial diet assays, the means were separated using the paired *t*-test ($P < 0.05$). MS-Excel was used to perform the Student's *t*-test with two tailed distribution.

Table 2.1 Sequence of primers used for PCR and RT-PCR analysis

TPS11-start	GGGGTACCATGTCGCCGGAATCTTGG
TPS11-stop	GAGCTCCTAGCTTTCTTTCGACAT
TPS11-C-F	ATGTCGCCGGAATCTTGG
TPS11-C-R	CTATGCTTTCTTTCGACATTGTC
TPS11-F	GGGTTAATCCTTGGGACGTT
TPS11-R	AGATGCCCTCTCCAAATCCT
tps11-G-F	GAATTGTGAAGTTATGTGATTATG
tps11-G-R	CTAACATCACAATGTTCCCC
tps11-1-F	ATGTCGCCGGAATCTTGG
tps11-1-R	AAAGTACCTAGTGAAATAACCATG
T-DNA-L	ATTTTGCCGATTTCGGAAC
ACT8F	ATGAAGATTAAGGTCGTGGCA
ACT8R	TCCGAGTTTGAAGAGGCTAC
PAD4-F	ACCGAGGAACATCAGAGGTAC
PAD4-R	AAATTCGCAATGTCGAGTGGC

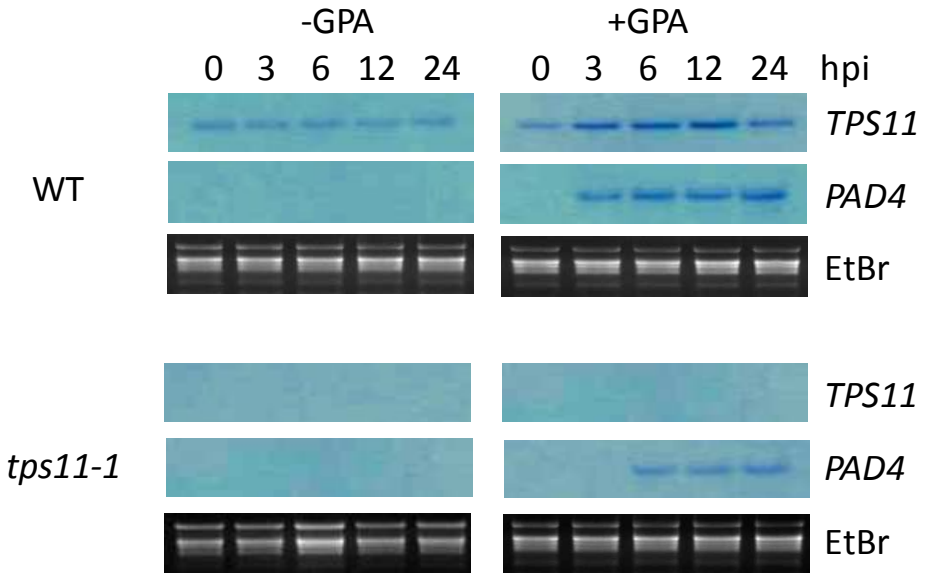


Figure 2.1. *TPS11* expression is transiently induced in *Arabidopsis* leaves infested with GPA

Northern blot analysis of *TPS11* and *PAD4* expression in uninfested (- GPA) and GPA-infested (+GPA) wild-type (WT) and *tps11-1* plants. EtBr, ethidium bromide-stained total RNA; hpi, hours post-infestation.

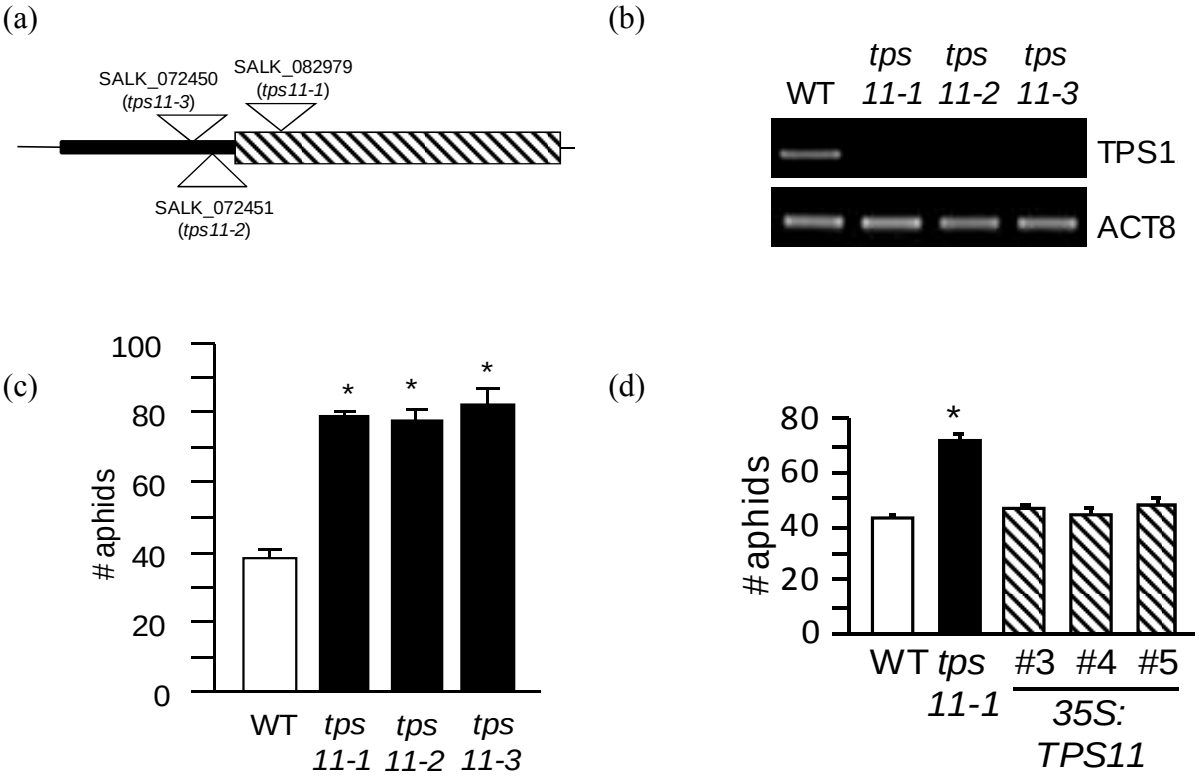
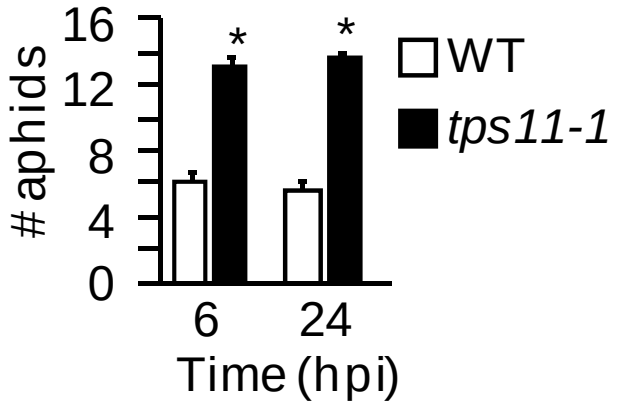


Figure 2.2. *TPS11* is required for *Arabidopsis* defense against GPA

(a) Diagrammatic representation of the *TPS11* gene, indicating locations of the T-DNA inserts in the SALK_092979 (*tps11-1*), SALK_072451 (*tps11-2*) and SALK_072450 (*tps11-3*) alleles. The solid black bar represents the predicted *TPS11* promoter and the hatched bar represents the coding region. (b) RT-PCR analysis of *TPS11* expression in leaves of the WT, *tps11-1*, *tps11-2* and *tps11-3* mutant plants. Gene-specific primers were used for the PCR step. The ACT8 gene provides a control for RT-PCR. (c) No-choice test. Total insect numbers on WT, *tps11-1*, *tps11-2* and *tps11-3* mutant plants 48 h after release of 20 adult apterous (wingless) aphids on each plant. (d) No-choice test. GPA numbers on WT, *tps11-1* and three 35S:*TPS11* transgenic lines (in the *tps11-1* genetic background) 48 h after release of 20 adult apterous aphids on each plant.

In (c) and (d), the values are mean aphid counts on ten plants of each genotype. Error bars represent SE. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the WT.

(a)



(b)

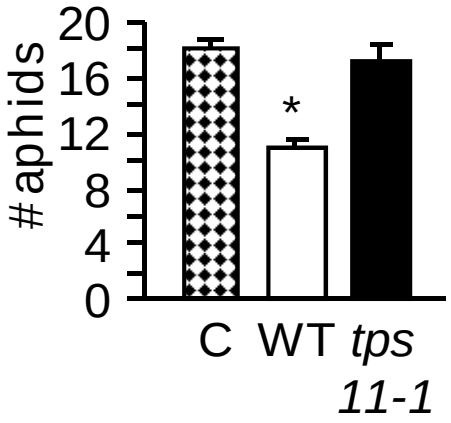


Figure 2.3. *TPS11* is required for antixenosis and antibiosis against GPA in *Arabidopsis*
(a) Choice test. GPA was given the choice between WT and the *tps11-1* mutant plants. Twenty aphids were released equidistant from a WT plant and a *tps11-1* mutant plant, growing in the same pot. The number of adult GPA that had settled on each plant was determined at 6 and 24 hpi. Values are mean aphid numbers on eight plants of each genotype for each time point. Error bars represent SE. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the WT. (b) Artificial diet assay. Comparison of GPA numbers on a synthetic diet (C) and a diet supplemented with WT and *tps11-1* petiole exudates. Three adult apterous aphids were introduced into each feeding chamber and allowed to feed on the diet. The total numbers of GPA (adults + nymphs) in each feeding chamber were determined 4 days later. Values are the means of three replicates for each treatment. Error bars represent SE. The asterisk indicates a significant difference ($P < 0.05$; *t*-test) from the synthetic diet control (C).

Figure 2.4. TPS11 protein exhibits homology to *Saccharomyces cerevisiae TPS1* and *TPS2* genes, which encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively

(a) ClustalW was used to align the *Arabidopsis* TPS11 (AtTPS11) amino acid sequence with the

S. cerevisiae TPS1-encoded trehalose-6-phosphate synthase (ScTps1). (b) ClustaW alignment of the *Arabidopsis* TPS11 (AtTPS11) amino acid sequence with the *S. cerevisiae TPS2*-encoded trehalose-6-phosphate phosphatase (ScTps2). An asterisk (*) denotes identical amino acids, two dots (:) denotes a conservative change, and a single dot (.) denotes semi-conservative change.

