

**The Effects of *Arabidopsis thaliana* Sulfotransferase 2a (AtST2a)
Over-expression on Tuber Formation**

Faraz Kazmi

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ABSTRACT

The Effects of *Arabidopsis thaliana* Sulfotransferase 2a (AtST2a) Over-expression on Tuber Formation.

Faraz Kazmi

This thesis describes the study of the effects of *Arabidopsis thaliana* *Sulfotransferase 2a* (*AtST2a*) over-expression in potato plants (*Solanum chacoense*). *AtST2a* encodes a sulfotransferase, that functions to reduce the endogenous levels of a hydroxylated product of jasmonic acid, 12-hydroxyjasmonate (12-OHJA) also known as tuberonic acid. Through phenotypic analysis of transgenic potato lines, *AtST2a* over-expression was shown to delay tuberization. In addition, tubers in the transgenic lines were smaller and more numerous than in the wild type control lines. Furthermore, regulation studies showed that 12-OHJA induces tuber-specific genes *in planta*, where, tuber induction previously was only demonstrated *in vitro*. Tuberonic acid sulfate, the product of the AtST2a sulfonation reaction was detected and quantified in potato leaf segments. This indicates the presence of a potato homologue of *AtST2a*. Six potential potato candidates were identified, of which two showed the greatest potential (TC77024 & TC87873), based on time course treatment assays. However, substrate specificity experiments showed that TC77024 and TC87873 are not hydroxy-jasmonate sulfotransferases.

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LIST OF ABBREVIATIONS

Ac	Acetyl
bp	base pairs
BSA	Bovine serum albumin
CTAB	Hexadecyltrimethyl ammonium bromide
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene diamine tetraacetate
EST	Expressed sequence tag
GC	Gas chromatography
HPLC	High performance liquid chromatography
IAA	Isoamyl alcohol
JA	Jasmonic acid
kDa	kilo daltons
LC	Liquid chromatography
Me-JA	Methyl jasmonate
MS	Mass spectrometry
12-OH-JA	12-hydroxyjasmonic acid
11-OH-JA	11-hydroxyjasmonic acid
12-OH-JA sulfate	12- hydroxysulfonyloxyjasmonic acid
OPDA	Oxo-phytodienoic acid
PAGE	Polyacrylamide gel electrophoresis
PAPS	3'-Phosphoadenosine 5'-phosphosulfate
PCR	Polymerase chain reaction
RT	Reverse transcription
SDS	Sodium dodecyl sulfate
TA	Tuberonic acid
TAG	Tuberonic acid glucoside

INTRODUCTION

Sulfate conjugation mediated by sulfotransferases is an important reaction in the transformation of xenobiotics, modulation of steroid hormone biological activity, and neurotransmitters in mammals (Falany, 1997b). In plants, sulfation plays an important role in signalling processes as indicated by the sulfation of brassinosteroids which abolishes their biological activity (Rouleau et al; 1999). An *Arabidopsis thaliana* sulfotransferase, AtST2a, was found to sulfonate 12-hydroxyjasmonic acid (Gidda et al; 2003). Jasmonic acid and its derivatives, referred to as jasmonates, play important roles in plant development, plant response to wounding, pathogen defense, plant flowering and tuberization (Wasternack and Parthier, 1997). A hydroxylated jasmonic acid derivative, 12-hydroxyjasmonic acid, also referred to as tuberonic acid, has been shown to induce tuberization *in vitro* (Yoshihara et al; 1989). This study uses *AtST2a* over-expression in transgenic potato plants, to modulate *in planta* levels of 12-hydroxyjasmonic acid, demonstrating its effect on tuberization. The isolation of 12-hydroxyjasmonic acid sulfate from potato leaf segments indicates the presence of a potato homologue of AtST2a. Thus, several putative potato sulfotransferases were investigated through expression pattern studies under substrate treatment and photoperiod treatment. This allowed us to identify candidates for a putative potato hydroxyjasmonate sulfotransferase. While this study was not successful in finding such a sulfotransferase, the presence of 12-OHJA sulfate shows that such an enzyme does exist in potato.

CHAPTER 1

LITERATURE REVIEW

Introduction

This thesis presents the results of the expression of the *Arabidopsis thaliana* gene *AtST2a* encoding a 12-hydroxyjasmonate sulfotransferase in *Solanum chacoense*, regulation studies of tuber specific genes in response to various treatments and lastly the potential identification of a *Solanum chacoense* 12-hydroxyjasmonate (12-OHJA) sulfotransferase encoding gene. The literature review begins with a description of the state of our knowledge on mammalian and plant sulfotransferases, followed by an overview of jasmonates and the jasmonate biosynthetic pathway. Finally, tuberization as well and the factors governing tuber formation are discussed.

Sulfotransferases

Sulfotransferases (STs) are a family of enzymes, which are responsible for catalyzing the transfer of a sulfonyl group (-SO₃-) from an activated sulfonate donor to a particular substrate (Falany, 1997b). There are many different sulfotransferases in bacteria, plants and animals that catalyze the sulfonation of many different substrates. In mammals, endogenous substances such as hormones, neurotransmitters and various xenobiotics are usually targets of the sulfonation reaction (Falany, 1997b). The active universal donor in these reactions is 3'-phosphoadenosine 5'-phosphosulfate (PAPS).

Compounds that are conjugated with a sulfonyl group are more water soluble than their precursor molecule, this facilitates their transport or excretion (Klaassen and Boles, 1997). Sulfotransferases can be divided into two classes. The first consists of membrane bound proteins which are responsible for the sulfonation of glycoproteins, glycosaminoglycans and tyrosine residues on proteins (review by Kehoe and Bertozzi, 2000). The second group consists of cytosolic soluble STs that act upon minor organic molecules such as flavonoids, steroids, glucosinolates as well as hydroxyjasmonates (Gidda et al; 2003; Varin et al; 1997b; Falany, 1997a). In bacteria and fungi, sulfotransferases are mostly involved in the sulfonation of sugars and polysaccharides (Mougous et al; 2002).

Membrane Bound Mammalian Sulfotransferases

Located primarily in the trans golgi network (TGN), the post-translational modification by sulfonating of protein tyrosyl residues is important in protein-protein interactions in many systems (review by Kehoe and Bertozzi, 2000). This reaction is catalyzed by the tyrosyl protein sulfotransferases (TPSTs). They catalyze the sulfonation of proteins travelling through the lumen of the TGN. Accordingly, the catalytic domains of TPSTs are facing towards the lumen of the TGN. Several proteins have been shown to become sulfonated through this pathway, several of which include plasma membrane and secreted proteins. Their sulfonation has been shown to be physiologically relevant in protein-protein interactions for leukocyte adhesion and chemokine signalling (review by Kehoe and Bertozzi, 2000). Thus by promoting protein-protein interactions related to

secretory proteins, receptor interaction, as well as plasma membrane protein localization, TPSTs play an important physiological role.

Membrane Bound Plant Sulfotransferases

Although extensive work has been done on their mammalian counterparts, little is known about membrane bound plant sulfotransferases. The gallic acid glucoside sulfotransferase, isolated from plasma membrane preparations of *Mimosa pudica*, is an example of a plant membrane bound sulfotransferase (Varin et al; 1997a). *M. pudica* (Leguminosae) can perform seismonastic (touch response) and nyctinastic (night leaf closure) movements. The leaves of this plant decline and close when stimulated by touch. In the absence of any stimulus, the leaves will reopen in a matter of minutes. This movement response was shown to be under the influence of electrical and chemical signals. As early as 1916, it was shown that *M. pudica* extracts as well as extracts of other plants that exhibit nyctinastic movement, could induce leaf closure when applied to a sliced *M. pudica* stem. The specific leaf closing factor was identified as gallic acid 4-*O*- (β -D-glucopyranosyl)-6'-sulfate, and named the Periodic Leaf Movement Factor 1 (PLMF-1) (Schildknecht et al; 1981). Gallic acid glucoside sulfotransferase is involved in the biosynthesis of PLMF-1, specifically catalyzing the final reaction. Tissue distribution analysis showed that the gallic acid glucoside sulfotransferase and the site of synthesis of PLMF-1 are restricted to the plasma membrane preparations of the primary, secondary,

and tertiary pulvini (a mass of large thin-walled cells surrounding a vascular strand at the base of a petiole) (Varin et al; 1997a).

Cytosolic Mammalian Sulfotransferases

Extensive work has been done on human cytosolic sulfotransferases (SULTs). This class of sulfotransferases consists of a super family of enzymes that sulfonate relatively small endobiotics and exobiotics (Strott, 2002). The SULT super family is divided into five distinct well-characterized families. Each is shown to be distinct based on amino acid sequence identity. The SULT1 family comprises of sulfotransferases which sulfonate phenolic drugs and catecholamines (SULT1A; human chromosome 16), estrogenic steroids (SULT1E) and thyroid hormones (SULT1B; human chromosome 4), and xenobiotics (SULT1C; human chromosome 2). The SULT2 family consists of members that sulfonate sterols (SULT2B; human chromosome 19), and neutral steroids (SULT2A). The SULT3 family has been implicated in the formation of sulfamates, while the SULT4 and SULT5 families consist of cDNAs yet to be fully characterized (Strott, 2002). Sulfonation increases water solubility and generally decreases biological activity of a compound (Falany, 1997b). As a result, the SULTs play a key role in the detoxification and inactivation of drugs and xenobiotics. It is also important to note that certain procarcinogens are converted by sulfonation into highly reactive intermediates which can bind covalently to DNA and act as chemical mutagens and carcinogens (Strott, 2002).

Cytosolic Plant Sulfotransferases

While plant cytosolic STs have not been as extensively studied as mammalian STs, they have been shown to be involved in reactions with important plant metabolites. Among the first cytosolic plant STs to be characterized were flavonol STs. Flavonoids are a class of compounds which are recognized as having an important role in plant metabolic processes, such as response to UV irradiation and microbial attack (Varin et al; 1992). The two first plant STs for which the cDNA clones were isolated and characterized were the 3- and 4'-ST of *Flaveria chloraefolia*. Structure function studies of these two enzymes have revealed the amino acid domains specific for catalysis, as well as substrate and co-substrate binding (Varin et al; 1995).

Similarly to humans, plants use steroids to regulate their growth and development. Three plant genes coding for brassinosteroid STs were isolated from *Brassica napus* (Rouleau et al; 1999). BnST1, BnST2 and BnST3 were isolated from the *B. napus* genomic library using AtST1 (previously known as RaRO47) as a probe. AtST1 (RaRO47) was the first putative ST isolated from *A. thaliana*. These BnST genes shared 85-87% similarity with AtST1, suggesting that they were possible orthologs of the *A. thaliana* gene. Biochemical analysis of BnST3 showed that it catalyzed the *O*-sulfonation of brassinosteroids and mammalian estrogenic steroids (Rouleau et al; 1999). Specifically, BnST3 catalyzed the sulfonation of a hydroxyl group of 24-epicathasterone, which is an intermediate in brassinolide biosynthesis. Sulfonation of 24-epicathasterone

abolished its biological activity, suggesting that plants like mammals modulated steroid activity by sulfonation (Rouleau et al; 1999).

Jasmonates are important metabolites in several plant processes, specifically involving stress, plant defense, growth and senescence. A hydroxyjasmonate ST, named AtST2a was isolated from *A. thaliana* and characterized (Gidda et al; 2003). This is the first reported ST involved in the sulfonation of jasmonates. This enzyme catalyzes the sulfonation of 12-hydroxyjasomate (12-OHJA). It was shown to have a high specificity for 12-OHJA and to a lesser extent 11-hydroxyjasmonate. It has been postulated that AtST2a serves to regulate the biological activity of 12-OHJA or possibly inactivate excess jasmonic acid (JA) through hydroxylation and subsequent sulfonation (Gidda et al; 2003). 12-OHJA has been shown to be involved in flower induction as well as tuber formation. This thesis will discuss the results of over-expression of AtST2a to decrease the levels of 12-OHJA *in planta*.

Jasmonic Acid (JA)

Derived from linolenic acid or hexadecatrienoic acid, jasmonic acid is a signalling compound involved in many plant processes. These processes include tuberization, wound response, defense against pathogens, as well as plant maturation (reviewed by Turner et al; 2002). The methyl ester of jasmonic acid, methyl-jasmonate (MeJA), was first isolated as a primary component in the aroma of the essential oils of *Jasminum grandiflorum* and *Rosemarinus officinalis* (Demole et al; 1962; Crabalona, 1967). JA

was first isolated in the culture medium of the fungus *Botryodiplodia theobromae* as a plant growth inhibitor (Aldridge et al; 1971).

Jasmonates are important signalling molecules proposed to be part of a signal transduction pathway that mediates the induction of plant defense genes in response to pathogen and insect attack (Doares et al; 1995). Jasmonates have been found to resemble mammalian prostaglandins structurally. Prostaglandins are fatty acid derivatives produced in many tissues of the human body and have many physiological effects. They are often found as secondary messengers within cells that have been targeted by other hormones. Prostaglandins are synthesized in the cell membrane from arachidonate, through the action of phospholipase A₂.

