AUGMENTATION DE LA RÉSISTANCE À LA GALE COMMUNE CHEZ DES VARIÉTÉS DE POMMES DE TERRE PRODUITES AU QUÉBEC

par

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INCREASING RESISTANCE TO COMMON SCAB IN POTATO VARIETIES CULTIVATED IN QUEBEC

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SOMMAIRE

La gale commune est une maladie de la pomme de terre (*Solanum tuberosum* L.) d'importance mondiale qui réduit la qualité des tubercules et diminue leur valeur marchande. Cette maladie caractérisée par la formation de lésions superficielles, profondes ou surélevées sur la surface du tubercule est causée par la bactérie *Streptomyces scabies* (syn. *scabiei*). *S. scabies* synthétise une toxine, la thaxtomine A, qui est essentielle pour le développement des symptômes de la maladie. La meilleure approche pour la gestion de la gale commune est le développement de variétés de pomme de terre résistantes. Cependant, les facteurs qui contribuent à la résistance à la gale commune ne sont pas bien connus. L'augmentation de la résistance à la thaxtomine A peut être un moyen d'augmenter la résistance à la maladie.

L'utilisation de la thaxtomine A comme agent de sélection directe des cellules somatiques a permis la production de variétés de pomme de terre ayant une plus grande résistance à la gale commune. Dans ce projet, nous avons utilisé une stratégie basée sur l'adaptation progressive des cals de pomme de terre à la thaxtomine A suivie de la régénération de plantules à partir d'embryons somatiques. Nous avons ainsi adapté à la thaxtomine A plusieurs variétés de pommes de terre cultivées ou nouvellement sélectionnées au Canada tel que Kennebec, Envol, Chieftain, Rubiconde et Belle d'Août. Au total, 92 variants somaclonaux adaptés à la thaxtomine A ont été produits après l'ajustement des conditions de régénération. Des somaclones sélectionnés au hasard ont été testés pour leur résistance à la gale. Pour une première étude de la résistance à la gale commune, nous avons développé une nouvelle méthode basée sur l'infection de mini tubercules de pomme de terre se développant à partir de bourgeons foliaires. L'augmentation de la résistance des somaclones sélectionnés à l'aide de ce test a été confirmée par l'infection de plantes en pots.

En second lieu, l'adaptation de la varieté de pomme de terre Russet Burbank à la thaxtomine A (0,5 µM) a produit le variant somaclonal RB9, qui possède une résistance augmentée à la gale commune. L'analyse protéomique a montré qu'une amélioration de la résistance de RB9 à la gale commune est associée à des modifications du protéome du tubercule. Les changements comprenaient une accumulation accrue des protéines principales du tubercule, telles que les patatines, les lipoxygénases et les inhibiteurs des protéases (type Kunitz). L'abondance de certaines protéines associées au stress oxydatif a également été modifiée. Nous avons constaté que l'infection par *S. scabies* provoque des changements dans l'abondance des patatines, des lipoxygénases et des inhibiteurs de protéase de type Kunitz. Ces protéines ont tendance à s'accumuler pendant les premiers stades de l'infection dans les tubercules de Russet Burbank et de RB9. Les tubercules de RB9 ont montré une accumulation constante des lipoxygénases en réponse à *S. scabies* durant toute la période d'infection, alors que dans les tubercules de la variété d'origine, l'infection a provoqué l'accumulation des lipoxygénases uniquement dans les premiers jours de l'infection. De plus, les jeunes tubercules de RB9 avaient un périderme renforcé, avec plus de couches des cellules suberisées et une morphologie cellulaire modifiée par rapport aux tubercules de la variété d'origine. Ainsi, l'amélioration de la résistance à la gale commune provoquée par l'adaptation à la thaxtomine A a été associée à des changements protéomiques et morphologiques.

La thaxtomine A est un facteur essentiel pour le développement des symptômes de la gale commune. Lorsqu'elle est appliquée sur des tranches de tubercules, elle provoque un brunissement de la chair des tubercules. Une coloration au Bleu de toluidine O des tranches de chair de tubercule traitées à la thaxtomine A a ici permis d'observer une coloration bleue typique des composés phénoliques. Par contre, la superficie du brunissement ne reflètait pas la résistance des variétés Russet Burbank et Yukon Gold à la gale commune, mais correspondait plutôt au niveau d'accumulation des phénols. Le traitement avec la thaxtomine A a provoqué la mort des cellules du parenchyme des

tubercules des deux variétés. Cependant, le degré de mort cellulaire, déterminé par une coloration au bleu d'Evans, n'était pas corrélée avec la superficie du brunissement. L'application d'acide 2-aminoindane-2-phosphonique, un inhibiteur compétitif de la phénylalanine-ammonia lyase, simultanément avec la thaxtomine A a réduit le développement du brunissement sur les tranches de pomme de terre. Ainsi, le brunissement des tranches de tubercule induit par le traitement à la thaxtomine A était provoqué, du moins en partie, par l'accumulation de composés phénoliques dans les cellules de la chair du tubercule.

Les résultats obtenus dans le cadre de ce projet permettent de mieux comprendre l'interaction entre des tubercules de pomme de terre et *S. scabies*. Ils mettent en évidence des facteurs protéiques qui pourraient être impliqués dans la résistance de la pomme de terre à la gale commune.

Mots clés : adaptation, gale commune, inhibiteurs des protéases, lipoxygénase, patatine, thaxtomine A, phénols, pomme de terre, protéomique, *Streptomyces scabies*.

ABSTRACT

Common scab is a globally important disease of potato (*Solanum tuberosum* L.) that reduces the quality of tubers and decreases their market value. This disease is caused by the bacterium *Streptomyces scabies* (syn. *scabiei*) which induce the formation of superficial, pitted or raised lesions on the tuber surface. *S. scabies* synthesizes a toxin, thaxtomin A, which is essential for the development of disease symptoms. The best approach for common scab management is the development of resistant potato varieties. However, little is known about the factors that contribute to common scab resistance. Increasing resistance to thaxtomin A may be a way to increase resistance to the disease.

The use of thaxtomin A as an agent for direct somatic cell selection had allowed the production of potato varieties with increased tolerance to common scab. The strategy used in this project was based on a progressive adaptation of potato calli to thaxtomin A before regeneration of plantlets from somatic embryos. We successfully adapted several potato varieties widely used or newly selected in Canada: Kennebec, Envol, Chieftain, Rubiconde and Belle d'Août. A total of 92 somaclonal variants adapted to thaxtomin A were produced after adjusting regeneration conditions. Randomly selected somaclones were tested for scab resistance. For an initial study of scab resistance, we developed a new method based on the infection of mini potato tubers developing from leaf buds. The increased resistance of the somaclones selected using this test was confirmed with *S. scabies* infection test *in planta*.

The adaptation of potato variety Russet Burbank to thaxtomin A (0.5 μM) had resulted in the production of a somaclonal variant, RB9, which had increased resistance to common scab. Here, LS-MS/MS proteomic analysis of soluble tuber proteins showed

that improved RB9 resistance to common scab was associated with changes in the tuber proteome. Changes included increased accumulation of major tuber proteins, such as patatins, lipoxygenases and serine protease inhibitors (Kunitz-type). The abundance of certain proteins associated with oxidative stress was also altered. We found *S. scabies* infection to cause changes in the abundance of patatins, lipoxygenases, and Kunitz-type protease inhibitors. These proteins tended to accumulate in the initial stages of infection in both Russet Burbank and RB9 tubers. The RB9 tubers showed a constant accumulation of lipoxygenases in response to *S. scabies* during the whole period of infection, while in tubers of the original variety, infection caused the accumulation of lipoxygenases only in the first days of infection. Moreover, young RB9 potato tubers had a reinforced periderm, with more periderm layers and an altered cell morphology compared to the Russet Burbank tubers. Thus, the improvement in scab resistance caused by adaptation to thaxtomin A was associated with both proteomic and morphological changes.

Thaxtomin A is an essential factor for the development of common scab symptoms. When applied to tuber slices, it causes browning of the tuber flesh. Toluidine blue O staining of tuber flesh sections treated with thaxtomin A here resulted in a blue coloration which is characteristic of phenolic compounds. The extent of the browning did not reflect the common scab resistance of Russet Burbank and Yukon Gold varieties but it corresponded instead to the level of phenols accumulation. Treatment with thaxtomin A caused the death of tuber parenchyma cells of both varieties. However, the degree of cell death, determined by staining with Evans blue, did not correlate with the extent of browning. The application of 2-aminoindan-2-phosphonic acid, a competitive inhibitor of phenylalanine-ammonia lyase, simultaneously with the thaxtomin A reduced the development of browning on potato slices. Thus, the darkening of the tuber slices caused by thaxtomin A treatment was caused at least in part by the accumulation of phenolic compounds in the tuber flesh cells.

Results obtained in the course of this project allow a better understanding of the interaction between potato tubers and pathogenic *S. scabies*. They also reveal the occurrence of protein factors that could be involved in potato resistance to common scab.

Keyword: adaptation, common scab, lipoxygenase, patatin, phenols, potato, protease inhibitors, proteomics, *Streptomyces scabies*, thaxtomin A.

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LISTS OF ABBREVIATIONS

Abbreviation	Full name
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ADP	Adenosine diphosphate
AIP	2-Aminoindan-2-phosphonic acid
AMVRT	Avian myeloblastosis virus reverse transcriptase
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ATP	Adenosine triphosphate
BAP	6-Benzylaminopurine
C4H	Cinnamate 4-hydroxylase
C4L	4 Coumarate:CoA ligase
cDNA	complementary DNA
CI	Callus induction media
CR	Callus regeneration media
CLR	Colonisation region
Cv	Cultivar
DES	Divinyl ether synthase
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDS1/PAD4	Enhanced disease susceptibility1/ phytoalexin deficient4
EH1	Epoxide hydrolase 1
GA_3	Gibberellic acid
GDP	Guanosine diphosphate
HCT	Hydroxycinnamoyl-Coenzyme A shikimate:quinate hydroxycinnamoyl-transferase
HRL	Hydroperoxide lyase

IAA	Indole-3-acetic acid
JA	Jasmonic acid
KTI	Kunitz-type inhibitor
LAH	Lipid acyl hydrolase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Lox	Lipoxygenase
Met, MetOH	Methanol
mRNA	Messenger RNA
NAA	α-Naphtaleneacetic acid
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PAI	Pathogenicity island
PAL	Phenylalanine ammonia-lyase
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Protease inhibitor(s)
PLA_2	Phospholipase A2
PPO	Polyphenol oxidase
qPCR	Quantitative PCR
RB	Russet Burbank
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TA	Thaxtomin A
TR	Toxicogenic region
YG	Yukon Gold
YME	Yeast malt extract medium
Zea	trans-Zeatin riboside

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CHAPTER 1
GENERAL INTRODUCTION

1.1 Potato horticulture and nutritional value

Potato is the most important tuber crop in the world and the third among the staple crops after wheat and rice (Birch et al., 2012). The total world potato production was estimated at 388,191,000 tonnes in 2017 (Statista, 2017). Potato horticulture is one of Canada's most important agricultural sectors. In Canada, potato crops were planted on an area of 141,300 hectares in 2018 and 145,910 hectares in 2019, with an average yield of 43,174.21 t/ha in 2018 (Statistics Canada, 2019).

Potato is a highly nutritious food rich in carbohydrates. Freshly harvested potato tubers contain about 77 percent water and 23 percent dry matter. About 60 to 80 percent of the dry matter is starch. The dry matter is also rich in protein and low in fat, which is comparable to cereals. Potato tubers are rich in several nutrients, including vitamins C, B1, B3 and B6 and minerals such as potassium, phosphorus and magnesium; it also contains folate, pantothenic acid and riboflavin (Figure 1.1 A).

1.2 Potato biology

Potato is an herbaceous perennial plant of the *Solanaceae* family with compound leaves. The potato plant can reach 60-100 cm in height. It is mostly cross-pollinated and produces green fruits that can contain about 300 seeds each. All above ground parts of potato plants contain the toxic alkaloids α-chaconine α-solanine. Glycoalkaloids are usually present at low levels in underground parts as potato tubers. However, they can accumulate to high levels in greened, stored and damaged tubers (Friedman and Dao, 1992).

Sugars, which are primarily manufactured in leaves, are transferred and deposited in the underground stems, called stolons, in the form of starch. With the influx of nutrients, the tips of the stolons swell, forming new tubers near the surface of the soil. At the end of the growing season, leaves and stems die and produced tubers detach from stolons. Tubers are the vegetative reproductive organs of potato plants that serve as reservoirs of nutrients, allowing the plant to survive adverse conditions in the state of dormancy. Each tuber contains buds (eyes), which are arranged in a spiral pattern. When conditions become favorable, buds sprout into shoots to generate a new plant (Vreugdenhil et al., 2007).

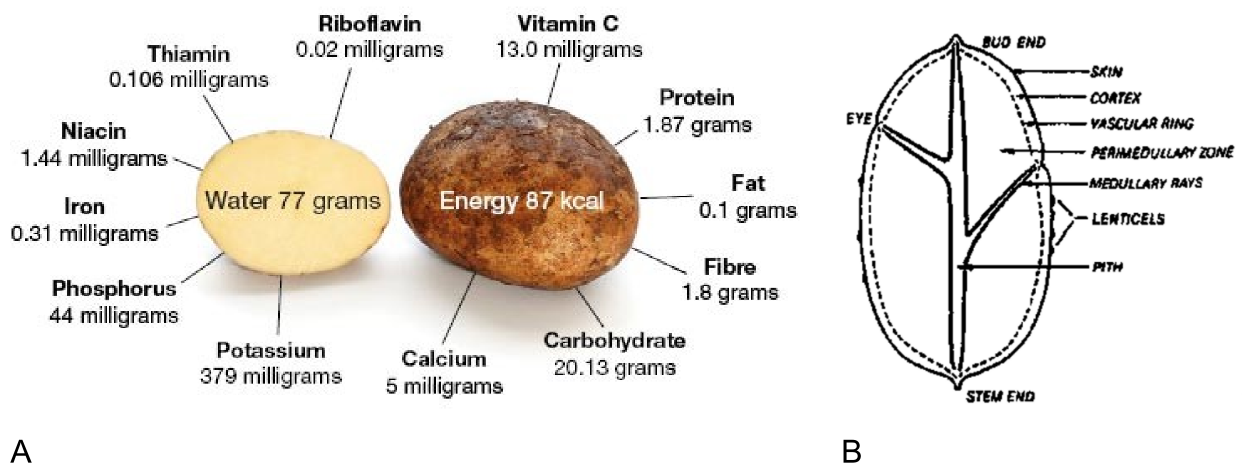

Figure 1.1. Nutritional value and morphology of the potato tuber.

A. Nutrient content of potato per 100 g of tuber after boiling in skin and peeling (FAO, 2008); B. Diagram of a longitudinal section of a potato tuber (Rastovski and van Es, 1981).

Potato tubers are highly variable in shape and colour. Tuber shapes are of four types: compressed, round, oval and long. Potato tuber skin varies in texture (russet, smooth, flaky, netted or rough) and in colour, from light yellow to pink or purple (CFIA; Custers, 2015). At the proximal end, the tuber is attached to the stolon, while the bud is located

at its distal end. Morphologically, a tuber consists of several distinct zones: the periderm (skin), the parenchyma, the ring of vascular bundles (xylem) and the medullar rays and medulla, also known as the pith. On average, the tuber periderm consists of 6-10 suberized cell layers. Lenticels or natural openings in the tuber periderm are formed by a circular group of suberized cells which are necessary for gas exchange, since the skin is almost impermeable to CO_2 or O_2. Buds or eyes are also present on the periderm surface as noted above. The tuber parenchyma is made of cortex cells and the perimedullary zone; it represents the main tissue of the tuber, as it contains reserve material in the form of starch and other nutrients (Figure 1.1 B) (Rastovski and van Es, 1981).

1.2.1 Major tuber proteins – content and functions

Tubers are a storage sink of potato, accumulating high amounts of carbohydrates mainly in the form of starch and significant amounts of reserve proteins (Fernie and Willmitzer, 2001). The main role of reserve proteins is to store nitrogen, carbon and sulfur in order to enable the plant’s survival under adverse conditions between the growing periods (Shewry, 2003). The soluble fraction of potato tuber protein predominantly consists of patatin and different classes of protease inhibitors (Weeda et al., 2009). Other major proteins of potato tubers are the lipoxygenases, annexin, glyoxalase I and enolase (Bauw et al., 2006).

1.2.1.1 Patatin

Patatin is the major storage protein of potato tuber and constitutes up to 40% of total soluble protein (Paiva et al., 1983). Patatin isoforms are part of a wide family of glycoproteins presenting a molecular weight of about 40 kDa. They have been shown to be localised in vacuoles of tuber cells (Paiva et al., 1983; Sonnewald et al., 1989). The coding sequences of patatins are conventionally divided into two classes (Class I

and Class II), which are represented in the potato genome in approximately equal parts but that show distinct expression patterns. Class 1 patatins are mainly expressed in potato tubers and, to a small extent in roots, while Class 2 patatins are mainly expressed in roots and, to a lesser extent (50-100 fold), in tubers (Mignery et al., 1988).

A characteristic feature of patatins is their enzymatic activities, which indicates that amino acid storage is not their only function (Mignery et al., 1988). One of these activities is their function as lipoacyl hydrolases, more precisely as phospholipid and lysophospholipid hydrolases (Senda et al., 1996; Hirschberg et al., 2001). Since it was shown that patatins were able to efficiently cleave fatty acids from various membrane lipids, it was suggested that they could participate in the wound response by providing a substrate for the synthesis of suberin and cytotoxic waxes, as well as secreting arachidonic acid, a potential phytoalexin elicitor (Bostock 1981; Mignery et al., 1988). Another type of catalytic activity reported for patatins is their esterase activity against PNP-laurate, PNC-acetate, α-naphthylaurate, α-naphthyl acetate, β-naphthyl acetate and phenyl acetate substrates (Racusen, 1986). Patatins also show hydrolase activity against β-1,3-glucans, suggesting their involvement in the pathogen response (Tonón et al., 2001). It has been proposed that β-1,3-glucanases play a role in protecting plants against fungal pathogens by cleaving β-1,3-glycans in the cell walls of fungal hyphae, thereby forming a pathogenesis-related protein response (Shewry and Lucas, 1997; van Loon and van Strien, 1999). The direct inhibitory activity of patatin has been shown against larvae of the corn root worm and the spore germination of fungal pathogen *Phytophthora infestans* (Strickland et al., 1995; Sharma et al., 2004; Bártová et al., 2019).

1.2.1.2 Protease inhibitors

Protease inhibitors (PI) are the most abundant tuber proteins, comprising up to 50% of the soluble proteins in tubers (Pouvreau et al., 2001; Schoenbeck et al., 2013). The

most abundant of these proteins are the Kunitz-type protease inhibitors (KTI), Protease inhibitors I and II (PI I and II) and Bowman-Birk trypsin/chymotrypsin inhibitors. PI are small proteins that are abundant in reproductive and storage organs, such as seeds and tubers (Fisher et al., 2015) where they are involved in a variety of protease-targeting processes. One of the most abundant tuber proteins is Protease inhibitor 2 (PI-II), which can reach up to 12% of total soluble tuber proteins (Pouvreau et al., 2001). In leaves, PI-II is expressed upon wounding, to inhibit insect gut proteases and increase resistance to insect pests (Graham et al., 1985; Johnson et al., 1989; Tamhane et al., 2009; Dunse et al., 2010). Bowman–Birk inhibitors have been shown to prevent early germination and act in plant immunity as their overexpression increases resistance to fungal pathogens (Qu et al., 2003). Potato multicystatin (a multidomain cysteine protease inhibitor), which regulates storage protein accumulation and mobilisation (Kumar et al., 1999), can act in plant defense by inhibiting proteases from insect digestive tracts, or suppressing the growth of pathogenic fungi (Orr et al., 1994; Siqueira-Junior et al., 2002).

1.2.1.2.1 Kunitz-type protease inhibitors

The most abundant fraction of protease inhibitors, reaching up to 22% of the soluble tuber proteins, are the Kunitz-type protease inhibitors (KTI) (Pouvreau et al., 2003). KTI are small proteins of about 17-24 kDa exhibiting a large sequence variability (Khalf et al., 2010; Fischer et al., 2015). KTI target various types of proteases, such as α-amylase, subtilisin-like protease, papain-like Cys proteases, trypsin, chymotrypsin and cathepsin D (Grosse-Holz and van der Hoorn, 2016). One of KTIs' functions is the regulation of germination as a result of inhibition of α-amylase which mobilizes storage carbohydrates during germination (Abdul-Hussain and Paulsen, 1989; Vallée et al., 1998). KTI are also involved in the defense response to pathogens (Fisher et al., 2015; Grosse-Holz and van der Hoorn, 2016). The functions of KTI have been well elucidated in relation to the inhibition of digestive proteases from the gut of insects and arachnids

(Arnaiz et al., 2018; Mendonça et al., 2019; Schlüter et al., 2010). It has also been shown that KTI can affect the susceptibility of plants to bacterial pathogens *Pseudomonas syringae* pv. *tomato* and *Erwinia carotovora* subsp. *carotovora* by controlling plant programmed cell death (PCD) during bacterial attack (Li et al., 2008).

In potato, KTI family genes are localised together on chromosome III at one complex locus which is linked with the locus for resistance to *P. infestans* (Heibges et al. 2003; Odeny et al. 2010). Since KTI are the second most highly accumulated proteins in potato tubers, they have been proposed to act as reserve protein themselves (Grosse-Holz and van der Hoorn, 2016) in addition to their role in protecting other storage proteins from endogenous proteases. In potato tubers, KTI may also be involved in the response to pathogen invasion, as it was shown that two forms of Kunitz-type serine PI accumulated in potato tubers in response to infection by *P. infestans* zoospores (Valueva et al., 1998). In addition, PI, including the KTI, have been proposed to act as modulators of protein catabolism in potato tubers affected by a Zebra-chip disease (Kumar et al., 2015).

1.2.1.3 Lipoxygenases

Lipoxygenases accumulate in large quantities in potato tubers (Bauw et al., 2006). These enzymes have been proposed to play a role in reserve metabolism, serving as storage protein in plant tissues and contributing to the mobilization of storage lipids during germination (Tranbarger et al., 1991; Feussner et al., 2001; Fischer et al., 1999).

Lipoxygenases are nonheme iron-containing enzymes catalyzing the dioxygenation of fatty acids. Depending on which fatty acid residue, C-9 or C-13, is oxygenated, plant lipoxygenases are divided into two categories, the 9S and 13S groups. In plants, lipoxygenases form a family of enzymes that catalyze the biosynthesis of reactive

chemicals known as oxylipins (Figure 1.2). Oxylipins include diverse linoleic and linolenic acid hydroperoxide derivatives involved in the physiological and pathological responses of plants (Vellosillo et al., 2007). 13S lipoxygenases catalyze the biosynthesis of jasmonates, which were shown to play a vital role in vegetative growth and development (Creelman and Mullet, 1997), plant fertility (Sanders et al., 2000; Stinzi and Browse, 2000) and mechanotransduction (Stelmach et al., 1998). The most characterized member of this subclass of oxylipins is jasmonic acid, which plays an important role in controlling resistance to necrotrophic and biotrophic pathogens as well as the wounding response (Antico et al., 2012). Oxylipins derived from 9S are known to be produced in response to bacterial infection (Vicente et al., 2012). They were suggested to act in cell wall modifications, brassinosteroid signalling activation, oxidative stress modulation and pathogen arrest (Lopes et al., 2011; Marcos et al., 2015; Vellosillo et al., 2007). Oxylipins of both classes exhibit direct antimicrobial activities against pathogenic bacteria, oomycetes and fungi (Prost et al., 2005).