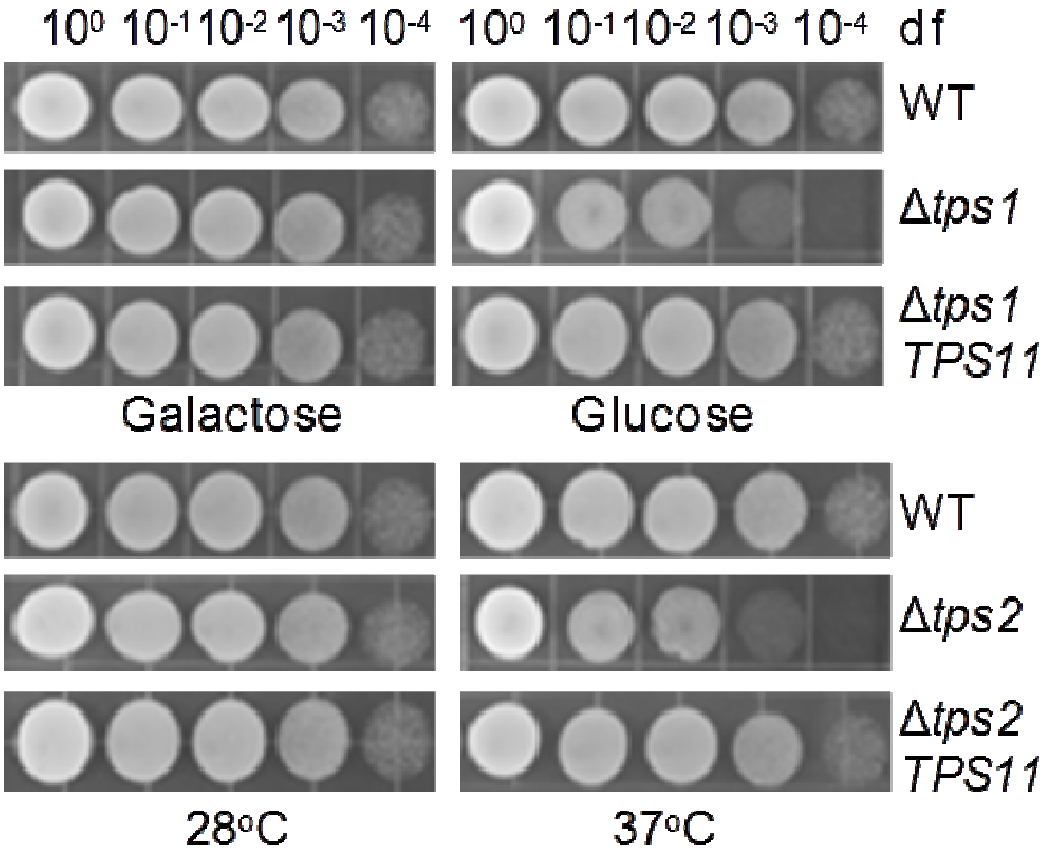


Figure 2.5. *TPS11* expression complements the glucose sensitivity of the *Saccharomyces cerevisiae tps1* mutant and the temperature sensitivity of the *tps2* mutant

To determine if TPS11 has trehalose-6-phosphate synthase (TPS) PS activity, *TPS11* was expressed in the TPS-deficient *tps1* mutant yeast, which is sensitive to growth on glucose, but not galactose, as the sole carbon source. The ability of *TPS11* to restore growth of the *tps1* mutant on glucose was determined, with growth on galactose providing the control. To determine if TPS11 has trehalose-6-phosphate phosphatase (TPP) activity, *TPS11* was expressed in the TPP-deficient *tps2* mutant yeast, which is sensitive to growth at 37ºC, but not at 28ºC. The ability of *TPS11* to restore growth of the *tps2* mutant at 37ºCwas determined, with growth at 28ºC providing the control. Serial tenfold dilutions of the yeast were spotted on agar plates. Photographs were taken 2 days later. WT, wild-type yeast; df, dilution factor.

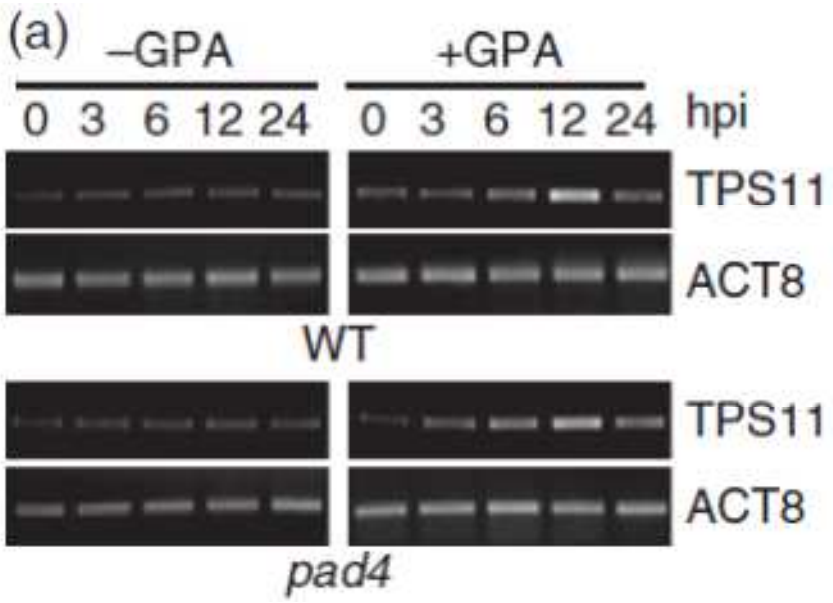


Figure 2.6. GPA induced expression of *TPS11* is not impacted by mutations in *pad4*

RT-PCR analysis of *TPS11* expression in leaves of uninfested (-GPA) and GPA-infested (+GPA) WT and *pad4* mutant plants.

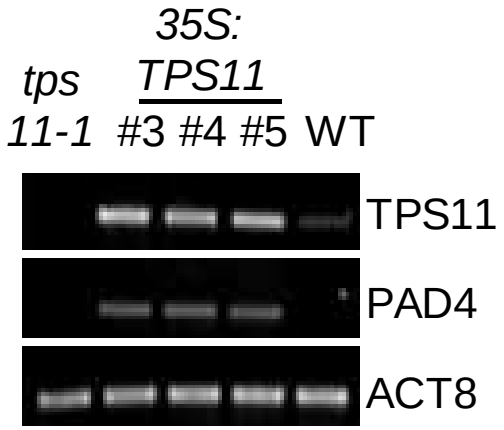


Figure 2.7. *PAD4* is constitutively over-expressed in *Arabidopsis* plants that overexpress *TPS11* Basal expression of *TPS11* and *PAD4* in transgenic 35S:*TPS11* plants which constitutively express *TPS11* from the *Cauliflower mosaic virus 35S* gene promoter in the *tps11-1* genetic background. The WT and *tps11-1* mutant plant provide the control genotypes for this experiment.

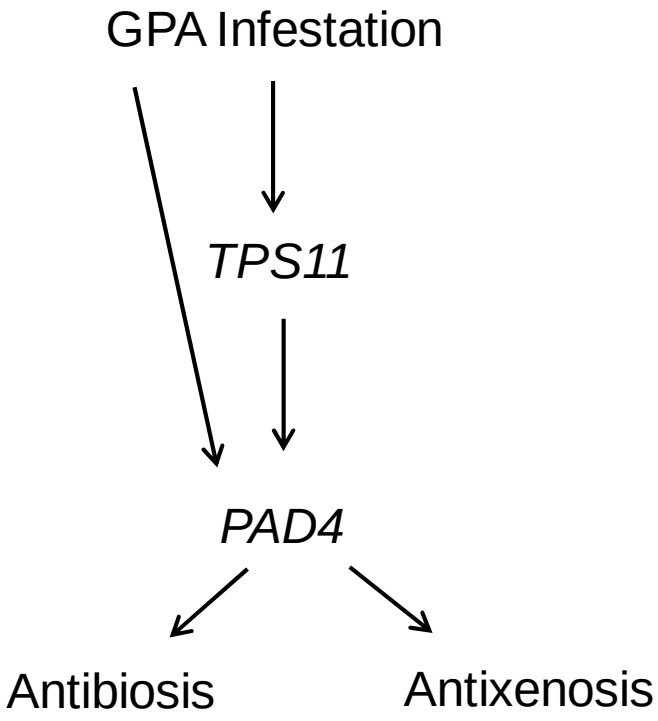


Figure 2.8. Model depicting the interaction between *TPS11* and *PAD4* in *Arabidopsis* defense against GPA

PAD4 has been demonstrated to influence both antibiotic and antixenotic defenses against GPA (Pegadaraju *et al.*, 2005). Induction of *PAD4* expression was found to be slower in GPA infested *tps11-1* plants (Figure 2.1) and at the same time *PAD4* was constitutively expressed in *Arabidopsis* plants overexpressing *TPS11* (Figure 2.7). It appears that *TPS11* expression is required to modulate *PAD4* expression in response to GPA infestation.

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CHAPTER 3

ARABIDOPSIS CLASS II TREHALOSE METABOLISM GENE*TPS11* AND TREHALOSE ARE REQUIRED FOR DEFENSE AGAINST GREEN PEACH APHID[2]

Abstract

Trehalose, a disaccharide similar to sucrose, is present in very minute quantities in higher plants. A role for trehalose in modulating plant growth and development and carbohydrate metabolism has been suggested. In *Arabidopsis* these are a total of 21 genes encoding for trehalose metabolism. In this chapter the role of *TPS11* in modulating trehalose levels in GPA infested leaves is defined. It was observed that GPA infested wild type plants transiently accumulate high levels of trehalose. By using transgenic and mutant *Arabidopsis* plants hyperaccumulating basal trehalose, it was established that trehalose accumulation has a detrimental effect on GPA fecundity. Similar effects were observed when trehalose was supplied exogenously to plants mutated in *TPS11* function. High levels of trehalose were also found to influence *PAD4* expression, a gene required for defense against GPA.

Introduction

Sugars are the major source of nutrition and storage in plants (Rolland *et al.,* 2006) and also regulate gene expression associated with growth and development (Koch, 1996) as well as

[2]Results presented in this chapter have been published in The Plant Journal, Singh *et al.,* 2011. 67 (1), 94-104. Used with permission from John Wiley and Sons

environmental stress (Roitsch, 1999). Sucrose is the major photosynthate that acts as the nutrient source between the source and sink tissues. The role of sucrose both as a nutrient source and signaling molecule has been established in plants (Rolland *et al.,* 2006). Recent studies have indicated at the emerging role of trehalose as a signaling molecule for controlling carbohydrate metabolism in plants (Paul *et al.,* 2008). Despite being present in trace amounts in most plants trehalose and its metabolic intermediate trehalose-6- phosphate (T6P) are emerging as key signaling players that control various physiological functions in plants (Paul *et al.,* 2008). Trehalose is a water soluble disaccharide composed of two molecules of glucose and is non-reducing in nature (Figure 1.1). The two glucose molecules are linked in an α, α- linkage. There are two more isomers of trehalose α, α- and α, β- trehalose, but only α, α-trehalose is present in biological systems (Elbein, 1974). Trehalose accumulates in spores of microorganisms and in response to a number of abiotic stresses such as heat and cold shock and dehydration in bacteria and fungi (Elbein *et al.,* 2003). In lower organisms it acts as a recalcitrant storage form for the much reactive glucose molecules and can be easily hydrolyzed to glucose during adverse growth conditions (Galinski, 1993). Among the known pathways of trehalose biosynthesis, higher plants including *Arabidopsis* utilize UDP-Glucose (UDPG) and glucose 6-phosohate (G6P) to synthesize T6P by the enzyme trehalose-6-phosphate synthase (TPS). Subsequently, T6P is de-phosphorylated by trehalose-6-phosphate phosphatase (TPP) resulting in the formation of trehalose. Most plants also express the trehalose degrading enzyme trehalase which regenerates glucose (Paul *et al.,* 2008). Due to its high solubility and ability to form hydrogen bonds with sugar and phosphate molecules, trehalose acts as a protectant for cellular membranes and

proteins during stress conditions (Peterbauer *et al.,* 2002) and has been utilized as a commercial food and enzyme protectant (Colaco *et al.,* 1992).

Trehalose application or accumulation has a positive impact on plant tolerance to a number of biotic and abiotic stresses. A number of reports have demonstrated that trehalose application can provide a higher level of resistance against fungi in plants. Spraying tomato plants with validamycin A, a trehalase inhibitor, protected them against tomato wilt caused by *Fusarium oxysporum* (Ishikawa *et al.,* 2005), and trehalose application enhanced resistance against powdery mildew in wheat (Reignault *et al.,* 2001). Trehalose also promotes tolerance against abiotic stress. For example, transgenic rice plants expressing gene fusion of *E. coli otsA/otsB* were found to be resistant to damage by photo-oxidative damage during drought conditions as compared to control plants (Garg *et al.,* 2002), and rice plants over-expressing the native *TPP1* gene were found to be tolerant to cold and salt stress (Ge *et al.,* 2008). Drought tolerance was also enhanced in tobacco plants by constitutive over-expression of yeast *TPS1* (Holmström *et al.,* 1996; Romero *et al.,* 1997; Karim *et al.,* 2007). However, constitutive expression of yeast *TPS1* also resulted in smaller leaves (Holmström *et al.,* 1996; Romero *et al.,* 1997), suggesting that constitutive activation of trehalose synthesis, while promoting stress tolerance also carries a developmental cost.