Much of what we know of jasmonate signalling comes from work on *A. thaliana* and tomato. Yet there seems to be discrepancies between the proposed JA signalling pathways between these two species, and it is unclear whether these simply represent knowledge gaps or fundamental differences in mechanism (Turner et al; 2002). In *A. thaliana*, mutants in JA biosynthesis or perception are deficient in defense responses and are male sterile (Feys et al; 1994; McConn and Browse, 1996; Vijayan et al; 1998). In contrast, tomato mutants in JA biosynthesis or perception are also deficient in defense however they are male fertile (Howe et al; 1996; Li et al; 2001). Likewise, in tomato the systemic induction of JA mediated responses follows the well characterized systemin signal pathway (Ryan et al; 2002). However there is no data indicating a similar pathway in *A. thaliana*, despite the demonstration of systemic signalling (Kubigsteltig et al; 1999).

It is important to note however that male sterile *A. thaliana* plants that are JA biosynthesis deficient can be rescued by exogenous application of 12-OHJA (Levitin, 2003).

Jasmonate Biosynthesis

The two proposed pathways for the biosynthesis of JA are the well studied octadecanoid pathway from linolenic acid (18:3), and a more recent hexadecanoid pathway from hexadecatrienoic acid (16:3) (Hedden and Phillips, 2000).

Octadecanoid Pathway

The octadecanoid pathway begins with the release of α -linolenic acid (LA) from chloroplast membrane lipids (Fig 1.1). It is possible that LA is released as a result of local events such as wounding or systemic signals. In tomato, the 18 amino acid polypeptide hormone systemin is a crucial wound response signal. Systemin and its receptor regulate a series of cellular cascade events which activates a phospholipase A₂ and releases LA from lipid membranes (Ryan, 2000). The next step involves the incorporation of molecular oxygen by lipoxygenase (LOX) resulting in 13-hydroperoxy LA. In potato, the LOX gene family includes three members, LOX1 through LOX3. Each of these genes differs in enzymatic properties, tissue expression patterns as well as organ specific expression. LOX1 is expressed in the roots and tubers, LOX2 in the leaves and LOX3 in both the leaves and roots. Antisense suppression transgenics of potato LOX1 (*POTLX-1*) strongly suggest that LOX1 is involved in the control of tuber

morphogenesis (Kolomiets et al; 2001). *POTLX-1* antisense suppression transgenics were showed to have reduced LOX1 transcript levels as well as reduced LOX1 activity in tubers and stolons. These suppression transgenics also showed specific changes in tuber development. Namely, a large fold reduction in tuber yield as well as a marked reduction in average tuber size was observed (Kolomiets et al; 2001).

13-hydroperoxy LA is subsequently converted to an unstable epoxide by allene oxide synthase (AOS). AOS was first purified from flax (Song et al; 1993), and then in other species such as *Arabidopsis* (Laudert et al; 1996), barley (Maucher et al; 2000) and tomato (Howe et al; 2000). The flax AOS was found to be a cytochrome P450 of the CYP74 family (Laudert et al; 1996). Over-expression of the flax AOS in potato plants showed an increase in levels of JA, possibly indicating that AOS is a rate limiting step in JA biosynthesis (Harms et al; 1995). Over-expression of *A. thaliana* AOS in *A. thaliana* and tobacco did not alter basal JA levels, however when these transgenic plants were wounded they exhibited peak jasmonate levels 2-3 times higher than control unwounded plants. This suggests that AOS may be involved in controlling defense in *A. thaliana* and tobacco (Laudert et al; 2000).

The unstable allene oxide is cyclized to *cis* -(+)-(9*S*, 13*S*)-12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase (AOC). In the absence of AOC, the unstable allene oxide can decay rapidly in aqueous solution by hydrolysis to α and γ -ketols and 12-OPDA (Hause et al; 2003). Due to the short half life of allene oxide, the absence of α and γ -ketols in plant tissues (Weiler EW, 1998) suggests that the reaction of AOS and

AOC is one that is closely coupled possibly in a complex (Schaller, 2001). AOC has also been shown to be chloroplast localized in tomato (Ziegler et al; 2000).

OPDA is then transported to the peroxisomes via an unknown mechanism. The 10, 11-double bond in the cyclopentanone ring of 12-OPDA is reduced by 12-oxo-phytodienoic acid reductase (OPR3) to form dihydro-12-OPDA. Three isoforms of 12-OPDA reductases have been characterized thus far in *Arabidopsis* (Schaller and Weiler, 1997; Biesgen and Weiler, 1999; Müssig et al; 2000). Biochemical studies showed that of the three reductases, OPR3 was the only one which was highly effective at reducing *cis*-(+)-OPDA, the natural precursor to JA, whereas OPR1 and OPR2 showed specificity to *cis*-(-)-OPDA (Schaller et al; 2000). Thus OPR3 is involved in jasmonate biosynthesis, while the physiological roles of OPR1 and OPR2 are not yet understood. *OPR3* mutants are male sterile due to pollen development defects and a delay of pollen grain release (Weber, 2002). However this mutant phenotype can be rescued through the exogenous application of MeJA, generating plants which are fertile.

The final three steps are successive β -oxidation reactions, which generate jasmonic acid. The only known places β -oxidation reactions occur in plants are the peroxisomes (Gerhardt, 1983). Thus it is probable that these reactions are localized in the peroxisomes, however this has not been investigated to date.

Hexadecanoid Pathway

Plant extract analysis of potato and *Arabidopsis* identified a novel compound similar to 12-OPDA and was named dinor-oxo-phytodienoic acid (Dn-OPDA) (Weber et al; 1997). This was especially unusual since in plants, octadecanoid derived signal compounds are well characterized, yet few hexadecanoid derived compounds have been studied. Dn-OPDA cannot be detected in the *Arabidopsis* mutant *fad5*, which cannot synthesize hexadecatrienoic acid (16:3). This suggests that Dn-OPDA is synthesized from plastid (16:3) rather than from the β -oxidation of 12-OPDA which contains 18 carbons. Treating *Arabidopsis* with low levels of Dn-OPDA increased the conversion of linolenic acid to α -ketol octadecanoid acid, suggesting that the compound can regulate its own biosynthesis (Weber et al; 1997).

Oxylipin Signature

The family of jasmonates consists of JA and cyclopentenones such as OPDA and Dn-OPDA. The levels of these compounds in a plant vary from species to species. The favoured accumulation of certain octadecanoids or their derived compounds gave rise to the suggestion that each plant may have a distinct pattern or 'oxylipin signature' (Weber et al; 1997). This term has been used to show the intricacy of jasmonate signalling, where single or multiple members derived from the hexadecanoid and octadecanoid pathways may be involved. Generally oxylipins are synthesized *de novo* in response to

wounding or pathogen attack, as well as other environmental and developmental factors (Howe and Schilmiller, 2002).

Hydroxylation of JA

While AtST2a catalyzes the sulfonation of a hydroxylated jasmonate (Gidda et al; 2003), the process that catalyzes the conversion of jasmonate to 12-OHJA is yet to be identified. Prostaglandins, which are structural analogs of jasmonates, are hydroxylated by hydroxylases in microsomes (Theoharides and Kupfer, 1981). *Arabidopsis* cytochrome P450s are good candidates for such a reaction. There are 272 cytochrome P450 genes present in the *A. thaliana* genome, of which members of the CYP94 and CYP86 have been shown to be fatty acid hydroxylases (Werck-Reichhart, 2002). Currently our lab is investigating possible putative hydroxylases that are involved in this reaction.

Tuberonic Acid

Jasmonates are integral to tuber development in tuber forming plant species. Tuberization normally occurs under specific inducing conditions, which are governed by a multitude of factors, which shall be discussed in detail in a further section. Two important factors are photoperiod and temperature in the *Solanum* species. *Solanum tuberosum* ssp. *Andigena* are dependent on short day conditions as well as night time temperatures to induce tuber formation. Grafting experiments by Gregory (1956) and

Chapman (1958) showed that this tuberization stimulus originated in the leaves under short day conditions and was transported to underground parts inducing tuber formation. Many attempts were made to identify this stimulus (review by Ewing, 1995), yet most studies were concentrating on known plant hormones and their effects on tuberization. Through culturing single-node segments of potato stems *in vitro*, two acidic substances were identified in the leaves of *Solanum tuberosum* which had tuber inducing activity (Koda and Okazawa, 1988). One such specific tuber-inducing substance was identified from the leaves of potato plants, and was found to induce tuberization *in vitro* of Jerusalem artichoke plants (Koda et al; 1988). This substance was identified as 3-oxo-2-(5'- β -D-glucopyranosyloxy-2'-Z-pentenyl)-cyclopentane-1-acetic acid (Yoshihara et al; 1989). The aglycone of this glucoside is 12-OHJA (Fig 1.2) and named tuberonic acid (TA) by the authors. There are four stereoisomers of TA, though naturally occurring TA is found as the *cis* isomer. While JA has inhibitory effects on plant growth such as leaf senescence, *cis*-TA as well as the glucoside of TA do not exhibit similar effects on plant growth (Koda, 1992).

The effect of photoperiod on JA hydroxylation was reported by Helder et al. (1993). Short day grown *Solanum demissum* was found to have 12-OHJA and 11-OHJA present in leaves of wild type plants that were tuberizing. However in long day grown plants no tubers formed, and these compounds were not detected. 11-OHJA is structurally similar to 12-OHJA and may be a possible tuber inducing substance. However no studies have shown that 11-OHJA has tuber inducing properties.

Radio-labelled JA (2-[¹⁴C]-JA) was applied to the leaves of *Solanum tuberosum* with plants grown under short and long day conditions, and was found to be converted within 10 days to tuberonic acid glucoside (TAG), with a migration of radioactivity throughout the plant, regardless of photoperiod length (Yoshihara et al; 1996). Importantly, the labelled TAG in potato stolons was found to be ten times higher in short day grown plants than long day grown ones. Conversely, labelled TAG was significantly higher in the flower buds of long day grown plants. Long day conditions are flower inducing but not tuber inducing. Thus it was concluded that JA is converted to TAG and transported throughout the plant while photoperiod affects localization of TAG (Yoshihara et al; 1996). This build up of TAG in stolons and flower buds may induce tuber and bud formation. The aforementioned work suggests that TA and TAG form through direct hydroxylation and glycosylation of JA.

JA and TA have also been found to induce expression of genes independently from each other. Treating barley leaf segments with JA showed that JA induced the expression of JIP6, a protein coding for thionin, and JIP23 in leaves (Miersch et al; 1999). However, treatment with TA did not induce JIP6 and JIP23, suggesting that TA and TAG may play a specific role in plants, which may be related to tuber formation.

Florigen and Tuberigen Hypothesis

Studies of photoperiod-responsive plants led to the development of the florigen hypothesis. It states that leaves perceive inductive photoperiods and as a result produce a signal that is translocated from the leaves to shoot apical meristems (SAMs) to initiate floral transition (Chailakhyan, 1936). This signal was referred to as 'florigen', though its chemical nature remains unknown. Although this term implies a unique single compound (a flowering hormone), this signal may be a mixture of several components (Bernier et al; 1993). Grafting experiments suggest that translocatable signals regulate flowering (Zeevart, 1984), as well as tuberization (Gregory, 1956; Chapman, 1958). Molecules such as cytokinins, gibberellins (GA) and carbon assimilates have been presented as candidates for the florigen (Bernier et al; 1993). However these molecules behave differently in different species (review by Levy and Dean, 1998).

Tuber development

Potatoes (tubers) are one of the most important crops world-wide ranking fourth in annual production behind rice, wheat, and barely (Ferne et al; 2001). Thus the factors governing tuber development have been under study for some time. Contrary to popular belief, tubers are not modified roots, but in fact modified stems. They are derived from lateral underground buds that form at the base of the main stem, which due to diagravitropical growth develop into stolons (Fig 1.3). Tuber induction, initiation, enlargement, dormancy and sprouting constitute the life cycle of a typical potato tuber.

Various environmental and endogenous factors are involved in the formation of tubers. Tubерization can be manipulated by manipulating nitrogen levels available to the plant. Experiments where the level of nitrogen supplied to hydroponic plants was precisely controlled showed that a continuous supply of nitrogen between 1 and 3 mM completely inhibited or severely delayed tuberization in plants grown under inducing conditions (Krauss et al; 1985). Interestingly, high nitrogen supply to potato leaves did not inhibit tuberization, despite being comparable to plants receiving high nitrogen through the roots. Reducing nitrogen levels in non inducing conditions such as long day or high temperatures did not result in tuberization, suggesting that nitrogen may not be involved in tuber induction, but be able to repress tuberization after induction. While it is not known how nitrogen levels cause inhibition of tuberization, there are reports showing that nitrogen withdrawal affects phytohormone levels, reducing gibberellic acid (GA) and increasing levels of abscisic acid (ABA) (Krauss et al; 1985).