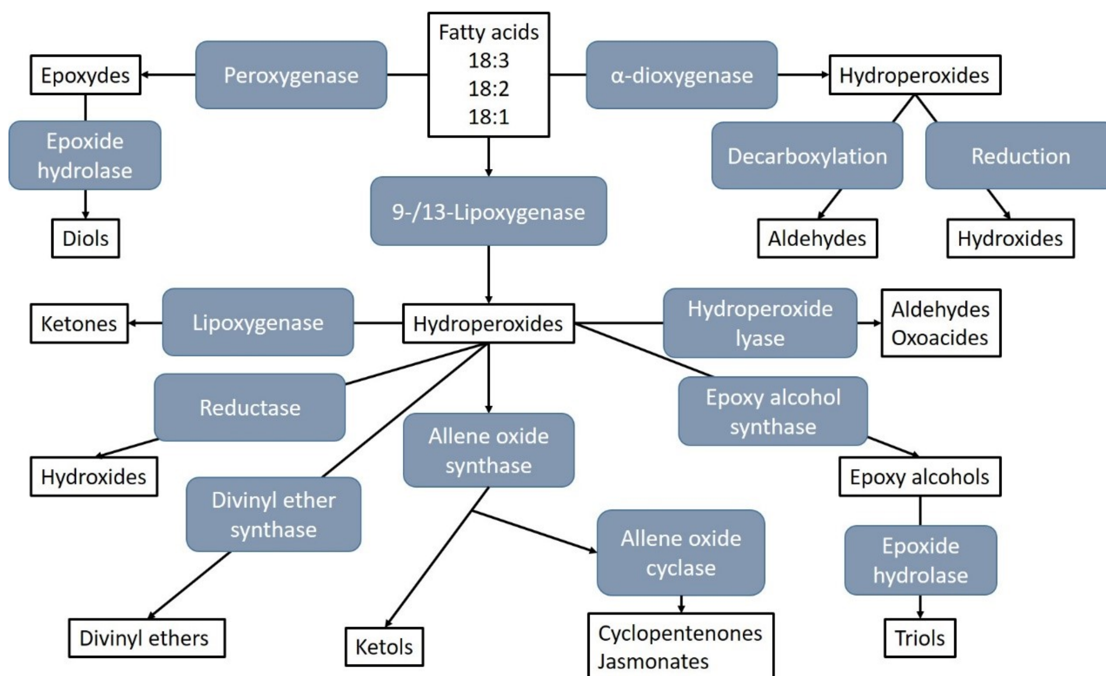

Figure 1.2. Oxylipin biosynthesis pathway (modified from Vellosillo et al., 2007).

In potato, lipoxygenases (Lox) are represented by three classes of enzymes, i.e., Lox1, Lox 2, and Lox 3, depending on their amino acid sequences. The expression of lipoxygenase-encoding genes is organ-specific: *lox1* genes are expressed mainly in tubers and roots, *lox2* are expressed in leaves, and *lox3* are expressed in both leaves and roots. Expression of Lox genes in bacterial cells revealed the substrate specificity of different Lox classes (Royo et al., 1996; Kolomiets et al., 2001). Lox1 enzymes predominantly use as a substrate linoleic acid, which is abundant in membrane lipids of tubers, leading to the production of 9S oxylipins. Members of the Lox2 and Lox3 classes preferred substrate is linolenic acid, which is prevalent in leaves, resulting in the biosynthesis of 13S oxylipins (Royo et al., 1996). Tuber-associated class 1 lipoxygenases are involved in tuber growth and development. The accumulation of Lox1 class mRNA in stolons and developing tubers positively correlates with the initiation and growth of tubers. Lox1 Class antisense suppression in transgenic mutants exhibited reduced tuber size, yield and had a disrupted tuber formation (Kolomiets et al., 2001).

1.3 Potato common scab

Potato common scab is a soil-borne disease affecting potato underground parts and stimulating the formation of unsightly corky lesions on the tuber skin. Common scab infection causes significant economic losses in potato crops by reducing the market value of tubers. According to a mail survey of Canada potato growers conducted in 2003, common scab causes average losses of about 7500–8500 Canadian dollars per farm, which is equivalent to a loss of 90–102 Canadian dollars per hectare. The resulting economic loss caused by common scab was estimated at 15.3 to 17.3 M of Canadian dollars in 2005 (Hill and Lazarovitz, 2005).

1.3.1 Pathogenic *Streptomyces ssp.*

Common scab is caused by pathogenic species of soil-inhabiting Gram-positive filamentous Actinobacteria of the genus *Streptomyces*. Among the 900 species of *Streptomyces* that have been described, most are saprophytic bacteria while only a dozen of species are plant pathogenic. The main species that have been implicated in common scab disease are *Streptomyces scabies*, *Streptomyces acidiscabies* and *Streptomyces turgidiscabies*, with the most prevalent being *S. scabies*. Common scab-causing *Streptomyces* spp. are found in many potato-growing regions throughout the world, including North America (USA, Canada), Europe, Japan, Korea and China (Figure 1.3) (Dees and Wanner, 2012).

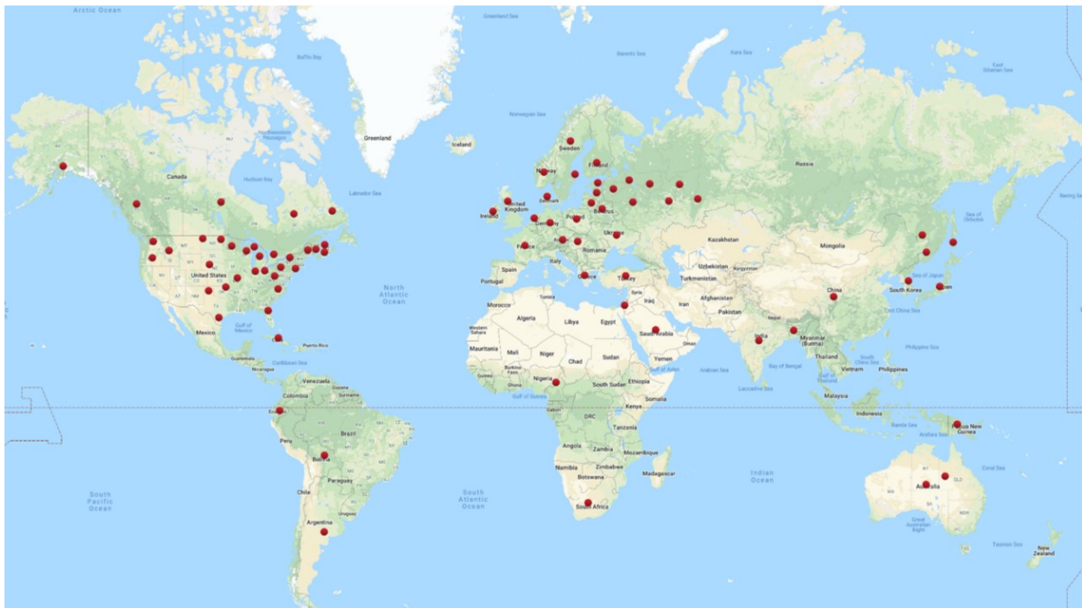

Figure 1.3. Common scab occurrence in the world.
Picture created by author using the Maptive software. Data taken from: AAFC, 2019; CIP program report, 1995; Gouws, 2006; Gultyaeva et al., 2004; Jackson, 2017; Lehtonen et al., 2004; Naher et al., 2013; Navarrete et al., 2017; Salazar, 2006; Vismara et al., 2017; Wanner, 2009.

While common scab causes the greatest losses in potato, it also affects other root crops such as beet (*Beta vulgaris*), parsnip (*Pastinaca sativa* L.), radish (*Raphanus sativus* L.), carrot (*Daucus carota*), sweet potato (*Ipomoea batatas*) and turnip (*Brassica rapa*) (Goyer and Beaulieu, 1997).

1.3.2 The disease cycle of *Streptomyces scabies*

S. scabies forms a branched mycelium with corkscrew sporogenous hyphae. After the formation of cross walls, the sporogenous hyphae split into individual spores which can be further spread by water, wind, soil movement and farm machinery. When a spore comes into contact with a suitable host, it germinates (Wharton et al., 2007). The infection usually occurs in the early tuberisation stages and affects expanding tissues, approximately 0-6 weeks after tuber initiation (Khatri et al., 2010, 2011). Invasion by the pathogen occurs through lenticels, wounds and any natural openings on the tuber surface. After penetration, *S. scabies* can grow through several layers of potato periderm, causing cell death and feeding with dead cell material. In response to pathogen invasion, potato tuber produces several layers of suberized cells to isolate the infected area but peridermal cells above this suberized layer die and provide additional nutrients to the pathogen. The pathogen then penetrates through the new formed suberized cell layer, repeating the cycle and causing the formation of scab lesions (Loria et al., 2003; Wharton et al., 2007). Common scab lesions stop expanding when the tuber skin is mature (Loria et al., 2006).

1.3.3 Virulence mechanisms of *Streptomyces scabies*

1.3.3.1 Thaxtomin A

The potential virulence factors of *S. scabies* can be categorized in three groups: phytotoxins, phytohormones, and secreted proteins (Li et al., 2019). The most described phytotoxins, which are considered as the main factor determining pathogenicity, are thaxtomins. Among the eleven known analogues of thaxtomins, the most important is thaxtomin A (TA), which is essential for the development of disease symptoms (Figure 1.4). It was shown that TA can induce the formation of scab-like lesions on the tuber surface (Lawrence et al., 1990) while *S. scabies* EF35 mutants deficient in producing the toxin were unable to invade potato tubers (Goyer et al., 1998; Joshi et al., 2007b). In addition, there are no pathogenic *Streptomyces* species that have been found not synthesizing the TA or other common scab inducing toxins (fridamycin E and desmethylmensacarcin) (Lapaz et al., 2018; Natsume et al., 2018; Wanner, 2009), and it was shown recently that the lower virulence of tree species of common scab-causing *Streptomyces* (*S. scabiei* ME01-11h, *S. stelliscabiei* NY02-1C, NY02-1A and *Streptomyces* sp. IdahoX ID01-12C) was associated with a lower TA production (Clarke et al., 2019). Although the specific mechanism of TA action is not known, this compound was shown to exhibit several biological effects. Purified TA causes tissue browning and cell death on excised potato tissues, while TA application on aseptically cultured potato tubers causes the formation of scab-like lesions similar to those caused by *S. scabies* (Lawrence et al., 1990). Treatment of plant seedlings with TA has different effects, including shoot and root swelling, cell hypertrophy, necrosis and PCD. TA also induces alterations in Ca^{2+} and H^{+} ion influx, accumulation of the antimicrobial phytoalexin scopoletin and lignin deposition (Bischoff et al., 2009; Brochu et al., 2010; Duval et al., 2005; Errakhi et al., 2008; Fry and Loria, 2002; Leiner et al., 1996; Lerat et al., 2009a; Meimoun et al., 2009; Scheible et al., 2003; Tegg et al., 2005).

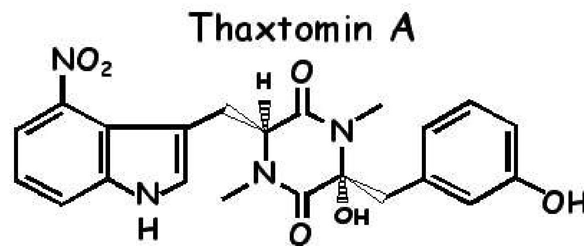

Figure 1.4. The chemical structure of thaxtomin A.

DNA microarray analyses revealed that TA induces similar gene expression profile (more than 75% of coincidence of upregulated genes) in *Arabidopsis thaliana* suspension cells as isoxaben, which is a known inhibitor of cellulose synthesis (Duval and Beaudoin, 2009). As well, induction of lignin and cell wall biosynthesis gene expression was reported in thaxtomin A habituated hybrid poplar cells (Brochu et al., 2010).

Thaxtomin A is a cyclic peptide containing 4-nitrotryptophan and phenylalanine residues. Biosynthesis of thaxtomin is driven by a biosynthetic gene cluster consisting of seven genes, six of which (*txtA*, *txtB*, *txtC*, *txtD*, *txtE* and *txtH*) are involved in thaxtomin biosynthesis while the remaining one, *txtR*, encodes a cluster-localized regulator. Two non-ribosomal peptide synthetases encoded by the *txtA* and *txtB* genes are responsible for the formation of the N-methylated cyclic dipeptide bone of TA. A P450 monooxygenase encoded by *txtC* gene is required for further hydroxylation steps (Loria et al., 2008). The nitric oxide synthase TxtD produces nitric oxide from L-arginine that is used for nitration of L-tryptophan by the cytochrome P450 monooxygenase TxtE (Kers et al., 2004; Barry et al., 2012). Finally, the *txtH* gene encodes a MbtH-like family protein that is required for proper functioning of the nonribosomal peptide synthetases TxtA and TxtB (Zhang et al., 2016; Li et al., 2019).

Induction of TA biosynthesis in the bacterium is controlled by several factors. These include the *bld* (bald) gene family, involved in morphological differentiation and/or the secondary metabolism of *Streptomyces* (Bignell et al., 2014); the pathway-specific transcriptional regulator TxtR, positively regulated by cellobiose (Joshi et al., 2007b); and the cellulose utilisation repressor CebR. The DNA-binding function of CebR is inhibited by cellobiose and cellotriose, resulting in increased expression of the *txtA*, *txtB* and *txtR* genes (Francis et al., 2015). In addition to cellobiose and cellotriose, suberin triggers TA production through stimulation of the onset of secondary metabolism (Lausier et al., 2008; Lerat et al., 2010; Wach et al., 2007). Taken together, these facts suggest that cellobiose, cellotriose and suberin may help the pathogen sensing the proximity of fast-growing plant tissue, which is the main site of action of TA (Joshi et al., 2007b).

In *S. scabies* 87-22, whose genome has been sequenced, the virulence genes are located on the so-called 'pathogenicity island', or PAI. The PAI is represented by two separate regions called toxicogenic region (TR) and colonisation region (CLR), which are localised in two separate regions of the chromosome. Genes involved in the biosynthesis of TA are located in the TR while other putative virulence factors-coding genes (*nec1* and *tomA*) are situated in the CLR (Lerat et al., 2009b; Huguet-Tapia et al., 2014; Zhang et al., 2016). The TR region includes two areas with different functions: TR1 and TR2. TR1 includes a complete cluster of genes involved in TA synthesis while TR2 is possibly involved in the mobilization of the TR region, as it contains putative integrative and conjugative elements (Zhang et al., 2016; Chapleau et al., 2016).

1.3.3.2 Coronafacoyl phytotoxins

Plant-pathogenic *S. scabies* bacteria are also known to synthesise coronafacoyl phytotoxins, non-host-specific phytotoxins composed of bicyclic hydrindane ring-based polyketide coronafacic acid, conjugated to an amino acid or amino acid derivative that

is linked via an amide bond (Bignell et al., 2018). *S. scabies* 87-22 was shown to produce N-coronafacoyl-L-isoleucine, which belongs to this phytotoxin family (Fyans et al., 2015). A suggested role for N-coronafacoyl-L-isoleucine, which exhibits a variety of biological activities, is the activation of the jasmonic acid (JA) signaling pathway, which in turn leads to the suppression of salicylic acid signaling critical to protect the plant against the pathogen (Xin and He, 2013). Recently, it was shown that the level of coronafacoyl phytotoxin production positively correlates with the severity of common scab symptoms (Cheng et al., 2019).

1.3.3.3 Concanamycins

The production of concanamycins A and B has also been detected in some *S. scabies* strains. Concanamycin refers to an 18-membered plecomacrolides, specific inhibitors of V-ATPases (Huss et al., 2002). It has been suggested that these phytotoxins contribute to the differences between the types of common scab lesions formed on potato tubers (Natsume et al., 2017).

1.3.3.4 Phytohormones

S. scabies bacteria produce the phytohormone indole-3-acetic acid (IAA), which is a well-known plant auxin. Although the exact role of IAA during infection has not yet been identified, it was discussed that *S. scabies* mutants with reduced IAA production caused reduced necrosis on radish roots (Li et al., 2019). It is known that IAA biosynthesis in *S. scabies* is stimulated by the addition of tryptophan, and at the same time negatively regulating the biosynthesis of TA. In this regard, it was suggested that regulation of the synthesis of IAA and TA by low concentrations of tryptophan could have a beneficial effect on plant development (Legault et al., 2011).

1.3.3.5 Secreted proteins

For the penetration and colonization of host plant tissues, pathogenic bacteria can secrete extracellular enzymes and pathogenesis promoting proteins. One of the proteins secreted by *S. scabies* is Nec1, which is encoded by the gene *nec1*. While Nec1 is not essential for pathogenicity, it exhibits necrogenic activity on excised potato tissue and was also proposed to function in the suppression of plant defense responses (Joshi et al., 2007a). Another protein that could potentially be involved in pathogenesis is Tomatinase A, encoded by the *tomA* gene. Tomatinase functions as a glycosyl hydrolase that detoxifies antimicrobial molecules produced by plants known as phytoanticipins such as tomato α-tomatine. However, it was shown that tomatinase A produced by *S. scabies* is not active against potato glycoalkaloids, which leaves its function still unexplained (Seipke and Loria, 2008). The scabin enzyme is another protein secreted by *S. scabies* that could potentially function as a virulence factor. Scabin belongs to the mono-ADP-ribosyltransferase family of proteins. It possesses NAD^+ glycohydrolase and ADP ribosyltransferase activity against potato genomic DNA, which could be involved in the induction of apoptosis (Lyons et al., 2016). *S. scabies* was shown to secrete other proteins that could be involved in the degradation of polymer compounds. *S. scabies* synthesises esterase (*estA*) and suberinase (*sub1*) enzymes involved in the degradation of suberin, which is found in the outer part of the potato tuber periderm. These and other enzymes that degrade cell walls could facilitate the introduction of the pathogen into the tissue of the tubers (Komeil et al., 2013; Li et al., 2019).

1.4 Mechanisms of potato resistance to common scab

In response to pathogens, plants can defend themselves by the production of defensive compounds, such as phytoalexins, glycoalkaloids and phenolics. These compounds play a role in pathogen resistance and are synthesized in potato tubers during pathogen

bacteria, fungi or virus infection (Andreu et al., 2001; Li et al., 2015). In general, these substances accumulate more rapidly in plants that are resistant to pathogens than in susceptible plants (Maina et al., 1984; Andreu et al., 2001).

Molecular mechanisms implicated in potato resistance to common scab are poorly understood and no genetic determinants of common scab resistance have been identified yet. This precludes the use of a genetic approach involving molecular modifications of potato plants for the production of highly resistant varieties (Dees and Wanner, 2012). Hence, investigations of potato plant defense response in the *S. scabies* – potato interaction are greatly needed.

1.4.1 Role of the tuber periderm during infection

There are many types of potato pathogens, such as insects, nematodes, bacteria, fungi and virus. In tubers, the well-developed periderm represents a primary barrier against pathogen attack. The tuber periderm consists of three cell layers: the phellem, the phellogen and the phelloderm (Figure 1.5). The phellem is the outer layer of the periderm, made of rectangular dead cells surrounded by a suberin-containing cell wall. Phellem cells are organized in 4-10 cell layers, depending on genotype, environment and growth stage. Suberin consists of suberin poly(phenolics) and suberin poly(aliphatics) that are cross-linked by glycerol, embedded with soluble waxes and attached to the inner side of the plant cell wall. The phellogen (or cork cambium) is a secondary meristem forming a layer of intensive outwardly dividing cells, which give rise to the phellem and the phelloderm. During tuber development, the outer surface of the periderm can remain either smooth (for red and white potatoes) or become rough to generate rough surface textures called netted skins. In some dark-skinned potato varieties (e.g. cv. Russet), the phellem is thicker with cracked cork layers in the outer side. This type of heavily netted skin is called russet (Bethke et al., 2014; Serra et al., 2010; Vreugdenhil et al., 2007, p 472, 473).

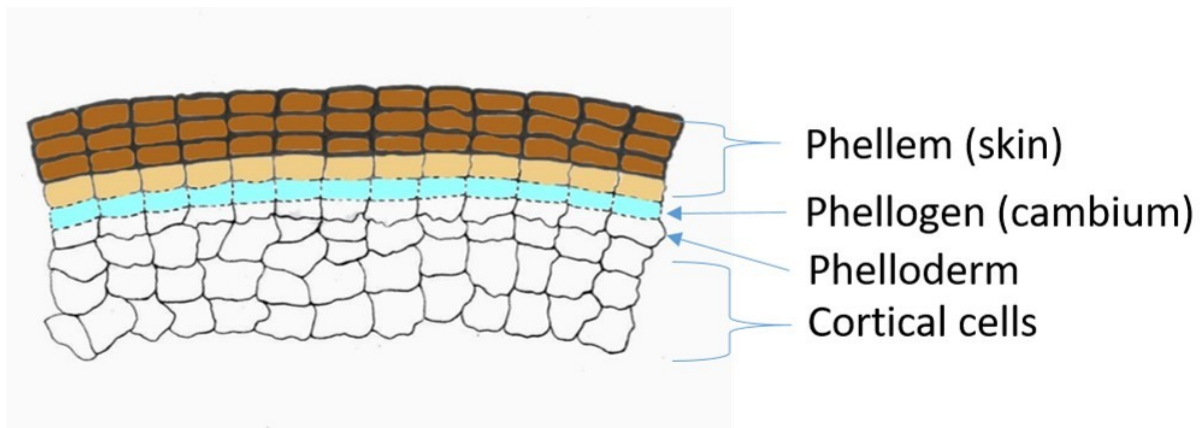

Figure 1.5. Periderm morphology of potato tuber (according to Vreugdenhil et al., 2007)**.**

Physiological differences in the tuber skin texture can partly explain the differences in variety susceptibility to soil born disease and common scab. It was reported that the number of periderm layers and extensive suberisation of tuber lenticels could determine resistance to common and powdery scab (Tegg et al., 2013).

A correlative analysis between skin types among potato varieties, scab resistance data from the Canadian Food Inspection Agency potato database and emnpirical data from Parent (2008) suggests that the russet skin texture would be an important factor contributing to common scab resistance (Figure 1.6).

Upon tuber damage, as a result of wounding or in response to different biotic and abiotic stresses, tuber cells induce various responses, including the induction of wound suberization. Wound signals induce suberization in 2-3 layers of parenchymal cells to form a closing layer. At this time, a wounding phellogen is formed under the closing layer. Wounding phellogen produces additional suberized cells, called the wound phellem (Figure 1.7) (Lulai and Corsini, 1998).

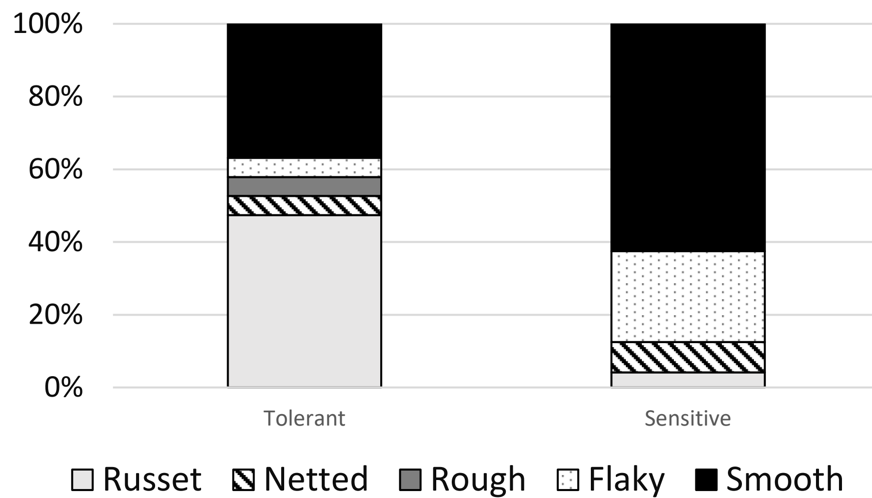

Figure 1.6. Skin texture and common scab susceptibility of potato varieties grown in Quebec.

In total 69 potato varieties were analysed: 15 very sensitive to common scab, 16 sensitive, 7 mid-sensitive, 6 mid-tolerant, 16 tolerant and 9 very tolerant (Data from CFIA, 2015 and Parent, 2008).

Induction and regulation of suberization are poorly understood but several plant defense regulators have been proposed to regulate the suberization process. For instance, it was shown that ethylene production is stimulated by tuber wounding. However, ethylene produced in response to tuber wounding does not appear to be required for wound-induced suberization of the closing layer or subsequent suberization associated with wound periderm development (Lulai and Suttle, 2004). It has been shown that treatment with IAA and cytokinin inhibits wound healing responses (wax deposition) characterized by the loss of water vapor in wound healing tissue. On the other hand, wound-healing responses are stimulated by abscisic acid (ABA) treatments (Soliday et al., 1978; Lulai et al., 2008). ABA treatment of potato cultured cells was shown to result in an increased accumulation of suberin components, enzymes of suberin biosynthesis and waxes (Cottle and Kolattukudy, 1982).

Peroxidases and H_2O_2, which are induced upon wounding, are also required for suberization. It is possible that these compounds act in facilitating cross-linking of phenolics within the suberin polyphenolic domain and attachment to the cell wall (Espelie et al., 1986; Bernards et al., 1999, 2004; Bernards and Razem, 2001; Razem and Bernards, 2003). While it is known that JA synthesis is induced upon wounding, there is no evidence that JA would have a regulatory role in tuber wound healing and suberization (Negrel et al., 1995; Vreugdenhil et al., 2007).