An emerging role of trehalose is its ability to act as a signal in controlling carbohydrate metabolism in plants. Is has been suggested that trehalose may be required for sugar transport and allocation in plants (Wingler *et al.,* 2000; Schluepmann *et al.,* 2004). Treatment of *Arabidopsis* seedlings with 25 mM trehalose resulted in the accumulation of starch in cotyledons along with reduced starch storage in the roots (Wingler *et al.,* 2000). Addition of trehalose at higher concentrations (100 mM) arrests *Arabidopsis* seedling root growth that could be rescued

by the addition of other metabolisable sugars (Schluepmann *et al.,* 2004). Trehalose applied at high concentrations is toxic to plants as *Arabidopsis* seedlings grown in the presence of 30 mM trehalose displayed the accumulation of stress detoxification enzymes (Bae *et al.,* 2005).These studies suggest that trehalose is toxic at higher a concentration which explains the low amounts at which this sugar is present in higher plants (Schluepmann *et al.,* 2004).

Amongst plants, the occurrence of trehalose was first recorded in lower plants like pteridophytes (Bianchi *et al.,* 1993). Presence of the trehalose biosynthetic pathway in angiosperms was first evident from experiments with validamycin A treated plants. Validamycin A is an inhibitor of trehalase. Tobacco plants treated with validamycin A accumulated high levels of trehalose thus indicating that tobacco has the biochemical machinery to synthesize trehalose (Goddijn *et al.,* 1997). The *Arabidopsis* genome sequence revealed the presence of 21 genes that exhibit homology to genes involved in trehalose metabolism. Based on their sequence similarity to yeast *TPS1* (TPS) and *TPS2* (TPP) these genes have been classified in to three different classes along with a single gene encoding for trehalase (Leyman *et al.,* 2001). Among the class I TPSs (*AtTPS1-4*) only TPS1 has been shown to be a functional TPS by its ability to complement the yeast *tps1* mutant that lacks a TPS activity (Blazquez *et al.,* 1998). Function of the remaining class I genes is not known besides the observation with microarray analysis that *TPS2* and *4* are expressed in developing seeds (Lunn, 2007). The class II TPSs (*AtTPS5-11*) can potentially encode for both trehalose-6-phosphate synthase and phosphatase domains. The basis for this classification is that class I TPS do not contain the typical phosphatase sequences within their C-terminal phosphatase domains whereas the class II genes have retained these phosphatase boxes (Leyman *et al.,* 2001). It was believed that the class II TPSs are not functional (Vogel *et al.,* 1998) but recent studies have shown that *AtTPS6* (Chary *et al.,* 2008) and *AtTPS11* (Singh *et*

al., 2011) can complement both *tps1* and *tps2* mutant strains of yeast, which lack TPS and TPP respectively. Sequence analysis of class II TPS genes shows that codon substitutions at the nucleotide level are the same at the level of proteins indicating towards conserved protein functions (Avonce *et al.,* 2006).

Amongst the class II genes, *AtTPS5* is required for thermo-tolerance in *Arabidopsis* plants and has been demonstrated to interact with the transcriptional co-activator MBF1c (Suzuki *et al.,* 2008). *AtTPS8-11* have conserved sited for SnRK1 mediated phosphorylation and can be phosphorylated by crude *Arabidopsis* leaf extracts (Glinski and Weckwerth, 2005). In a similar manner, AtTPS5, 6 and 7 are expected to be phosphorylated by SnRK1 and have been shown to bind to 14-3-3 proteins (Harthill *et al.,* 2006). SnRK1 are plant serine-threonine protein kinases with conserved domains related to the Snf1 (Sugar non-fermenting1) of *Saccharomyces cerevesiae* and AMPK (AMP-activated protein kinases) of animals (Baena-González *et al.,* 2007). The *Arabidopsis*, *KIN10* and *KIN11* genes display the highest similarity to SnRK1 (Baena-González and Sheen, 2008). SnRK1 have been demonstrated to play a central role in carbohydrate metabolism as overexpression of KIN10 resulted in the altered expression of a number of carbohydrate metabolism genes (Baena-González *et al.,* 2007). It appears that SnRK1 regulates the expression of genes that aid the plant in coping with stress conditions as genes for protein synthesis are downregulated while those involved in providing alternative sources of energy under sugar starvation conditions are upregulated (Baena-González *et al.,* 2007). SnRK1 are known to regulate a number of key carbohydrate metabolism enzymes like sucrose synthase in potato where the antisense expression of SnRK1 resulted in downregulation of the enzyme (Purcell *et al.,* 1998). SnRK1 also plays an important role in regulation of starch synthesis by regulating the post-translational activation of ADP-glucose pyrophosphorylase (AGPase), the

first committed enzyme of starch biosynthesis (Tiessen *et al.,* 2003). Starch synthesis in barley pollen grains has also been shown to be under the influence of SnRK1 (Zhang *et al.,* 2001). The central role of SnRK1 in regulating carbohydrate metabolism is indicated by the observation that both glucose-6-phosphate and T6P are able to inhibit SnRK1 activity (Toroser *et al.,* 2000; Zhang *et al.,* 2009).

Aphids belong to the order Hemiptera or true insects and utilize a unique dietary source in the form of phloem sap and are able to alter the source-sink relationship of the host plant by directly ingesting the nutrient source (Blackman and Eastop, 2000; Walling, 2000). Since the phloem sap has a high concentration of sucrose, aphids have evolved mechanisms to avoid the osmotic constraints accorded by the high sugar solute concentration of their diet (Walling, 2008). With the exception of the recent reports demonstrating a biological function for *AtTPS6* and *AtTPS11* (Chary *et al.,* 2008; Singh *et al.,* 2011), the role of other class II TPS genes is not clear. In the present chapter the role of trehalose in providing defense against GPA has been discussed. It was observed that the transient accumulation of trehalose, observed in GPA infested *Arabidopsis* plants, is under the control of *TPS11*. The role of *PAD4* in providing defense against GPA has been previously established (Pegadaraju *et al.,* 2005; Pegadaraju *et al.,* 2007; Louis *et al.,* 2010). In this chapter evidence is provided to show that *PAD4* expression is also modulated by the accumulation of trehalose.

Results

TPS11 is Essential for the Transient Increase in Trehalose in GPA-Infested Leaves

Trehalose levels in uninfested WT and *tps11* mutant plants were comparable (approximately 2 µg/g fresh weight), suggesting that *TPS11* does not contribute in any significant manner to the basal trehalose content in *Arabidopsis* leaves. As GPA infestation induces *TPS11* expression, trehalose levels were also monitored in aphid-infested leaves to determine whether *Arabidopsis* responds to GPA infestation by increasing content of trehalose. GPA infestation resulted in a transient increase in trehalose in GPA-infested WT plant (Figure 3.1a). This increase in trehalose in the WT plant was limited to the aphid-infested leaves and was not observed in uninfested leaves of plants in which other leaves were exposed to aphids (Figure 3.1b). However, the transient increase in trehalose that was localized to the GPA-infested leaves of the WT plant was not observed in the *tps11-1* mutant (Figure 3.1a and Figure 3.1b).

In addition, basal trehalose content was elevated in the 35S:*TPS11* plant compared to the WT plant (Figure 3.2). These results confirm that *TPS11* is involved in trehalose metabolism leading to the accumulation of trehalose in GPA-infested leaves. These results also show a strong correlation between the ability to accumulate trehalose and the level of GPA resistance.

Trehalose Hyper-Accumulating Plants Display Enhanced Resistance Against GPA

To further test the relationship of trehalose with plant defense against GPA, the impact of trehalose on plant resistance was further tested in no-choice assays performed with the *tre1* mutant, which accumulates ten times more trehalose than the WT (Figure 3.3a) due to deficiency in the trehalose degrading enzyme, trehalase. GPA numbers were significantly lower on the *tre1* mutant than the WT (Figure 3.3b). Similarly, GPA numbers were significantly lower on a transgenic plant that accumulates elevated trehalose levels due to expression of a TPP encoded by the bacterial *otsB* gene (Figure 3.3a,b) (Schluepmann *et al.,* 2003).

Taken together, the above results confirm that constitutive hyper-accumulation of trehalose enhances resistance against GPA in *Arabidopsis*. When taken together with the need of *TPS11* for trehalose accumulation in GPA-infested plants and for basal resistance against GPA, the above results strongly implicate a role for *TPS11*-determined trehalose accumulation in *Arabidopsis* defense against GPA.

Exogenous Application of Trehalose is Detrimental to GPA Fecundity

The transient increase in trehalose levels in response to GPA feeding in *Arabidopsis* leaves suggests an important role for trehalose in providing defense against GPA. To test a direct role of trehalose against GPA, *tps11-1* and wild type *Arabidopsis* plants were injected with 10 µM trehalose and subjected to a no-choice test with GPA. Trehalose injected *tps11-1* mutant plants were found to be more resistant to GPA as compared to the water injected control plants (Figure 3.3a). However, in case of the wild type plants the exogenous application of trehalose did not accord any heightened resistance against GPA as compared to the control plants (Figure 3.4). These results implicate trehalose deficiency in *tps11* as the cause of the heightened performance of GPA on the *tps11* mutant plants. Further proof that trehalose in the diet may be detrimental to GPA was provided by comparing insect performance on artificial diets supplemented with trehalose. Addition of 0.5 µM trehalose to the artificial diet significantly reduced the number of GPA compared to the control diet (Figure 3.5). Further increase in trehalose concentration (10 µM) resulted in a more severe impact on aphid fecundity (Figure 3.5). By contrast to trehalose, a similar adverse effect on GPA fecundity was not observed with glucose (Figure 3.5) suggesting that the antibiotic effect of trehalose against is not a general effect of sugar toxicity or osmolarity. The impact of exogenous trehalose application on both, the *in planta* and artificial

diet experiments, suggests that dietary trehalose has a direct negative impact on GPA, in addition to its impact on plant defenses.

Trehalose Accumulation Modulates *PAD4* Expression in GPA-Infested Plants

PAD4, which, like *TPS11*, is required for antixenosis and antibiosis against GPA, is a key regulator of gene expression in aphid-infested *Arabidopsis* (Pegadaraju *et al.,* 2005, 2007; Louis *et al.,* 2010a). As shown in Figure 2.6, the aphid infestation-induced expression of TPS11 was unaffected in the *pad4* mutant, indicating that *PAD4* does not regulate *TPS11* expression. However, studies with the *tps11* mutant had indicated that *TPS11* modulates PAD4 expression (Fig. 2.7). To determine if trehalose influences *PAD4* expression, *PAD4* expression was monitored in the trehalose hyperaccumulationg *otsB* plant and in plants treated with trehalose. As shown in Figure 3.6, the basal expression of *PAD4* was elevated in the *otsB* plant, compared to the WT plant. In addition, *PAD4* expression was also stimulated in *Arabidopsis* leaves treated with trehalose (Figure 3.6b), suggesting that PAD4 expression is under the control of trehalose metabolism. *PAD4* expression was also constitutively higher in the *TPS11* over-expressing plants (Figure 3.6c). These results indicate that a second role of *TPS11* or the transient trehalose generated via the same in plant defense against GPA is to promote the expression of *PAD4*.

Discussion

Trehalose increases in GPA-infested leaves (Figure 3.1a) paralleled the transient increase in *TPS11* expression (Figure 2.1) suggesting that this increase in trehalose content is a direct result of increased *TPS11* expression in GPA-infested leaves. Indeed, in comparison to WT plants, constitutively elevated expression of *TPS11* under the control of the *35S* promoter

resulted in a higher basal content of trehalose. However, the available evidence does not rule out the possibility that *TPS11* indirectly affects trehalose content in GPA-infested leaves by influencing the expression/activity of other trehalose biosynthetic genes. The basal trehalose levels were not significantly different between *tps11-1* and wild type plants, suggesting that *TPS11* does not contribute to basal trehalose content in *Arabidopsis*.