High temperatures also have an inhibitory effect on tuber induction. It is postulated that high temperatures influence the separation of assimilates by decreasing the amount available to the tubers and increasing the amounts to other plant parts. It was shown that high temperatures given to the shoots had the most significant inhibitory effect on tuber induction. While high temperature did not affect the creation of the inducing signal; it did prevent stolons from forming tubers (Ewing and Stuik, 1992).

High light and high sucrose levels have the effect of promoting tuberization. The effect of low light intensities on tuberization mimics the effect of high temperatures, and high light levels can reverse the inhibitory effects of high temperatures (Menzel, 1985). Low light intensities have been shown to increase acidic GA amounts in potato leaves (Woolley and Wareing, 1972). Thus it is possible that the effect of irradiance may be mediated through a GA controlled process. Tuberization *in vitro* is very dependent on sucrose concentration, and sucrose has been shown to induce several genes that are tuber specific (Xu et al; 1998). It has been demonstrated that tips of stolons growing in media containing 1% sucrose had elevated levels of GA when compared to stolons growing in 8% sucrose (Xu et al; 1998). As a result the authors suggested that sucrose can modulate endogenous GA levels in the stolon tip.

Photoperiod Regulated Tuberization

Potato (*S. tuberosum*) is a short day (SD) plant that requires day lengths of 12 hours or less to tuberize. In the photoperiodic response, the length of the dark period is more important than the length of the light period. This was demonstrated through an interruption of a long dark period (SD) with red light treatment that prevented tuber induction, and was partially reversed by subsequent far-red light treatment (Batutis and Ewing, 1982). The site of perception of the photoperiodic response is in the leaves, and was observed in single leaf cuttings (Ewing and Wareing, 1978). Photo-reversibility is a characteristic of phytochrome mediated responses. Phytochromes are protein kinases,

which communicate light stimuli to other molecules via light dependent enzymatic activity. There have been five types of phytochromes identified in tomato and *Arabidopsis*, ranging from PHYA through PHYE. It has also been shown that phytochromes exist in most if not all higher plants (Wang and Deng, 2002). PHYB level was reduced in *S. tuberosum* transgenic antisense plants, which allowed for the plants to tuberize in both short and long day conditions (Jackson et al; 1996). These antisense plants have lost their ability to inhibit tuberization in long day (LD) conditions, suggesting that PHYB may have a role in long day tuberization inhibition. PHYA has been demonstrated to be involved in the photoperiodic flowering control in *A. thaliana* and may also be involved in other photoperiod responses such as tuberization.

The effect of photoperiod on tuber induction might be mediated in part by gibberellic acid (GA) treatment, which prevents or delays tuber induction in short day conditions. Inhibiting GA biosynthesis with an inhibitor such as ancymidol, allows for tuber induction in long day (non inducing) conditions (Jackson and Prat, 1996). Over expression of GA oxidase (an enzyme involved in GA biosynthesis) gave rise to transgenic potato plants that needed longer short day photoperiods to induce tuber formation. Conversely, antisense inhibition of GA oxidase led to plants that formed tubers earlier than control plants grown under short day conditions (Carrera et al; 2000).

Flowering and tuberization are both distinct reproduction strategies which involve the sensing of photoperiod and the generation of a signal in the leaves. While there are several distinct flowering pathways, one of the best studied genes of the photoperiod

dependent pathway is *Arabidopsis* *CONSTANS* (*CO*). *CO* codes for a Zn-finger transcription factor and up regulates the transcription of several meristem identity genes (Puterhill et al; 1995). Over expression of *CO* in *Arabidopsis* gave rise to early flowering plants that were insensitive to day length (Puterhill et al; 1995; Simon et al; 1996; Samach et al; 2000; Suárez-López et al; 2001). Two tuber formation pathways have been proposed, a photo dependent pathway and a gibberellin (GA) dependent pathway (Martínez-García et al; 2001). To investigate the photo dependent pathway, *CO* was over expressed in transgenic potato plants via a strong 35S promoter (Martínez-García et al; 2002). It was found that plants over expressing *CO* had delayed tuber induction, and that the tubers formed were small and poorly developed. Photoperiod perception was not altered by *CO* over expression and the transgenic plants exhibited phenotypes opposite to the ones found in transgenic antisense *PHYB* potato plants (Martínez-García et al; 2002; Jackson et al; 1996).

Tuberization was found to be inhibited in response to *CO* over expression in leaves. This was determined through grafting experiments involving grafting of scions (aerial portion) from wild type and transgenic *CO* over expressing plants onto wild type and transgenic *CO* stocks (root portions) (Martínez-García et al; 2002). The grafted plants were then grown in short day tuber inducing conditions. Wild type scions grafted onto transgenic stocks resulted in tuberization identical to wild type plants, while transgenic scions grafted onto wild type stocks tuberized like the transgenic control plants. This suggested that *CO* expression in the leaves is sufficient to inhibit tuber formation, while *CO* expression in the stolons is not (Martínez-García et al; 2002). The

authors proposed a model (Fig 1.4) for the role of *CONSTANS* in tuberization. The *StCOL1* gene in potato shares significant homology to *CO* and strongly suggests the existence of a *CONSTANS* functionality in potato (Martínez-García et al; 2002).

CHAPTER 2
MATERIALS AND METHODS

Materials

12-hydroxyjasmonate (12-OHJA) was provided by Dr. O. Miersch, from the Leibniz Institute of Plant Biochemistry, Halle, Germany. Methyl jasmonate was purchased from Bedoukian Research Inc. USA. *Solanum chacoense* wild type and transgenic plants expressing *AtST2a* were provided by Dr. N. Brisson, Department of Biochemistry, Université de Montréal; Montréal; Canada. pBluescript II SK(+) clones of TC77024 (GB#: CK851577 and CK716670) and TC87873 (GB#: CK861810 and CK862405) were provided by Dr. Barry Flinn from the Canadian Potato Genome Project – BioAtlantech, Fredericton, Canada. Putative potato STs were selected via BLAST analysis of *AtST2a* against the TIGR potato EST database. All other reagents were of analytical or molecular biology grade.

Transgenic Phenotype Analysis

S. chacoense plants were grown in soil in a greenhouse during an 8-10 hour photoperiod, at a day time temperature of 22-25° C and a night time temperature of 17-20° C. Tubers had been stratified for at least two months at 4 degrees prior to planting. Plants were watered every 3-4 days until senescence began to occur (evidenced by leaves changing to a yellow color). For phenotypical analysis, plants were observed every 3-4

days for tuber formation after first flowering. This was done through surface observation of plant root portions after pulling them out of their growing pots. Tubers were harvested from each dead plant, and the number of tubers per plant as well as the weight of each tuber was recorded.

Time Course Assays and Dark Treatments

S. chacoense plants were grown in a growth chamber during an 8 hour photoperiod, at a day time temperature of 22° C and a night time temperature of 18° C. Two week old shoots (with at least one leaf) were cut and placed in microfuge tubes containing either 100 µM of 12-OHJA or 50 µM of methyl jasmonate (MeJA). Control shoots were placed in microfuge tubes containing water. Tissue samples were taken at different time intervals (t = 0h, 1h, 2h, 4h, 8h). The samples were placed in liquid nitrogen and stored at -80° C.

Plants that had been grown for a period of one month in a growth chamber under the above growth conditions were placed in a separate growth chamber with no light for a period of 24 hours. During this period, tissue samples were taken in the dark from the plants at various time intervals (t = 0h, 1h, 4h, 8h, 12h, 24h). The samples were frozen in liquid nitrogen and stored at -80° C.

Western Blot Analysis

For the detection of AtST2a protein in various experiments, Western blotting was performed. Plant samples were ground to a fine powder in liquid nitrogen and boiled in 2X SDS sample buffer for total protein extraction. To confirm the integrity of the proteins and equal loading of each sample, protein extracts were also electrophoresed on SDS-PAGE and stained with Coomassie blue (Laemmli, 1970). Protein extracts were separated by SDS-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and transferred onto nitrocellulose membrane. AtST2a was immunodetected using anti-AtST2a polyclonal antibodies (dilution 1:1000) and goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000; BioRad).

Preparation of Anti-AtST2a Antibodies

Anti-AtST2a polyclonal antibodies were raised in rabbits using PAP agarose purified recombinant enzymes expressed in *E. coli* (Gidda, 2001). Purified protein (100 µg) was injected subcutaneously into a rabbit in Freund's complete adjuvant. The rabbit was injected 3 more times 21, 42, 70 days after the first injection, with 50 µg of purified protein in Freund's incomplete adjuvant. The rabbit was bled 10 days after the last injection and serum extracted. This procedure was performed by H el ene Ste-Croix at the McGill University animal house facility.

Total RNA extraction

For expression analysis of various genes, total RNA was extracted via a modified protocol by A. Cashmore (1982). Plant material was ground into a fine powder in liquid nitrogen, and transferred to an equal volume of extraction buffer (For 50 ml: 10 ml Tris-HCl 1M pH 9.0, 4 ml NaCl 5M, 1 ml MgOAc 1M, 12.5 ml Sucrose 2M, 0.25 ml DTT 1M, 5 ml 10% SDS, 2.5 ml EDTA 400 mM, and 16.25 ml ddH₂O-DEPC). The samples were mixed well and 1 volume of phenol : chloroform : IAA (25:24:1 v/v/v) was added. The samples were then agitated vigorously for 20 minutes and then subsequently centrifuged for 15 minutes at 3200 X g. The aqueous phase was collected and again treated with an equal volume of phenol : chloroform : IAA (25:24:1 v/v/v), agitated for 20 minutes and centrifuged for 15 minutes at 3200 X g. The aqueous phase was collected and then precipitated with 0.6 volume of isopropanol for 30 minutes on ice. Samples were then centrifuged for 12 minutes at 4° C and 12 000 X g. The aqueous phase was discarded and the pellet was re-suspended in a final concentration of 2M LiCl, and left to precipitate overnight at 4° C. Samples were then centrifuged for 20 minutes at 4° C and 3200 X g, and then re-suspended in 0.5 ml DEPC H₂O. One tenth volume of 3M NaOAc (pH 5.2) and 2 volumes of 95% ethanol were then added to the samples and left to precipitate overnight at -20° C. Samples were centrifuged for 20 minutes at 12 000 X g and 4° C. The pellets were washed with 70% ethanol, centrifuged for 5 minutes (12 000 X g), dried and re-suspended in 20 µl of DEPC H₂O and stored at -80° C.

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

Total RNA (2.5 µg) was treated with 20U of DNase I (Roche Molecular Biochemicals) in 50 µl of 0.1 M sodium acetate, 5mM MgSO₄, pH 5.0 for 10 min at 37°C. DNase I was heat inactivated at 95°C for 5 min and RNA was ethanol precipitated. cDNA was synthesized using Moloney Murine Leukemia Virus reverse transcriptase (New England Biolabs) in a 25 µl reaction volume as recommended by the manufacturer. RT reaction product (1 µl) was used for PCR with Ex Taq DNA polymerase (Takara Biochemicals).

DNA Extraction

For genomic DNA extraction, tissue was ground in liquid nitrogen and dissolved in extraction buffer (3% CTAB, 1.4 M NaCl, 52 mM β-mercaptoethanol, 20 mM EDTA and 100mM Tris-HCl, pH 8.0) preheated at 60 °C. The extract was incubated for 30 min at 60 °C, with occasional shaking. The aqueous phase was extracted twice with chloroform, and precipitated with isopropanol. The precipitate was washed with 95% ethanol, dried and re-suspended in sterile water.

Sequencing of Clones

To determine if the clones provided and created were full length, sequencing of DNA from plasmid preparations was done. Plasmids were purified from an overnight

culture of XL-1 Blue transformed *E. coli* with a QIAprep Spin Miniprep Kit (Qiagen). Sequencing of pBluescript clones as well as pQE30 clones was done at the Genome Québec Innovation Centre, Montréal; Canada.

Sub-Cloning of TC77024 & TC87873

The pBluescript clones TC77024 and TC87873 were used as templates for polymerase chain reaction (PCR). For TC77024, oligonucleotide 5'-GCGCGGATCCATGTCAAATCTCAAACCTTCTCC-3' was designed to introduce a *Bam*HI site at the 5' end of the gene, while oligonucleotide 5'-GCGGGGTACCTTAAATATATGAAAATTTTCAGTCCA-3' was designed to introduce a *Kpn*I site at the 3' extremity of the gene. Similarly for TC87873, oligonucleotides 5'-GCGCGGATCCATGTCAAATCTCAAACCTTC-3' and 5'-GCGGGGTACCTTAGATGTATGAAAACCTTTATTCCA-3' were designed to introduce *Bam*HI and *Kpn*I sites at the 5' and 3' ends respectively. TC77024 and TC87873 were both amplified from pBluescript clones via PCR with Ex-Taq DNA polymerase (Takara).