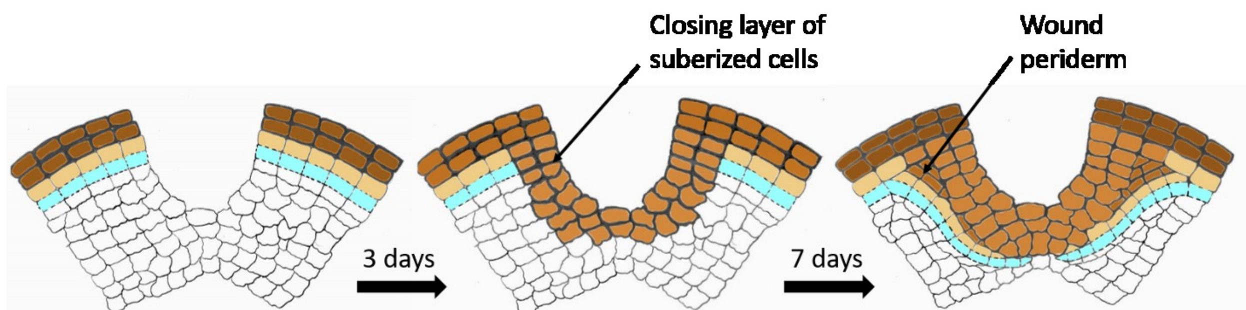

Figure 1.7. Wound periderm formation (according to Vreugdenhil et al., 2007)**.**

1.4.2 Phenylpropanoids and phenolics

One of tuber responses to *S. scabies* infection is the formation of lesions formed through suberisation of the wound periderm (Spooner and Hammerschmidt, 1992). Suberized cells have been shown to act as an antimicrobial barrier against invasion of bacterial and fungal pathogens (Lulai and Corsini, 1998; Kamula et al., 1995). Suberisation of cell walls involves the synthesis and crosslinking of phenylpropanoid derivatives such as the hydroxycinnamic acid amides, which lead to strengthening of the polyphenolic cell wall barrier (King and Calhoun, 2005 and 2010; Novo et al., 2017). The suberin of potato tuber periderm has aliphatic and phenolic domains. A recently proposed model for suberin suggests that the polyphenolic domain, made of hydroxycinnamic acids, is embedded in the primary cell wall. The polyphenolic domain

is covalently linked to a glycerol-based polyaliphatic domain located between the primary cell wall and the plasma membrane (Bernards, 2002).

The primary precursor of suberin is phenylalanine resulting from the biosynthetic phenylpropanoid pathway. At the first steps of this pathway, phenylalanine is converted to various intermediates, such as cinnamic, coumaric, caffeic and ferulic acid by the action of the following enzymes: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4 coumarate:CoA ligase (C4L), and hydroxycinnamoyl-coenzyme A shikimate:quinate hydroxycinnamoyl-transferase (HCT). Biosynthesis of the suberin polyaliphatic domain is initiated by the production of 16:0 and 18:0 fatty acids. Further transformation of these compounds includes oxidation, elongation, and other reactions that lead to the formation of hydroxycinnamoyl coenzyme A derivatives (Bernards, 2002).

In addition to suberin, the phenylpropanoid pathway leads to the formation of various classes of secondary metabolites such as monolignols, anthocyanins, isoflavonoids, and stilbens. Phenylpropanoids are involved in defense responses in the form of salicylic acid and defensive phytoalexins (Dixon and Paiva, 1995; Dixon et al., 1996). In potato, accumulation of nicotiflorin, rutin and chlorogenic acid in response to a concentrated culture filtrate of *P. infestans* was related to differential resistance to *Pectobacterium atrosepticum* and *P. infestans* (Kröner et al., 2012). It was previously noted that potato resistance to common scab could be related to a high level of chlorogenic acid in the tuber (Johnson and Schaal, 1957).

The accumulation and oxidation of phenolic compounds produced by the chemical conversion of phenylalanine leads to the browning of plant tissues, in particular potato tuber parenchyma (Vitti et al., 2011; Teoh et al., 2016). Injury of potato tuber leads to an increase in the activity of PAL, polyphenol oxidase (PPO) and peroxidase (POD) that results in the enzymatic browning of tissues as a response to stress (Saltveit, 2000;

Teoh et al., 2016). PPO activity plays an important role in the plant defense response to pathogens and numerous insect pests (Constabel and Barbehenn, 2008). Regarding bacterial plant diseases, it has been shown that PPO-ovexpression in tomato plants regulates susceptibility to *Pseudomonas syringae* pv. *tomato* (Li and Steffens, 2002; Thipyapong et al., 2004).

1.5 Agronomic methods to cope with common scab

Several agronomic approaches were developed for common scab management, such as a crop rotation, management of planting dates, decrease of soil pH, increased irrigation during early tuber development, soil treatment with fungicides, leaf treatments with 2,4-D (2,4-Dichlorophenoxyacetic acid) and use of potato varieties with increased resistance (Thompson et al., 2013). Unfortunately, each of these control strategies has disadvantages that reduce its efficiency in practice.

1.5.1 Crop rotation

Crop rotation is used to reduce diseases caused by some plant parasitic microorganisms. This method implicate management of soil microbial communities that enhance pathogen inhibitory activities leading to disease suppression. Several types of crop rotation systems involving canola, rapeseed, winter rye rotations (Larkin et al., 2010, 2011), corn or alfalfa (Wiggins and Kinkel, 2005) were proposed for common scab reduction. However, this approach was restricted by the ability of *Streptomyces* ssp. to survive for up to 10 years in the soil as a saprophyte (Kritzman et al., 1996).

1.5.2 Management of planting dates

Management of planting dates implies that planting and harvesting dates of potato could reduce tuber damage by common scab. The optimum temperature for the growth

and development of *S. scabies* is about 25-30°C. Planning the timing of planting and harvesting can reduce the exposure of the potato crop to temperatures favorable to this pathogenic microorganism. It was shown that minimizing the period that potato tubers spend in the ground (early planting combined with early harvest) can reduce tuber grade-out due to common scab damage. Unfortunately, this approach may affect potato yield (Waterer, 2002).

1.5.3 Soil pH

Growth and development of *S. scabies* is affected by soil acidity. Decreasing soil pH (lower than 5.2) inhibits growth of the pathogen. However, this condition may facilitate the growth of other scab-causing pathogens (*S. acidiscabies* and *S. turgidiscabies*) that are favoured by acidic pH (Dees and Wanner, 2012). In particular, pH affects the chemical solubility and availability of essential plant nutrients and the solubility of fertilizers (Marschner et al., 1987).

1.5.4 Irrigation

It was reported that abundant irrigation during the early phases of tuber formation can reduce the damage caused by common scab (Lapwood et al., 1973). However, other investigators observed an increased infection incidence during application of this method (Larkin et al., 2011). This difference between the two studies could be explained by a shift to an alternative infecting agent such as *S. turgidiscabies*, which can tolerate higher levels of soil wetness. Also, netted scab (*Streptomyces reticuliscabiei*) was shown to increase with the increased humidity (Wilson et al., 2001).

1.5.5 Soil fumigation

Soil treatment with chemical compounds remains the most widely used method to control soil pathogens even though it is expensive and has a negative impact to the environment. Chloropicrin, a broad-spectrum antimicrobial agent currently used as a fungicide, herbicide, insecticide and nematicide, is effective against scab (Al-Mughrabi et al., 2016). Being widely non-specific, chloropicrin has an enormous negative impact on soil biomass and soil microbial diversity (Rokunuzzaman et al., 2016). Chloropicrin is a toxic substance that can induce bacterial mutagenicity (Schneider et al., 1999). In Ontario, some efficacy has been demonstrated against common scab for chloropicrin-based fungicides. There are several limitations for chloropicrin application in northern territories that makes it inefficient such as temperature of application (above 7°C) and restrictions for planting within 30 days after treatment as well as high cost of the fungicide and non-lasting protection. In addition, it was shown that fungicides can reduce tuber size and yield (Dees and Wanner, 2012). Another widely used fungicide that gave good suppression of soil-borne inoculum, pentachloronitrobenzene, is carcinogen and its application was forbidden (Thompson et al., 2013).

1.5.6 Treatments with 2,4 D

2,4-Dichlorophenoxyacetic acid (2,4-D) is an auxin-type herbicide, commonly used to control weeds. Leaf treatment with sublethal concentrations of 2,4-D during the early stages of plant development was recently proposed as a way of controlling common scab (Tegg et l., 2008). Unfortunately, this method could affect plant development and reduce tuber size and yield (Thompson et al., 2013). Despite the suppression of scab symptoms, the mechanism of action of this substance is not understood. It was found that 2,4-D does not directly inhibit pathogenic bacteria, but would instead enhance the resistance of potato plants (Tegg et al., 2008).

1.5.7 Use of potato varieties with increased resistance

The most environmentally beneficial and effective approach to control common scab could be the use of common scab resistant potato varieties. Unfortunately, no commercial potato variety yet is completely resistant to the disease (Dees and Wanner, 2012).

1.6 Somaclonal variation to achieve target traits and properties of potato plants

Somaclonal variation of potato plants was shown to induce stable changes in valuable agronomic traits of potato, including reduced incidence of common scab. The obtained traits may remain stable during three years of consecutive tests in the field (Evans et al., 1986).

The occurrence of somaclonal variation is based on the fact that most plant cells are totipotent and can be reprogrammed for development into a new organism. Any differentiated plant cell can dedifferentiate in meristem-like cells and differentiate again in specialized cells. This developmental process is guided by a ratio of plant hormones – cytokinins and auxins *in planta*. High cytokinin to auxin ratio promotes shoot formation while a high auxin to cytokinin ratio induces root formation. The *in vitro* culture of totipotent plant cells and their differentiation into specialized structures such as shoots or somatic embryos are frequently accompanied by genetic and epigenetic changes in the plant genome, which may lead to change in phenotype, a phenomenon called somaclonal variation. Consequently, somaclonal variations may lead to the production of clonally propagated plant descendants that are morphologically, genetically and/or epigenetically different from the initial clone (Kaeppler et al., 2000).

The process underlying inheritance of tissue culture induced variation can take place at different levels of genome organisation, including ploidy changes, chromosome rearrangements, sequence variation, activation of transposable elements and changes in DNA methylation patterns. Activation of quiescent transposons and retrotransposons can be induced by tissue culture in many plant species, supporting the notion that cell dedifferentiation of cells lead to a certain derepression of epigenetically silenced DNA sequences (Kaeppler et al., 2000).

In practice, tissue culture becomes through the production of somaclonal variants a tool that can lead to the expression of desirable traits in plant varieties. In most cases, new phenotypical traits are stable and often inherited stably through sexual generations. Somaclonal variation is generally considered as a somatically and meiotically stable event (Kaeppler et al., 2000).

1.6.1 Selection and adaptation (or habituation) to TA as a tool to increase common scab resistance

While no genetic determinants of scab resistance have been identified yet, other strategies have been proposed to increase potato resistance. A selection method of potato cells resistant to the key pathogenicity determinant of *S. scabies*, the toxin thaxtomin A (TA), has been described. Using somatic cell selection to TA as a positive selection agent, 13 potato lines of cv. Iwa with increased resistance to common scab were regenerated (with 85-86 % lower disease score) by Calum Wilson's group (Wilson et al., 2009). Unfortunately, using this approach, the frequency of generation of scab-resistant clones was very low, i.e. one event per 1.9×10^5 cells treated with TA. Moreover, more than half of the plants regenerated after the selection with TA did not exhibit common scab resistance. The same research team reported in 2010 the generation of potato variants cv. Russet Burbank with strong to extreme resistance to common scab using the same somatic cell selection. Later on, it was shown that Russet

Burbank lines highly resistant to common scab also showed higher resistance to powdery scab in glasshouse and field experiments (Tegg et al., 2013). Enhanced resistance to both diseases suggests that resistance could be mediated by an unspecific defense mechanism.

Viviane Brochu, in our lab, described another somatic cell selection approach that was based on the habituation (adaptation) of cell culture to gradually increased concentrations of TA. Adaptation of a culture of hybrid poplar cells to TA toxin led to changes in the composition of the cell wall and induced changes in the expression of genes involved in cell wall synthesis and modification, flavonoid and lignin synthesis. Induced modifications caused long-term resistance to TA, as well as to other cellulose biosynthesis inhibitors in poplar cell culture (Brochu et al., 2010). Our laboratory also showed that the TA-habituated method described above could be successfully implemented to habituate undifferentiated potato cells to TA, and to regenerate potato lines with higher resistance to TA and common scab than the original variety (Ducharme, 2013). Potato calli from stem segments of Russet Burbank and Yukon Gold varieties were adapted to TA. The results showed that some somaclonal variants of both varieties appeared to be more resistant to common scab than the initial varieties (Beaudoin, 2012).

The TA-selection and adaptation approaches can improve scab resistance in potato varieties with valuable agronomic characteristics. TA-adapted plants with increased resistance to common scab would also be an interesting tool for a better understanding of the mechanism of potato protection against pathogenic *S. scabies*.

1.7 Research hypothesis and objectives

Since TA is a key pathogenicity determinant of *S. scabies*, we suggested that increasing resistance to TA in potato may lead to a better resistance to common scab

disease. Previously, it was shown in our laboratory that TA adaptation of Russet Burbank variety calli may lead to an improvement of scab resistance of the produced somaclonal variants. In particular, the Russet Burbank somaclone 9 (RB9) showed up to 20% increased resistance to common scab during tests both under controlled conditions in growth chambers and in subsequent field tests (Beaudoin, 2012; 2017).

In this project, we hypothesise that **the method of calli adaption to TA can be applied to various varieties of potato**. We also suggest that, in practical terms, adaptation to TA will improve scab resistance in other potato varieties. To address this hypothesis, we have chosen five potato varieties that are widely used or newly selected in Québec: Envol, Rubiconde, Belle d'août, Chieftain and Kennebec.

Research objectives for this part of the project were : 1. To set up culture conditions for callogenesis, adaptation to TA and regeneration for the different potato varieties selected; 2. To develop a screening method for selecting of potato somaclones with improved resistance to common scab; 3. To confirm resistance to common scab in somaclones adapted to TA using standard methods involving plant infection in pots.

In a second part of the project, we hypothesize that **TA adaptation causes morphological changes and/or changes in the level of proteins associated with resistance to common scab**.

To confirm this second hypothesis, we pursued the following research objectives : 1. To characterise changes in protein composition and abundance induced by adaptation to TA by comparing the tuber proteomes of Russet Burbank and somaclone RB9; 2. To determine whether *S. scabies* infection affects the content of proteins accumulated differently in the tubers of the original Russet Burbank and TA adapted somaclone RB9; 3. To investigate changes in the periderm of Russet Burbank and somaclone RB9 tubers.

In a third part of the project, we investigated the effects of TA on tuber tissues. When applied on potato tuber slices, TA caused the browning of the tuber parenchyma, an effect that had long been described as necrosis (Loria et al., 1995, 1997; Tegg et al., 2008). The intensity of browning had also been associated with the level of resistance to TA and to common scab. However, when analyzing the collection of varieties known to show common scab resistance, it was found that the intensity of the browning did not reflect the level of resistance of these varieties to common scab (Tegg and Wilson, 2010). We found TA to cause a strong browning of tuber tissues of the common scab moderately resistant Russet Burbank variety, while inducing no darkening on slices of Yukon Gold tubers, a variety very sensitive to common scab. This contradiction led us to suggest that browning was probably a response of potato tuber to TA and not a direct indication of susceptibility to the toxin.

It is known that darkening of plant tissues upon infection by pathogenic organisms is often associated with the accumulation and oxidation of phenolic compounds in damaged tissues (Nicholson and Hammerschmidt, 1992). It was shown also that TA treatment may cause the accumulation of phenolic compounds in *Arabidopsis* hypocotyls and tobacco leaves (Lerat et al., 2009a; Bischoff et al., 2009). Here we put forward a third working hypothesis for the projet stating that **TA-induced tissue browning occurs as a result of phenolic compounds accumulation in tuber parenchyma cells.** According to this hypothesis, TA would induce a higher accumulation of phenolic compounds in the parenchyma cells of Russet Burbank tubers than in those of Yukon Gold tubers.

To address this hypothesis, we set the following objectives: 1. To study whether observed differences in parenchyma browning caused by TA in tubers of cvs. Russet Burbank and Yukon Gold are related to the accumulation of phenolic compounds; 2. To determine whether the level of cell death caused by TA correlates with the intensity of browning on tuber sections of Russet Burbank and Yukon Gold; 3. To investigate

whether inhibition of the phenylpropanoid pathway affects the development of TA-induced browning.

Three scientific manuscripts were produced as an output of the project, each of them addressing one of the three working hypotheses proposed for this work. These manuscripts are presented in the subsequent parts of this thesis.

CHAPTER 2

Thaxtomin A adaptation improves resistance to common scab in potato varieties cultivated in Quebec

2.1 Article introduction and contribution of authors

In the first chapter of the work, we show that adaptation of calli to the phytotoxin thaxtomin A (TA) may improve common scab resistance of potato varieties Kennebec, Envol, Belle d'août and Chieftain. We have adjusted media and culture conditions for the regeneration of selected potato varieties upon adaptation to TA. In addition, we propose a novel common scab resistance screening method, based on the infection with *S. scabies* of potato mini-tubers developing from axillary buds on potato stem segments. This technique allows for a fast evaluation of TA-adapted somaclones for resistance to common scab and could be adapted for screening of common scab resistance in newly developed varieties. Using this approach, we confirmed improved resistance of the adapted somaclones to common scab, as well as the validity of our new method compared to usual tests with infected potato plants in pots.

The contributions of each author in this manuscript is as follows: NB and II designed the experiments, analysed and interpreted data. II performed all experiments. NB supervised the project. NB and II wrote the manuscript. Both two authors approved the final version of the manuscript.

The manuscript presented below has been prepared for submission in the journal In Vitro Cellular and Developmental Biology – Plant (IVP).

A NOVEL ASSAY FOR EVALUATION OF COMMON SCAB RESISTANCE IN POTATO SOMACLONAL VARIANTS ADAPTED TO THAXTOMIN A.

Iauhenia Isayenka and Nathalie Beaudoin*

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2.2 Abstract

Thaxtomin A is the key pathogenicity factor of the predominant potato common scab pathogen *Streptomyces scabies* (syn. *S. scabiei*). In order to increase resistance to common scab, cell cultures of potato varieties Envol, Kennebec, Belle d'août, Rubiconde and Chieftain were gradually adapted to increasing concentrations of the phytotoxin thaxtomin A. A total of 92 somaclonal variants adapted to thaxtomin A were derived from original varieties. TA-adapted somaclones were tested for common scab resistance using a new test based on infection of potato mini-tubers developing in the auxiliary buds of potato stem segments. According to this test, two somaclonal variants produced from Belle d'août, one somaclone from Chieftain, two somaclones from Envol and two somaclones from Kennebec showed a significant increase in common scab resistance compared to parental varieties. These results were confirmed using a standard scab resistance test in planta.

Key words

Solanum tuberosum, common scab, thaxtomin A, leaf bud, *Streptomyces scabies*

2.3 Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
CI	Callus induction media
CR	Callus regeneration media
GA_3	Gibberellic acid
NAA	α-Naphtaleneacetic acid
PCD	Programmed cell death
TA	Thaxtomin A
YME	Yeast Malt Extract medium
Zea	trans-Zeatin riboside

2.4 Introduction

Common scab is one of the most important potato diseases in the world, diminishing market value of tubers (Dees and Wanner 2012). The economic loss caused by common scab in Canada was estimated between 15.3 and 17.3 millions of Canadian dollars in 2002 (Hill and Lazarovits 2005). While several agronomic approaches have been developed for common scab management, the most environmentally beneficial and effective approach is the use of common scab resistant potato varieties (Thompson et al. 2013). Unfortunately, molecular mechanisms of potato resistance to common scab are poorly understood. This makes genetic approach and molecular modification of plants unsuitable for production of highly resistant varieties (Dees and Wanner 2012).

The prevalent common scab inducing agent is the soil-inhabiting actinobacterium *Streptomyces scabies* (Dees and Wanner 2012). This pathogen infects tubers through lenticels, wounds or other natural openings, leading to the formation of scabby lesions

on the tuber surface. *S. scabies* secretes a toxin, thaxtomin A (TA), which is essential for the development of disease symptoms (Goyer et al., 1998). TA is a cellulose biosynthesis inhibitor which causes different physiological changes in plants. These changes include shoot and root stunning, cell hypertrophy, necrosis and PCD (programmed cell death), alterations in Ca^{2+} and H^{+} ion influx, and the accumulation of the antimicrobial phytoalexin scopoletin (Duval et al. 2005, Errakhli et al. 2008, Fry and Loria 2002, Leiner et al. 1996, Lerat et al. 2009, Meimoun et al. 2009, Scheible et al. 2003, Tegg et al. 2005, Bischoff et al. 2009).

Recently, several methods have been proposed for selecting TA-resistant plant cells that can be used to increase plant resistance to common scab. In 2009, Wilson's research group reported production of Iwa variety plants with increased resistance to common scab using somatic cell selection to TA (Wilson et al. 2009). The same research team reported in 2010 that generation of potato variants cv. Russet Burbank using the somatic cell selection resulted in enhanced resistance to common scab (Wilson et al. 2010). Brochu et al. (2010) described an approach that involved the adaptation of hybrid poplar cell culture to gradually increasing concentrations of TA (0.1-1.3 μM) within a period of 12 months. TA tolerance was associated with numerous changes in gene expression including genes involved in chromatin and DNA modifications, suggesting the implication of epigenetic changes in the process (Brochu et al. 2010). This method was successfully applied to potato tissue culture by adapting Russet Burbank calli to TA (Ducharme 2013). One somaclonal variant of the cv. Russet Burbank adapted to TA showed increased resistance to common scab in the greenhouse and field tests (2015-2016) (Beaudoin 2017).

In this article, we describe the *in vitro* conditions that are required for the adaptation of the potato varieties Envol, Rubiconde, Chieftain, Kennebec and Belle d'août calli to thaxtomin A and the regeneration of common scab resistant somaclones. Assessing potato resistance to common scab is a lengthy process that is performed in the field, in

the growth chamber or in the greenhouse and, therefore, requires a large space. To carry out commonly used tests in pots or in the field, a large number of prpagated plants or tubers are needed, which makes these experiments lengthy and costly. To make the screening of the collection of somaclonal variants more effective, we have developed a new approach that allows the screening of a large number of mini-tubers using reduced number of plants.This new method involves infection by *S. scabies* of mini-tubers produced by plant leaf buds that reduces drastically the number of plants to be tested, the areas for testing, as well as the costs and time for plant propagation. We show that results obtained with this test are comparable to infection tests performed using tubers produced from plants grown in pots.

The proposed approach for the production of toxin-adapted potato plants and the express-screening method can be used to increase pathogen resistance in registered potato varieties, as well as for quick and effective screening of plants for scab resistance in classical potato breeding programs.

2.5 Materials and methods

2.5.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Company unless otherwise indicated.

2.5.2 Plant material and growth conditions

Potato varieties were kindly provided by Les semences Elite du Québec Inc. and Les Buissons Research Center Inc. Potato varieties used for adaptation to TA and common scab analysis have different levels of susceptibility to common scab: Belle d'août (moderately resistant to common scab), Envol (susceptible), Chieftain (moderately

resistant), Rubiconde (common scab resistance not determined), Kennebec (susceptible).

Potato plants were maintained and propagated on MS (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% bacto-agar (BD Difco), with pH adjusted to 5.7. Light conditions were set to 60-75 µmol m^{-2} s^{-1} with a 16/8 hours light/dark period and constant temperature at 22°C in Sanyo plant growth cabinet. Plants were subcultured as one-node segments every 8 weeks.

2.5.3 TA extraction and purification

Production and purification of thaxtomin A were performed from oat bran broth inoculated with *S. scabies* EF-35 according to Beauséjour (Beauséjour et al. 1999).