The ability of *TPS11* to control trehalose content and curtail insect infestation, and the ability of trehalose to complement the *tps11* defect, and of plants hyper-accumulating trehalose to efficiently curtail GPA infestation suggest an important role for trehalose in contributing to *Arabidopsis* defenses against GPA. Supplementation of artificial aphid diet with 0.5 and 10 µM trehalose further suggests that trehalose has a direct detrimental effect on GPA. At both concentrations, it was observed that GPA fecundity was negatively impacted by trehalose. In addition, injection of 10 µM trehalose into *tps11-1* leaves restored wild type level of resistance against GPA. However, trehalose injection in wild type plants did not provide any added resistance against GPA. Neither did the elevated trehalose levels observed in *35S*:*TPS11* plant result in hyper-resistance against GPA, thus suggesting that the small increase in trehalose observed in GPA infested wild type leaves is sufficient to induce resistance against aphid infestation. However, when *Arabidopsis* plants accumulate trehalose to concentrations that are substantially higher than that found in the GPA-infested plants and in the *35S:TPS11* plants, as in the *tre1* mutant and the *otsB* plants, the plants become hyper-resistant to GPA. These results suggest that a threshold amount of trehalose is needed for basal resistance against GPA, beyond which plants become hyper-resistance. Part of this hyper-resistance could be due to the direct toxic effect of dietary trehalose on GPA (Glinski and Weckwerth, 2005).

The relatively low levels of trehalose and the transient nature of the trehalose increase in GPA-infested plants, argue against a requirement for trehalose as an energy source or as an osmoprotectant in *Arabidopsis* defense against GPA. Instead, it can be speculated that *TPS11* regulates host defense against GPA by providing a threshold level of 'signaling' trehalose. The ability of *TPS11* and trehalose to influence *PAD4* expression (Figure 3,4a, b, c) in the GPA-infested leaves supports this hypothesis. However, as a small and delayed induction of *PAD4* expression is observed in *tps11-1* mutant plants, therefore the role of *TPS11*-independent factor(s) also contributing to the overall induction of *PAD4* expression in GPA-infested plants cannot be ruled out. The importance of trehalose in controlling the severity of aphid infestation in *Arabidopsis* was counterintuitive given that aphid hemolymph contains millimolar amounts of trehalose, which serves as an energy source (Moriwaki *et al.,* 2003). It is plausible that, unlike trehalose in the hemolymph, a dietary source of trehalose is detrimental to the insect. Indeed, inclusion of trehalose (0.5 or 10μM) in a synthetic diet containing sucrose as the major carbon source limited the size of the insect population, compared to a diet lacking trehalose (Figure 3.5). In contrast to trehalose, glucose added to the synthetic diet did not adversely affect the insect population. A previous study has shown that, although trehalose provided as the sole sugar was utilized by GPA, it adversely affected the survival of nymphs (Mittler *et al.,* 1970). Trehalases are present in the aphid mid-gut (Cristofoletti *et al.,* 2003), suggesting that aphids do encounter plant-derived trehalose. However, given the transient nature of the trehalose increase in the insect-infested leaves, and the uncertainty over whether sufficient trehalose is consumed by the insect from the plant, the relevance of the toxic effect of dietary trehalose in insect populations feeding on plants is not clear. Instead, as discussed above, trehalose regulating plant defense is

the major mechanism by which trehalose controls the severity of GPA infestation on *Arabidopsis*.

Materials and Methods

Plant and insect materials

Green peach aphid (GPA; *Myzus persicae* Sülzer) (Kansas State University, Museum of Entomological and Prairie Arthropod Research, voucher specimen #194) was reared as previously described (Pegadaraju *et al.,* 2005) on a 50:50 mixture of commercially available radish (*Raphanus sativus*) and mustard (*Brassica juncea*) plants. Approximately 4-week-old *Arabidopsis* plants cultivated at 22ºC under a 14 h light (100 μE m^{-2} sec^{-1})/10 h dark regime were used for all experiments. The *pad4-1* (CS3806), *tre1* (SALK_147073C) mutant plants and the *otsB* expressing transgenic line (Schluepmann *et al.,* 2003) are all in the accession Columbia.

No-choice and choice tests

In no-choice assays (Pegadaraju *et al.,* 2005), a total of 20 adult apterous (wingless) aphids were released on each plant, and the total numbers of insects counted 2 days later. For caging experiments, five insects were released on each leaf at the center of a cage constructed using double-sided foam tape. Leaf tissue from the caged region and the upper insect-free leaves were analyzed for trehalose, sucrose and starch content.

Carbohydrate estimation

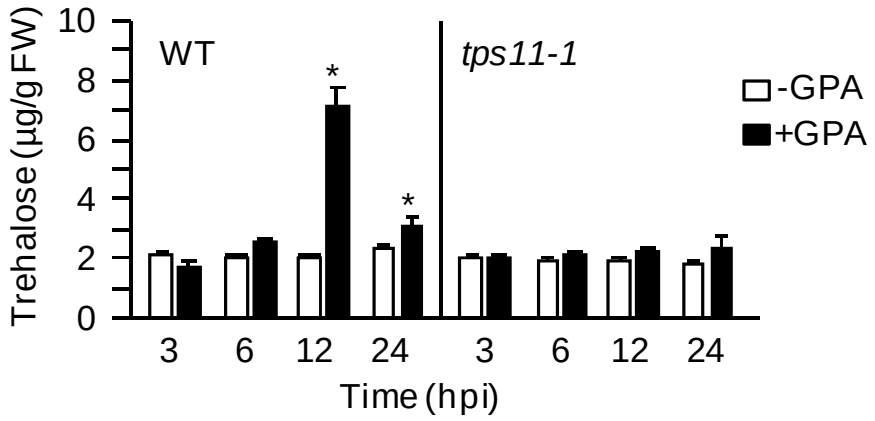
Leaf samples from infested and uninfested plants were collected at 3, 6, 12 and 24 hpi. Sugar extraction and analysis were performed on leaves after removal of aphids, as previously

described (Srivastava *et al.*, 2008). Sucrose was resolved at 30ºC on a CarboPac PA20 column (Dionex, http://www.dionex.com) using a flow rate of 0.5 ml min^{-1} and 50 mM NaOH eluent with pulsed amperometric detection. Trehalose levels were determined after separation of the samples on a CarboPac MA1 column (Dionex) using 480 mM NaOH eluent.

Statistical analysis

For the no-choice assays, artificial diet assays and carbohydrate estimations, the means were separated using the paired *t*-test ($P < 0.05$). MS-Excel was used to perform the Student's *t*-test with two tailed distribution.

(a)



(b)

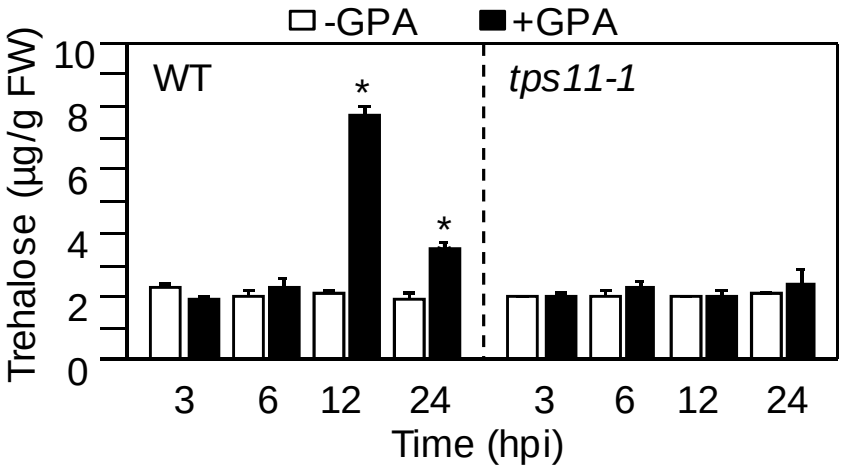


Figure 3.1. *TPS11* is essential for the transient increase in trehalose in GPA-infested leaves (a) Trehalose content in uninfested and GPA-infested WT and *tps11-1* plants. Values are the means of three replicates. Error bars represent SE. Asterisks indicate significant differences ($P < 0.05$) from the uninfested sample for that time point. (b) Trehalose content in GPA-infested and distal uninfested leaves of *Arabidopsis* plants that were locally challenged with aphids. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the uninfested sample for that time point.

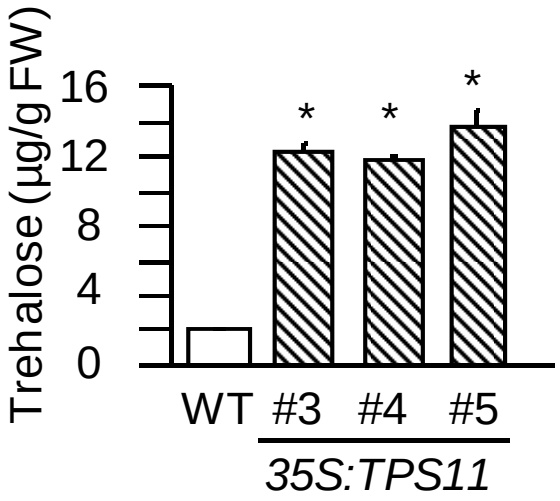
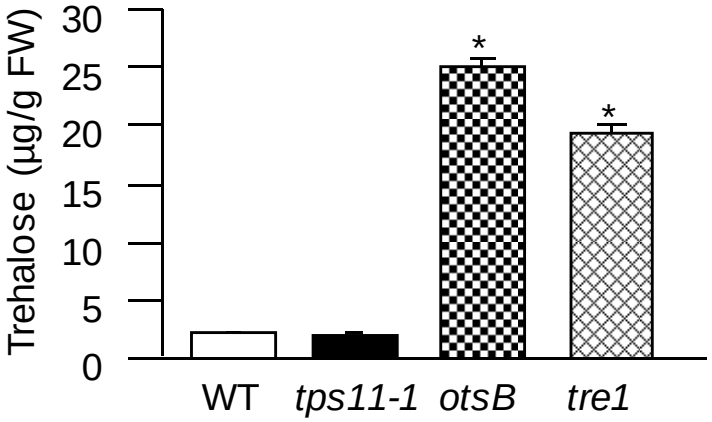


Figure 3.2. Constitutive over-expression of *TPS11* results in high basal levels of trehalose
Basal trehalose content in WT and three independently derived 35S:TPS11 lines in the tps11-1 genetic background. Values are the means of three replicates. Error bars represent SE. Asterisks indicate significant differences ($P<0.05$; *t*-test) from the wild type.

(a)



(b)

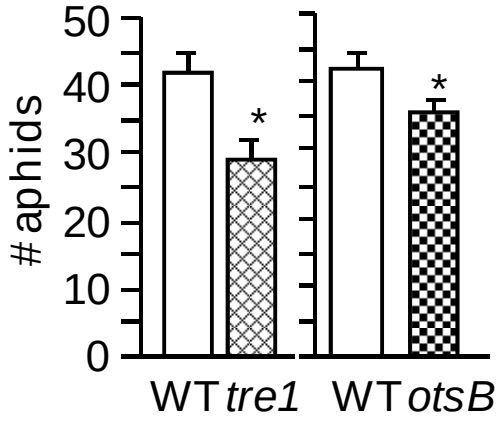


Figure 3.3. Trehalose hyperaccumulating *otsB* and *tre1* plants display enhanced resistance against GPA

(a) Basal trehalose content in leaves of WT, *tps11-1* and *tre1* mutants, and *otsB*-expressing transgenic plants. Values are the means of three replicates. Error bars represent SE. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the WT. (b) No-choice test. Total insect numbers on WT, *tre1* and *otsB* plants 48 h after release of 20 adult apterous aphids on each plant. Values are mean aphid counts on ten plants of each genotype. Error bars represent SE. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the WT.