The amplified products were then ligated into the blunt end vector pGEM-T (Promega). Clones were selected based on blue/white selection, plasmid purified and digested with *Bam*HI and *Kpn*I restriction endonucleases. The digests were electrophoresed on a 1% agarose gel to separate plasmid and insert fragments. Digested inserts were then excised from the agarose gel and purified with QIAquick Gel Extraction Kit (Qiagen). The purified digested inserts were then ligated into the *Bam*HI and *Kpn*I

sites of the bacterial expression vector pQE30 (Qiagen). Clones containing TC77024 and TC87873 were verified by restriction endonuclease analysis and by DNA sequencing. All enzymes used for cloning were from New England Biolabs and were used under the conditions recommended by the manufacturer.

DNA sequence analysis

DNA and protein sequence alignments were performed using the ClustalW program (<http://www.ebi.ac.uk/clustalw/>) as well as NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the similarity/identity values determined from the pairwise comparisons of various potato STs relative to *Arabidopsis* STs. This was done by performing both nucleotide and amino acid sequence alignments of each putative potato ST against the known 18 *Arabidopsis* STs.

Expression of recombinant TC77024 and TC87873

A fresh culture of *E. coli*, strain XL1-blue harboring TC77024 or TC87873 (O.D600 = 0.7) was induced with 1mM isopropylthio- β -D-galactopyranoside (IPTG) for 10 hours at 20 °C. Bacterial cells were collected by centrifugation, resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 0.3M NaCl and 14 mM 2-mercaptoethanol (lysis buffer). The cells were lysed by sonication and the recombinant proteins recovered in the soluble fraction by centrifugation at 12000 X g for 15 min at 4 °C. The supernatant was applied to a nickel-nitrotriacetic acid agarose matrix (Qiagen) equilibrated in buffer A. The resin was washed with 50 mM sodium phosphate (pH 6.0), 0.3 M NaCl and 14 mM 2-mercaptoethanol (wash buffer) and the proteins were eluted

with the same buffer containing 0.5 M imidazole (elution buffer). The Ni-agarose purified protein was desalted in 25 mM bis-Tris, pH 6.5. Protein concentration was estimated using the Bradford Reagent (Bio Rad) with bovine serum albumin (BSA) as the reference protein.

SDS-Polyacrylamide Gel Electrophoresis

In order to verify the solubility and evaluate the level of purity of the recombinant protein after chromatography on nickel-agarose, aliquots of the recombinant enzyme were subjected to 12% polyacrylamide gel electrophoresis according to the method of Laemmli (1970). The proteins were visualized by Coomassie Blue staining.

Sulfotransferase Assay

Analysis of substrate specificity was performed by testing enzymatic activity with three different concentrations (1, 10 and 100 μ M) of acceptor substrate (12-OHJA). The reaction mixture (50 μ l) contained 50 pmol [35 S] PAPS (Perkin Elmer) and approximately 0.25 mg of Ni-agarose purified recombinant TC77024 or TC87873 in 50 mM Tris (pH 7.5). The reactions were allowed to proceed for 10 min at 20° C. The TC77024 or TC87873 sulfated reaction product was extracted with 1-butanol saturated with water and an aliquot was counted for radioactivity in scintillation fluid.

Detection and quantification of JA, OPDA, dn-OPDA, 12-OH-JA, 12-O-Gluc and 11-OH-JA from *Solanum chacoense*

This experiment was performed by the laboratory of Dr. O. Miersch, Leibniz Institute of plant Biochemistry, Halle, Germany. Fresh plant material (1g) was homogenized with 10 ml methanol and 100 ng of (²H₆) JA, 12-(²H₃)OAc-JA and 11-(²H₃)OAc-JA (prepared by Dr. O. Miersch) were added as internal standards. The filtrate was evaporated and acetylated with Pyridine/Acetic acid anhydride (2:1) at 20° C overnight. The reaction mixture was evaporated, resuspended in ethyl acetate and loaded on a silica (SiOH) column (50mg; Machery- Nagel). The flow-through containing JA and acetylated forms 11-OH-JA and 12-OH-JA was collected and evaporated. The evaporated mixture was resuspended in 5 ml methanol and loaded on a 3 ml DEAE-Sephadex A25 column (acetylated-form in methanol). The column was washed with 3 ml of methanol followed by 3 ml of 0.1 M acetic acid in methanol. The jasmonates were eluted with 5 ml of 1 M acetic acid in methanol (Fraction A), evaporated and separated on preparative HPLC for GC-MS analysis.

The SiOH column was washed with methanol and the flow through (Fraction B) was collected for analysis of 12-OH-JA sulfate. Fraction B was evaporated, resuspended in 10% acetonitrile and chromatographed by reverse phase HPLC (Method gradient, 10% to 90% acetonitrile in 15 min at a flow rate of 1 ml/min). Fractions were collected from 4.5 to 7 min, evaporated and resuspended in 50 µl methanol and analyzed by LC-MS.

Preparative HPLC: Fraction A eluted from the DEAE-Sephadex A25 column was subjected to preparative HPLC column, Eurospher 100-C18 (5 μm , 250 x 4 mm). Jasmonates were eluted with methanol-0.2 % acetic acid in H_2O (1:1) at a flow rate of 1 ml/min and UV detector at 210 nm. Fractions between R_t 9.15 and 11 min containing 11-OAc-JA and 12-OAc-JA and between 12 and 13.30 min containing JA were collected and evaporated. The samples were dissolved in 200 μl chloroform/*N,N*-diisopropylethylamine (1:1) and derivatized with 10 μl pentafluorobenzylbromide at 20 $^\circ\text{C}$ overnight. The evaporated derivatized samples were dissolved in 5 ml *n*-hexane and passed through a SiOH-column (500mg; Machery- Nagel). The pentafluorobenzyl esters were eluted with 7 ml of *n*-hexane/diethylether (2:1), evaporated, dissolved in 100 μl acetonitrile and analyzed by GC-MS.

GC-MS: (GCQ Finnigan, 70 eV, NCI, ionization gas NH_3 , source temperature 140 $^\circ\text{C}$, column Rtx-5 (30 m x 0.25 mm, 0.25 μm film thickness), injection temperature 250 $^\circ\text{C}$, interface temperature 275 $^\circ\text{C}$; Helium 40 cm s^{-1} ; splitless injection; column temperature program: 1 min 60 $^\circ\text{C}$, 25 $^\circ\text{ min}^{-1}$ to 180 $^\circ\text{C}$, 5 $^\circ\text{ min}^{-1}$ to 270 $^\circ\text{C}$,1 min 270 $^\circ\text{C}$, 10 $^\circ\text{ min}^{-1}$ to 300 $^\circ\text{C}$, 25 min 300 $^\circ\text{C}$).

Retention time of 12-($^2\text{H}_3$)OAc-JA-pentafluorobenzyl ester: 20.61 min, 12-OAc-JA-pentafluorobenzyl ester: 20.66 min, using fragments m/z 270 (standard) and m/z 267 for quantitation.

Retention time of 11-(²H₃)OAc-JA-pentafluorobenzyl ester: 18.40 min, 11-OAc-JA-pentafluorobenzyl ester: 18.38 min, using fragments *m/z* 270 (standard) and *m/z* 267 for quantitation.

Retention time of (²H₆) JA -pentafluorobenzyl ester: 14.66 min, JA-pentafluorobenzyl ester: 14.72 min, using fragments *m/z* 215 (standard) and *m/z* 210 for quantitation.

Detection of 12-OH-JA sulfate by LC MS/MS

Performed by the laboratory of Dr. O. Miersch, Leibniz Institute of plant Biochemistry, Halle, Germany. The negative ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4 kV; heated capillary temperature 220°C; sheath gas: nitrogen) coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a RP18-column (4 μm, 1x100 mm, Ultrasep). For the HPLC, a gradient was used starting from H₂O: acetonitrile (90:10; containing 0.2% acetic acid) to 10:90 in 15 min followed by a 10 min isocratic period at a flow rate of 70 μl/min. The collision-induced dissociation (CID) mass spectra during the HPLC run were performed with a collision energy of 30 eV (collision gas: argon, collision pressure: 1.8 x 10⁻³ Torr). All mass spectra are averaged and background subtracted. The following results were obtained:

12-Hydroxysulfonyloxyjasmonic acid: Rt (LC-MS), 12.32 min, negative electrospray MS m/z (rel. int.): 305 ([M-H]⁻, 100); CID spectrum: 225 (93), 147 (9), 97 (100), 59 (58).

For the determination of 12-hydroxysulfonyloxyjasmonic acid in plant material the daughter ions at m/z 225, 97 and 59 were measured in the selected ion monitoring (SIM) mode. The CID spectrum of 12-hydroxysulfonyloxyjasmonic acid displays significant ions at m/z 225, 97 and 59 reflecting the typical structural features of the compound. The ion at m/z 97 represents a key ion in the negative CID mass spectra of sulfated compounds (Boss et al; 1999).

Oligonucleotides used for gene amplifications in various PCR & RT-PCR

Oligonucleotides were designed using the OLIGO software (Molecular Biology Insights Inc.) and primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of oligonucleotides used in various experiments follows below.

Oligos for PCR Analysis of *S. chacoense* Samples:

- Primers used to amplify *ACTIN* of *S. tuberosum*

(Genebank Acc # U60487)

(Pot-Actin 5') 5'-CATGGAGAAGATTTGGCATCATA-3'

(Pot-Actin 3') 5'-TATCAGCAATGCCAGGAAACATA-3'

(Actin 5') 5'-TCCTACGTCTCGACCTTGCT-3'

(Actin 3') 5'-TCAGGAGGAGCAACCACTTT-3'

- Primers used to amplify tuber specific genes

(Genebank Acc # AF498099)

(Patatin 5') 5'-ACTTGGAGAACTCGTGTGCAT-3'

(Patatin 3') 5'-TAGAGCTTCCTCATAGGTTTCAGGA-3'

(Genebank Acc # AY135641)

(Pot-AOC 5') 5'-TGCCTTCCAAATTA CTCTTTCT-3'

(Pot-AOC 3') 5'-CAGGTGTTGGCTCTACCGTC-3'

(Genebank Acc # AY135640)

(Pot-AOS 5') 5'-ATTGTCTCTTTATCGGAAAAACCAA-3'

(Pot-AOS 3') 5'-AAGTCGTCTAGAACTTCGGAAGAC-3'

(Genebank Acc # U60200)

(Pot-Lox 5') 5'-AACAGATCCTAAAAGTGAAAGCAGG-3'

(Pot-Lox 3') 5'-AATCAATTGCTAGTGGCTTCAAA-3'

- Oligonucleotide primers used to amplify the putative *S. chacoense* STs

(TC77024 5') 5'-TGTCAAATCTCAA ACTTCTCCC-3'

(TC77024 3') 5'-GAAGCAAAGGGCAAATGAGT-3'

(TC87873 5') 5'-TATCCTGTTTTTGAACAAAATCACC-3'

(TC87873 3') 5'-TTTAAGCTGAAGTTTGGGCTG-3'

(TC88534 5') 5'-CAATACCTCAA ACTACTTCACCACA-3'

(TC88534 3') 5'-ACATATGAGTTGCCAAGAGTCTAGG-3'

(TC85921 5') 5'-GCAAGTTTATTCTCTACCCCTTAC-3'

(TC85921 3') 5'-TCTGGTAAACAATTGAAAGCAAGA-3'

(TC85524 5') 5'-GAGTGACAAAGAATCATCACCAA-3'

(TC85524 3') 5'-CGCGCAACAAAAAATTCATA-3'

(TC78605 5') 5'-GACTAATCCGGATGCTACTTCTTC-3'

(TC78605 3') 5'-TGTTGGTACACTGTATTGGTTCAAT-3'

- Primers used to clone TC77024 in pQE30

(TC77024-BamH1 5') 5'-CGCGGATCCATGTCAAATCTCAAACCTTCTCC-3'

(TC77024-Kpn1 3') 5'-GCGGGGTACCTTAAATATATGAAAATTCAGTCCA-3'

- Primers used to clone TC87872 in pQE30

(TC87873-BamH1 5') 5'-GCGCGGATCCATGTCAAATCTCAAACCTTC-3'

(TC87873-Kpn1 3') 5'-GCGGGGTACCTTAGATGTATGAAAACCTTATTCCA-3'

- Primer used for cDNA synthesis

(Poly T) 5'-TTTTTTTTTTTTTTTT-3'

- Primer used for pQE30 DNA sequencing

(pQE30-Sequencing 5') 5'-GCGGATAACAATTCACACAGA-3'

(pQE30-Sequencing 3') 5'-CTCCTGAAAATCTCGCCAAG-3'

CHAPTER 3

ANALYSIS OF OVER-EXPRESSION OF 12-HYDROXYJASMONATE

SULFOTRANSFERSE FROM *ARABIDOPSIS* IN POTATO

Results

Phenotypical Analysis

Five transgenic lines over expressing *AtST2a* (Fig 3.1) were obtained from the laboratory of Dr. Norman Brisson and were grown until senescence while being observed for phenotypical differences compared to wild type plants. In the first vegetative propagation of plants, the transgenic lines showed reduced size in average tuber weight and many tubers relative to wild type plants which had larger, fewer tubers (Fig 3.2). Two of these transgenic lines, 11 & 30, had the most severe phenotypic differences. The differences were shown to be statistically different from the wild type control lines though one way analysis of variance (ANOVA) ($\alpha=0.05$) and t-tests. A second propagation with a much larger pool of plants was grown for wildtype, line 11 and line 30. The results obtained with the second propagation were consistent with the previous results for the variable tuber weight (Fig 3.3).