2.5.4 Adaptation of potato calli to TA

Calli were induced on stem internodes of four-week-old *in vitro* plants on two callus induction (CI) media: JCI according to JayaSree (JayaSree et al. 2001) and WCI according to Wilson (Wilson et al. 2009) with some modifications. Both CI contained Murashige and Skoog (MS) basal salts and vitamins (Murashige and Skoog, 1962). JCI medium was supplied with 30 g L^{-1} sucrose, 0.2 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 2.26 mg L^{-1} 6-benzylaminopurine (BAP), 0.7% agar with pH adjusted to 5.7. WCI contained 5 g L^{-1} sucrose, 40 mg L^{-1} ascorbic acid, 500 mg L^{-1} casein enzymatic hydrolysate, 0.2 mg L^{-1} α-naphtaleneacetic acid (NAA), 2 mg L^{-1} BAP, 5 mg L^{-1} gibberellic acid (GA_3), 0.8% bacto-agar with pH adjusted to 5.7. Explants were incubated at 22°C in the dark. Induction of callus was done in three replications. After 4 weeks, calli were excised from differentiated tissues, transferred to the fresh callus induction (CI) media, containing 0.2 µM TA or the appropriate volume of methanol as a control and incubated at 22°C in the dark. Each 3-4 weeks, necrotic regions were

removed and calli transferred to the fresh CI media with higher (+0.1 µM each time) TA concentration. Upon each transfer (starting from 0.3 µM TA), part of TA habituated calli were transferred to callus regeneration (CR) media (MS salts and vitamins, 30 g L^{-1} sucrose and 0.7% agar, with pH adjusted to 5.7) supplemented with hormones, antioxidants and casein as an additional nitrogen source (Table 2.1). TA-habituated calli were incubated under reduced light intensity (45-60 µmol m^{-2} s^{-1}) at 20°C up to 16 weeks. During that period, calli were transferred to fresh CR media each 4 weeks. Regenerants were cut off (1 shoot per callus) and transferred to a hormone-free MS medium for regeneration of plantlets.

Table 2.1. Composition of regeneration media (CR) tested with the different potato varieties.

Medium / MS supplements	WM1	WM2	WM3	WM4	WM5	JM6	JM7	JM8	JM9
Ascorbic acid, mg L^{-1}	40	40	40	40	40	40	40	40	-
Casein hydrolysate, g L^{-1}	0.5	-	-	-	-	-	-	-	-
BAP, mg L^{-1}	2.00	2.00	2.00	2.00	2.00	2.25	2.25	2.25	2.25
NAA, mg L^{-1}	0.10	0.10	0.10	0.03	0.03	0.03	-	0.03	-
GA_3, mg L^{-1}	5	5	1.04* 10^{-4}	5	1.04* 10^{-4}	-	1.04* 10^{-4}	1.04* 10^{-4}	-
Zea, mg L^{-1}	8	8	8	8	8	8	8	8	8

BAP, 6-benzylaminopurine; NAA, α-naphtaleneacetic acid; GA_3 - gibberellic acid; Zea, trans-zeatin riboside (GoldBio); WM1-WM5, CR media modified from the original proposed by Wilson et al. 2009; JM6/JM7-JM9, CR media modified from the original proposed by JayaSree et al. 2001

Callus viability was defined as the percentage of proliferating calli 5 weeks after transfer to the regeneration medium for the total number of calli transferred.

2.5.5 Leaf bud assay

S. scabies EF-35 (Paradis et al. 1994) were propagated in liquid Yeast Malt Extract medium (YME) containing 4 g L^{-1} glucose, 4 g L^{-1} yeast extract (Fisher scientific) and 10 g L^{-1} malt extract (BD Difco) for 5-8 days (Pridham et al. 1956). Afterwards, bacteria were plated to solid YME, supplemented with 1.5% agar and 1 g L^{-1} of $CaCO_3$. SAY solution containing 20 g L^{-1} glucose, 1.2 g L^{-1} L-asparagine, 0.6 g K_2HPO_4, 10 g L^{-1} yeast extract (BD difco) was mixed with 300 mL of sterile vermiculite (Holyday, Montreal, Canada) in Magenta boxes (Goyer and Beaulieu 1997). Each Magenta box was inoculated with two pieces (about 1 cm^2) of solid YME with *S. scabies* mycelium and incubated 21 day at 30ºC in the dark (Fig. 2.1).

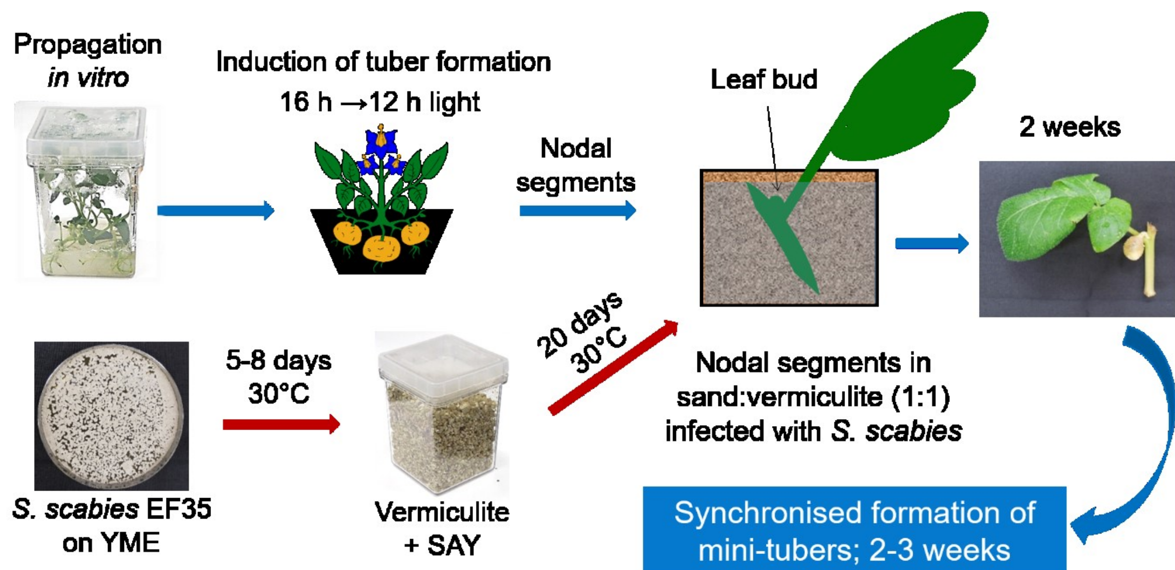

Figure 2.1. Schematic representation of leaf bud assay for evaluation of potato common scab resistance.
See text for description.

Four-week-old potato plants propagated *in vitro* were transferred to pots with soil mixture (soil:sand:vermiculite=2:2:1) and grown at 22/18 ºC under a 16-h photoperiod with light intensity adjusted to 60-75 µmol m^{-2} s^{-1} in a Conviron growth chamber (PGR15). After one month, the photoperiod was changed to 12/12 hours light/dark to stimulate tuber formation. The lower 2/3 of the stems of 2-month-old plants were cut into nodal segments containing the leaf. The nodal segments were placed in a moistened substrate consisting of sand and vermiculite mixture (1:1) so that the stem segment with the axillary bud was completely immersed in the substrate while the leaf blade remained on the surface (Struik and Wiersema 2012). A substrate not infected with bacteria was used as a control. For infection, the bacterial culture was added to the substrate in a dilution of one to forty. To equalise bacterial concentration in test conditions, the contents of all Magenta boxes were mixed together before being added to the sand-vermiculite substrate. Leaf buds were irrigated twice a week with 200 mL of water for every 3 liters of substrate. Mini-tubers were harvested three weeks after the start of the experiment and photographed from two sides. The area covered with scabs was measured using the ImageJ software (Schneider, Rasband and Eliceiri 2012) and expressed as a percentage relative to the total surface of the tuber. The relative area of the tuber covered with scabs was converted into disease index. The absence of lesions on the tuber surface corresponded to scab index 0. Lesions occupying less than 1 % of tuber surface correspond to the scab index of 0.5; 1 - 5 % =1; 6-10% = 2; 11-25% = 3; 26-50% = 4; 51-75% = 5 and 76-100% = 6.

2.5.6 Common scab test in pots

Bacteria *S. scabies* EF-35 were prepared as described above for the *Leaf bud test*. Four-week-old plants propagated *in vitro* were transferred to the substrate (sand:vermiculite 2:1) mixed with bacteria in dilution 1/40. A substrate not infected with bacteria was used as a control. Experiment was carried out in the greenhouse (test 2) or growth chamber (test 3). Plants were irrigated two times a week, one time in

combination with 100 ml of fertilizer (20:20:20). After 16 weeks of plant growth, tubers were harvested and analysed. Common scab was evaluated on potato tubers according to the recommendations of Canadian Food Inspection Agency, PI-009 with some modifications. Tuber surface covered by scab lesions was evaluated visually as a percentage and converted to common scab index as described above. The depth of lesions was measured by immersing a dissecting needle into lesions that developed into the tuber. The dissecting needle section that was immersed in the hole was marked and measured. The average value of the depth of all damages of the tuber was estimated.

2.5.7 Statistical analysis

The statistical significance of mean values was determined by Student's *t*-test after comparison of parental and habituated sample variances.

2.6 Results and discussion

2.6.1 Callus induction and adaptation to TA

For adaptation to TA, calli were induced from internodal segments from *in vitro* potato plants using different varieties and two different media. Callus formation on the internodal stem segments was observed after 3-4 weeks. Each segment produced a de-differentiated cell mass at one or both ends of the explant with an efficiency that varied according to the variety and the type of callus induction (CI) medium used. Calli induced from Rubiconde explants on both CI media and from Chieftain on WCI were formed not only on the cut edges of an explant but on the lateral side (Fig. 2.2).

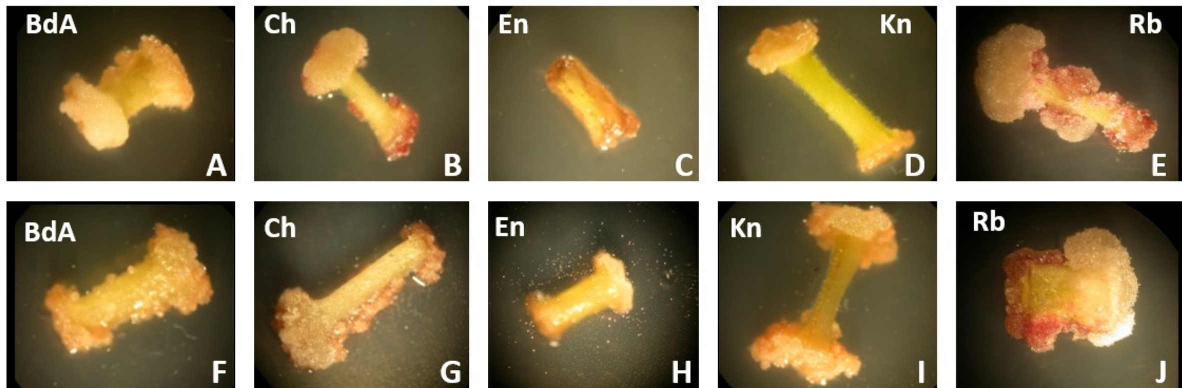

Figure 2.2. Callus induction in potato varieties Belle d'août (BdA), Chieftain (Ch), Envol (En), Kennebec (Kn), Rubiconde (Rb).

A-E explants on callus induction medium according to JayaSree (JCI) (JayaSree et al. 2001); F-J explants on callus induction medium according to Wilson (Wilson et al. 2009).

Table 2.2. Callus induction efficiency.

	JCI medium		WCI medium	
Variety	Callus induction efficiency	Total callus/explant	Callus induction efficiency	Total callus/explant
Belle d'Août	1.1±0.3*	77/69	1.3±0.5	87/69
Chieftain	1.3±0.2	56/43	2.1±0.2	50/24
Rubiconde	1.6±0.1	20/12	1.3±0.3	15/12
Envol	0	0/28	1.6±0.2	41/26
Kennebec	1.3±0.2	56/48	1.7±0.4	53/34

JCI, callus induction medium according to JayaSree (JayaSree et al. 2001); WCI, callus induction medium according to Wilson (Wilson et al. 2009). * Standard deviation of the mean.

The lowest efficiency was observed for Belle d'août with a mean of 1.1 ± 0.3 callus formed per explant on JCI medium (Table 2.2). Explants of Chieftain had the highest

callus formation efficiency of 2.1 ± 0.2 calli per explant on WCI. Callus was not induced on Envol explants on JCI, but normal callus formation occurred on WCI. In general, the efficiency of callus induction on WCI was slightly higher than that obtained on JCI (Table 2.2).

After 4 weeks on CI medium, calli were transferred to fresh CI media supplemented with TA (0.2 μM) or with the corresponding volume of methanol as a control. Each 4 weeks, calli were transferred to fresh CI media containing higher (+0.1 μM) TA concentration or the same volume of methanol (control). TA adapted calli starting from 0.3 μM TA were transferred to the regeneration induction (CR) media.

2.6.2 Regeneration of somatic embryos required modifications of hormonal media

In our preliminary experiments after TA adaptation, somatic embryogenesis was not induced on the original media proposed by Wilson (Wilson et al. 2009) and JayaSree (JayaSree et al. 2001). This indicated that the regeneration conditions needed to be optimized.

To induce regeneration in TA-adapted and methanol-adapted (control) calli, the hormonal composition of CR media was modified as shown in the Table 2.1. Calli adapted to TA or methanol (control) on WCI media were transferred for regeneration to WM1-5 media, which were modified from the initial regeneration medium proposed by Wilson. In the same way, calli that were adapted to TA or methanol (control) on JCI medium were subsequently transferred for regeneration on JM5/6-9 media, modified from the regeneration medium proposed by JayaSree.

In comparison to other varieties, Kennebec showed the highest regeneration ability. Calli regeneration occurred on all tested media (Table 2.3) whether they were adapted

or not to TA. On the WM2 medium, which differs from WM1 only in the absence of casein hydrolysate, the frequency of Kennebec regeneration decreased from 28.6% (WM1) to 6.7% (WM2), showing the positive effect of casein hydrolysate on the formation of Kennebec somatic embryos (Gray and Conger 1985). Calli cultivated on WM3 medium with a reduced content of gibberellic acid (GA_3) had the highest regenerating frequency, i.e., 72.4%. The opposite effect was observed on WM4 medium with a high content of GA_3 and reduced concentration of auxin NAA, where only 3.7% of Kennebec calli formed somatic embryos. Decline in GA_3 concentration in WM5 medium resulted in the increase of Kennebec regeneration up to 29.4%, indicating that high GA_3 content could negatively affect somatic embryogenesis and regeneration. For the JM6/7 system, we used a successive change of nutrient media that involved a first step with auxin for four weeks, followed by transfer to media containing gibberellin. This system was slightly more effective for Kennebec than other JM modifications with regeneration frequency of 58.7%. The regeneration was slightly lower for JM8 medium (44.6%) where the corresponding concentrations of auxin and gibberellin were added initially and for JM9 (50.0%) containing no auxin and gibberellin supply (Table 2.3).

Similar to Kennebec, the most effective regeneration medium for Belle d’août calli was WM3, with a regeneration efficiency of 41.7%. Interestingly, WM1 medium containing casein hydrolysate and a high GA_3 was the less effective for induction of Belle d’août regeneration. On this medium, the development of morphogenic callus was extremely low despite the high viability of calli (Table 2.3 and 2.4). Media with a low concentration of NAA and GA_3 (WM5, JM6/7 and JM8) induced Belle d’août regeneration to a lower extent with 5.4 %, 7.0 % and 14.7 % respectively. JM9 medium, which did not contain auxin and gibberellin, induced Belle d’août regeneration only in 3.4 % of calli (Table 2.3).

Table 2.3. Total regeneration efficiency (regenerating calli/total calli) of TA adapted and control (methanol-adapted) calli on different types of regeneration media.

	Regeneration efficiency (%)							
Varieties	WM1	WM2	WM3	WM4	WM5	JM6/7	JM8	JM9
Kennebec	28.6 (10/35)	6.7 (4/60)	72.4 (21/29)	3.7 (1/27)	29.4 (10/24)	58.7 (37/63)	44.6 (25/56)	50.0 (11/22)
Belle d'Août	0.8 (1/131)	-	41.7 (10/24)	-	5.4 (6/111)	7.0 (9/128)	14.7 (15/102)	3.4 (4/117)
Envol	23.3 (10/43)	15.2 (7/46)	13.8 (4/29)	0 (0/17)	0 (0/16)	-	-	-
Chieftain	0 (0/29)	0 (0/14)	-	0 (0/19)	0 (0/8)	4.4 (2/46)	0 (0/17)	5.9 (2/34)
Rubiconde	0.9 (2/218)	-	-	-	-	-	-	1.4 (1/73)

WM1-WM5, CR media modified from the original proposed by Wilson et al. 2009; JM6/JM7-JM9, CR media modified from the original proposed by JayaSree et al. 2001; Regeneration efficiency showed in %.

The same tendency was observed for both Kennebec and Belle d'août potato varieties: inhibition of regeneration on media with a high content of gibberellin and an increase in the regeneration frequency on the medium with a high concentration of auxin NAA.

Envol calli induced exclusively on WCI media were transferred to WM1-5 regeneration media. On WM1, WM2 and WM3 the frequency of Envol regeneration was 23.3, 15.2 and 13.8%, respectively. A decrease in NAA concentration in WM4 and WM5 media led to the complete loss of the regenerative ability, although calli viability was not affected (Table 2.3 and 2.4). These results suggest that increased NAA and additional nitrogen supply are favorable for regeneration of Envol calli.

Table 2.4. Callus viability ratio and total number of calli formed (callus/explant) on different CR media.

	Callus viability, % (viable calli/total calli)							
Varieties	WM1	WM2	WM3	WM4	WM5	JM6/7	JM8	JM9
Kennebec	54.3 (19/35)	63.3 (38/60)	89.7 (26/29)	81.5 (22/27)	76.5 (26/34)	82.5 (52/63)	48.2 (27/56)	63.6 (14/22)
Belle d'août	87.8 (115/131)	-	79.2 (19/24)	-	32.4 (36/111)	28.1 (36/128)	32.4 (33/102)	37.6 (44/117)
Envol	58.1 (25/43)	41.3 (19/46)	89.7 (26/29)	94.1 (16/17)	87.5 (14/16)	-	-	-
Chieftain	34.5 (10/29)	42.9 (6/14)	-	42.1 (8/19)	0 (0/8)	87.0 (40/46)	47.1 (8/17)	70.6 (24/34)
Rubiconde	100 (218/218)	-	-	-	-	-	-	46.6 (34/73)

WM1-WM5, CR media modified from the original proposed by Wilson (Wilson et al. 2009); JM6/JM7-JM9, CR media modified from the original proposed by JayaSree (JayaSree et al. 2001); 5 weeks after transfer.

Both red varieties of Chieftain and Rubiconde had very low morphogenic ability. Somatic embryogenesis and subsequent regeneration of Chieftain calli occurred in 4.4 and 5.9 % exclusively on JM6/7 and JM9 media. The frequency of Rubiconde regeneration on the two tested media was extremely low: 0.9% for WM1 and 1.4% for JM9, although callus viability was 100 and 46.6%, respectively (Table 2.4). Low morphogenic competence of red varieties Chieftain and Rubiconde could be due to faster tissue aging, associated with a high anthocyanin content. It has been suggested that plant tissue culture containing a high level of phenolic compounds, in particular anthocyanins, undergoes a faster aging and loss of morphogenic competence (Murashige and Nakano 1965; Bailey et al., 1994; Benson 2000).

2.6.3 Habituation to TA affects calli regeneration ability

To determine whether adaptation to thaxtomin A affects the regeneration ability of calli, the number of calli forming morphogenic structures was determined at various time intervals (1, 4, 8, 12 weeks) after transfer to regeneration induction media (Fig. 2.3).

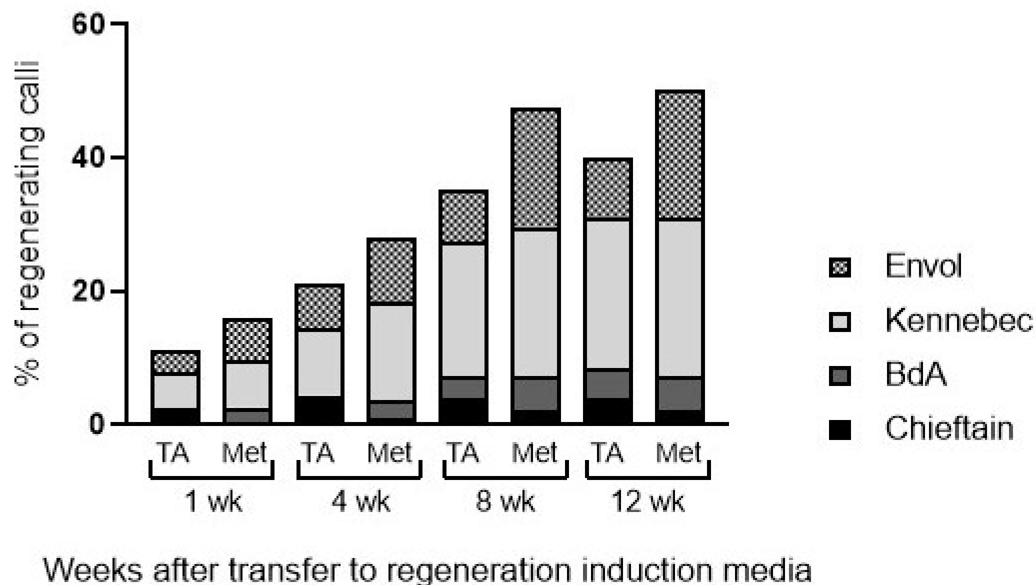

Figure 2.3. Regeneration of somatic embryos from TA-adapted and control (methanol-adapted) calli.

Morphogenesis was determined after 1, 4, 8, 12 weeks (wk) after transfer to regeneration induction media. Each column consists of the total regeneration of calli (%) of Envol, Kennebec, Belle d'août (BdA) and Chieftain varieties.

In general, TA adaptation had a negative effect on the regeneration ability of potato varieties. Adaptation to TA slightly decreased the regenerative ability of Kennebec calli during first 4 weeks, but later on frequency of regenerated calli was similar in both control and TA habituated samples. TA-habituated Envol calli regenerated to a lesser

extent than methanol-adapted control. Regeneration of Belle d'août calli adapted to TA was delayed compared to the control, starting after 4 weeks of maintenance on CR medium. In contrast, regeneration ability of Chieftain calli seemed to be increased after adaptation to TA. However, while morphogenic structures were formed, they never resulted in organ development (Fig. 2.3). The effect of the morphogenesis inhibition caused by TA in calli of various potato varieties is an intriguing phenomenon, possibly related to the responses induced by this toxin in plant tissues

2.6.4 Production of TA-habituated somaclones

A total of 92 somaclonal variants were produced: 55 from Kennebec, 27 from Belle d'août, 7 from Envol, 1 from Chieftain and 2 from Rubiconde adapted to different concentrations of TA (Table 2.5). Examples of regenerating calli of potato varieties underwent adaptation to TA or methanol as a control are presented in Figure 2.4.

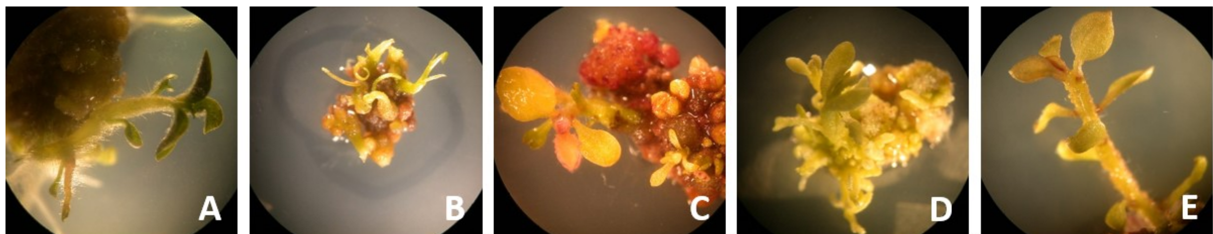

Figure 2.4. Calli regeneration after adaptation to TA or methanol as a control. **A** – Belle d'août regenerating on JM6/7 after habituation to TA 0.4 μM; **B** – Envol habituated to 0.3 μM of TA on WM1; **C** – Rubiconde control on JM9; **D** – Kennebec on JM8 after habituation to TA 0.3 μM; **E** – Chieftain on JM9 habituated to TA 0.3 μM.

Table 2.5. Number of regenerants produced according to the level of adaptation to the TA and the regeneration medium.

Variety	Effective regeneration medium	Regenerants number according to the level of adaptation to thaxtomin A (μM)						Total
		0.3	0.4	0.5	0.6	0.7	0.9	
Belle d'août	JM6/JM7; JM8; WM3; WM5		8 (14 wk)	13 (13 wk)	6 (13 wk)			27
Kennebec	WM1; WM2; WM3; WM4; WM5; JM6/JM7; JM8; JM9	5 (11 wk)	6 (5 wk)	11 (6 wk)	21 (8 wk)	12 (6 wk)		55
Envol	WM1; WM2; WM3	6 (8 wk)	1 (16 wk)					7
Chieftain	JM9	1 (12 wk)						1
Rubiconde	WM1						2 (13 wk)	2

WK, weeks on CR medium; WM1-WM%, CR media modified from the original proposed by Wilson et al. 2009; JM6/JM7-JM9, CR media modified from the original proposed by JayaSree et al. 2001.