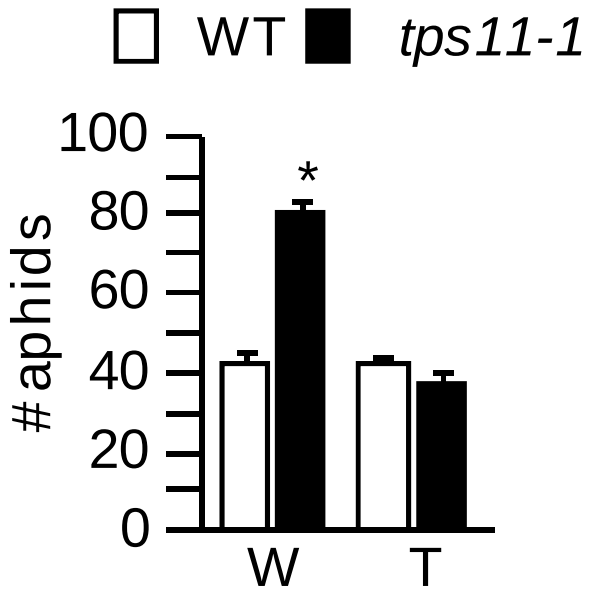


Figure 3.4. Trehalose application complements *tps11* defect

No-choice test. GPA numbers at 2 dpi on WT and *tps11-1* plants that were pre-treated with water (W) or 10 μM trehalose (T). Values are the means of 10 replicates. Error bars represent SE. The asterisk indicates a significant difference ($P < 0.05$; *t*-test) from the WT.

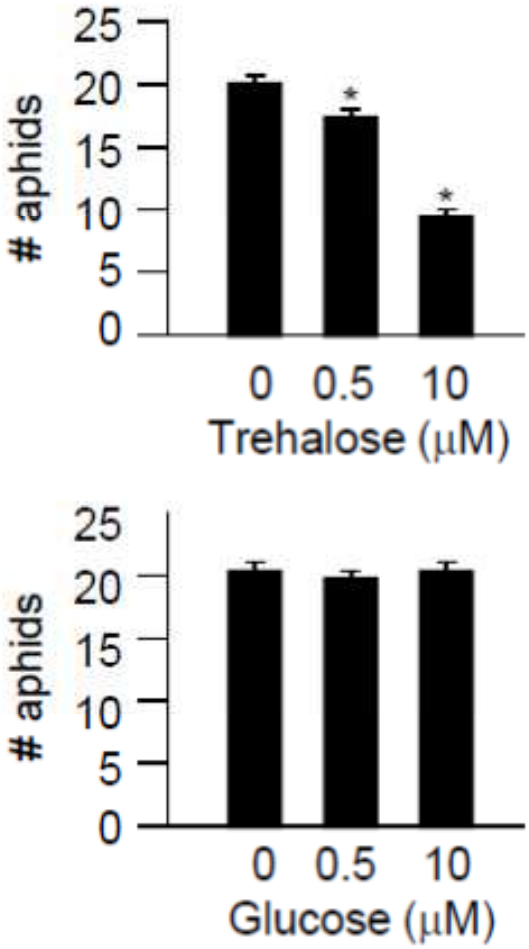
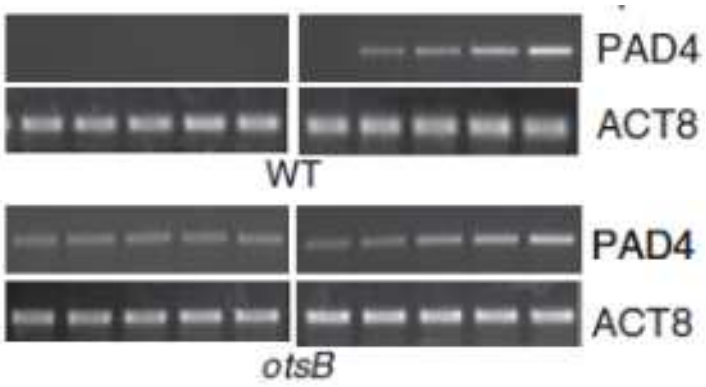


Figure 3.5. Ingested trehalose has a detrimental effect on GPA fecundity

Impact on aphid fecundity of trehalose and glucose added to an artificial diet. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the control diet.

(a)



(b)

(c)

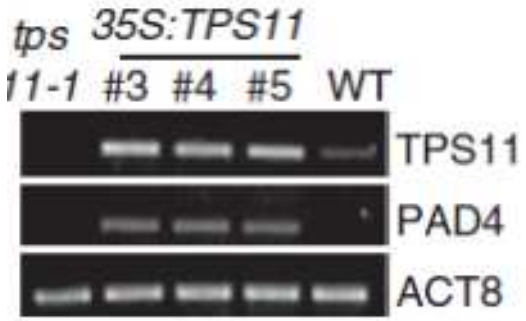


Figure 3.6. Trehalose modulates *PAD4* expression

(a) *PAD4* expression in leaves of uninfested and GPA-infested WT and *otsB* plants. (b) *PAD4* expression in leaves of WT *Arabidopsis*, 12 h after treatment with 10μM trehalose (T) or water (W). (c) Basal expression of *TPS11* and *PAD4* in WT, *tps11-1* and 35S:*TPS11* transgenic plants

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CHAPTER 4

ARABIDOPSIS CLASS II TREHALOSE METABOLISM GENE *TPS11* MODULATES SUCROSE AND STARCH METABOLISM IN RESPONSE TO GREEN PEACH APHID INFESTATION[3]

Abstract

Plant productivity is negatively affected by aphid feeding as the insect is able to feed directly from the phloem sap. The loss of carbon resources, mainly in the form of sucrose leads to changes in the source-sink nature of the infested tissue. In this chapter the role of *Arabidopsis thaliana TPS11* gene in modulating both sucrose and starch levels in response to GPA feeding is defined. GPA infested wild type leaves were found to contain increased levels of both sucrose and starch whereas GPA infested *tps11-1* mutant plants had high levels of sucrose and lower levels of starch as compared to the wild type leaves. Transgenic or mutant plants which accumulate higher than wild type levels of basal trehalose were found to accumulate higher levels of starch. The direct role of starch accumulation in defense against GPA was evident from the larger size of GPA population on the starch-deficient *pgm1* mutant and the smaller size of GPA population on the starch hyper-accumulating *ssIII* mutant than the wild type plant.

Introduction

Sucrose is the main form of carbohydrate that is utilized by plants for long distance transport from the source to sink tissues. In most plants sucrose is also the main component of

[3]Portions of the results (Figs. 4.1-4.5a and 4.8) presented in this chapter have been published in The Plant Journal, Singh *et al.,* 2011. 67 (1), 94-104. Used with permission from John Wiley and Sons

the phloem sap that is consumed by phloem feeding insects like aphids (Douglas, 2003). The crucial role of sucrose in aphid diet is clear from the observation that it acts as the main feeding stimulant and is responsible for the high osmotic pressure encountered by the aphids while feeding the phloem sap (Douglas, 2006). Potato plants expressing the antisense sucrose transporter *StSUT1* displayed a lower concentration of sucrose as compared to the control plants (Pescod *et al.,* 2007). These plants supported lower growth of *Macrosiphum euphorbiae* (Potato aphid) as compared to non transgenic potato plants (Pescod *et al.,* 2007). The requirement for sucrose by aphids has also been demonstrated by its requirement in the artificial diet (Mittler and Meikle, 1991). However, most plant species utilize sucrose for transport of photosynthates and as a ready energy source. The daily cycling of sucrose is under diurnal control (Geigenberger, 2011) and the C is transiently stored in the form of starch during the day and remobilized to sucrose by starch degradation, during the night (Zhang *et al.,* 2005). This dynamic nature of starch allows it to be utilized as a dynamic sink for the storage of sucrose that is produced via photosynthesis during the day.

Starch is the linear polymer of glucose molecules linked via α-1, 4- glycosidic bonds. Insoluble in nature, it is composed of the linear amylose or the branched amylopectin (Geigenberger *et al.,* 2004). In the first committed step of starch synthesis, glucose-1-phosphate (G1P) and ATP are converted to ADP-Glucose, by the action of ADP-Glucose Pyrophosphorylase (AGPase), which then acts as the glucosyl donor to starch synthase (Kolbe *et al.,* 2005). The activity of plastidial phosphoglucomutase (PGM) converts the intermediates of the Calvin cycle (Light-independent cycle) into G1P which is fed into starch synthesis. Thus, a direct mutation in the *pgm1* gene of *Arabidopsis* leading to the loss of function of PGM results in plants which fail to accumulate starch in their leaves (Yu *et al.,* 2000).

In most plant species the AGPase is plastid localized whereas in the case of cereal endosperm the enzyme is localized in the cytosol (James *et al.,* 2003). AGPase, in *Arabidopsis*, is a heterotetramer composed of two large and two small subunits (Okita *et al.,* 1990). The expression of AGPase has been demonstrated to be influenced positively by carbohydrate levels (Tiessen *et al.,* 2003), whereas increase in nitrate levels has a negative impact on AGPase expression (Scheible *et al.,* 1997). It has also been shown that 3-phosphoglyceric acid (3PGA) levels also influence AGPase activity by inducing the enzyme whereas inorganic phosphate inhibits the enzyme activity (Geigenberger *et al.,* 1998). Regulation of AGPase by 3PGA indicates towards its regulation by light, which indeed has been shown to be the case (Hendriks *et al.,* 2003; Stitt *et al.,* 2010). It was demonstrated in both pea and *Arabidopsis* that illumination of leaves and isolated chloroplast led to the redox activation of AGPase (Hendriks *et al.,* 2003). Similar observations were made by Stitt and co-workers (2010) in case of *Arabidopsis* plants where AGPase was found to be redox activated by light and completely inactivated in the dark (Stitt *et al.,* 2010).

A strong relation between changes in sugar levels in the leaves and the rate of starch biosynthesis has been demonstrated (Sulpice *et al.,* 2009; Stitt *et al.,* 2010). Any perturbations in the rate of photosynthesis, due to light intensity or stress conditions, leading to changes in the carbon balance are reflected in the modulation of starch synthesis (Gibon *et al.,* 2009). Photosynthates accumulated during the day, in the form of starch, are mobilized to supply carbon to the plant tissues during the night time (Sulpice *et al.,* 2009). Therefore, starch has been envisioned as a central regulator of plant growth (Sulpice *et al.,* 2009). Another mode of regulation of AGPase that has recently emerged is its redox activation by T6P, the phosphorylated intermediate of trehalose metabolism (Kolbe *et al.,* 2005). It has been suggested

that T6P may act as the link between sucrose levels in the cytoplasm and AGPase in the chloroplast. Addition of T6P to intact *Arabidopsis* chloroplasts led to the redox activation of AGPase (Kolbe *et al.,* 2005). Reductive activation of AGPase has also been demonstrated to be under the control of SnRK1 in *Arabidopsis* (Jossier *et al.,* 2009) and potato tubers (Tiessen *et al.,* 2003). Although the mechanism has not been completely deciphered at the molecular level, it was shown recently that SnRK1 activity in young *Arabidopsis* leaves may be inhibited by T6P (Zhang *et al.,* 2009). This provides a light independent platform for the regulation of AGPase by T6P.

The ADP-Glucose generated by the action of AGPase acts as the donor of glucosyl moieties to the growing starch chain which is synthesized by starch synthase (SS). Based on their amino acid sequence, five classes of starch synthases have been identified in plants (Zhang *et al.,* 2005). The granule-bound SS (GBSS) is associated with the chloroplast and is mainly responsible for amylose synthesis. SSI, SSII, SSIII and SSIV-V form the other members of the different classes of SS (Zhang *et al.,* 2005). These different classes have distinct functions with respect to amylopectin biosynthesis and in *Arabidopsis* SSIII and SSIV may be required for the initiation of starch granules (Szydlowski *et al.,* 2009). SSIII has also been demonstrated to be a negative regulator of starch synthesis, since *Arabidopsis* plants with mutation in the *ssIII* gene accumulate high levels of starch even at the end of the dark period as compared to the wild type control plants (Zhang *et al.,* 2005).