In addition to tuber weight, we wished to observe if there were any differences in the timing of tuber formation, between the wild type and transgenic lines. We hypothesized that the transgenic lines would show a delayed tuber formation phenotype. In the first propagation of plants, we observed a delay in tuber formation between wild

type control plants and the various transgenic lines of 7 days for lines 11 & 4, to 11 days for lines 20, 13 & 30 (Fig 3.4). Single factor ANOVA analysis ($\alpha=0.05$) showed that the results obtained with transgenic lines are significantly different from the ones of the control lines. The appearance of tubers in the second propagation was delayed in the transgenic lines relative to wild type plants (Fig 3.5). This second propagation had a larger sample set than the first, consisting of 9-18 plants per line, and showed similar results validated by single factor ANOVA ($\alpha=0.05$).

Furthermore, it is important to note that the transgenic lines 20, 11 and 30 showed significant differences in the number of tubers obtained per plant. These lines gave a large number of smaller tubers, as compared to the wild type plants which had larger tubers (Fig 3.6).

Quantification of Jasmonate compounds in Potato

In order to determine if the endogenous levels of 12-OHJA were different in wild type plants as compared with the transgenic plants over-expressing *AtST2a* in the sense orientation, various jasmonate like compounds were quantified. Three individual lines were tested in triplicate: wild-type, transgenic line 11, and transgenic line 30. The jasmonates tested were as follows: 12-oxo-phytodienoic acid (OPDA) and dinor-oxo-phytodienoic acid (dn-OPDA) which are precursors of jasmonic acid, jasmonic acid (JA), tuberonic acid (12-OHJA), tuberonic acid sulfate (12-HSO₄JA), tuberonic acid glucoside (12-O-Gluc-JA or TAG), and 11-OHJA which is structurally similar to 12-OHJA.

GC-MS analysis of wild type *S. chacoense* leaf samples showed the presence of both 12-OHJA and 12-HSO₄JA (the sulfated product of 12-OHJA via AtST2a sulfonation). This indicates the existence of an AtST2a homologue in potato plants. GC-MS analysis (Table 3.1) of the transgenic plants over-expressing *AtST2a* showed that these plants contained a lower level of 12-OHJA as compared to wild type plants. This corroborates similar experiments performed on other AtST2a transgenic plant species, such as *N. tabaccum* which also showed lower overall 12-OHJA levels in the transgenics when compared to wild type plants (Levitin, 2003). While 12-O-Gluc-JA levels remain relatively unchanged, OPDA, dn-OPDA and 11-OHJA levels are overall higher in the transgenic plants than the wild type. JA levels are drastically lower in the transgenic plants, and this can most likely be attributed to the rapid conversion of 12-OHJA to 12-HSO₄JA, driving the conversion of JA to 12-OHJA. This suggests that the hydroxylated jasmonates may regulate JA levels.

Discussion

Over-expression of AtST2a has been tested in flowering plant species such as *A. thaliana* (Gidda, 2001) and *N. tabaccum* (Levtin, 2003), however never before on a tuber forming plant species such as potato. In the previous studies, AtST2a over-expression resulted in a delayed flowering phenotype. In the present study, we found that *S. chacoense* over-expressing AtST2a exhibits a delay in tuber formation, a reduction in tuber size and an increase in the number of tubers. It has been proposed that AtST2a regulates the biological activity of 12-OHJA or possibly participates in the inactivation of excess jasmonate (JA) through hydroxylation and subsequent sulfonation (Gidda et al; 2003). Thus we would expect the pool of available JA and 12-OHJA to be depleted by the over-expression of AtST2a in the transgenic plants. As expected, the depletion of these two key tuber inducing compounds in the transgenic lines led to a delay in tuber formation. However the fact that this expected phenotype was coupled with a reduction in tuber size and an increase in tuber numbers is interesting. Both jasmonic and tuberonic acid have been implicated as tuber inducing compounds (Koda et al; 1991; Pelacho and Mingo-Castel, 1991). Jasmonic acid has also been shown to be involved in the induction of radial cell expansion in tubers (Takahashi et al; 1994) and tuber buds (Castro et al; 1999). As was discussed in the introduction, an antisense suppression mutant of potato lipoxygenase 1 (*POTLOX1*) also showed a decrease in tuber size, and tuber number, as well as an additional phenotype of malformed tubers (Kolomiets et al; 2001). Lipoxygenase is involved early in the jasmonate biosynthesis pathway, the product of the reaction being 13-hydroperoxy LA. It was suggested that the POTLOX1 suppression

mutant phenotype was due to the fact that the sink strength was reduced of individual tubers due to the inhibition of their enlargement, and that the transgenic plants compensated in inductive conditions by initiating more tubers (Kolomiets et al; 2001). Our data suggest that 12-OHJA also plays a key role in determining the enlargement and formation of tubers. By reducing the pool of oxylipin products such as JA and 12-OHJA, it is possible that cell expansion during tuber enlargement is impaired. While the *POTLOX1* antisense mutants also exhibited malformed tubers, we did not observe any malformation in the *AtST2a* transgenic plants, with the exception of a reduction in relative size. The suppression of *POTLOX1* likely disrupts the endogenous levels of oxylipin compounds such as JA and 12-OHJA, because *POTLOX1* is involved in the initial steps of jasmonic acid biosynthesis. The over-expression of *AtST2a* has a negative effect on the endogenous levels of JA and 12-OHJA. Since a malformation phenotype was not observed in the *AtST2a* transgenic plants, it is possible that *POTLOX1* may be involved in maintaining proper radial expansion, while 12-OHJA may specifically be related to the initiation of tuber formation and on the growth maintenance of the tubers. The phenotype of the *AtST2a* transgenic plants suggests that depleting the endogenous levels of 12-OHJA disrupts tuber formation, and delays tuber formation even under inducing environmental conditions.

The presence of 12-HSO₄JA in wild type potato leaves indicates the presence of a potato homologue of *AtST2a*. The search for the *AtST2a* potato homologue is presented in the next chapter of this thesis. It is interesting to note that OPDA, dn-OPDA and 11-OHJA levels were elevated in the transgenic lines when compared to wild type

plants. OPDA is a precursor of jasmonic acid and is reduced by 12-oxo-phytodienoic acid reductase (OPR3) to form dihydro-12-OPDA. On the other hand, dn-OPDA is a JA precursor in the hexadecanoid pathway (Weber et al; 1997). It is possible that elevated levels of 12-HSO₄JA are affecting the oxylipin signature of the plant, where levels of different oxylipin like compounds are altered. More studies will be required to understand why the accumulation of 12-OHJA sulfate leads to a reduction of JA and the accumulation of the precursors OPDA and dn-OPDA. It seems that the sulfated derivative blocks the conversion of OPDA to JA. This hypothesis has to be confirmed.

CHAPTER 4

REGULATION OF TUBER SPECIFIC GENES IN RESPONSE TO 12-HYDROXYJASMONATE AND THE IDENTIFICATION OF A *SOLANUM CHACOENSE* HOMOLOGUE OF *ARABIDOPSIS* SULFOTRANSFERASE 2A

Results

Tuber Specific Gene Regulation

While tuberonic acid has been identified as a tuber inducing compound *in vitro* (Koda et al 1991a), there has been no demonstration of the *in vivo* function of this compound in potato. Thus we set out to investigate what effects 12-OHJA has on the expression of several potato specific genes. Several genes were selected, and are as follows: *Actin*, *Patatin*, *POTLOX1*, *AOC* and *AOS*. The sequences of these genes were obtained from the National Center for Biotechnology (NCBI) database. Primers were designed to give PCR products of approximately 700 bp. The gene *Actin* was used as a control, since endogenous levels of actin transcript are constant and unaffected by jasmonate treatments (Gidda, 2001). Patatin is a potato storage protein glycoprotein, while lipoxygenase (*POTLOX1*), allene oxide cyclase (*AOC*), and allene oxide synthase (*AOS*) are all involved in jasmonic acid biosynthesis. Wild type potato plant shoots (with at least one leaf) were treated for zero to eight hours with 12-OHJA and methyl jasmonate (MeJA). RT-PCR was then performed, with the gene specific primers. The results show (Fig 4.1) that of the four genes selected (*Actin* being the fifth as a control), that *AOS* and *POTLOX1* are induced through time course treatment by 12-OHJA and

MeJA. In the case of *AOS* increase is discernable after two hours of treatment, while in the case of *POTLOX1* induction is discernable after four hours of treatment. Both genes are induced by 12-OHJA and MeJA.

Identification of a Potential Potato Homologue of *AtST2a*

Based on the accumulation of 12-OHJA sulfate in wild type potato, a homologue of *AtST2a* must be present in this plant. To find the homologue, the *AtST2a* nucleotide and protein sequences (Accession number: NM_120783) were aligned against The Institute for Genomic Research's (TIGR) *S. tuberosum* EST database using the BLAST tool. The six best putative genes were selected as candidates for the *AtST2a* homologue (refer to Table 4.1 for their percent identities). They were named as follows: TC77024, TC87873, TC85524, TC78605, TC85921, and TC88534. Since *AtST2a* expression is up regulated by MeJA and 12-OHJA (Gidda, 2001), the expression patterns of the six genes following 12-OHJA and MeJA treatments was studied. Primers were designed to give PCR products of approximately 450 bp for each gene. Figure 4.2 shows treatments for two hours and four hours with both 12-OHJA and MeJA. Of the six genes, TC77024 and TC87873 showed induction, while TC85921 gave constitutive expression. The remaining genes showed no induction.

AtST2a gene expression is also regulated by photoperiod. *AtST2a* mRNA could be detected in samples from plants that had been in the dark for more than 8 hours (Levitin, 2003). Since *AtST2a* is regulated by photoperiod, we would expect that a potato

homologue would also be subject to a similar regulation. The six genes were tested for expression in the dark with samples taken at various intervals (Fig 4.3). We can see from the RT-PCR results that TC77024 and TC87873 showed discernable induction in dark treated plants, particularly after longer dark treatments. TC85524 showed very faint induction throughout the treatment, suggesting that it might also be light regulated. However TC85524 did not show any induction following treatments with 12-OHJA and MeJA, whereas TC77024 and TC87873 showed both induction in the dark and with 12-OHJA and MeJA.

On the basis of induction upon treatment with 12-OHJA and MeJA, as well as induced expression in the dark, TC77024 and TC87873 were sub-cloned from pBluescript (clones provided by the Canadian Potato Genome Project) into the vector pGEM-T. The clones were then excised with restriction endonucleases and sub-cloned into the expression vector pQE30. Both TC77024 and TC87873 clones were sequenced to determine if they were indeed full length clones. Figure 4.4 shows the full length alignment of both TC77024 and TC87873 amino acid sequences against *AtST2a*. TC77024 has an open reading frame that codes for a 324 amino acid protein, while TC87873 has an open reading frame coding for a 335 amino acid protein.

Substrate Specificity

In order to determine if TC77204 or TC87873 is a homologue of *AtST2a*, we tested the two potato genes to see if one of them encodes a protein that would accept 12-OHJA as a substrate. The histidine tagged recombinant potato proteins were isolated

from *E.coli*. The nickel agarose-purified enzymes were used to test 12-OHJA as an acceptor molecule, using radio-labelled PAPS as a cofactor. However both enzymes did not accept 12-OHJA as a substrate. Figure 4.5 shows the recombinant protein fractions collected during the purification of the TC77204 and TC87873 proteins. Table 4.2 displays the counts per minute (CPM) obtained when the enzymes were assayed with 12-OHJA as substrate.

Discussion

The *in vitro* effects of tuberonic acid on potato and its ability to induce tuberization are well documented (Koda et al 1991a), however no potato study *in planta* has been performed before. We saw that treatment with 12-OHJA and MeJA induced the *AOS* and *POTLOX1* genes, while the genes *AOC* and *Patatin* were constitutively expressed. This suggests that 12-OHJA is involved in the induction of the jasmonate biosynthetic pathway in potato. Lipoxygenase has been demonstrated to be a key gene in the control of potato tuber development (Kolomiets et al; 2001), and its induction along with that of *AOS* indicates that 12-OHJA may possibly play a pivotal role in the formation of tubers through the induction of tuber specific genes.