Overall, these results suggest that the type of regeneration medium used for each potato variety is critical to be able to regenerate somatic embryos. While TA-adaptation caused for most varieties a slight or moderate decrease in regeneration efficiency, TA-adapted calli were able to regenerate in CR medium found to be efficient for non-adapted calli.

2.6.5 Common scab resistance analysis

Scab resistance of TA-adapted somaclonal variants was determined using a new method based on infection of potato plant leaf buds. Initially, common scab rates resulting from leaf bud infection of original varieties Kennebec, Belle d'août, Chieftain and Envol were compared with known levels of common scab resistance. According to

the CFIA, Kennebec and Envol varieties are susceptible to common scab whereas Chieftain is moderately resistant (CFIA). According to producer studies, Belle d'août has been reported as a moderately scab resistant variety (Pommes de terre Bérubé web site).

In the leaf bud test, *S. scabies* infection of mini-tubers produced from common scab sensitive varieties Envol and Kennebec lead to significantly more damages than those observed in moderately resistant varieties Belle d'août and Chieftain (Fig. 2.5).

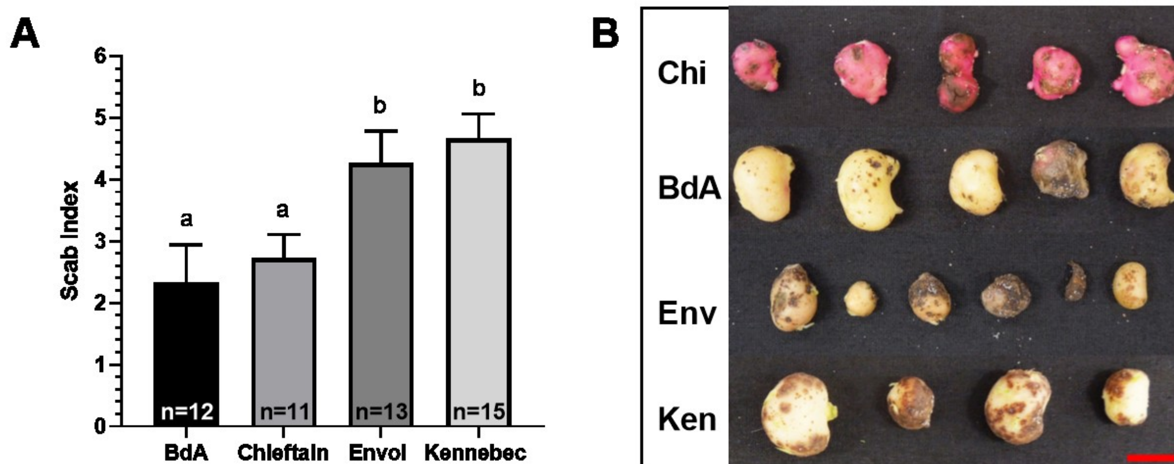

Figure 2.5. Common scab resistance of varieties Kennebec, Belle d'août, Chieftain and Envol evaluated by leaf bud test.

A. Common scab index; error bars represent the standard error of the mean; different letters signify significantly different samples according to unpaired *t*-test ($p \leq 0.05$); n - number of analysed samples; **B.** Representative images of mini-tubers infected with *S. scabies* EF35 of potato varieties Chieftain (Chi), Belle d'août (BdA), Envol (Env), Kennebec (Ken). Red bar is 1 cm.

Common scab-susceptible varieties Kennebec and Envol had common scab indices of 4.7 and 4.2, respectively. Moderately resistant varieties Chieftain and Belle d'août had significantly lower common scab index values, 2.7 and 2.3 respectively. These results showed that the common scab index data obtained in the leaf bud test reflected the reported common scab index obtained in tubers tested in the field. This indicates that this is a reliable test to determine the level of common scab sensitivity.

Several somaclones adapted to TA were randomly selected for subsequent common scab resistance analysis (Table 2.6). Based on the results of the leaf bud infection, we selected three of the six TA-adapted Belle d'août somaclones with reduced symptoms of common scab infection. Belle d'août somaclones were selected in two independent leaf bud tests (Leaf bud test-A and Leaf bud test-B). In leaf bud test-A, somaclone 23-1 had lower common scab index of 1.1 compared to the 2.3 common scab index of the parental variety. In the leaf bud test–B, somaclones 10–6 and 12–4 had a scab index of 0.4 and 0.3, respectively, which were significantly lower than the common scab index of the original variety at 1.8.

Subsequent analysis in pots confirmed that the trend for lesion development remained similar to that previously described using the leaf bud test. In the first test in pots (test in pots-1) tubers of all TA-adapted somaclones were less affected by scabs. Common scab indices for somaclones 10-6 and 12-4 from Belle d'août were significantly lower than that of the original variety. The scab lesion depth was significantly reduced for all three somaclones comparing to Belle d'août tubers (Table 2.7).

Table 2.6. Resistance to common scab of somaclonal variants habituated to TA.

Variety	Somaclonal variant (TA conc. μM)	Common scab index		
		Leaf bud test	Test in pots-1	Test in pots-2
Belle d'août	Parent	(A) 2.3±0.6 (n=12)	2.6±0.4 (n=16)	0.9±0.2 (n=28)
		(B) 1.8±0.4 (n=13)		
	23-1 (0.4)	(A) 1.1±0.4 (n=6)	2.1±0.4 (n=15)	-
	10-6 (0.5)	(B) 0.4±0.1[b] (n=9)	1.3±0.3[c] (n=12)	0.2±0.1[b] (n=8)
	12-4 (0.5)	(B) 0.3±0.1[b] (n=10)	0.8±0.2[a] (n=7)	0.6±0.1 (n=35)
Envol	Parent	4.3±0.5 (n=13)	1.2±0.5 (n=7)	1.2±0.3 (n=21)
	61-1 (0.3)	3.4±0.4 (n=11)	0.9±0.4 (n=12)	1.0±0.2 (n=21)
	61-4 (0.3)	0.9±0.2[a] (n=13)	3.2±0.8 (n=6)	1.1±0.3 (n=33)
Chieftain	Parent	2.7±0.4 (n=11)	-	-
	55-1 (0.3)	1.4±0.4[c] (n=13)	-	-
Kennebec	Parent	4.7±0.4 (n=15)	3.4±0.4 (n=31)	3.1±0.5 (n=17)
	78-4 (0.7)	2.5±0.3[a] (n=14)	2.6±0.5 (n=12)	1.8±0.2[c] (n=31)
	39-2 (0.4)	3.9±0.2[d] (n=15)	2.1±0.2[b] (n=51)	-
	47-1 (0.5)	2.7±0.5[b] (n=12)	-	3.1±0.4 (n=20)

Data are presented as an average common scab index of the sample ± standard error; n - number of analysed tubers in the sample; the significance of differences between somaclonal variants and parental varieties was evaluated by *t*-test in accordance with the analysis of sample variances; data were considered significantly different from parental variety if P value was ([a]) - $p<0.001$; ([b]) - $p<0.01$; ([c]) - $p<0.05$; ([d]) - $p<0.1$; (A) and (B) - two individual experiments conducted with somaclones and parental Belle d'août plants.

In the second test in pots (test in pots-2) the scab index appeared low for both Belle d'août and TA-adapted plants. Still, the development of lesions was reduced for TA-habituated plant's tubers both on the surface and in depth (Table 2.6 and 2.7). Thus, the results demonstrated that infection of leaf buds in the variety Belle d'août was a reliable test to identify common scab resistant somaclones.

The potato variety Envol, which is susceptible to common scab, showed a common scab index of 4.3 according to the bud infection test, while the somaclone 61-1 was less sensitive to common scab, with a reduced disease index of 3.4. Similar results were observed in pot tests, where tubers of the somaclone 61-1 were less infected

(with a scab index of 0.9 in the test 1 and 1.0 in the test 2), than the parent variety which had a common scab index of 1.2. Moreover, the depth of scab lesions of 61-1 tubers was reduced comparing to the original variety in both tests conducted in pots (Table 2.6 and 2.7).

Table 2.7. Tuber lesion depth induced by *S. scabies*.

	Somaclonal variant	Test in pots -1, lesion depth, mm	Test in pots - 2, lesion depth, mm
Belle d'août	Parent	2.1±0.6 (n=16)	0.4±0.2 (n=28)
	23-1	0.5±0.2[b] (n=15)	-
	10-6	0.5±0.3[d] (n=12)	0.0±0.0[b] (n=8)
	12-4	0.4±0.4[c] (n=7)	0.2±0.1 (n=35)
Envol	Parent	0.9±0.5 (n=7)	1.4±0.5 (n=21)
	61-1	0.7±0.3 (n=12)	0.9±0.3 (n=21)
	61-4	3.0±0.9[c] (n=6)	0.7±0.3 (n=33)
Kennebec	Parent	2.1±0.4 (n=31)	2.0±0.4 (n=17)
	78-4	1.9±0.4 (n=12)	0.8±0.2[c] (n=31)
	39-2	0.2±0.1[a] (n=51)	-
	47-1	-	1.3±0.3 (n=20)

Data is presented as a mean depth of common scab lesions induced on the tuber surface ± standard error; comparison the somaclones to parents was done by *t*-test in accordance with the analysis of sample variances; n - number of analysed tubers in the sample; data were considered significantly different from parental variety if P value was ([a]) - p<0.001; ([b]) - p<0.01; ([c]) - p<0.05; ([d]) - p<0.1; (-) - the test was not conducted.

The somaclonal variant 61-4 was found to be more resistant to common scab using the leaf bud test, with a drastic reduction of tuber surface damage. On average, less than 1% of the surface of 61-4 tubers was covered with common scab lesions, which corresponds to a common scab index of 0.9. However, this data was not confirmed in the infection tests in pots. In the first test in pots, somaclone tubers were more susceptible to scab than the original variety, possibly due to heat stress caused by high summer temperatures in the greenhouse. In the second pot test, the infected 61–4

tuber area was similar to that of the parent variety, but the depth of the scab lesions tended to decrease. It is possible that somaclone 61-4 may be more sensitive to heat stress, which could potentially affect its level of resistance to common scab.

The somaclonal variant 55-1 produced from the Chieftain variety in leaf bud test had a scab index of 1.4 (1-5% damage) which was significantly lower than that of the parental variety with an index of 2.7. Despite the increased resistance shown in the leaf bud test, the somaclone 55-1 was screened out from tests in pots, since 55-1 plants were smaller than parental Chieftain and had a stunted phenotype.

Plants of Kennebec variety, which is sensitive to common scab, had the highest common scab index (4.7) according to the leaf bud infection test. Kennebec somaclones adapted to TA were significantly more resistant than the original variety with different scab resistance levels. Somaclone 78-4 had the highest resistance according to the infection of leaf bud. 78-4 tubers were damaged on only 6-10% of the surface that correspond to the index 2.5. In subsequent tests in pots, 78-4 also showed less scab sensitivity than the original variety, both in terms of scab-covered area and in the depth of lesions. Somaclone 39-2 was significantly more resistant to common scab according to the results of leaf bud test with an index of 3.9. These results were confirmed in a subsequent pot test, where 39-2 tubers showed significantly reduced surface damage and reduced lesion depth. The least pronounced resistance effect was found in somaclone 47-1 tubers, which were damaged in the leaf bud test by 6-10% (scab index 2.7). However, in the pot test, 47-1 damaged area of the tubers corresponded to that of the parental variety, although the depth of lesions tended to decrease.

2.7 Conclusions

Overall, our results show that TA-adaptation of calli from Kennebec, Envol, Belle d'août, and Chieftain may lead to the production of plants with increased resistance to common scab. This technique allows for the improvement (i.e., for increased resistance to common scab) of varieties already recognized for their advantageous agronomic traits. However, further tests in the field should be performed to confirm enhanced resistance to common scab and evaluate agronomic characteristics. The TA-adapted somaclones more resistant to common scab also represent a valuable tool for unraveling the mechanism of potato resistance to common scab, as well as elucidating the interactions of potato plants with *S. scabies.* Finally, in this work, we have developed a new method of *S. scabies* infection using leaf buds that allows the quick screening of large potato collections for common scab resistance in limited spaces and shorter period. The efficiency of this method was confirmed using plant infection with *S. scabies* in pots. This method could be useful for determination of common scab resistance in TA-adapted plant collections and newly selected potato varieties.

2.8 Acknowledgements

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2.9 Conflict of interest

The authors declare that they have no conflict of interest.

2.10 References

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CHAPTER 3

Common scab resistance in thaxtomin A adapted potato.

3.1 Article introduction and contribution of authors

Even though the pathogenic determinants of common scab causing *S. scabies* are relatively well characterized, potato resistance factors to this pathogen remain uncharacterized. In this part of the thesis, we discuss changes in the proteome of scab-resistant somaclone RB9 produced from Russet Burbank calluses adapted to thaxtomin A. We show, among other changes, that the main tuber proteins patatins, lipoxygenases and Kunitz-type protease inhibitors accumulate in large quantities in the tuber flesh of scab-resistant somaclone RB9 compared to the original variety. We also show the abundance of major potato tuber proteins to increase in the presence of *S. scabies*. These results suggest that these proteins could play a role in pathogen defense, along with a structural adaptation to thaxtomin A leading to a reinforcement of the RB9 periderm via the production of a higher number of suberized cell layers. We discuss how these morphological and protein changes in the tuber may contribute to their increased resistance to common scab.

The contributions of each author to this manuscript were as follows: NB and II designed most experiments, analysed and interpreted data. DM contributed to proteomic data and western blot data analysis and interpretation. CB contributed to common scab data analysis and interpretation. Labeled LC-MS/MS and bioinformatic analysis of the raw data were performed at the Proteomics platform of the Quebec Genomic Center. NB supervised the project. NB and II wrote the manuscript.

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Proteomic changes associated with enhanced common scab resistance in Russet Burbank somaclone adapted to thaxtomin A

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One sentence summary: Tuber proteins accumulated in potato plants adapted to thaxtomin A are involved in the response to pathogenic *Streptomyces scabies*.

N.B. and I.I. designed most experiments, analysed and interpreted data; D.M. contributed to proteomic data and western blot data analysis and interpretation; C.B. contributed to common scab data analysis; N.B. supervised the project. I.I. and N.B. wrote the manuscript.

3.2 Abstract

Potato common scab is one of the most widespread diseases of potato. It has been found in 80% of cultivation sites in Canada. Common scab caused by the actinobacterium *Streptomyces scabies* (syn *S. scabiei*) is characterized by the formation of lesions on the potato tuber surface that reduce their marketability. Calli made from the potato variety Russet Burbank were adapted to the main virulence factor of *S. scabies*, thaxtomin A (TA), in order to regenerate potato plants with higher resistance to common scab. The somaclone RB9, originating from the cv Russet Burbank, was found to be more resistant to common scab than the original Russet Burbank variety, and this increased resistance was stable over several generations. Label-free LC/MS/MS proteome analysis of RB9 tuber flesh revealed changes that occurred at the protein level. The most significant changes were the increased accumulation in RB9 tubers of proteins involved in the metabolism of lipids (lipoxygenases), reserve proteins (patatins) and serine protease inhibitors (Kunitz-type). Infection with *S. scabies* also induced the accumulation of these proteins in potato mini-tubers of both RB9 and original variety. Young RB9 potato tubers had a reinforced periderm, with more periderm layers compared to the original Russet Burbank tubers. These changes could contribute to the increased common scab resistance in RB9 potato tubers.

Key words: potato, thaxtomin A, common scab, lipoxygenases, patatins, protease inhibitors

One sentence summary: Tuber proteins accumulated in potato plants adapted to thaxtomin A are involved in the response to pathogenic *Streptomyces scabies*

3.3 List of abbreviations

AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ATP	Adenosine triphosphate
DES	Divinyl ether synthase
EH1	Epoxide hydrolase 1
GDP	Guanosine diphosphate
HRL	Hydroperoxide lyase
JA	Jasmonic acid
KTI	Kunitz-type inhibitor
LAH	Lipid acyl hydrolase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
Lox	Lipoxygenase
qPCR	quantitative PCR
RB	Russet Burbank
RB9	Russet Burbank 9
TA	Thaxtomin A

3.4 Introduction

Common scab is a widely spread disease of potato (*Solanum tuberosum* L.), causing the formation of superficial, raised or deep corky lesions on the potato tuber periderm (Goyer and Beaulieu, 1997). Common scab is caused by the soil-inhabiting Gram-positive actinobacteria *Streptomyces scabies* (syn. *S. scabiei*) (Dees and Wanner, 2012). The infection usually occurs during the early tuberisation stages affecting expanding tissues, approximately 0-6 weeks after tuber initiation (Khatri et al., 2010, 2011). Invasion of pathogen occurs through lenticels, wounds and any other natural openings on the tuber surface. *S. scabies* secretes a toxin, thaxtomin A (TA), which is essential for the development of disease symptoms. TA is the main pathogenicity factor of *S. scabies*. It was shown that TA can induce the formation of scab-like lesions on the tuber surface (Lawrence et al., 1990). In addition, *S. scabies* mutants lacking TA production are unable to infect potato tubers (Goyer et al., 1998; Joshi et al., 2007). TA inhibits cellulose biosynthesis and deposition, causing different changes in plants, including shoot and root stunning, cell hypertrophy, necrosis and PCD (programmed cell death), alterations in Ca^{2+} and H^{+} ion influx, and the accumulation of the antimicrobial phytoalexin scopoletin (Duval et al., 2005; Errakhli et al., 2008; Fry et al., 2002; Leiner et al., 1996; Lerat et al., 2009; Meimoun et al., 2009; Scheible et al., 2003; Tegg et al., 2005; Bischoff et al., 2009). The mode of TA action remains unclear but the main effects of TA are most probably the results of cell wall perturbations. Microarray analysis revealed that TA induces similar gene expression profile (more than 75% similarities in upregulated genes) in *Arabidopsis* suspension cells as those induced by isoxaben, which is a known cellulose synthesis inhibitor (Duval and Beaudoin, 2009).

The most environmentally beneficial and effective approach to cope with common scab is the use of resistant potato varieties. Unfortunately, until now, there is no commercial potato variety that is totally resistant to common scab (Dees and Wanner, 2012). Molecular mechanisms implicated in the resistance of potato plant to common scab are

poorly understood and no genetic determinants of common scab resistance have been identified. That makes genetic approach and molecular modification of potato plants unsuitable for production of highly resistant varieties (Dees and Wanner, 2012). Thus, the improvement of potato common scab resistance by cell selection methods looks promising. One of the proposed approaches involved the gradual adaptation of plant cells to TA (Brochu et al., 2010). TA-adapted poplar cells were tolerant to toxin concentrations that were lethal to the initial cell culture. TA tolerance was associated with changes in the expression of numerous genes including genes involved in chromatin and DNA modifications, suggesting the implication of epigenetic changes (Brochu et al., 2010).

A similiar approach was used to improve potato resistance to common scab and generate common scab tolerant potato varieties. Potato somaclonal variants habituated to TA were produced with the potato cv. Russet Burbank (RB) by habituation of potato tissue culture to gradually increased toxin concentrations. Potato somaclonal variants with increased resistance to common scab were selected on the basis of *S. scabies* infection in pots and in the field. As a result of this screening, TA habituated potato variant RB9 with higher resistance to common scab (up to 20%) was selected (Beaudoin, 2012, 2017).

Until now, molecular determinants that induce response to *S. scabies* infection in potato as well as tuber resistance determinants are unknown. It this article, we describe the changes that occurred in the proteome of RB9 tubers and discuss the involvement of these changes in response and resistance to *S. scabies.*

3.5 Results

3.5.1 Adaptation to TA induced changes in tuber flesh protein content

To determine the changes induced by the adaptation of potato plants to TA, we conducted a proteomic analysis of RB and RB9 tuber flesh using a label-free LC-MS/MS method. In total, label free LC-MS/MS analysis detected 1234 protein IDs in both RB and RB9 tuber flesh protein samples. Among these proteins, 825 protein IDs were detected in two or more samples and thus considered as quantifiable. A total of 801 protein IDs (97.1% of all quantifiable proteins) were present both in the tubers of the TA-adapted line (RB9) and in the tubers of the original Russet Burbank variety (Fig. 3.1). Protein IDs exclusively detected in RB tubers constituted only 2.1% of quantifiable proteins (17 IDs), while only 7 protein IDs (0.8%) were found exclusively in RB9 (Fig. 3.1). According to UniProt gene ontology, proteins detected exclusively in RB were predominantly involved in structural components of ribosomes and transcription factors, organization of nucleosomes and ubiquitin-ligase system, or represented enzymes carrying hydrolase and serine peptidase activities (Table S3.1). In addition, some proteins were involved in the glycolytic process, the synthesis of trehalose and serine, brassinosteroid mediated signaling pathway. Proteins found exclusively in RB9 tubers included integral components of the membrane and enzymes with hydrolase and transferase activity (Table S3.2).

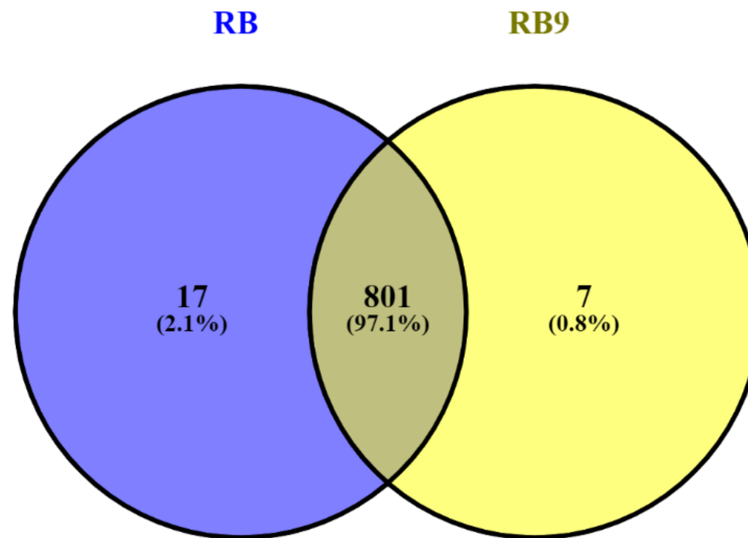
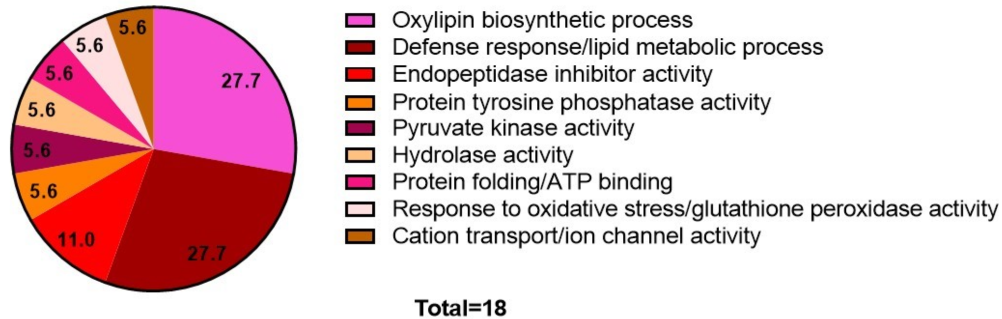


Figure 3.1. Changes in the protein occurrence in RB9 and RB tubers caused by adaptation to thaxtomin A.

Proteins detected in Russet Burbank tubers are in blue; proteins detected in RB9 tubers are in yellow.

Only quantifiable proteins whose abundance differed 1.5 or more times in RB9 compared to the original variety (p-value ≥ 0.1) were selected from the proteomic data. Selected proteins were classified in different functional groups by gene ontology using UniProt database. Proteins more abundant in RB9 tubers, than in Russet Burbank appeared to be involved predominantly in lipid metabolism (Fig. 3.2 A). Proteins involved in oxylipin biosynthesis constituted 27.7%, another 27.7% were implicated in lipid metabolism associated with a stress response. Proteins with endopeptidase inhibitor activity accounted for 11.0%. Other groups were minor: they included various enzymes, such as protein tyrosine phosphatase, pyruvate kinase, hydrolase, and glutathione peroxidase. The two remaining groups included proteins involved in folding and cation transport processes (Fig. 3.2 A).