In the present chapter the role of *TPS11* in modulating the levels of sucrose and starch in response to GPA feeding is demonstrated. *Arabidopsis* plants were found to accumulate high levels of both sucrose and starch in GPA infested leaves. However, the amount of sucrose accumulated in the GPA infested *tps11-1* mutant plants was significantly higher than the wild

type plants. At the same time starch levels were found to be higher in the wild type GPA infested leaves as compared to the *tps11* mutant plants. Expression of genes responsible for starch biosynthesis was also found to be altered by GPA feeding. It was observed that GPA population size was smaller on mutant and transgenic plants accumulating high levels of starch than in wild type plants. The results presented in this chapter show that GPA feeding on *Arabidopsis* alters the sucrose to starch transition in the infested leaves and that *TPS11* generated T6P/trehalose is responsible for controlling the sucrose levels available to the aphids.

Results

TPS11 Modulates Sucrose and Starch Levels in GPA Infested *Arabidopsis* Leaves

Aphid infestation resulted in a substantial increase in sucrose levels in the WT plant (Figure 4.1a). The sucrose content at 24 hpi was 40–50% higher in GPA-infested leaves of the *tps11-1* mutant compared with the WT plant (Figure 4.1a), suggesting that *TPS11* adversely affects sucrose accumulation in the aphid-infested leaves. Since the C in sucrose is transiently stored as starch, the level of starch in GPA infested leaves was also monitored. An increase in starch levels was also observed in the GPA infested leaves of both wild type and *tps11-1* mutant plants. The amount of starch accumulated in the infested wild type leaves was significantly higher as compared to the *tps11-1* plants. Starch increases in the insect infested leaves were also observed in plants kept in dark (Figure 4.1b) which suggests that starch accumulation in response to GPA feeding is independent of the light activation of starch biosynthesis. To determine if the C required for sucrose and starch accumulation was coming from the other leaves, a caging experiment was conducted, in which insects were caged to individual leaves of a plant with a

double-sided tape barrier. Sucrose and starch levels were measured over a 24h period in the caged and the distal leaves. Plants that did not contain insects provided controls for this experiment. As shown in Figure 4.2, no increase in sucrose or starch was observed in the un-infested leaves of WT and *tps11-1* plants that had insects caged on other leaves, suggesting that the C for the high sucrose and starch levels in GPA-infested leaves was predominantly coming from other internal sources. Since the upregulation of *PAD4* in GPA-infested plants is regulated by *TPS11* and trehalose (see Chapters 2 and 3), starch levels were also monitored in GPA-infested leaves of the *pad4-1* mutant to determine if, like *TPS11*, *PAD4* was required for controlling starch accumulation in GPA- infested leaves. Similarly treated wild type plants provided the controls for this experiment. As shown in Figure 4.3, comparable amount of starch accumulated in the GPA-infested wild type and *pad4-1* leaves indicating that *PAD4* is not critical for controlling starch accumulation in GPA-infested tissues.

Trehalose Over-Accumulation Stimulates Starch Accumulation in *Arabidopsis* Leaves

Since *TPS11* is involved in the synthesis of T6P and trehalose, the above results suggest that TPS11-derived T6P and/or trehalose modulate starch accumulation in GPA-infested leaves. Indeed, other studies have reported that trehalose application result in increased starch accumulation in *Arabidopsis* seedlings (Wingler *et al.,* 2000). Similar observation was made in both wild type and *tps11-1* plants injected with 10µM trehalose (~100µl in each leaf). The trehalose injected leaves accumulated significantly higher levels of starch as compared to either sucrose or water injected leaves, thus confirming that trehalose is capable of promoting starch accumulation in *Arabidopsis* (Figure 4.4a).

To further test if increased trehalose levels can promote starch accumulation *in planta*, basal levels of starch were monitored in plants that constitutively accumulate elevated trehalose levels. As described in Chapter 3, the transgenic *35:TPS11* and bacterial TPP *otsB* expressing plants, and the *tre1* mutant, which lacks trehalase activity, all accumulate significantly higher levels of trehalose than the wild type plant. These plants also accumulated nearly double the amount of starch than the wild type and *tps11-1* mutant plant (Figure 4.4b), thus confirming that increase in trehalose content in plants promote *in planta* accumulation of starch.

Starch Accumulation Negatively Affects GPA Fecundity

To determine whether starch accumulation has a role in plant defense, insect numbers on wild type and starch-deficient *pgm1* mutant plants were compared (Caspar *et al.,* 1985). In a no-choice assay, GPA numbers were higher on the *pgm1* mutant than the WT (Figure 4.5a). By contrast, GPA numbers were lower in the starch hyper-accumulating *ssIII* mutant plant (Zhang *et al.,* 2005) than the wild type plant (Figure 4.5b). These results strongly suggest that starch accumulation in *Arabidopsis* contributes to controlling GPA infestation.

GPA Feeding Alters the Expression of *Arabidopsis* Genes Responsible for Starch Metabolism

AGPase is the enzyme responsible for the first committed step of starch biosynthesis and *APL3* encodes for one of its large subunits in *Arabidopsis* (Geigenberger, 2011). Increased expression of *APL3* was observed in GPA infested wild type leaves (Figure 4.6) and the expression mirrored the transient expression of *TPS11* in response to GPA feeding (Figure 2.1). However, the induction of *APL3* expression in the GPA infested *tps11-1* plants was weaker as compared to the wild type plants (Figure 4.6).

To further test the role of starch metabolism in *Arabidopsis* defense against GPA, no-choice tests were conducted with *Arabidopsis* mutants that have T-DNA insertions in individual genes encoding amylose synthesizing starch synthase (*GBSSI*) and those involved in amylopectin synthesis (*SSI*, *SSII* and *SSIV*) starch synthase and those encoding amylases (*AMY1*, *AMY2* and *AMY3*). The starch-overproducing *ssIII* mutant plant, which exhibits enhanced resistance to GPA, was used as a control along with the wild type plant. As shown in Figure 4.7, GPA numbers were significantly higher on the *gbss1* mutant, which is deficient in the synthesis of amylase. By contrast, insect numbers on the *ssI*, *ssII*, *ssIV*, *amy1*, *amy2* and *amy3* mutants was comparable to that on the wild type plant.

These results suggest that the plastid localized GBSS1 is likely the major enzyme involved in starch synthesis in GPA-infested plants. However, this needs to be verified by estimating starch at both the quantitative and qualitative levels in the GPA-infested leaves of the different starch synthesis mutant plants. A distinction in the levels of amylase and amylopectin levels would clarify the role of GBSS1 in starch synthesis during aphid infestation. EPG analysis of GPA feeding on the starch biosynthesis mutants would also provide insight into the quality of starch contributing to defense against GPA. The absence of any impact on GPA numbers on the α-amylase *amy1*, *amy2* and *amy3* mutants could indicate that although starch accumulation is important, starch hydrolysis may not be critical. Alternatively, these AMY proteins may not be responsible for breakdown of the transient starch. However, it is more likely that these genes encode redundant functions. Indeed, the individual *amy1*, *amy2* and *amy3* mutants accumulate wild type levels of starch (Yu *et al.,* 2005). Future studies with double and triple *amy* mutants are needed to fully understand the contribution of the *AMY* genes, if any, to starch turnover and defense against GPA.

Discussion

In *Arabidopsis*, GPA feeding causes an increase in the mRNA levels of a monosaccharide symporter, which is indicative of an increase in the sink strength of the insect infested tissue (Moran and Thompson, 2001). Similarly, ***M. nicotianae*** infestation in tobacco resulted in changes in expression of genes related to source–sink balance, and the insects preferred to settle on young leaves, which act as strong sinks (Voelckel ***et al.,*** 2004). Furthermore, bioassays performed in the presence of ^{14}C showed that the infested galls formed by the galling aphid *Pemphigus betae* on narrowleaf cottonwood (*Populus angustifolia*) exhibit increased influx of sugars in to the galls (Larson and Whitham, 1991), suggesting that the gall is a physiological sink. Our results indicate that GPA feeding leads to an increase in the sucrose content in GPA-infested leaves (Figure 4.1a and 4.1b), suggesting that GPA infestation is accompanied by an increase in the sink strength of the infested leaves. Increased sink strength probably benefits the insect by maintaining a continued supply of nutrients to the infested leaf. The increase in sucrose content was higher in the *tps11-1* mutant than the WT plants (Figure 4.1a and b and Figure 4.2), suggesting that *TPS11* negatively affects sucrose increases in GPA-infested leaves. One mechanism by which *TPS11* could negatively affect sucrose accumulation in aphid-infested leaves is by promoting the flux of carbon into starch. This is evident from the lower starch content in the GPA-infested *tps11-1* leaves compared to the WT leaves (Figure 4.1a and b and Figure 4.2). Another possibility, not tested in this study, is that aphid feeding results in the increased retention of sucrose in the infested tissue and that the heightened levels of sucrose are not due to an influx of sucrose.

Trehalose application also promotes starch accumulation in *Arabidopsis* leaves (Figure 4.4a). Other studies have also reported increases in starch content and in expression of genes

involved in starch synthesis in trehalose-treated plants (Wingler *et al.,* 2000; Kolbe *et al.,* 2005). Starch accumulation in GPA-infested leaves could result from an increase in the synthesis of starch, a reduction in starch turnover, or a combination of both. The general consensus is that T6P promotes starch synthesis by redox activation of AGPase (Paul *et al.,* 2008). In addition, starch turnover is adversely affected by trehalose (Ramon *et al.,* 2007). Starch increases are not limited to the *Arabidopsis*–GPA interaction. Such increases have also been observed in *Sorghum bicolor* infested with the yellow sugarcane aphid *Sipha falva* (Gonzales *et al.,* 2002). The larger size of the GPA population on starch-deficient *pgm1* mutant and a lower population on the *ssIII* mutant, which hyperaccumulates starch compared to WT plants (Figure 4.3 a, b) suggests that starch accumulation does contribute to *Arabidopsis* defense against GPA. Starch metabolism plays a very important role in controlling carbon utilization by plants, and starch accumulation acts as a dynamic sink for the carbon that is fixed by photosynthesis. Due to tight regulation of its metabolism, starch can control both the carbon usage and growth patterns of a plant (Sulpice *et al.,* 2009).

In aphid-infested leaves, starch accumulation at the expense of sucrose could result in a 'secondary sink', thus countering the insect's efforts to manipulate the host physiology to increase nutrient availability in the infested leaves. In addition, as starch has an inhibitory effect on GPA feeding (Campbell *et al.,* 1986), starch accumulation could make the leaf less desirable to GPA, thereby contributing to overall resistance. The direct role of starch in providing defense against GPA is also made clear from the observation that GPA population size was larger on the *gbssI* mutant, which lacks a plastidic amylase synthesizing activity, than on the wild type plant (Figure 4.7). Since trehalose has a negative effect on starch turnover (Ramon *et al.,* 2007) it was expected that amylase mutants of *Arabidopsis* would support a lower aphid growth. However, no

significant difference was observed in GPA populations supported by *amy1*, *amy2* and *amy3* mutants when compared to wild type plants. This observation is validated by the previous observations that these α-amylase encoding genes may not be involved in the degradation of the transitory starch accumulated during the day (Yu *et al.*, 2005). Alternatively, the *AMY1*, *AMY2* and *AMY3* genes may have redundant activities. Further studies of double and triple mutants will be required to further test the contribution of these three *AMY* genes to starch accumulation in GPA-infested plants and overall resistance of *Arabidopsis* to GPA.