The quantification of 12-HSO₄JA in potato leaf segments indicates the presence of a potato *AtST2a* homologue. Of the six potential candidates we identified, two seemed promising, TC77024 and TC87873, both of which showed 12-OHJA induction and dark period induction. However based on the results obtained from the substrate specificity assay, these two enzymes are not hydroxy-jasmonate sulfotransferases. They are however potato sulfotransferases, based on their sequence comparison with *AtST2a* and the presence of key conserved domains. Since the potato genome project is currently incomplete, it is entirely possible that there are other sulfotransferases of potato that have not been identified yet, and that one of these is the potato homologue of *AtST2a*.

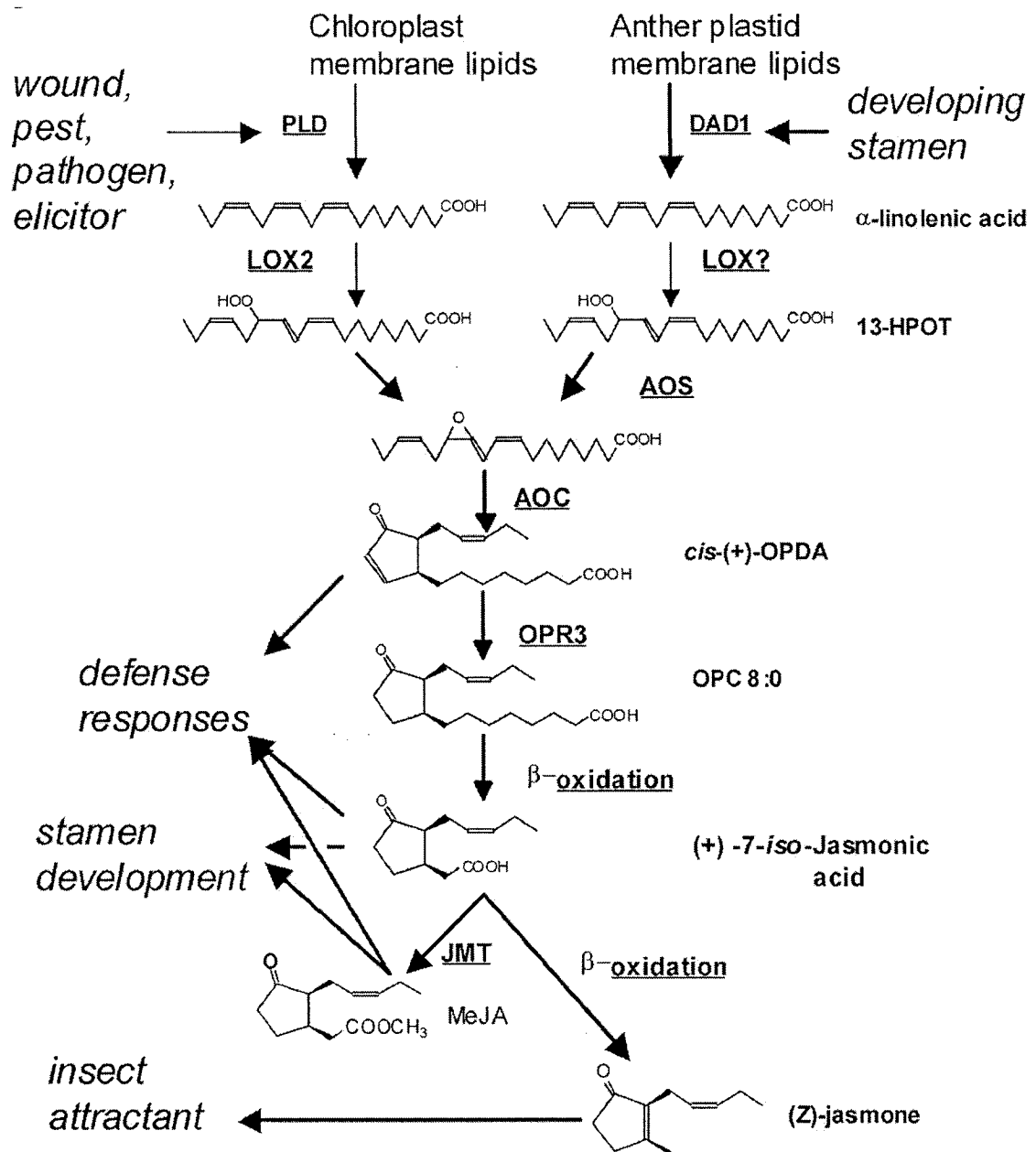


Figure 1.1 Model of Biosynthesis of Jasmonic Acid

Adapted from J. Turner (Turner et al; 2002)

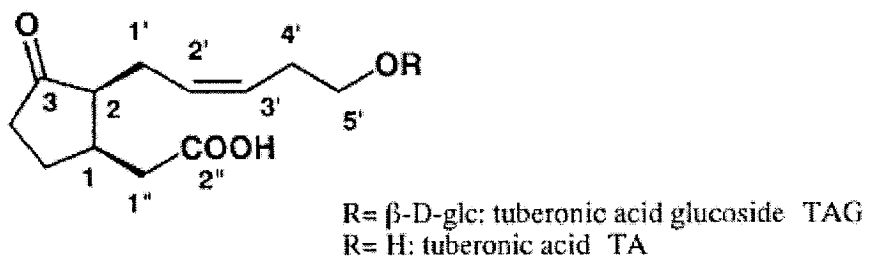


Figure 1.2 Structures of Tuberonic Acid (TA) and Tuberonic Acid Glucoside (TAG)

Adapted from H. Matsuura (Matsuura et al; 2000)

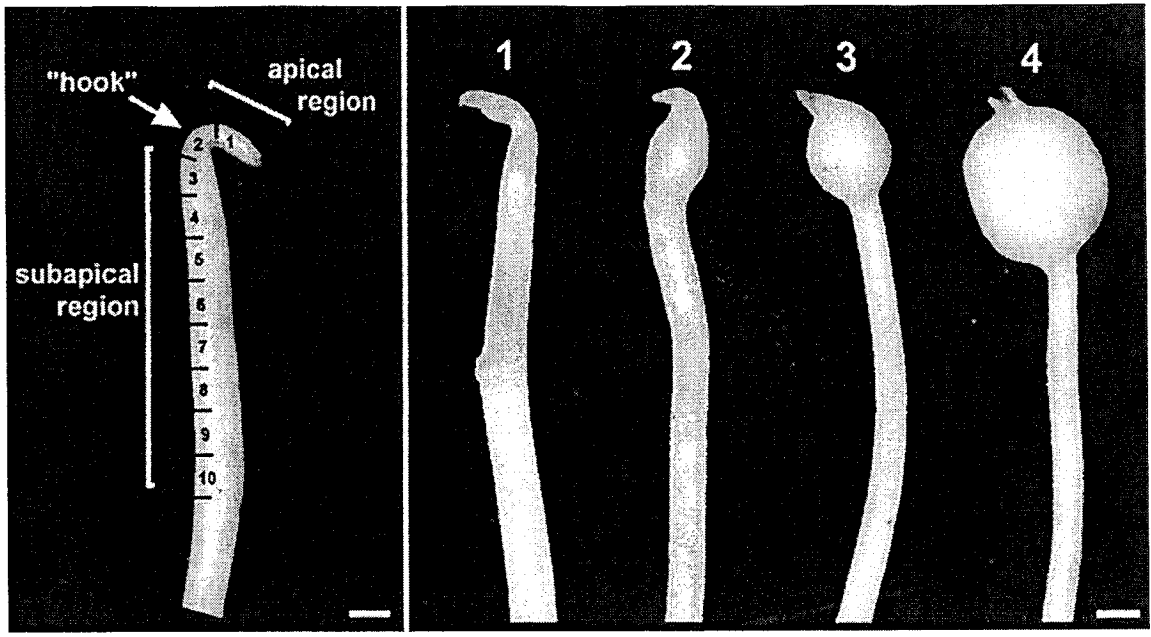


Figure 1.3 Stages of tuberization ranging from swelling to prominent subapical swelling.

Adapted from Viola et al; (2001).

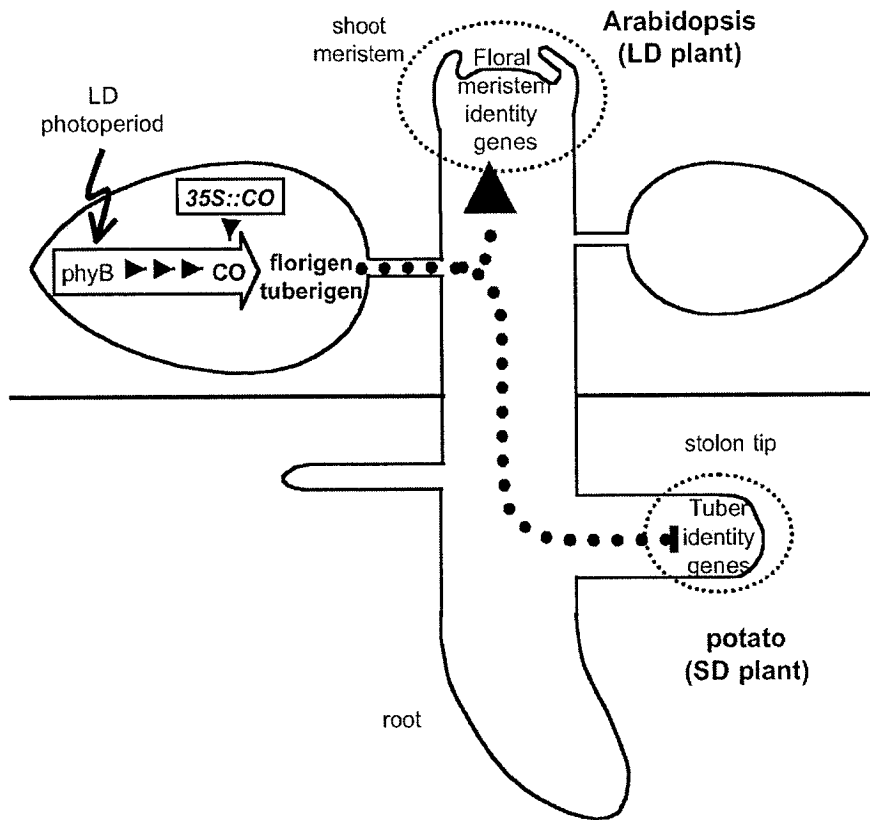


Figure 1.4 Model of the Action of *CONSTANS* in Photoperiodic Responses.

Adapted from J.F. Martínez-García (Martínez-García et al; 2002).

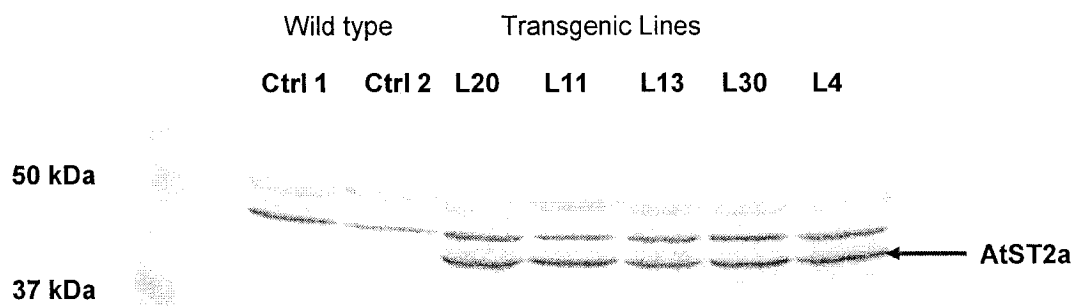


Figure 3.1

Western blot analysis of wild type control lines and transgenic lines, showing the expression of the AtST2a protein in the transgenic lines.

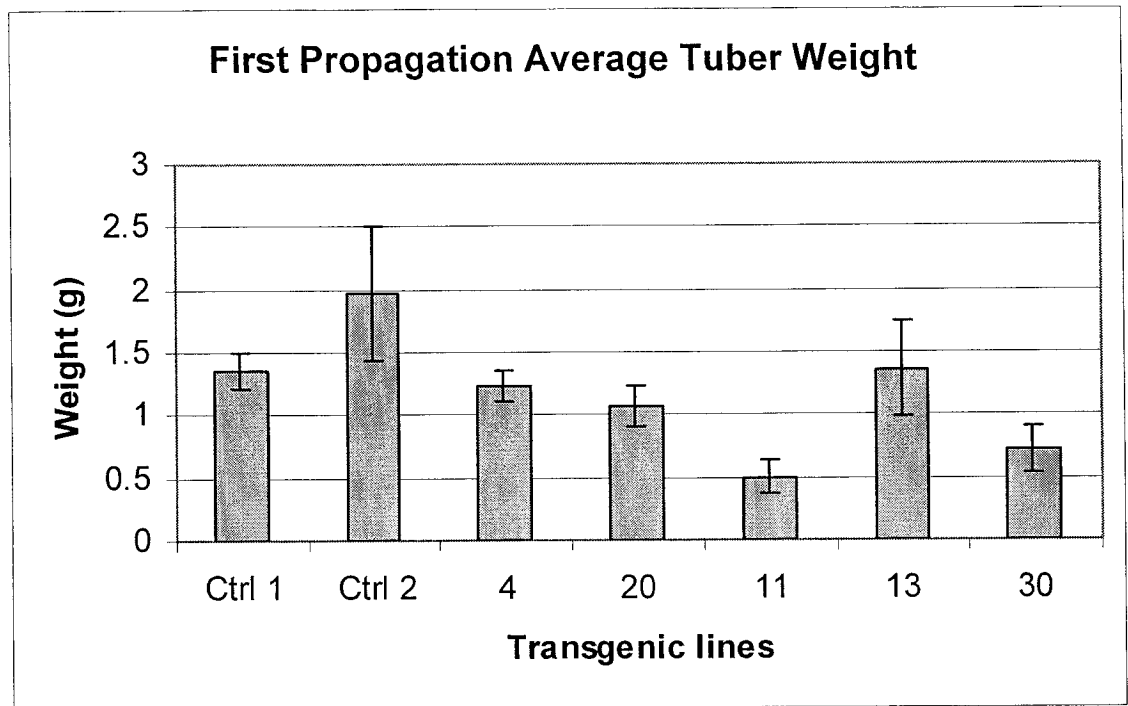


Figure 3.2

Graph of the average tuber weight per line for the first propagation of tubers. Wild type potato lines are represented by Ctrl 1 and 2 respectively, while the transgenic lines are numbered. Lines 11 & 30 show reduced weight phenotype as verified through one way ANOVA ($\alpha=0.05$) and t-tests. The sample size consisted of approximately 3-4 plants per line.