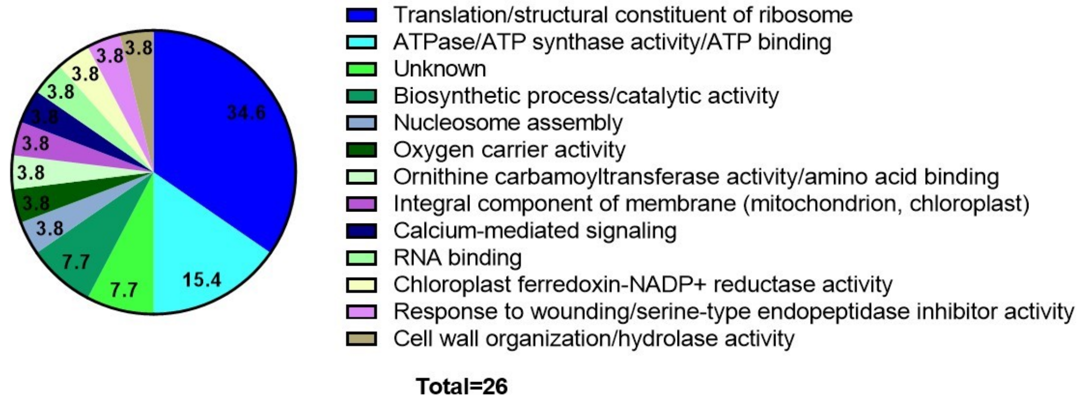


Figure 3.2. Functional groups of differentially accumulated in the tuber flesh of RB9 somaclone.

Functional classification was done for the proteins whose abundance varied more than 1.5 times in RB9 compared to Russet Burbank ($p \leq 0.1$) using gene ontology tool of UniProt database. **A.** Proportion (%) of proteins that were more abundant in RB9 vs RB. **B.** Proportion (%) of proteins that were less abundant in RB9 vs RB.

Less abundant proteins in RB9 tubers, were predominantly involved in biosynthetic processes and energy metabolism (Fig. 3.2 B). Ribosome structural proteins made up 34.6% of this group. Proteins involved in the synthesis, binding and decomposition of ATP accounted for 15.4%, while proteins involved in biosynthetic processes and having

catalytic properties constituted 7.7%. Some other proteins less abundant in RB9 were involved in nucleosome assembly, oxygen carrier activity, calcium-mediated signaling, binding of RNA and amino acids. Finally, minor protein group that was less abundant in RB9 included integral component of chloroplast and mitochondrion membrane, proteins involved in cell wall organization or in wounding reaction.

In order to understand which of these proteins could be involved in the response to TA or common scab pathogen, we identified those that could be implicated in various types of stress (Table 3.1). Several proteins with altered abundance in RB9 tubers were involved in oxidative stress, such as glutathione peroxidase, glutamate-glyoxylate aminotransferase 2, GDP-mannose pyrophosphorylase and chloroplastic ferredoxin-NADP reductase (Chen et al., 2004; Lin et al., 2011; Palatnik et al., 1997; Rodriguez et al., 2007; Verslues et al., 2007). Other proteins, less abundant in RB9, such as Proteinase inhibitor I and pectin acetylesterase were implicated in wounding and mechanical stress (de Souza et al., 2014; Graham et al., 1986; Orfila et al., 2012; Pawar et al., 2013). As well as other proteins that could play a role during stress application as hemoglobin, calmodulin isoform 1 and ornithine carbamoyltransferase, were less abundant in RB9 tubers As well as other proteins that may play a role during stress exposure, such as hemoglobin, calmodulin 1 isoform, and ornithine carbamoyltransferase, were less common in RB9 tubers (Table 3.1) (Sowa et al., 1998; Knight and Knight, 2001; Bolwell et al., 2002; Dordas et al., 2003; Legay et al., 2009; Phean-O-Pas et al., 2005).

Table 3.1. Stress-related proteins differentially abundant in RB9 vs RB.

Protein names (IDs)	Fold Change RB vs RB9	*t*-test RB vs RB9	Stress type implication
Probable inactive patatin-3-Kuras 1; Patatin proteins (group E, F, O, P) (Q3YJS9; Q2VBJ3; Q2VBJ2; Q2VBI3 etc.)	2.29	0.037	defense response; lipid catabolic process
Patatin-2-Kuras 3 (Q42502)	2.90	0.049	
Patatin-1-Kuras 2; Patatin group A-1; A-2; A-3; Patatin-01; Patatin-16 (Q3YJT4, Q2MY60; Q2MY59; Q2MY58; Q2MY50; Q41487)	1.66	0.064	
Patatin-2-Kuras 1 (Q3YJT3)	2.87	0.069	
Patatin group J-1 (Q2MY54; Q7DMV4)	5.08	0.100	
Linoleate 9S-lipoxygenase 1 (P37831)	1.78	0.079	biotic/abiotic stress; oxylipin biosynthetic process
Linoleate 9S-lipoxygenase 2 (O24379)	1.80	0.071	
Probable linoleate 9S-lipoxygenase 3 (Q43189; Q9SAP1)	1.83	0.071	
Lipoxygenase (O49150)	2.04	0.082	
Lipoxygenase (Q9SC16; O22507; O24378)	1.93	0.031	
Kunitz-type inhibitor D (M1AG22)	2.07	0.037	biotic/abiotic stress; PCD
KTI-D protein (M1AN26; A0A097H1A8)	5.33	0.046	
Glutathione peroxidase (M1AWZ7)	1.56	0.085	oxidative stress
Proteinase inhibitor I (K7WNW8; Q3S492)	3.52	0.095	wounding
Pectin acetylesterase (M1C8D8; M1C8D9)	1.84	0.099	mechanical stress; biotic stress
Calmodulin isoform 1 (M1D7F9; D0V3Y6; C5IJ81; P13868)	1.59	0.048	biotic/abiotic stress; PCD
Glutamate-glyoxylate aminotransferase 2 (M1BLM3; M1BLM4; M1BCN2)	2.00	0.060	ABA; oxidative stress [1]
GDP-mannose pyrophosphorylase (Q9ZTW5; M1B9V2; M1B9V1; M1AHI2)	1.92	0.084	oxidative stress [2]
Ornithine carbamoyltransferase, chloroplastic (M0ZWK1; M1B932)	2.68	0.024	salt stress [3]
Ferredoxin-NADP reductase, chloroplastic (M0ZY46; M1AUU9; M0ZY47)	3.54	0.087	oxidative stress
Hemoglobin (non-symbiotic class 1) (Q8GV42; M1D1V)	2.80	0.005	hypoxic stress

Proteins with possible stress implication were selected according to UniProt gene ontology and [1-]Verslues et al., 2007; [2-]Lin et al., 2011; [3-]Legay et al., 2009. Change in the protein content with an increase in the content in the tubers of RB9 compared to the original variety is indicated in red, a change with a decrease in the protein content in the tubers of RB9 compared to RB is shown in blue; fold change 1.5 times and more, $p \leq 0.1$.

3.5.2 Increased abundance of patatins, lipoxygenases and Kunitz-type protease inhibitors in RB9 was confirmed by western blot analysis

To confirm the results obtained in the proteomic analysis, we carried out western blotting of some of the proteins whose abundance was increased in B9 tubers as compared with the RB tubers. Accumulation of Patatins, 9S-Lipoxygenases, and Kunitz-type serine protease inhibitors (KTI) was confirmed by western blot (Fig. 3.3 A).

Using a specific anti-patatin polyclonal primary antibodies against potato patatin, two bands, corresponding to a 42-kDa glycosylated form and a 40-kDa form, were observed confirming the presence of patatin. The brightness of both bands was calculated as a whole, which represented the total content patatins. In total, patatins were approximately 1.7 times more abundant in RB9 tubers than in RB (Fig. 3.3 A).

Similarly, we used an anti-Lox polyclonal primary antibody for plant lipoxygenase Class I (from *Glycine max*) detection to perform western blot analysis of proteins extracts from RB9 and RB. We detected a band of approximately 100 kDa, which corresponded to potato lipoxygenases (Lox). In RB9 mature tubers, Lox were accumulated 2.2 times comparing to RB (Fig. 3.3 A). For detection of KTI we used anti-*Sl*CD1 loop polyclonal antibodies (Khalf et al., 2010). KTI proteins were detected as small proteins approximately from 18.5 to 20 kDa. More abundant were the 18.5 and 19 kDa KTI. The 19 kDa and 20 kDa proteins were respectively 2.2 and 6.9 times more abundant in RB9 tubers than in RB. No difference was observed in the band intensity corresponding to the 18.5 kDa protein. Thus, the results of proteomic analysis regarding the accumulation of patatin, lipoxygenase, and KTI in RB9 tubers were confirmed by western blot.

3.5.3 The accumulation of patatins and KTI proteins correlated with gene expression

To determine whether changes in protein abundance correlated with changes in gene expression, we performed qPCR analysis for genes corresponding to these proteins. Increased accumulation of Patatin and KTI proteins was associated with increased expression of particular gene loci. The expression of patatin pPATB1 locus coding for Patatin-2-Kuras 3 protein (Q42502) combined with Patatin B1 (X13179.1) was significantly increased in RB9 with the fold change of 1.3 (Fig 3.3 B). Since this slight change in expression could not explain the drastic accumulation of patatin in RB9 tubers, we analyzed the total expression pattern of patatin genes (groups 1, 2, 8, 12, J1, D1, D2, D3, A1 and A2). The total expression of these genes (Pat-1) was increased in RB9 with a fold change of 1.5. Transcript level of locus Lox1.2 (102602192) coding for Linoleate 9S-lipoxygenase 2 was not different in RB and RB9 (fold change 0.97 for RB9) (Fig 3.3 B).

Relative gene expression of Kunitz-type serine protease inhibitor DrTI-like (102593157) locus coding for KTI-D protein (M1AN26) was induced (3.5 fold change) compared to RB (Fig 3.3 B). The accumulation of Patatin-2 Kuras-3 correlated with an increase in the expression of the PATB1 gene as well as a general increase in the expression of the patatin genes. The accumulation of KTI proteins was associated with an increase in gene expression of DrTI-like locus coding for KTI class D. Lipoxygenase protein accumulation was not associated with the tested loci.

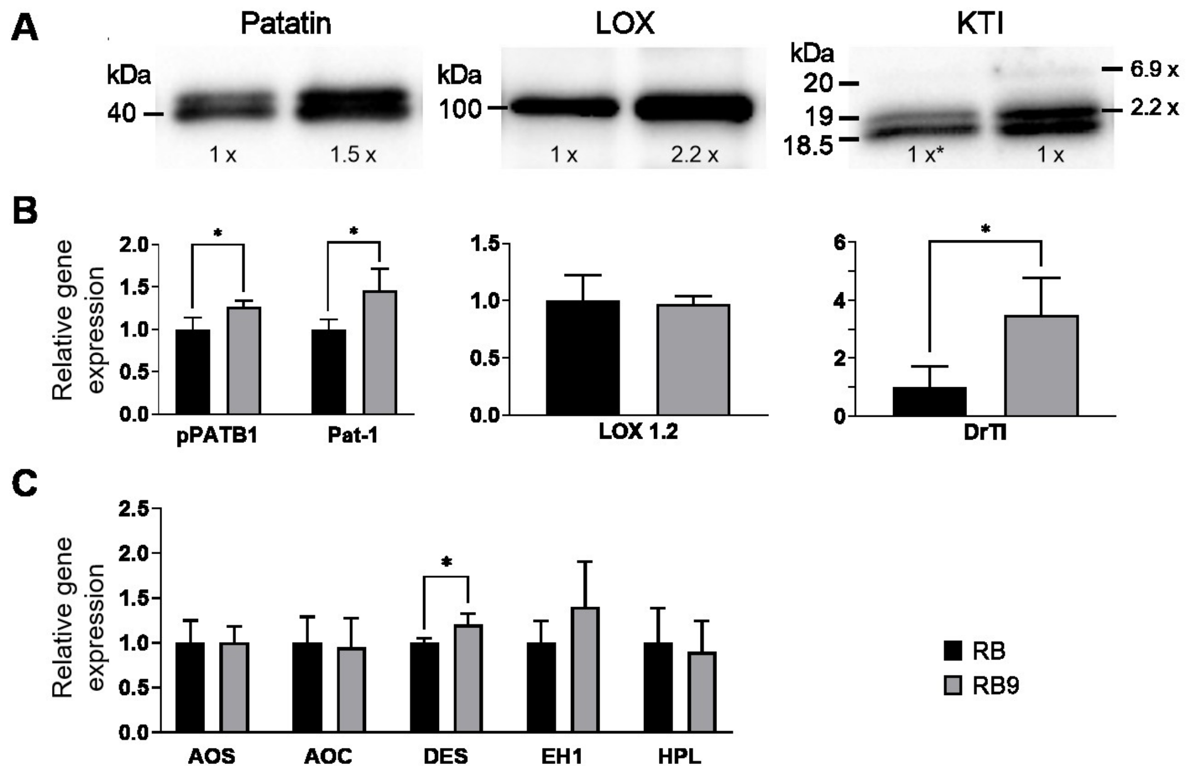


Figure 3.3. Analysis of major protein abundance and gene expression changes in Russet Burbank and TA-adapted somaclone RB9 tuber flesh.

A. Western blot analysis of patatin, lipoxygenase (Lox) and Kunitz-type protease inhibitors content in Russet Burbank and RB9. **B.** Relative expression of loci coding for Patatin pPATB1 and Pat-1 for patatin groups: 1, 2, 8, 12, J1, D1, D2, D3, A1 and A2; lipoxygenase coding for Lox1.2 and Kunitz protease inhibitor DrTI. **C.** Relative expression of genes involved in oxylipin biosynthesis: AOS – allene oxide synthase, AOC – allene oxide cyclase; DES – divinyl ether synthase; EH1 – epoxide hydrolase 1 and HRL – hydroperoxide lyase; gene *18S* was used for normalisation; chart bars are standard deviation; asterisk signifies significantly different samples according to unpaired *t*-test (p≤0.05).

Lipoxygenases in plants are involved in the biosynthesis of oxylipins, which include the well-known jasmonic acid (JA) as well as defense-related compounds. To determine how the oxylipin metabolic pathway may be implicated in RB9 tubers, we analysed the relative expression of genes involved in the JA biosynthesis (allene oxide synthase and allene oxide cyclase), as well as genes involved in other branches of oxylipin biosynthesis (divinyl ether synthase (DES), epoxide hydrolase (EH) and hydroperoxide lyase (HRL)). The relative gene expression of the allene oxide synthase (AOS) and allene oxide cyclase (AOC) genes in RB9 tubers remained at the same level as in the parental variety. A slight increase in expression was observed for DES and epoxide hydrolase 1 (EH1) genes of RB9 with a fold change of 1.2 and 1.4, respectively. The increased expression was only statistically significant for DES. Expression of the hydroperoxide lyase gene was slightly reduced in RB9 compared to the expression in the parent variety (Fig 3.3 C). There results suggest that changes in the accumulation of lipoxygenases did not lead to JA biosynthesis, but rather may be directed towards the production of divinyl esters.

3.5.4 *S. scabies* infection increased patatins, lipoxygenases and Kunitz-type protease inhibitors accumulation in tuber flesh

To investigate whether the accumulation of patatins, lipoxygenases and KTI may be important in plant defense, we evaluated the expression of these proteins during *S. scabies* infection. We used the method of potato leaf bud infection, which allows the synchronized production of mini-tubers. First, we confirmed that mini-tubers formed in the axils of RB9 leaf buds showed enhanced common scab resistance compared to RB mini-tubers. A preliminary infection of RB9 and RB leaf buds with two dilutions (1/20 and 1/40) of *S. Scabies* EF-35 was carried out. When using a high bacteria concentration (1/20), scab lesions covered an average of 92.2 ± 3.8% of RB mini tuber surface. However, only 59.1 ± 17.6% of the surface of RB9 mini-tubers showed common scab lesions. Bacterial dilution 1/40 resulted in a decrease of the average

surface covered by lesions to 84.0 ± 9.9%, while for RB9 mini-tubers, the average damaged surface was about 52.3 ± 15.5% (Fig. 3.4 A).

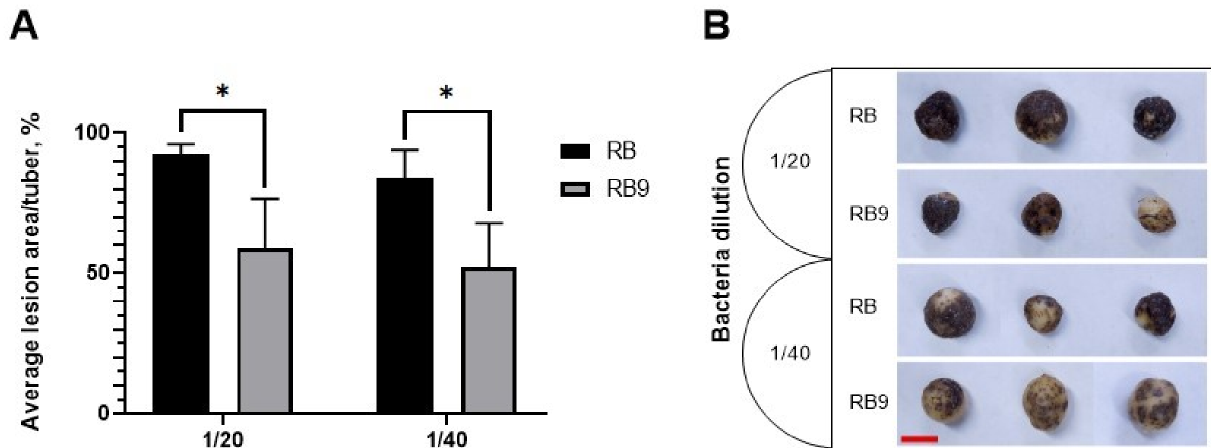


Figure 3.4. Somaclone RB9 is more resistant to common scab than parental Russet Burbank variety in the leaf bud infection test.

A. Average tuber area affected by common scab lesions; error bars represent the error of the mean; asterisk reflects significantly different data sets according to *t*-test ($p<0.05$) Number of analysed samples (n): RB 1/20 (n=5), RB 1/40 (n=5), RB9 1/20 (n=5) and RB9 1/40 (n=4); **B.** Mini-tubers of RB and RB9 formed in the axils of leaf buds infected with *S. scabies* EF-35 in dilutions 1/20 and 1/40. Red bar in the bottom of the picture is 1 cm.

Next, RB and RB9 leaf buds with 7 day-old developing mini-tubers were infected with *S. scabies* EF-35 using a dilution of 1/40. The presence of patatins, Lox, and KTI proteins in RB and RB9 mini-tubers was analyzed by western blotting with appropriate antibodies on days 3, 7, and 14 of infection (Fig. 3.5). The intensity of the bands on the membrane was measured using ImageJ program. For each studied protein, the value of the band intensity for the control conditions of the RB sample on day 14 after the start of the test was set up as 100%. Consequently, the relative intensity of the bands

at other time points for all conditions was determined by comparing with the intensity of the 14 days control and was expressed as a percentage of the 14 day RB control.

Since the infection was delayed by 7 days compared with the induction of tuberisation, scab lesions on mini-tubers surface developed to a lower extent than in the previous test and formed only on the 14th day of the experiment (Fig. 3.5). During the mini-tuber development, the stable accumulation of the major storage tuber protein patatin was observed in both RB9 and original variety.

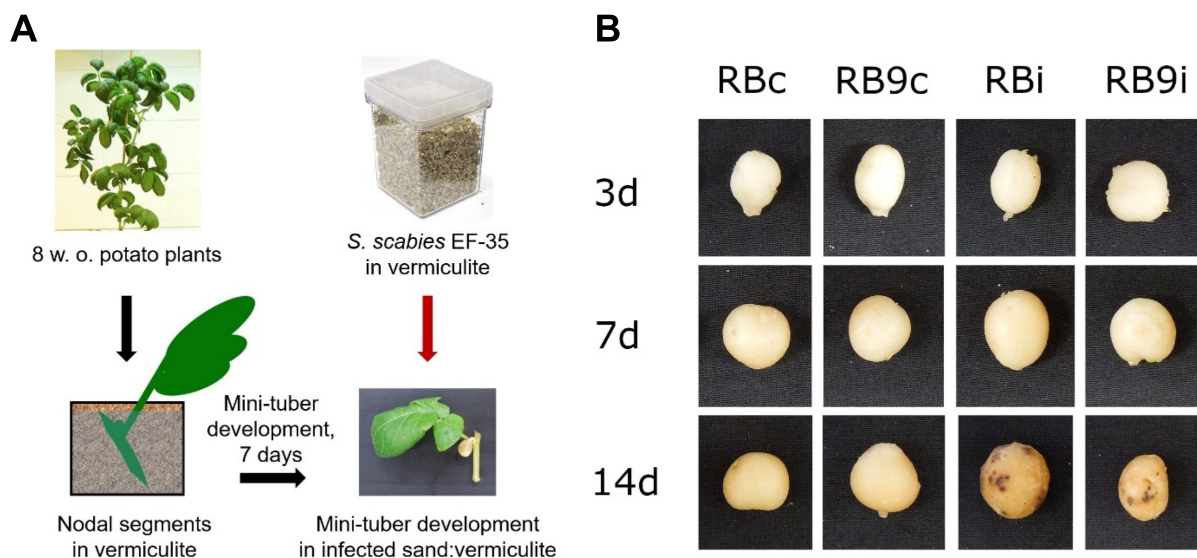


Figure 3.5. Changes in the content of the main potato tuber proteins during infection by *S. scabies*, experiment organisation.

A. Schematic representation of the infection of mini-tubers formed in the axillary buds of potato stem segments. **B.** Representative pictures of mini-tubers at 3, 7 and 14 days (d) harvest time-point of the infection test.

Under control conditions, patatins accumulated in accordance with an increase in the intensity of the respective protein bands. At the initial time point of the experiment (0

days of infection or 7 days from the beginning of mini-tuber development), the intensity of the two bands corresponding to patatin was 2.3 and 3.7% for RB and RB9, respectively, relative to the intensity of the two patatin bands that was set at 100% at 14 days for the original RB variety (Fig. 3.6 A and B). By the 3rd day of the test, which corresponded to 10 days of tuber development, the intensity increased to 12.2 and 13.8%, and continued to increase to 39.1 and 48.6% on the seventh day for RB and RB9, respectively. At the final time point (14 days from the test start), the intensity of patatin bands for tubers of RB and RB9 was 100 and 98.6%, respectively. The presence of bacteria stimulated the accumulation of patatins on the 7th day of the test, since the intensity of the corresponding bands increased by 16.1 and 22.1% compared to non-infected mini-tubers for RB and RB9 respectively (Fig. 3.6 A and B). Despite the fact that patatins were in a large extent accumulated in developing mini-tubers of both RB and RB9, the presence of *S. scabies* influenced its content, causing an even larger increase on the 7th day of infection.

The development of mini-tubers was also associated with the accumulation of lipoxygenase proteins. Class 1 lipoxygenase proteins amounted to only 0.2% at day 0 for mini-tubers of both RB and RB9, according to the intensity of the corresponding bands (Fig. 3.6 C and D). Subsequently, the intensity of the bands corresponding to lipoxygenases increased to 45.5 and 31.8% on the third test day and to 107.3 and 96.6% on the seventh day, for RB and RB9 respectively. On day 14th, the intensity was 100% for RB and 91.2% for RB9. The presence of bacteria caused a significant increase in the intensity of the bands corresponding to lipoxygenases on the 3rd test day for both RB and RB9 samples. The intensity of lipoxygenase bands increased by 42.5% for RB and by 45% for RB9 compared to the corresponding uninfected mini-tubers.

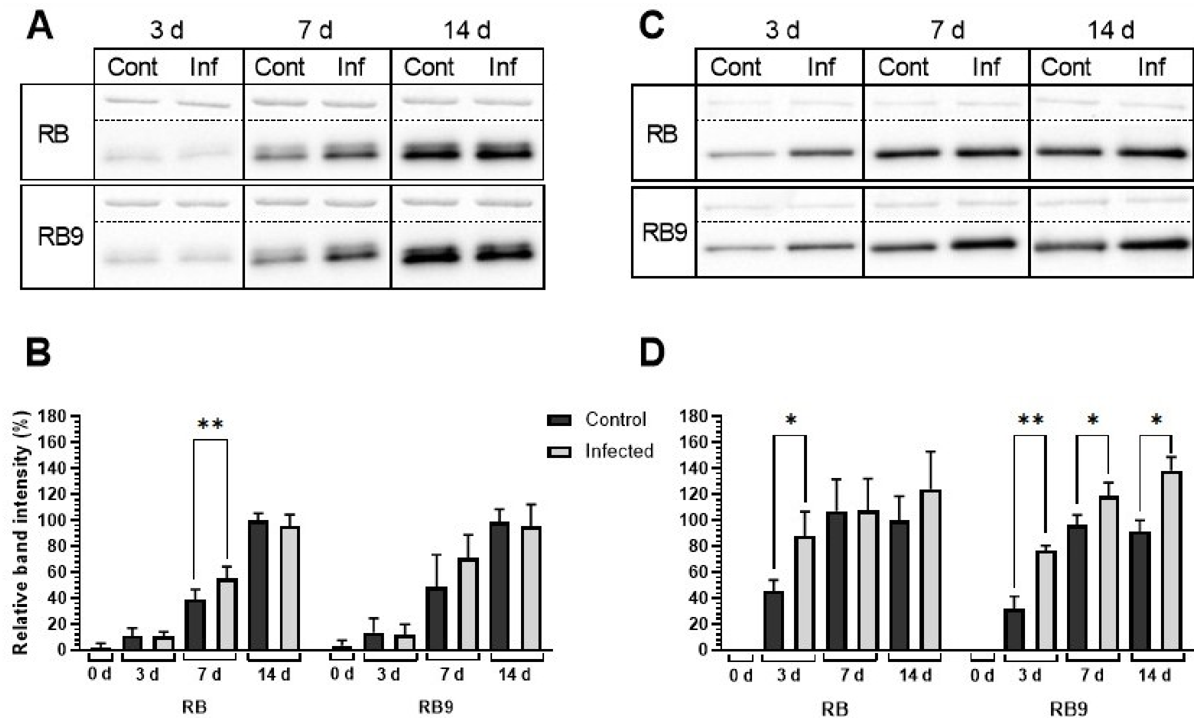


Figure 3.6. Changes in patatins and lipoxygenases content during *S. scabies* infection in mini-tubers of Russet Burbank and somaclone RB9 adapted to TA.