Based on the results presented in this dissertation a model for the role of *TPS11* in defense against GPA and its role with *PAD4* can be envisioned (Figure 4.8). GPA infestation induces *PAD4* expression, and transiently induces *TPS11* expression and trehalose accumulation in GPA-infested leaves. In addition, GPA infestation also results in sucrose and starch accumulation in GPA infested leaves. In this model, both *PAD4* and *TPS11* are required to curtail GPA infestation. *TPS11*-dependent trehalose metabolism independently promotes *PAD4* expression and starch accumulation in the GPA-infested leaves. A *TPS11*-independent mechanism is also shown as promoting *PAD4* expression, as some induction of *PAD4* expression was retained in the GPA-infested *tps11-1* mutant. Several lines of evidence indicate that *TPS11* and trehalose regulate *PAD4* expression. Loss of *TPS11* function in the *tps11-1* mutant abolishes the GPA infestation-induced accumulation of trehalose and attenuates the induction of *PAD4* expression. In contrast, constitutive over-expression of *TPS11* from the 35S promoter resulted in higher basal expression of *PAD4*. *PAD4* expression was also induced by trehalose treatment, and was higher in the *otsB* transgenic plant, which has high trehalose content. This model also suggests a role for the *TPS11*-promoted starch accumulation in plant defense. The larger size of the insect population on the starch-deficient *pgm1* and *gbss1* mutants and a smaller population

size on the starch hyper-accumulating *ssIII* mutant than in the wild type plant support this hypothesis. Other effects of TPS11 on *Arabidopsis* defense against GPA cannot be ruled out at present.

Materials and Methods

Plant and insect materials

Green peach aphid (GPA; *Myzus persicae* Sülzer) (Kansas State University, Museum of Entomological and Prairie Arthropod Research, voucher specimen #194) was reared as previously described (Pegadaraju *et al.,* 2005) on a 50:50 mixture of commercially available radish (*Raphanus sativus*) and mustard (*Brassica juncea*) plants. Approximately 4-week-old *Arabidopsis* plants cultivated at 22_C under a 14 h light (100 μE m^{-2} sec^{-1})/10 h dark regime were used for all experiments. The following T-DNA insertion mutants were sourced from the ABRC stock center: *gbssI* (Salk_047731), *ssI* (Salk_259021), *ssII* (Salk_035782), *ssIII* (Salk_151477), *ssIV* (Salk_096130), *amy1* (Salk_014462), *amy2* (Salk_008656), *amy3* (Salk_005044). All the T-DNA insertional mutants are in the accession Columbia.

No-choice and choice tests

In no-choice assays (Pegadaraju *et al.,* 2005), a total of 20 adult apterous (wingless) aphids were released on each plant, and the total numbers of insects counted 2 days later. For caging experiments, five insects were released on each leaf at the center of a cage constructed using double-sided foam tape. Leaf tissue from the caged region and the upper insect-free leaves were analyzed for trehalose, sucrose and starch content.

RT-PCR analysis

For the expression analysis of starch metabolism genes, primers listed in table 4.1 were employed. PCR using gene-specific primers was performed under the following conditions: 95ºC for 5 min, followed by 25 cycles of 95 ºC for 30 sec, 54 ºC for 30 sec and 72 ºC for 30 sec, with a final extension of 72 ºC for 7 min.

Carbohydrate estimation

Leaf samples from infested and uninfested plants were collected at 3, 6, 12 and 24 hpi. Sugar extraction and analysis were performed on leaves after removal of aphids, as previously described (Srivastava *et al.,* 2008). Sucrose was resolved at 30ºC on a CarboPac PA20 column (Dionex, http://www.dionex.com) using a flow rate of 0.5 ml min^{-1} and 50 mM NaOH eluent with pulsed amperometric detection. For starch estimation, leaves from which sugars had been extracted were ground in 80% ethanol and washed once with 80% ethanol. The ground material was centrifuged (12 000 g at room temperature for 10 min) and the pellet was resuspended in 1 ml of water and boiled for 30 min. Starch content was determined using a starch kit (#10 207 748 035, R-Biopharm, http://www.r-biopharm.com).

Chemical treatment of plants

Trehalose and sucrose solutions (10 µM) in water were infiltrated into the abaxial surface of leaves using a needle-less syringe.

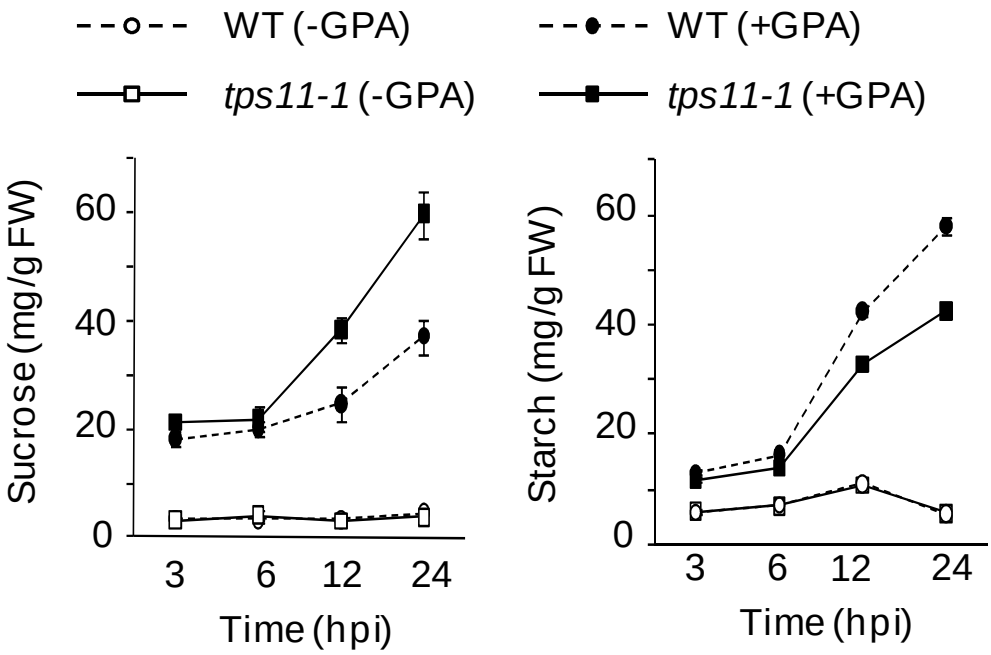
Statistical analysis

For the no-choice assays, choice assays and artificial diet assays, the means were separated using the paired *t* test ($P < 0.05$). MS-Excel was used to perform Student's *t*-test with two tailed distribution.

Table 4.1: Primer sequences used in this study

APL3-F	TTGTTCAGCACCATGTCGAT
APL3-R	TTTCAGATCGATGCCAGTTG

(a)



(b)

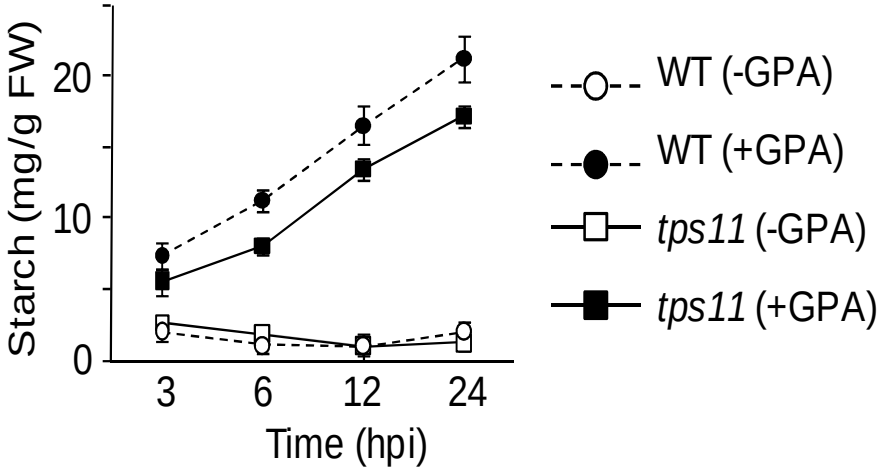


Figure 4.1. *TPS11* modulates sucrose and starch levels in GPA infested *Arabidopsis* leaves (a) Sucrose and starch content in leaves of uninfested and GPA-infested plants. Values are the means of three replicates. Error bars represent SE. (b) Starch content during the dark period in GPA-infested *Arabidopsis*. Values are the mean of 10 leaf samples for each time point. Error bars represent SE

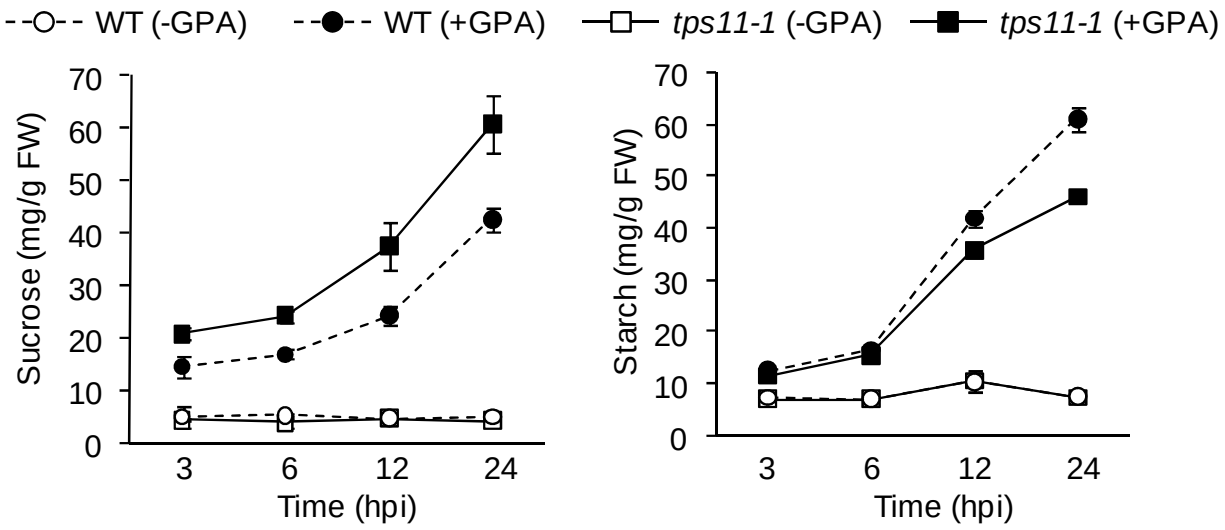


Figure 4.2. Sucrose and starch accumulation in GPA-infested and systemic un-infested leaves

Sucrose and starch contents in GPA-infested and distal uninfested leaves of *Arabidopsis* plants that were locally challenged with aphid. Values are the means of two replicates. Error bars represent SE.

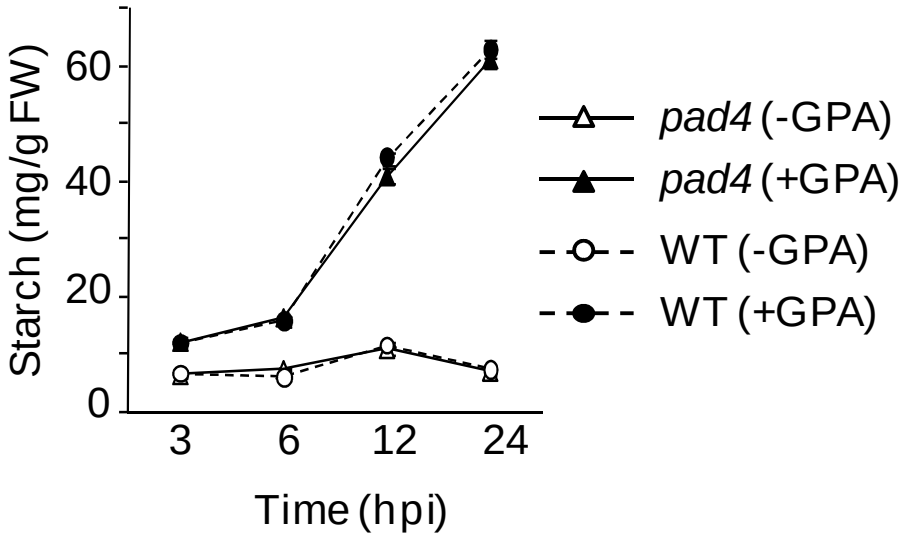
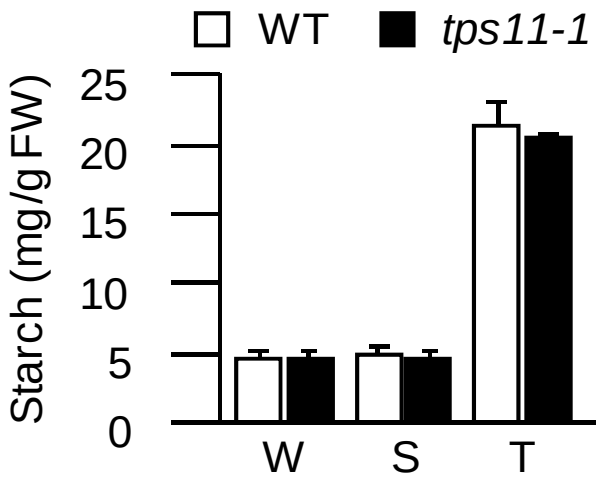


Figure 4.3. PAD4 is not required for starch accumulation in GPA-infested leaves
Starch content in leaves of uninfested and GPA-infested WT and *pad4* plants. Values are the means of three replicates. Error bars represent SE.