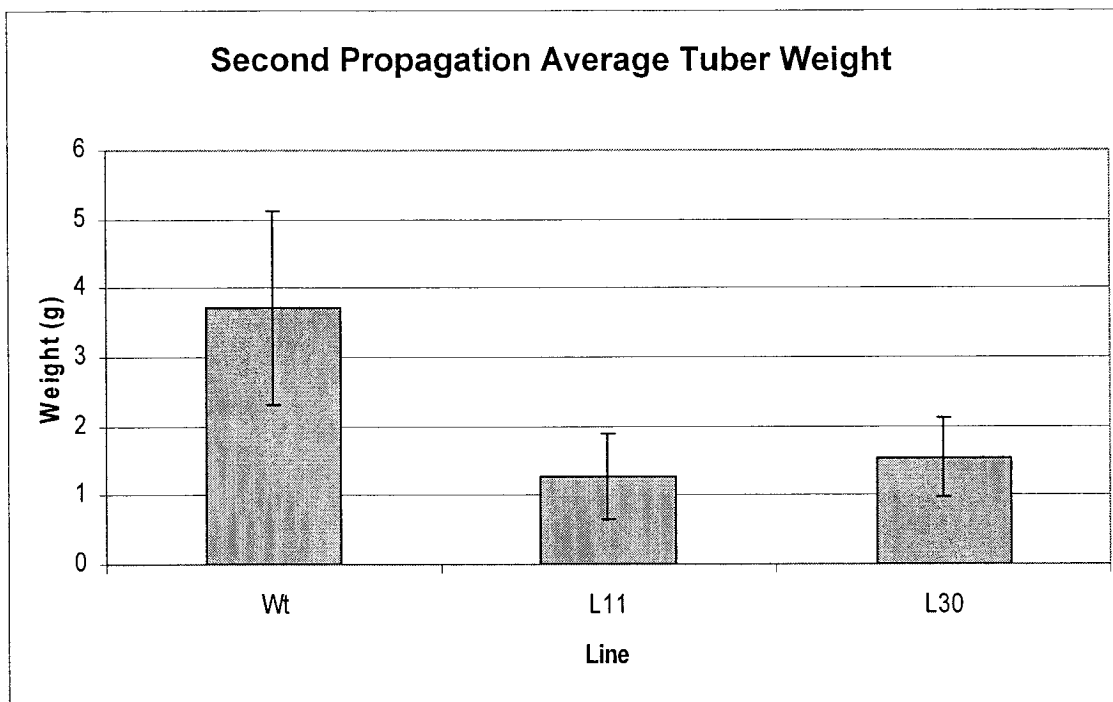


Figure 3.3

Graph of the second propagation of tubers grown with a larger sample set (9-18 plants per line). The results for the wild type was shown to be statistically different the results for the transgenic lines via one way ANOVA ($\alpha=0.05$).

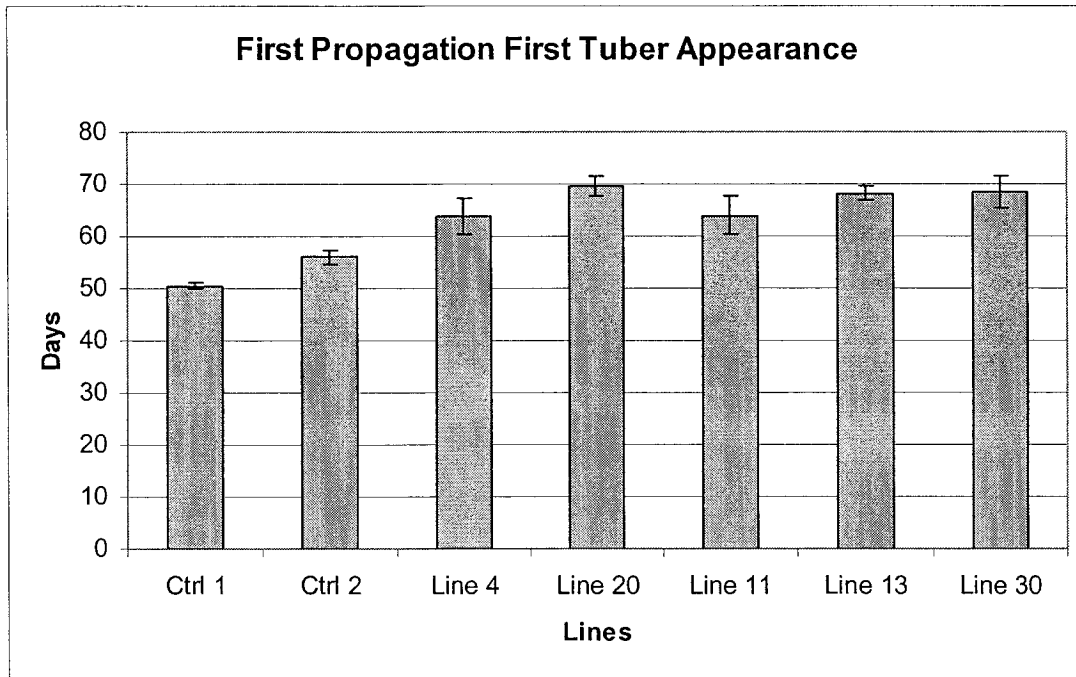


Figure 3.4

Graph depicting the first appearance of tubers in various potato plant lines showing a marked difference between control wild type plants and the transgenic lines, verified via one way ANOVA ($\alpha=0.05$).

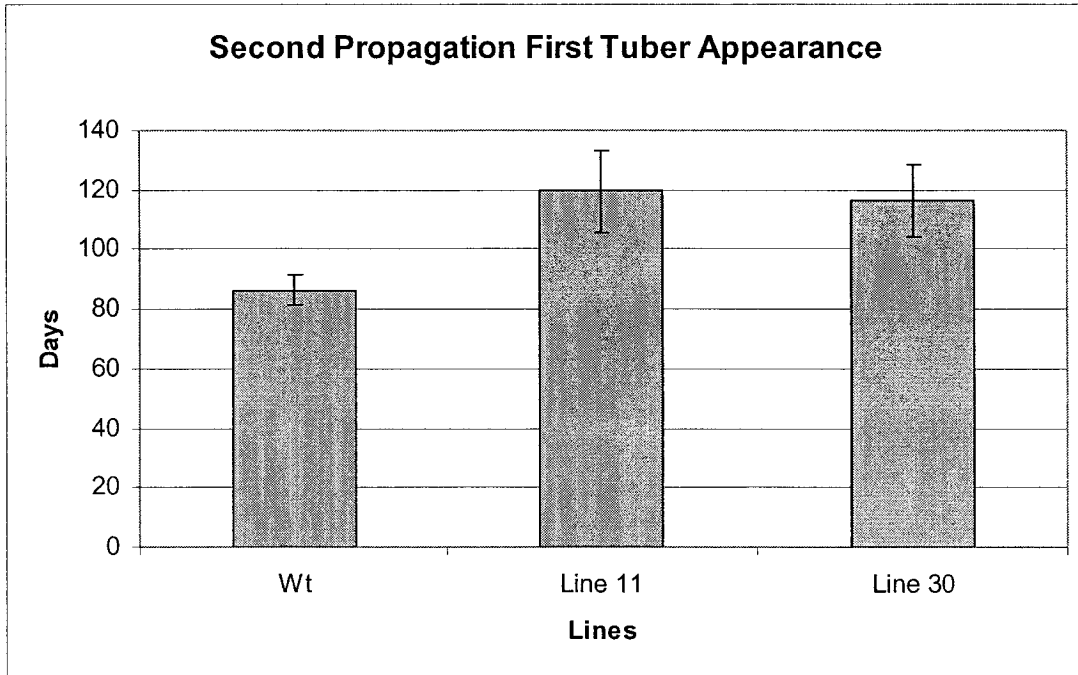


Figure 3.5

Graph showing the first appearance of tubers in the second propagation of plants with the transgenic lines forming tubers approximately 14 days after the wild type plants. Results were shown to be statistically significant via single factor ANOVA ($\alpha=0.05$).

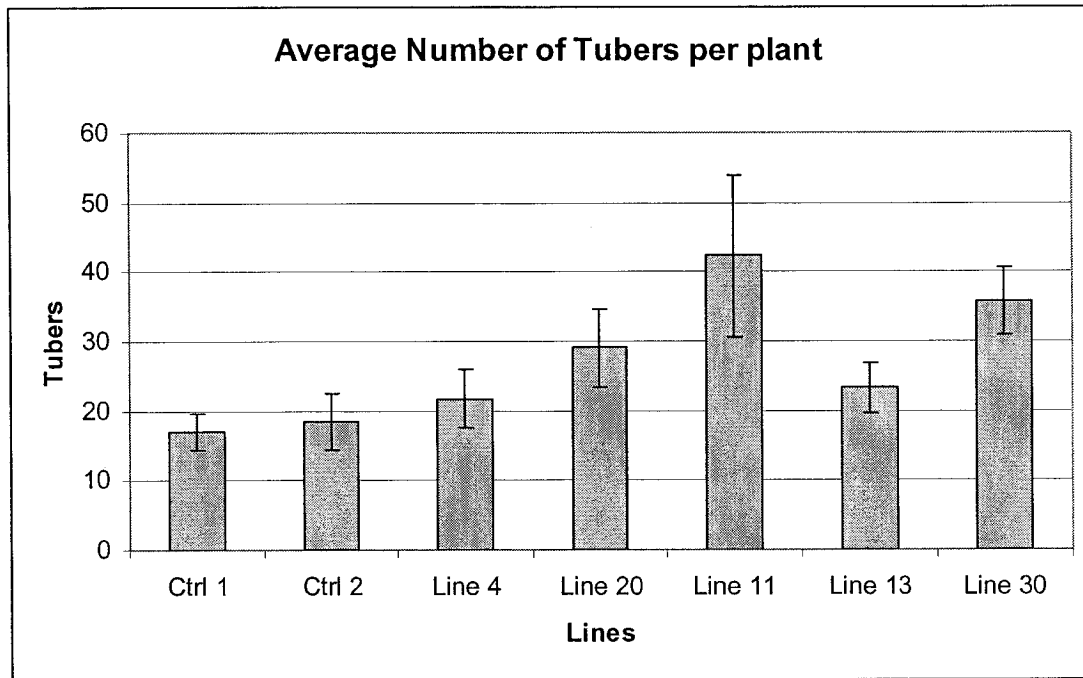


Figure 3.6

Average number of tubers per plant in the first propagation. Lines 20, 11 and 30 produced a significantly larger number of tubers relative to the wild type plants (represented as Ctrl 1 and 2 in the figure), as shown by single factor ANOVA ($\alpha=0.05$).