A, C Photographs of western blots probed with anti-patatin (**A**) and anti-Lox (**C**) Ab for Russet Burbank and TA adapted RB9 somaclone. The upper lines are pictures of membranes after Ponceau S staining, representing a loading control. **B**, **D.** Intensity of the bands corresponding to patatins (**B**) and lipoxygenases (**D**). 0.1 µg of total soluble protein was loaded in (**B**) and 5 µg of total soluble protein was in (**D**) in control and infected mini-tubers at 3rd, 7th and 14th days of experiment. The intensity of each band is expressed as a percentage from the band intensity of the corresponding protein accumulated in Russet Burbank mini-tubers under control conditions on the 14th day of the experiment. The band intensity is the average of six (patatin) or three (lipoxygenase) western blots analysed using the ImageJ program after background subtraction. Chart error bars are standard deviation; asterisk (*) reflects significantly different data sets according to unpared *t*-test (p<0.05), (**) p<0.01.

In contrast to RB, this tendency persisted for the remaining time points of the test for RB9 mini-tubers, in which bacteria significantly stimulated the accumulation of lipoxygenases compared to uninfected tubers. The intensity of lipoxygenase bands increased by 22% and by 46.5% on the seventh and fourteenth day, respectively in RB9 samples (Fig. 3.6 C and D). S. scabies provoked a rapid response in the form of lipoxygenase accumulation on the third day of RB and RB9 infection. In tubers of RB9, a strong accumulation of lipoxygenases was observed in response to the pathogen presence on the following days as well.

KTI are a group of proteins with different molecular weights that are involved in various processes, such as the growth and development, defense, and can also act as a storage proteins in potato tubers (Bergey et al., 1996; Grosse-Holz, 2016; van der Hoorn, 2008). During the mini tubers infection test, we detected Kunitz-domain containing proteins corresponding to molecular weights of approximately 18.5, 19, and 20 kDa.

The 19 kDa-band was the dominant of all bands detected by the anti-KTI antibodies. The intensity of this band increased from 8.6 and 11.8% on the third day, reached 62.1 and 36.1% on the 7th day and continued to increase up to 14 days, amounting to 100 and 82.7%, respectively, for RB and RB9 (Fig. 3.7 A and C). Pathogen infection caused an increase in the intensity of this band, which was most prominent on the 7th day with an intensity increased by 14.8% for the original variety and by 21.5% for RB9 (Fig. 3.7 C). These differences in the band intensity in the control and infection conditions were significant for RB9. It is possible that the 19-kDa Kunitz-type proteins could act in the response to scab-inducing *S. scabies*.

The smallest of the detected KTI proteins corresponded to 18.5 kDa band. The 18.5 kDa band intensity gradually increased under control conditions, starting from 4.9 and 3.3% on the third day and reaching 44.0 and 26.5% on the seventh day and 100 and

86.8% on day 14 for RB and RB9, respectively (Fig. 3.7 D). The intensity of the 18.5-kDa band was similar to control in the presence of the scab pathogen, suggesting that this protein may work as a reserve protein.

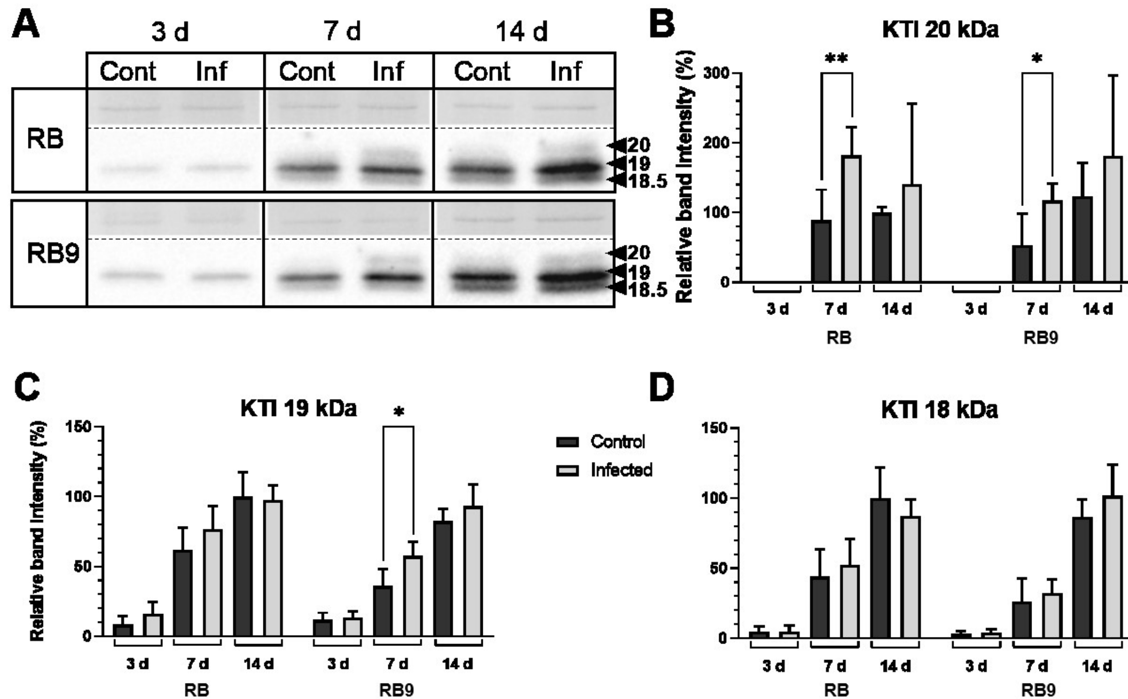


Figure 3.7. Changes in 20, 19 and 18.5 kDa Kunitz-type protease inhibitors content in control and during infection of RB and RB9 mini-tubers.

A. Photographs of western blots probed with anti-Kunitz Ab for proteins extracted from Russet Burbank (RB) and TA adapted RB9 somaclone, 15 µg of total soluble tuber protein was loaded to PAAG. The upper line represents photograph of membranes after Ponceau S staining (loading control). **B.** Intensity of the bands corresponding to a Kunitz-type protease inhibitor of 20 kDa in control and infected mini-tubers at 3rd, 7th and 14th days of experiment. **C.** Intensity of the bands corresponding to a Kunitz-type protease inhibitor of 19 kDa in control and infected mini-tubers at 3rd, 7th and 14th days of experiment. **D.** Intensity of the bands corresponding to a Kunitz-type protease inhibitor of 18.5 kDa in control and infected mini-tubers at 3rd, 7th and 14th days of

experiment. The relative intensity of each band is expressed as a percentage of the band intensity of the same protein detected in samples from the original variety in control conditions on day 14 of the experiment. The band intensity is the average of the six analyzed membranes measured using the ImageJ program after background subtraction; chart error bars are standard deviation; asterisk (*) reflects significantly different data sets according to unpared *t*-test ($p<0.05$), (**) $p<0.01$.

3.5.5 The number of cell layers increased in tuber periderm of TA adapted plants

The periderm of potato tubers is the primary barrier to the invasion of the pathogenic bacteria. Previously, the enchanced formation of phellem cell layers was shown in the tuber periderm of plants with induced resistance to common scab (Thangavel et al., 2016).Tuber periderm of RB9 and RB plants was examined using UV microscopy, which allows the visualisation of suberin polyphenols, auto fluorescent under UV light.

The number of peridermal layers observed in RB9 tubers was significantly higher than that of the original RB variety. The tubers of RB9 in the weight category from 0.3 to 0.8 g had an average of 13.3 ± 0.9 layers, while the corresponding tubers of the parental variety contained an average of 11.5 ± 0.8 layers (Fig. 3.8 A).

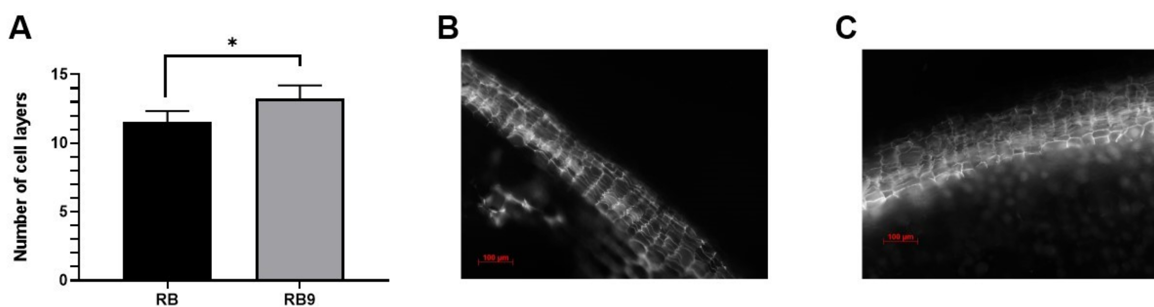


Figure 3.8. Periderm reinforcement in RB9 somaclone tubers after TA adaptation.

A. Increase in suberized cell layers number in the periderm of RB9 tubers in comparison with the parent variety of Russet Burbank. Values represent mean of four biological replications. Error bars in the graph represent the standard error of the mean. **B, C.** Photographs of suberin-containing auto-fluorescent cells in the periderm of potato tubers less than 1 gram in size, in the original variety Russet Burbank (**B**) and in somaclone RB9 (**C**). The scale bar located in the lower left corner of the periderm images B and C is 100 µm; asterisk (*) reflects significantly different data sets according to unpared *t*-test ($p<0.05$).

Microscopic observation of phellem cells in RB9 tubers also revealed changes in morphology, with cells that looked mishappened in RB9 compared to the mostly rectangular cells observed in RB tuber (Fig 3.8 B and C).

3.6 Discussion

The enhanced common scab resistance detected in somaclone RB9 was acquired as a result of Russet Burbank calli adaptation to thaxtomin A, the key factor in the pathogenicity of *S. scabies*. Increased resistance to common scab was stable in RB9 plants over several years, as confirmed by tests conducted in the light chamber, in pots, in the field as well as on mini-tubers formed from the axillary buds of potato stems. In

this work, we investigated the changes that occurred at the protein level as a result of adaptation to thaxtomin A and that could be involved in enhancing resistance to common scab.

3.6.1 Proteomic analysis

According to the proteomic analysis, changes in the presence and abundance of several groups of proteins occurred in the flesh of RB9 tubers. Adaptation to TA caused a slight change in the proteome, since 97.1% of the proteins were present both in the tubers of the parental variety and in the tubers of RB9.

Most of the proteins with higher abundance or detected exclusively in RB9 tubers were potentially involved in the response or reaction to various types of stress and pathogen defense. The most substantial changes in RB9 tuber protein were associated with the accumulation of proteins implicated in lipid metabolism, i.e., lipoxygenases and patatin proteins. These protein groups made up a total of 55.4% of the differentially accumulated proteins. Another important change was the accumulation of serine-type protease inhibitors bearing the Kunitz domain. Western blot and qPCR analysis of genes coding for patatin and KTI confirmed the proteomic data regarding changes in the abundance of patatins, lipoxygenases and KTI. Proteins not detected or less abundant in RB9 tuber flesh were involved in translation, transcription, ATP metabolism, and other biosynthetic processes. These changes may indicate a general decrease in metabolic activity and cell energy charge in RB9 tubers (Sowa et al., 1998).

Since the most significant changes have occurred in the content of proteins associated with lipid metabolism, i.e. lipoxygenases and patatins, as well as serine-type protease inhibitors, our work is focused on the involvement of these protein groups during the interaction of tubers with common scab inducing bacteria. Patatins, lipoxygenases, and KTI can be actively involved either in the inhibition of bacterial growth, neutralization of

pathogen lytic enzymes, or pathogen response signaling pathways. Significant changes in the abundance of these proteins occurred in the presence of *S. scabies* in RB and RB9 tubers formed by leaf buds.

3.6.2 Patatin

Various forms of patatins accumulated in large amounts in RB9 tubers. Since patatin is the main reserve protein of potato tubers with the content that can reach 40% of the total tuber protein, a 1.5-fold change in the total patatin content observed in these studies indicates an important accumulation of this protein in RB9 tubers, which therefore could influence the abundance of other tuber proteins (Mignery et al., 1988, Prat et al., 1990). In addition to its function as a reserve protein, patatin is involved in the protection against pathogenic organisms due to its enzymatic activities as phospholipase A_2 (PLA_2), lipid acyl hydrolase (LAH) and esterase (Andrews et al., 1988; Hirschberg et al., 2001; Racusen, 1984; Rosahl et al., 1987; Senda et al., 1996). For instance, patatin was shown to inhibit spore germination and the development of pathogenic microorganisms (Sharma et al., 2004). Patatin implication in the potato Zebra-chip disease was also proposed, since accumulation of patatins was detected in areal plant parts and decreased in potato tubers infected with *Candidatus* Liberibacter *solanacearum* (Alvarado et al., 2012; Kumar et al., 2015). Due to their functions in the defense response, the accumulation of active forms of patatin in RB9 tubers could inhibit germination of *S. scabies* spores, thereby delaying the spread of the pathogen in the tuber tissues and, thus, reducing the lesion area.

Significant changes in patatin abundance were observed on the 7th day of infection in RB and RB9 tubers. A delayed patatin response to the infection could be due to the fact that the isoforms actively involved in the response to the pathogen were minor comparing to the total mass of accumulated patatin. On the 14th day, the accumulation of patatin slowed down in infected tubers compared with healthy tubers for both RB

and RB9. At this point, it is possible that tuber metabolism was switching to a mechanism of energy conservation as a result of infection. Alternatively, this change may be explained by the beginning of proteolytic process described by Kumar et al. (Alvarado et al., 2012; Kumar et al., 2015). Significant accumulation of patatin during infection suggests that some patatin isoforms may be involved in the processes underlying the resistance of somaclone RB9 to common scab.

3.6.3 Lipoxygenases

The Lipoxygenases (Lox) that accumulated (up to 2.1 fold change) in RB9 tuber flesh belong to 9S-Lox group. Lox are enzymes that add oxygen to the acyl chain of polyunsaturated fatty acids (PUFAs) to produce 9- or 13-hydroperoxy derivatives. The primary product can be subsequently modified by diverse enzymes to generate an extensive metabolite family, called oxylipins. Oxylipins exhibit the protective activity either due to their signaling abilities or as direct antimicrobial compounds that are toxic to pathogen organisms (La Camera, 2004; Griffiths, 2015). Since lipoxygenases were accumulated in the tubers of scab-resistant somaclone RB9, it is possible that antimicrobial oxylipins were synthesized at a higher level in RB9, thus affecting the viability of *S. scabies* cells and reducing the symptoms of common scab.

Changes in abundance of 9S-lipoxygenases could also be associated with the induction of oxidative stress in plant cells. 9S-hydroperoxide products can induce the accumulation of reactive oxygen species (ROS) and may be involved in ROS signalling (Vellosillo et al., 2007; López et al., 2011). This suggests that changes in the abundance of proteins could be directly or indirectly associated with oxidative stress. Reduction in glyoxylate aminotransferase and GDP-D-mannose pyrophosphorylase in RB9 tubers as well as accumulation of glutathione peroxidase could suggest an increase in peroxide content in potato cells (Chen et al., 2004; Lin et al., 2011; Verslues et al., 2007).

The presence of *S. scabies* stimulated the accumulation of lipoxygenases, which was observed from the third day after the onset of infection. This increase in the content of lipoxygenases in the presence of the pathogen was maintained in RB9 mini-tubers during the entire experiment. In contrast, accumulation of lipoxygenase in infected parental mini-tubers was only noticeable on the third day of infection. A strong response in the form of lipoxygenase accumulation was a hallmark of scab-resistant somaclone RB9, which may indicate the involvement of these changes in increasing resistance to bacterial infection (Hwang and Hwang, 2010).

It was previously shown that patatin proteins can be involved in fatty acids mobilization from structural lipids to provide precursors for oxylipin biosynthesis (La Camera et al., 2004; La Camera et al., 2009). Lipoacylhydrolase activity (LAH) correlated with the expression of patatin coding NtPAT3 gene in the response triggered by specific stresses and infections. The NtPAT-LAH response could provoke plant cell death, inducing the strong accumulation of 9-hydroperoxy fatty acids (La Camera et al., 2004). Lox-generated fatty acid hydroperoxides could be further metabolised not only with a production of cytotoxic, or signaling compounds but could also generate substances with antimicrobial properties. This suggests that the patatin–LAH activity leading to oxylipin accumulation could contribute to increasing resistance to common scab.

3.6.4 Protease inhibitors

The accumulation of protease inhibitors in RB9 tubers may contribute to enhancing common scab resistance. Protease inhibitors are known to control the action of proteases that are vital for the growth and development of the organism (van der Hoorn, 2008). Tuber protease inhibitors are generally reported to act as storage and defense proteins (Bergey et al., 1996; Grosse-Holz et al., 2016). KTI were reported to have specific or broad target specificities with role in defense against lytic enzymes produced by herbivores and microbial pathogens attacks (Kim et al., 2005; Speransky, 2007; Li

et al., 2008). An increase in KTI abundance in RB9 tubers possibly could act in the inhibition of lytic proteases of *S. scabies* thus protecting tuber living cells from degradation. On the other hand, it was shown that KTI could act as antagonists of the PCD in plants triggered by phytopathogens and mixotoxin fumonisin b1 (Li et al., 2008). The suggested KTI role is intriguing, as it may imply their involvement in counteracting the PCD caused by thaxtomin A in the process of infection of potato tubers.

KTI have a double role as proteins that protect cells from pathogen proteases, and at the same time can be used as reserve proteins in potato tubers (van Der Hoorn, 2008). It was mentioned that KTI abundance was important during potato Zebra chip disease. Decrease in KTI abundance and induction of proteases induced by Zebra chip pathogen affected tuber protein content (Kumar et al., 2015). We observed that the 18 kDa KTI level was not changed in the presence of bacteria, which could indicate its role as a reserve protein. In contrast, KTI with a molecular weight of 20 and 19.5 kDa tended to increase under infection both in RB9 and Russet Burbank mini-tubers. The accumulation of the 19 and 20 kDa KTI during infection may indicate that they could be involved in the response to common scab infection.

A definite relationship can be discerned between the accumulation of lipoxygenases and KTI. It was shown that, the production of oxylipin derivatives of JA pathway can activate the transcription of genes encoding protease inhibitors (Farmer and Ryan 1992). These data were reported by da Silva Fortunato et al. who suggested that KTI could be activated by lipid hydroperoxide products (da Silva Fortunato et al., 2007). We cannot exclude the possibility that lipoxygenases and KTI could be implicated in one metabolic mechanism in response to *S. scabies* infection.

3.6.5 Common scab resistant somaclone has a reinforced periderm

Since the periderm is known as a primary barrier to pathogenic bacteria, changes at the periderm level may affect the susceptibility of potato tubers to common scab. As it was mentioned earlier, selection with thaxtomin A caused increased suberization of potato tuber lenticels (Thangavel et al., 2016). The gradual adaptation to thaxtomin A caused a significant increase in the number of suberin containing cell layers in the periderm of RB9 somaclone. An increase in the number of suberized cell layers in RB9 tuber periderm may serve as an additional barrier to the penetration of pathogenic bacteria. Thus, reinforced periderm could have a positive effect on tuber resistance to common scab.

3.7 Conclusions

Increased resistance to common scab in TA-adapted somaclone RB9 was associated with proteomic and morphological changes in RB9 tubers. In general, proteomic analysis revealed changes in the abundance of proteins related to lipid metabolism, oxidative stress, and inhibition of serine proteases. The main metabolic changes in RB9 tuber were associated with patatin, 9S-ipoxygenases and KTI accumulation. We found that *S. scabies* infection of mini-tubers induced the accumulation of patatin, 9S-lipoxygenases and KTI in RB9 and Russet Burbank, suggesting their involvement in pathogen response. The *S. scabies*-mediated induction of 9S-lipoxygenases in RB9 tubers may be implicated in a mechanism underlying resistance to common scab. Another factor possibly contributing to scab resistance could be changes in the outer periderm layer which showed an increase in the number of suberin-containing cell layers that may prevent pathogen entry.

3.8 Materials and methods

3.8.1 Chemicals

All chemicals were purchased from Sigma-Aldrich Company unless otherwise indicated.

3.8.2 Plant material and growth conditions

Potato in vitro cuttings were kindly provided by Les semences Elite du Québec Inc. and Les Buisson Research Center Inc. Potato plants were maintained and propagated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% sucrose, 0.7% agar (BD Difco) and pH 5.7. Light conditions were set at 60-75 µmol m^{-2} s^{-1} with a light period of 16 h day/8 h night and constant temperature 20-22°C in SANYO growth cabinet. Plants were subcultured as one-node segments every 8 weeks.

3.8.3 Proteomic analysis

Label-free LC-MS/MS of RB and RB9 tuber flesh (tuber size 1.5-2.5 g) was conducted in Quebec genomic center (Proteomics platform). 1ug of each sample was injected in LC-MS MS Orbitrap Fusion and resolved for 300 min with 270 min gradient. Data dependent acquisition was performed with MS MS higher-energy collisional dissociation (HCD). Bioinformatic analysis was performed using MaxQuant / Andromeda. Protein accession numbers were retrieved from the Uniprot Databank for *Solanum tuberosum* taxon TaxID 4113 (TAX_SolaTube) with fixed modifications: Carbamidomethylation (C), and variable modifications: Acetylation (Prot N-term), Oxidation (M). Quantification was performed with normalized LFQ values on unmodified peptides only. Changes in protein accumulation in RB9 compared to RB

tubers were considered as significant if *t*-test p-value was ≤ 0.10 and Fold Change ≥ 1.5.

3.8.4 Western blot analysis

Proteins were extracted from tuber flesh of cv. Russet Burbank with 0.1 M Tris-base (Fisher Sci.) adjusted to pH=8.0 with HCl (Ricca), 5% sucrose (m/v), 2% (m/v) SDS (Fisher Sci), protease inhibitors (Protease inhibitor cocktail; PMSF 1mM). Protein concentration was determined by RC DC (BioRad) assay. Samples were heated 95°C 5 min, and from 0.1 µg to 15 µg of total protein were resolved in 12 or 15% SDS-PAGE gels, as indicated. Proteins were electroblotted to PVDF membrane (Amersham) for 1h-1.5h using tank transfer. Blots were blocked with a skimmed milk (Selection) 4% (m/v) in T-TBS (1.5h) at RT with agitation. Primary antibodies were applied overnight in dilution 1:5000 with agitation at 4°C. For patatin and lipoxygenase detection, we used the primary polyclonal anti-Patatin antibodies (AS12 1842) purchased from Agrisera, Sweden and anti-Lox (CLAS06-128A), CEDARLANE, Canada, produced in rabbit. Detection of KTI was performed with anti-*Sl*CDI loop polyclonal antibodies (Khalf et al., 2010). After washing with T-TBS 2 times, membrane was incubated with secondary antibodies. Goat Anti-Rabbit IgY HRP conjugate (Transgen biotech) were used for anti-patatin and anti-Lox and Goat Anti-Chicken IgY HRP conjugate (Life technologies) for anti-CD1 binding in dilution 1:10000 for 1 hour at RT. Blot was washed as above and developed with ECL (Clarity Western ECL Substrate, BioRad, 170-5060) for 5 minutes. Membranes were photo documented with Bio-Rad ChemiDoc™. Band density was estimated with ImageJ program after background subtraction (Schneider et al., 2012).