(a)



(b)

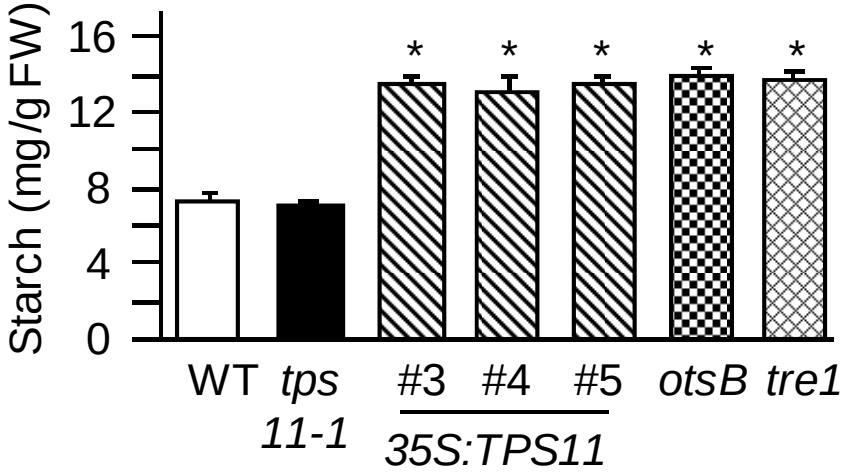
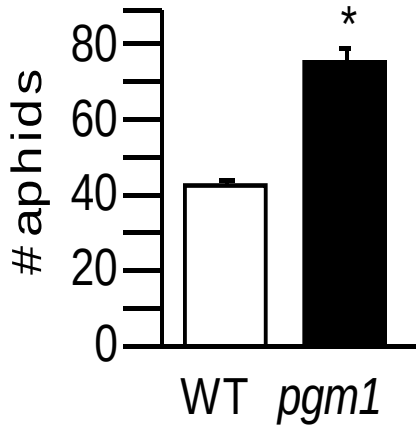


Figure 4.4. Trehalose application and over-accumulation stimulates starch accumulation in *Arabidopsis* leaves

(a) Starch content 12 h after treatment of WT and *tps11-1* leaves with 10 μM trehalose (T) or with 10 μM sucrose (S) and water (W) as negative controls. Values are the means of three replicates. Error bars represent SE. (b) Basal starch content in the WT, *tps11-1*, 35S:*TPS11*, *otsB* and *tre1* plants. Three independently derived transgenic 35S:*TPS11* plants were evaluated. Values are the means of three replicates. Error bars represent SE. Asterisks indicate significant differences ($P < 0.05$; *t*-test) from the WT.

(a)



(b)

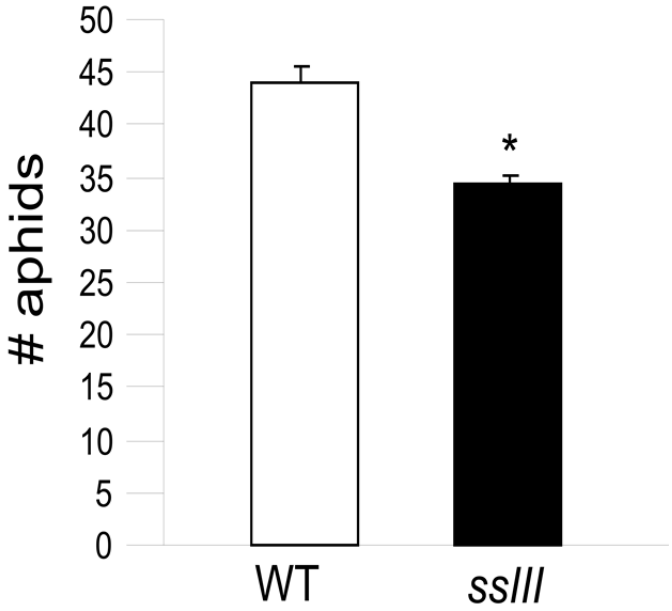


Figure 4.5: Starch accumulation adversely impacts GPA fecundity

(a) No-choice test. Total GPA numbers at 2 dpi on WT and *pgm1* plants. Values are mean aphid counts on ten plants of each genotype. Error bars represent SE. The asterisk indicates a significant difference ($P < 0.05$; *t*-test) from the WT. (b) No-choice test. Total GPA numbers at 2 dpi on WT and *ssIII* plants. Values are mean aphid counts on ten plants of each genotype. Error bars represent SE. The asterisk indicates a significant difference ($P < 0.05$; *t*-test) from the WT.

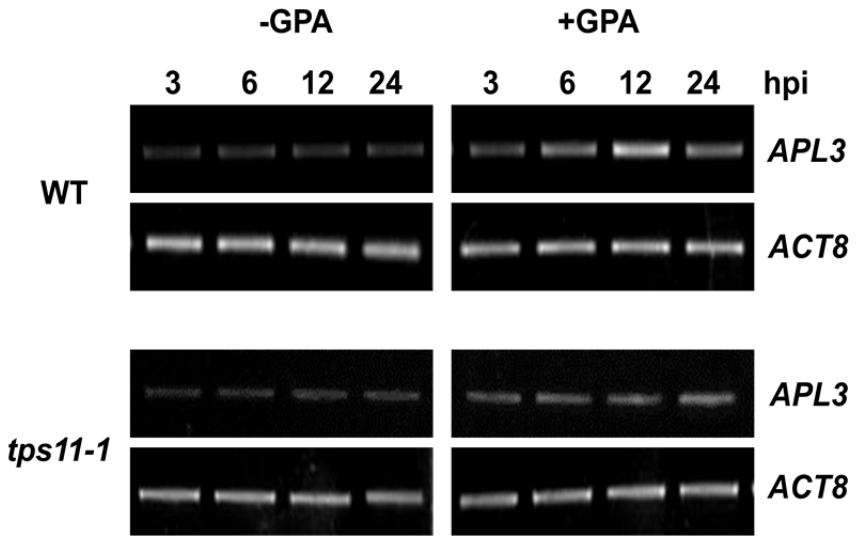


Figure 4.6: GPA feeding alters the expression of *Arabidopsis* genes responsible for starch metabolism RT-PCR expression analysis of *Apl3* in GPA infested (+GPA) and uninfested (-GPA) WT and *tps11-1* plants. *ACT8* was used as the internal control for equal loading of the samples.

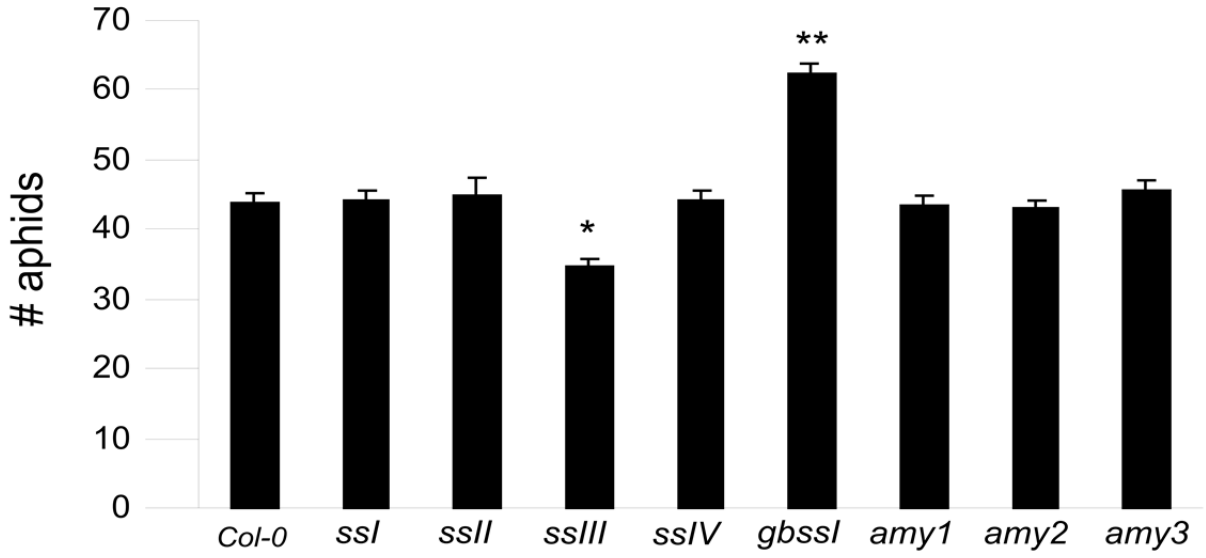


Figure 4.7: Mutations in chloroplastic starch synthase impacts GPA population size on *Arabidopsis*

No-choice test. Total GPA numbers at 2 dpi on wild type and *Arabidopsis* plants mutated for starch synthase and α-amylase function. Values are mean aphid counts on ten plants of each genotype. Error bars represent SE. The asterisk indicates a significant difference ($P < 0.05$; *t*-test) from the WT

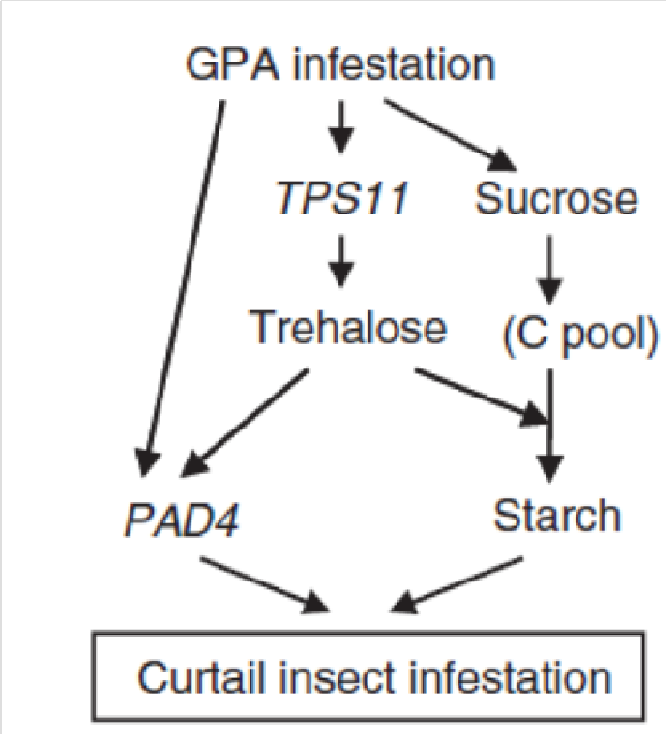


Figure 4.8. Model depicting the impact of *TPS11* on *Arabidopsis* defense against GPA

Arabidopsis class II trehalose metabolism gene, *TPS11* contributes to trehalose metabolism and is responsible for trehalose accumulation in response to GPA infestation. TPS11 can potentially modulate both carbohydrate metabolism to promote starch accumulation at the expense of sucrose in infested leaves. *TPS11* function is also required for the timely induction of *PAD4*, a gene that contributes to the overall defense against GPA in *Arabidopsis*.

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