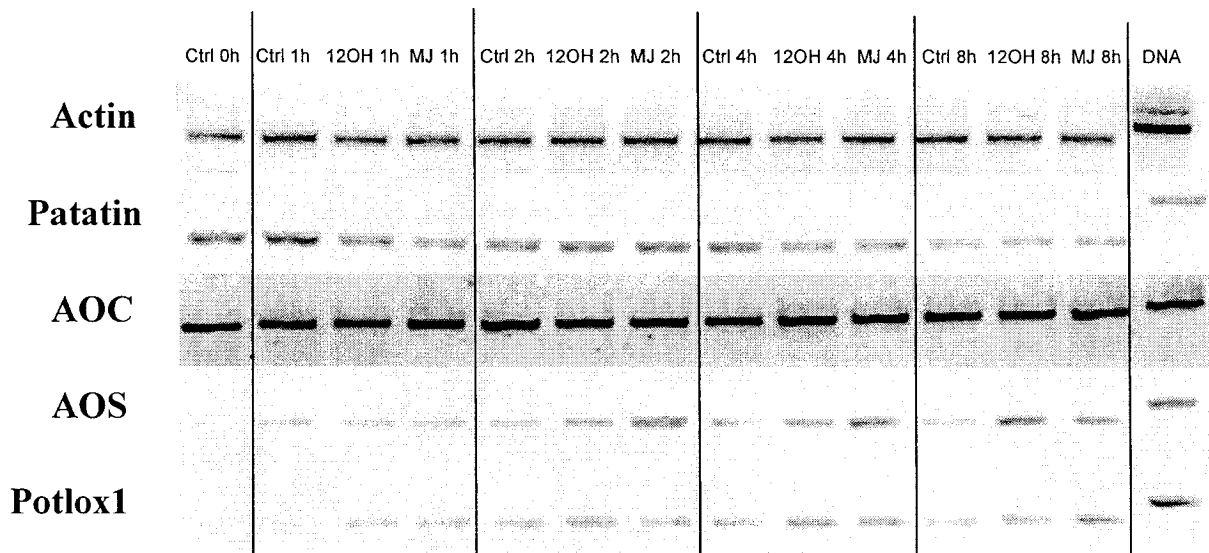


Figure 4.1

RT-PCR analysis of tuber specific gene expression in wild type plants, after treatment with 100 μ M 12-OHJA or 50 μ M MeJA over various time intervals, with *Actin* as an internal control. *Patatin* and *AOC* are expressed constitutively, while *AOS* and *POTLOXI* show induction with 12-OHJA and MeJA at later time points. Slight induction in *AOS* and *POLOXI* at later control time points can be attributed to jasmonate wound response. The controls for each time point are non treated shoots.

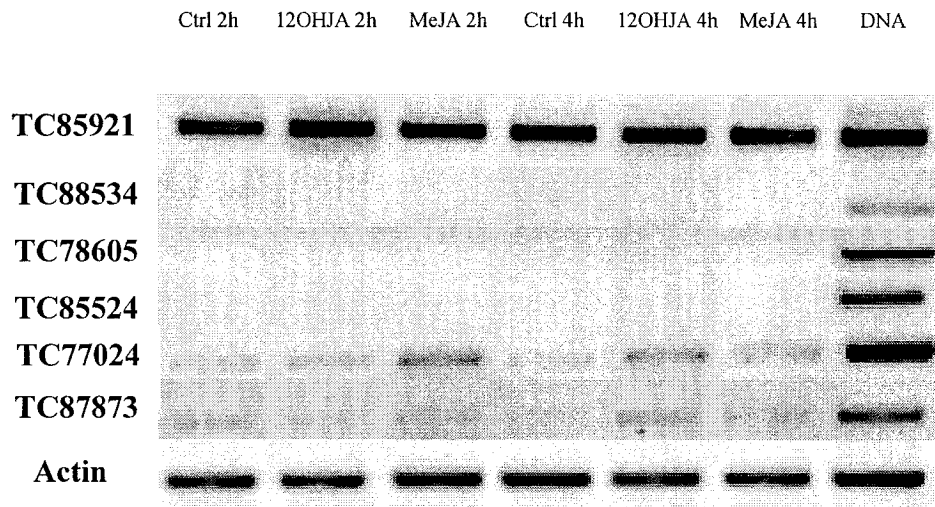


Figure 4.2

RT-PCR expression patterns of six potato putative ST genes after 2 hour and 4 hour treatments with 12-OHJA and MeJA. TC88534, TC78605, TC85524 showed no induction under treatments. TC85921 showed constant expression, while TC77024 & TC87873 showed induction under treatments.

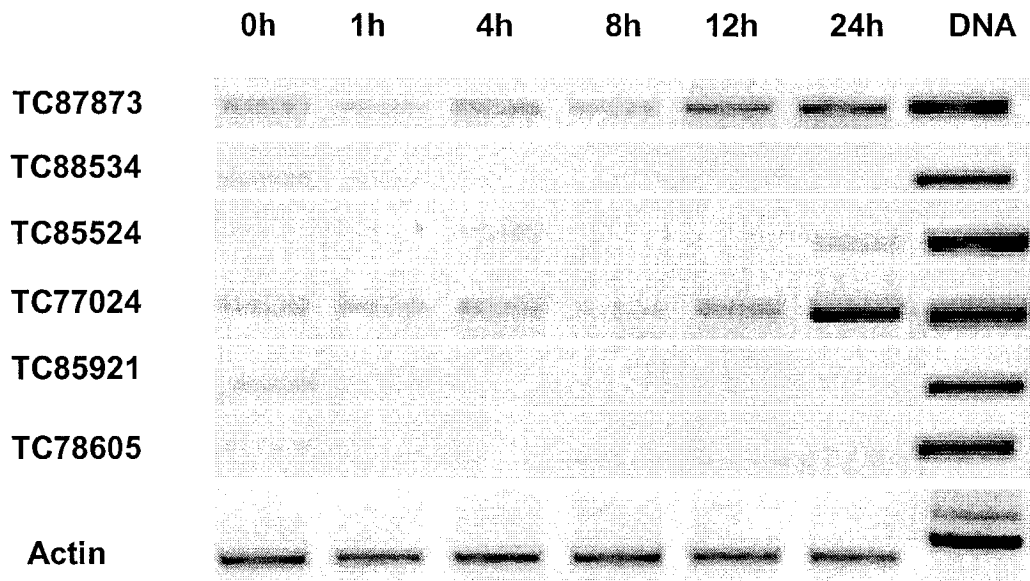


Figure 4.3

Dark Treatment expression patterns of the six potato genes. RT-PCR was conducted on dark treated samples ranging from one hour to twenty-four hours. Actin was used as an internal control. We can see that TC87873 and TC77024 show discernable induction while TC85524 shows faint induction in the dark.

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TC87873      MSKS--QTSPQIPPKY-----LQEDE-ITDECKKLLLTLPKERGWL 38
TC77024      MSKS--QTSPQIPPKY-----LQEDE-ISDECKKLLLTLPKERGWL 38
AtST2a       MATSSMKSI PMAIPSFMSCHKLELLKEGKTRDVPKAEEDGLSCEFQEMLDLSPKERGWR 60

TC87873      ASHMYNYQGFVWTPRIIQGVIAQCQHQFQAQSDIILVTPKSGTTLKSLFLVLN--RM 96
TC77024      TSHSYNYQGFWAIPRIIQAVMACQQQFQAQSDIILVTPKSGTTLKALLFGLVN--RV 96
AtST2a       TRYLYLFQGFWCQAKEIQAIMSFQKHQFQSLQSLNDVVLATIPKSGTTLKALTFTILNRHFR 120

TC87873      KYLVFEQNHPLLKPNPHDLIPFLEIIFYGDGLVPNFASFSTPRLLSTHMPFASLPKSVQD 156
TC77024      KHPIFEQNHPLLKPNPHDLVPPFELTLYVDGQVPDFSSFTTPRLLSTHLPFASLPKSVQD 156
AtST2a       DPVASSTNHPLFTSNPHDLVPPFQYKLYANGDVPDLGLASPRTFATHLPFGSLKETIEK 180

TC87873      SKTKLVYLCRNPKDTFISLWHFTNNLRLHKKDIHPIEEKIESFCMGVSLYGPFWNHVLDY 216
TC77024      SKTKLVYLCRNPKDTFISMWHFANNLMLHKKETKSIEDLDFRCKGISVYGPFDHVLVDY 216
AtST2a       PGVKVYLCRNPFDTFISSWHYTNNIKSESVSPVLLDQAFDLYCRGVIIGFPFWEHMLGY 240

TC87873      WKQSIKPNKVLFLMYEEIKVQPKLHLKRLAEFLECPFSIEEENCGVMDEILRMCSFENL 276
TC77024      WKQSIENPN-----EQPKMHLKRLAEFLECPFSIKEENCGVVDEILRMSFKNL 265
AtST2a       WRSLKRPEKVFFLRYEDLKDDIETNLKRLATFLELPFTEEEERKGVVKAIAELCSFENL 300

TC87873      SNLEVNANGKFSTGEENKAFERRKGEVGDWKNYFTTEMSEKLNHIIEQKFQSGIKFSYI 335
TC77024      SNLEVNNTGKLSSTGEENKAFERRRGEIGDWRNYFTTEMSEKLNHIIEQKFQSGIKFSYI 324
AtST2a       KKLEVNKSNKSIKNFENRFLERRKGEVSDWVNYLSPSQVERLSALVDDKLGSGLTFRLS 359

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Figure 4.4

Amino acid sequence alignment of AtST2a, TC77024 and TC87873, using CLUSTALW

1.8. The boxes indicate the conserved N-terminal portion (YPKSG(T/N)W) of the ST and the conserved C-terminal portion (RK(G/A)XXGDWK(N/T)XFT) which are involved in co-substrate binding and catalysis.

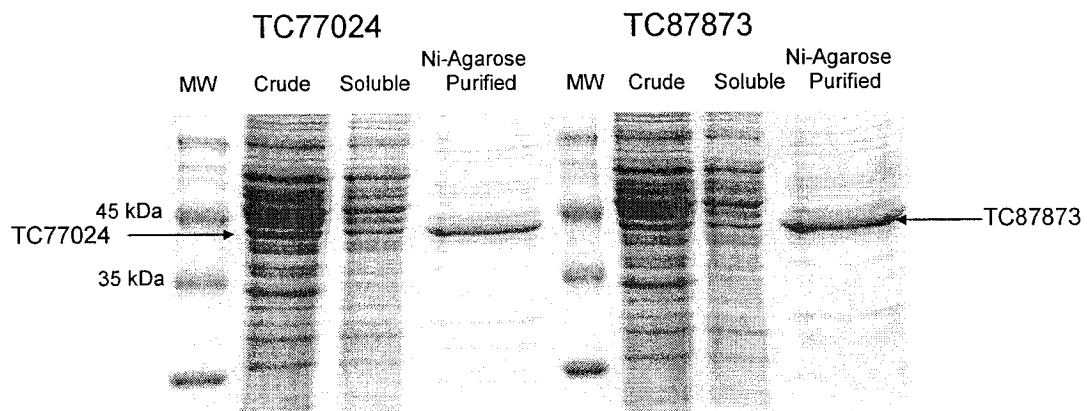


Figure 4.5

SDS-PAGE analysis of fractions collected during the purification of recombinant TC77024 and TC87873.

Table 3.1

Quantification of OPDA, dn-OPDA, JA, 12-OHJA, 12-HSO₄JA, 12-O-Gluc-JA, and 11-OHJA in *Solanum chacoense* wild type leaves as compared to transgenic lines over-expressing sense orientation *AtST2a*

Lines	Compounds (pmol/g fr. wt.)						
	<u>OPDA</u>	<u>dn- OPDA</u>	<u>JA</u>	<u>12-OH- JA</u>	<u>11-OH- JA</u>	<u>12-HSO₄-JA</u>	<u>12-O-Gluc- JA</u>
<u>Wild type</u>	356 ± 21	61 ± 3	268 ± 11	1816 ± 41	225 ± 12	10555 ± 252	8780 ± 250
<u>Line 11</u>	579 ± 44	120 ± 15	17 ± 3	1063 ± 48	963 ± 52	37212 ± 1298	10233 ± 913
<u>Line 30</u>	828 ± 81	303 ± 38	50 ± 9	777 ± 40	622 ± 35	27876 ± 1425	8461 ± 568

Note: Values represent the average of three samples from each line.

Table 4.1

Percent Amino Acid Sequence Identity of *AtST2a* and the Six Potato ST genes

	AtST2a	TC77024	TC87873	TC88534	TC85921	TC85524	TC78605
AtST2a	100%	47%	45%	37%	39%	31%	29%
TC77024	47%	100%	79%	74%	44%	28%	0%
TC87873	45%	79%	100%	74%	38%	0%	0%
TC88534	37%	74%	74%	100%	35%	0%	0%
TC85921	39%	44%	38%	35%	100%	33%	0%
TC85524	31%	28%	0%	0%	33%	100%	0%
TC78605	29%	0%	0%	0%	0%	0%	100%

TC77024 and TC87873 show the most percent identity to *AtST2a* at 47% and 45% respectively.

Table 4.2

Radioactive Counts per Minute Obtained for Substrate Specificity with 12-OHJA (1 μ M)

	CPM
TC77024	77.4
TC87873	118.4
AtST2a	7725.2
No Enzyme	148.5
TC77024 no PAPS	22.8
TC87873 no PAPS	15.6
AtST2a no PAPS	14.5
PAPS	266343.1

Note: We can see that AtST2a shows significant activity with its acceptor molecule 12-OHJA. TC77024 and TC87873 however do not show any significant activity with 12-OHJA as a substrate. This table shows the data for 1 μ M of 12-OHJA, similar results were obtained for 10 and 100 μ M of substrate.

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