3.8.5 RNA extraction and RT-qPCR

The search for the corresponding RNA sequences was carried out in NCBI-Gene database. Primers for qPCR listed in table S3.3, were generated using Primer-BLAST

(NCBI – NIH) program. Synthesised primers were validated by PCR. PCR fragment size was estimated in 2.5% agarose gel. RNA from potato tubers was extracted using phenol:chlorophorm extraction method (Kumar et al., 2007). To digest DNA, the samples were treated with TURBO DNase (Invitrogen). RNA was quantified with Spectrophotometer NanoDrop and RNA quality was determined using 1.5% agarose gel electrophoresis. 2 µg of extracted RNA were mixed with 1 µl of 0.5 µg/µl anchored oligo DT in the final volume of 10 µl and heated to 70°C for 5 minutes. After cooling down to +4°C, 5 µl of AMVRT reverse transcriptase (Promega), 1.5 µl of RNAsin (Promega), 1 µl of dNTPs (10 mM; IDT) and 2.5 µl of 5x AMVRT buffer (Promega) were added to RNA samples to the final volume of 25 µl. cDNA synthesis was performed using the program: 42°C for 1 hour, 70°C for 10 minutes. Afterwards samples were cooled down to +4°C and kept frozen (-20°C) until analysed. 1 µl of cDNA was mixed SYBR Green Master Mix (BioRad) and 0.25 µM of each of two primers (F+R) according to recommendations of the supplier. Real-time quantitative PCR was carried out using the program: denaturation 95°C for 3.00 min (1 cycle), annealing 95°C for 15 sec, 63°C for 30 sec (40 cycles), and extension 95°C 1 min, 60°C 30 sec, 95°C 30 sec (1 cycle) on the Mx3000P thermocycler (Agilent Technologies, Santa Clara, USA). Potato 18S rRNA was used as a reference (F: AATTACCCAATCCTGACACGGG; R: TTGCCCTCCAATGGATCCTCGTTA (Nicot et al., 2005)). Annealing temperature for majority of primers was set at 63 °C and 60°C for primers Pat-1 and 18S. The calculations of relative gene expression were made using the method delta delta Ct (Livak and Schmittgen, 2001).

3.8.6 Leaf bud assay

S. scabies EF-35 were propagated in liquid Yeast Malt Extract medium (YME) containing 4 g/L glucose, 4 g/L yeast extract (Fisher Sci), 10 g/L malt extract (BD Difco) for 5-8 days (Pridham et al., 1956). Afterwards, *S. scabies* EF-35 (Paradis et al., 1994) were plated to solid YME, supplemented with 1.5% agar (BD Difco) and 1 g/L of $CaCO_3$.

SAY solution containing 20 g/L glucose, 1.2 g/L L-asparagine, 0.6 g K_2HPO_4, and 10 g/L of (BD Difco) yeast extract (Labruyere, 1971; Goyer and Beaulieu, 1997) was mixed with 300 ml of vermiculite (Holyday, Montreal, Canada) in Magenta boxes under sterile conditions. Each Magenta box was inoculated with two pieces (about 1 cm^2) of solid YME with *S. scabies* mycelium and incubated 21 day at 30ºC in the dark.

Four-week-old plants propagated *in vitro* were transferred to pots filled with soil mixture (soil:sand:vermiculite=2:2:1) and grown at 22/16ºC under 16-h photoperiod with light intensity adjusted to 60-75 μmol m^{-2} s^{-1} in the Conviron plant growth chamber (PGR15). After one month, the photoperiod was changed to 12 h day/12 h dark to stimulate tuber formation for an additional month. The lower 2/3 of the stems of 2-month old plants were cut into nodal segments containing the leaf. The nodal segments were placed in a moistened substrate consisting of sand and vermiculite mixture (1:1) so that the stem segment with the axillary bud was completely dipped in the substrate with the leaf blade remaining on the surface (Struik and Wiersema, 2012). The bacterial culture was previously added to the substrate in a dilution 1/20 or 1/40 for experimental conditions. A substrate not infected with bacteria was used as a control. Leaf buds were irrigated twice a week with 200 ml of water for every 3 liters of substrate. Mini-tubers were harvested three weeks after the start of the experiment and photographed from two sides. The area covered with scabs was measured using the ImageJ program and expressed as a percentage relative to the total surface of the tuber.

3.8.7 Infection with *S. scabies*

Bacterial cultures and plant stem segments were prepared as previously described in Leaf bud assay. Freshly cut nodal segments were kept in a moistened vermiculite to assure tuber development. After 7 days of incubation, leaf buds were placed in a sand-vermiculite (1:1) containing bacteria *S. scabies* EF-35 in the amount of the fortieth (1/40) part of the total volume of the substrate. Samples were collected at day 0 before

the infection, and at 3, 7 and 14 days after the onset of infection. Each sample consisted of pooled mini-tubers in the amount of 10 on day zero, 4 on day 3 and 7, and 3 on day 10, so that the total weight reached 1.0 - 2.0 grams. Each pool was considered as a biological replication. Total protein was isolated from whole mini-tubers and analyzed as described above for Western blot analysis. Experiment was repeated two times. Results are presented as a mean of 6 biological replications analysed for Patatin and KTI and 3 biological replications for Lox.

3.8.8 Periderm analysis using fluorescent microscopy

The analysis of periderm organization and thickness was performed on tubers of 0.3 – 0.8 g (0.8 – 1.15 cm). From each tuber three slices were cut out in abaxial, middle and adaxial regions. Cross sections of periderm were cut on two sides of each slice with a razor blade. Autofluorescence of periderm layers containing suberin was visualized using a Zess AxioImager Z1 fluorescence microscope (excitation 365, 445/50 emission 461 nm; DAPI). The average number of cell layers was calculated from the results from at least 3 tubers.

3.8.9 Statistical analysis

The statistical analysis of data sets was performed using Student's *t*-test after a preliminary comparison of the variances of the parental and habituated sample.

3.9 Acknowledgments

We thank les semences Elite du Québec Inc. and Les Buisson Research Center Inc. for *in vitro* potato plants. We would like to acknowledge the MSc. student Marc-Antoine Turcotte for designing qPCR primers for general patatin gene expression analysis.

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3.10 Supplementary material

Table S3.1. Proteins exclusively detected by LC-MS/MS in RB tubers.

Entry name	Protein names	Gene ontology (GO)
Q2XPX2_SOLTU	40S ribosomal protein S3a	cytosolic small ribosomal subunit [GO:0022627]; structural constituent of ribosome [GO:0003735]; translation [GO:0006412]
M1CBS3_SOLTU M1CBS2_SOLTU	Uncharacterized protein	ribosome [GO:0005840]; structural constituent of ribosome [GO:0003735]; translation [GO:0006412]
M0ZY46_SOLTU M1AUU9_SOLTU M0ZY47_SOLTU	Ferredoxin--NADP reductase, chloroplastic (FNR) (EC 1.18.1.2)	chloroplast [GO:0009507]; ferredoxin-NADP+ reductase activity [GO:0004324]
M0ZWK1_SOLTU M1B932_SOLTU	Uncharacterized protein	amino acid binding [GO:0016597]; ornithine carbamoyltransferase activity [GO:0004585]
Q38HV0_SOLTU	Salt tolerance protein 5-like protein	
M1C9X0_SOLTU	Uncharacterized protein	chloroplast stroma [GO:0009570]; phosphopyruvate hydratase complex [GO:0000015]; magnesium ion binding [GO:0000287]; phosphopyruvate hydratase activity [GO:0004634]; glycolytic process [GO:0006096]; response to cytokinin [GO:0009735]; trichome morphogenesis [GO:0010090]
M0ZH89_SOLTU M0ZH90_SOLTU	Uncharacterized protein	nucleus [GO:0005634]; nucleosome assembly [GO:0006334]
M1CKH6_SOLTU	Uncharacterized protein	ribosome [GO:0005840]; structural constituent of ribosome [GO:0003735]; zinc ion binding [GO:0008270]; translation [GO:0006412]
M1C305_SOLTU M1C306_SOLTU M1CRP6_SOLTU	Glyco_transf_20 domain-containing protein	catalytic activity [GO:0003824]; trehalose biosynthetic process [GO:0005992]

Entry name	Protein names	Gene ontology (GO)
Q41415_SOLTU Q41414_SOLTU M1C5H3_SOLTU Q41412_SOLTU M1C5H2_SOLTU Q41413_SOLTU Q41416_SOLTU	Epoxide hydrolase	hydrolase activity [GO:0016787]
M1CR82_SOLTU M1CR83_SOLTU M1CR81_SOLTU	PHB domain-containing protein	endoplasmic reticulum [GO:0005783]; ubiquitin protein ligase binding [GO:0031625]; ubiquitin-dependent ERAD pathway [GO:0030433]
I6XKY2_SOLTU M1B080_SOLTU M1A5G2_SOLTU M1A5G3_SOLTU I6WZC5_SOLTU	Serine/threonine-protein phosphatase (EC 3.1.3.16)	nucleus [GO:0005634]; metal ion binding [GO:0046872]; protein serine/threonine phosphatase activity [GO:0004722]; brassinosteroid mediated signaling pathway [GO:0009742]
WHY1_SOLTU	Single-stranded DNA-binding protein WHY1, chloroplastic (DNA-binding protein p24) (PR-10a binding factor 2) (PBF-2) (Protein WHIRLY 1) (StWhy1)	chloroplast [GO:0009507]; nucleus [GO:0005634]; DNA-binding transcription factor activity [GO:0003700]; sequence-specific DNA binding [GO:0043565]; single-stranded DNA binding [GO:0003697]; defense response to fungus [GO:0050832]; positive regulation of transcription, DNA-templated [GO:0045893]
M0ZMF8_SOLTU	Phosphoserine aminotransferase (EC 2.6.1.52)	O-phospho-L-serine:2-oxoglutarate aminotransferase activity [GO:0004648]; L-serine biosynthetic process [GO:0006564]
M1AEU6_SOLTU M1AEU4_SOLTU M1AEU7_SOLTU M1AEU5_SOLTU M1AEU8_SOLTU	Peptidase_S9 domain-containing protein	serine-type peptidase activity [GO:0008236]
Q948Z3_SOLTU M1B703_SOLTU	Peroxidase (EC 1.11.1.7)	extracellular region [GO:0005576]; heme binding [GO:0020037]; metal ion binding [GO:0046872]; peroxidase activity [GO:0004601]; hydrogen peroxide catabolic process [GO:0042744]; response to oxidative stress [GO:0006979]
M0ZIP7_SOLTU	NAC-A/B domain-containing protein	nascent polypeptide-associated complex [GO:0005854]

Table S3.2. Proteins exclusively detected by LC-MS/MS in RB9 tubers.

Entry name	Protein names	Gene ontology (GO)
M1CRG7_SOLTU	Plug_translocon domain-containing protein	endoplasmic reticulum [GO:0005783]; integral component of membrane [GO:0016021]; protein transport [GO:0015031]
M1AMV5_SOLTU	C2 domain-containing protein	
M1AMV7_SOLTU M1AMV8_SOLTU	Uncharacterized protein	transferase activity, transferring acyl groups other than amino-acyl groups [GO:0016747]
M1C511_SOLTU	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1	endoplasmic reticulum membrane [GO:0005789]; integral component of membrane [GO:0016021]; plant-type cell wall [GO:0009505]; plasma membrane [GO:0005886]; vacuolar membrane [GO:0005774]; dolichyl-diphosphooligosaccharide-protein glycotransferase activity [GO:0004579]; protein N-linked glycosylation [GO:0006487]
M1CFR8_SOLTU M1CFR9_SOLTU	Uncharacterized protein	protein tyrosine phosphatase activity [GO:0004725]
M0ZRU4_SOLTU	M20_dimer domain-containing protein	hydrolase activity [GO:0016787]
M1CR44_SOLTU	Uncharacterized protein	

Table S3.3. Primers used in qPCR gene expression analysis.

Protein name	Protein number/locus	Primers (NCBI)
Kunitz-type serine protease inhibitor DrTI-like	M1AN26/102593157	F: CAGCAATAAACGGAGCCGGA R: GCAACACCTGAAAGGGACAGA
Latex serine proteinase inhibitor-like (KTI-D)	M1AG22, A0A097H1A8/ 102589278	F: GAGGCGTGAGGCTTGCTAA R: AGACTGCTACACCATTATCGAGG
Patatin-2-Kuras 3	Q42502/ pat2-k3 (pPATB1)	F: GGTCTCGGGTTGTAGTGGT R: CCAAAAGGTTACATAATCCAAGCAC
Patatin-1 (Pat-1)	Patatin groups: 1, 2, 8,12, J1, D1, D2, D3, A1, A2, A3	F: AAT CAT TCC GGG TAC CAT TCT C R: CTC CTG TAC TTG TTC CTC CAA TTA
Lox1.2 (Linoleate 9S-lipoxygenase 2-like)	O24379/102602192	F: TGGACTATTTGGTGGCCCTG R: ACCATTTGTAGGATCACCTTGAAC
DES	Q9AVQ1/ DES (LOC102588225)	F: GCAAGTCCGCTGTGTCCATA R: GTGTGTAGACTAGCCCCTGC
9-AOS	M0ZKX2/ AOS, AOS2 (102577479)	F: CGGAAACGGAAAGTCCGACA R: TCCCGACCTCGATCCAAAAC
AOC	Q8H1X5/102577822	F: GCTCAACAGATTCAACTAACACTGA R: AGATAAGCAGGGCTTCCACG
HPL	Q93X18/ Hpl (102577863)	F: CCAAATGTGGTGGCGGTTTT R: TCTTAATCTGGGCATGTTTAGGTTC
Epoxide hydrolase	Q41415/102577894	F: CTATCCCTGATGCTCCGGTT R: CCAGTTTATGGGTAAAGCACGG

CHAPTER 4

Thaxtomin A induces the production of phenolic compounds in potato tuber slices

4.1 Article introduction and contribution of authors

Thaxtomin A, a key virulence factor of *S. scabies*, was reported to induce the formation of symptoms similar to those induced by the bacterium on potato tubers. Application of thaxtomin A on potato tuber slices induces browning of the tuber parenchyma tissue, presumably due to necrosis. However, it was shown that the intensity of browning (the so-called "necrosis") caused by thaxtomin A did not correlate with the resistance to common scab in some potato varieties. Here, our objective was to determine why the browning effect of thaxtomin A is more pronounced on tuber slices of common scab-resistant potato variety Russet Burbank than on slices of the sensitive Yukon Gold variety. We showed that the observed symptoms of tissue browning are not due to cell death, as previously assumed, but rather correlate with the accumulation of phenolic compounds that cause a characteristic brown color. When using toluidine blue O staining on TA treated cells that had turned brown, we observed a blue coloration indicating the accumulation of phenolic compounds. The flesh of Yukon Gold tuber treated with thaxtomin A accumulated less phenolic compounds than Russet Burbank tissues treated with TA. Moreover, tissue browning could be reduced by the addition of a competitive inhibitor of the enzyme Phenylalanine-ammonia lyase (PAL), indicating that thaxtomin A induced the *de novo* synthesis of phenolic compounds when applied to tuber flesh.

The contributions of each author in the manuscript were as follows: NB and II designed the experiments, analysed and interpreted data. II performed all experiments. NB

supervised the project. NB and II wrote the manuscript. All authors approved the final version of the manuscript.

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Thaxtomin A induces the production of phenolic compounds in tuber slices

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4.2 Abstract

The application of phytotoxin thaxtomin A on potato tuber slices causes browning of the flesh tissue. Staining with toluidine blue O showed that this browning was due to the accumulation of phenolic compounds. Induced phenolic compounds accumulated to a greater extent in the tissues of the common scab resistant Russet Burbank than in those of the susceptible Yukon Gold variety, which does not correlate with the level of cell death caused by thaxtomin A. The addition of a competitive inhibitor of the phenylalanine ammonia-lyase enzyme reduced tissue darkening caused by thaxtomin A, indicating the induction of the phenylpropanoid pathway.

Keywords: *Streptomyces scabies*, thaxtomin A, potato, tuber slices, total phenolics

Highlights

1. Thaxtomin A induces phenols accumulation in potato tuber parenchyma.

2. Phenols accumulation does not correlate with cell death caused by thaxtomin A.

3. Tuber flesh darkening induced by thaxtomin A does not reflect resistance to common scab.

Abbreviations: AIP – competitive PAL inhibitor 2-aminoindan-2-phosphonic acid; EDS1/PAD4 – ENHANCED DISEASE SUSCEPTIBILITY1/ PHYTOALEXIN DEFICIENT4 plant defense pathway; PAL – phenylalanine ammonia-lyase; TA – thaxtomin A

4.3 Introduction

Thaxtomin A (TA) is a phytotoxin which is essential for the pathogenicity of the potato common scab inducing agent *Streptomyces scabies* (syn. *S. scabiei*). *S. scabies* infects young potato tubers during the intensive growth phase. The infection causes the formation of lesions on the tuber surface in the form of scabs or pits, which reduce the market value of the crop [1]. Although the exact mechanism of action of TA as a pathogenic factor during scab induction has not yet been elucidated, it was shown that TA overproduction or repression in *S. scabies* correlates with the pathogenicity of bacteria, since mutants impaired in the TA biosynthesis pathway did not infect plants [2, 3, 4].

TA was described as an inhibitor of cellulose biosynthesis [5], causing a variety of physiological effects on plant tissues and cells. This toxin was shown to induce an atypical programmed cell death (PCD) in Arabidopsis cell cultures and to provoke a rapid Ca^{2+} influx [6, 7]. Recently, TA was identified as a chemical activator of the EDS1/PAD4 signalling that plays an important role in plant basal immunity against virulent biotrophic pathogens and in effector-triggered immunity [8]. TA is known to cause defense responses, linked with the accumulation of phenolic derivatives in plants leading to ectopic lignification in etiolated Arabidopsis seedlings and accumulation of defensive phytoalexin scopoletin in tobacco leaves [9, 10]. TA treatment of Arabidopsis suspension cells induced defense-related phenylalanine-ammonia lyase (PAL) gene expression [11]. This variety of effects indicates the great influence of TA on various processes occurring in plant cells. However, it does not explain its specific method of action in the disease.

The use of TA in the selection or adaptation of plant cells, among others, revealed changes in the metabolism of phenolic compounds. Adaptation of hybrid poplar cells to TA modulated the expression of genes involved in cell wall and lignin biosynthesis [12].

Moreover, scab-resistant plants generated from TA-selected cells showed an extensive suberization of phellem layers beneath the tuber lenticels as a positive trait against pathogen invasion [13]. Furthermore, it was shown that in response to pathogen and in common scab-resistant potato somaclones, tubers produced more phellem cell layers and accumulated suberin polyphenols in the phellem cell walls [14]. Intensive suberization plays a role in the protective reaction of the tuber to the development of infection, since the accumulation of phenolic compounds in the periderm of the diseased tuber can contribute to the formation of a protective barrier that makes cell walls more resistant to enzyme attack [15].

TA has often been used for preliminary screening of potato germplasm for common scab resistance [16]. Darkening of the tuber parenchyma as a result of TA treatment has been described as necrotic lesions [13, 17, 18, 19, 20]. Unfortunately, conflicting results were obtained when analyzing a collection of potato varieties with a known level of common scab resistance. It was found that the Russet Burbank potato variety, which is moderately resistant to scab, was very sensitive to TA, while the scab sensitive variety Tasman showed a high tolerance to TA [13]. In addition, we found in preliminary studies that common scab sensitive Yukon Gold potato variety was extremely resistant to TA, while the Russet Burbank variety was very sensitive to TA, as reported before. Controversy in the response to TA in these varieties brought us to investigate the underlying events that cause the development of dark areas on the potato tuber surface as a result of TA application.

Taking into account that TA causes the accumulation of phenolic compounds in plant tissues, as described above, we hypothesized that the observed color change occurred as a result of the accumulation of phenol-containing substances. In this work, we show that Russet Burbank parenchymal cells of tuber slices treated with TA accumulated significant amounts of phenolic compounds, resulting in strong tissue browning. In contrast, TA applied on Yukon Gold tuber slices only induced a slight increase in

phenols accumulation. Although TA caused cell death in the tuber flesh cells, the level of cell death did not correlate with the development of the brown color. These results question the use of TA for testing common scab resistance.

4.4 Material and methods

4.4.1 Tuber production

In vitro grown potato plants of Russet Burbank and Yukon Gold varieties were kindly provided by Les semences Elite du Québec Inc. and Les Buisson Research Center Inc. Potato plants were propagated on Murashige and Skoog (MS) medium [21] supplemented with 3% sucrose, 0.7% agar (BD Difco) and pH 5.7. Light conditions were set to 60-75 µmol m^{-2} s^{-1} with a light period of 16 h day/8 h night and constant temperature 20-22°C in SANYO growth cabinet. Four-week-old plants propagated *in vitro* were planted in pots filled with soil mixture (soil:sand:vermiculite=2:2:1) and grown at 22/18 ºC under 16-h photoperiod with light intensity adjusted to 60-75 µmol m^{-2} s^{-1} in a Conviron plant growth chamber (PGR15). After one month, the photoperiod was changed to 12 h day/12 h dark to stimulate tuber formation. Irrigation was applied twice a week, one time in combination with fertilizer (20:20:20). After 16 weeks of plant growth, tubers were harvested and used for analysis.

4.4.2 TA extraction and purification

Production and purification of TA were performed from oat bran broth inoculated with *S. scabies* EF-35 according to Beauséjour [22].

4.4.3 TA test on tuber slices

This method was modified from Loria et al., 1995 [17]. Potato tubers were sterilised with 30% of bleach solution in water during 20 minutes, dried under sterile laminar flow and aseptically cut into slices. Tuber slices were placed on sterile wet filter paper in Petri dishes. Discs of filter paper (5 mm) were soaked in TA diluted in methanol or in methanol as a control and dried under sterile air flow. After being completely dry, the 5 mm filters were transferred to the potato slice surface and a water drop (20 µl) was added to the top of the disc to ensure tight connection with the tuber surface. Tuber slices were stored in the dark at room temperature for 4 days unless otherwise specified.

4.4.4 Microscopic study of the effect of TA on potato parenchyma cells

After conducting the TA test using 5 and 7 µM TA on tuber slices for 7 days, freehand sections were prepared from tuber slices of Russet Burbank and Yukon Gold varieties. Tuber slices were cut into sections perpendicular to the TA treated surface. Light microscopy was performed on tuber tissue sections using Leica stereomicroscope (M165FC).

4.4.5 Toluidine Bleu O staining

For phenolic staining, tuber flesh fresh hand-sections were incubated in 0.01% (w/v) Triton X-100 (Fisher Sci.) and 10% (v/v) commercial bleach for 24 h at room temperature to reduce pigmentation. Afterwards, sections were rinsed with distilled water and 100% ethanol [23]. Toluidine blue O 0.1% solution was applied to tuber flesh sections for 10-15 sec. Sections were rinsed with distilled water and observed with Leica stereomicroscope (M165FC) coupled with imaging system.

4.4.6 Evans Blue determination of TA-induced cell death

This method was modified for potato tubers from Nv et al., 2017 [24]. The tubers of Russet Burbank and Yukon Gold were washed and surface sterilized with 30% bleach for 20 min. Pieces of tuber flesh in the form of cylinders with a diameter of 10 mm were cut out aseptically with a cork borer. Each cylinder was split lengthwise with a scalpel into two parts and cut into slices of approximately 1 mm thick. Parenchymal slices were incubated in liquid MS (pH 5.7) medium containing either 5 μM TA, the corresponding volume of methanol, hydrogen peroxide (100 mM) or untreated (control) on an orbital shaker, 120 rpm. Samples were collected after 0, 24, or 48 h, frozen in liquid nitrogen, and stored at -20°C until further analysis.

Parenchyma slices were rinsed with distilled water and placed in 4.5 ml Evans Blue dye solution (2.5 mg/ml Evans Blue, $CaCl_2$ 0.1 M, pH 5.6) for 20 minutes on an orbital shaker. After staining, slices were washed three times with distilled water. The excess water was absorbed with filter paper. Evans Blue stain was extracted from 100 mg of tuber parenchymal tissue. Tuber flesh tissues were ground in liquid nitrogen using a mortar and pestle. The dye was extracted with 1 ml of a 1% SDS (Fisher Sci.) solution. After centrifugation, the optical density was measured at 600 nm using a microplate reader and compared with an Evans Blue standard curve.

4.4.7 Inhibition of phenylalanine ammonia-lyase activity

Slices of Russet Burbank potato tubers were treated with 5 μM TA as described above. For this assay, we added either 20 μl of water or 20 μl of 10 μM 2-aminoindan-2-phosphonic acid (SV ChemBioTech Inc.) to filter disk just after transferring the 5 mm filters containing TA to the surface of a potato slice. The discs were also wetted 24 hours after the start of the test. Color development on slices was observed and photo-documented after 5 days in the dark at room temperature.

4.4.8 Total phenols extraction and determination

Slices of potato tubers were treated with 5 and 7 µM TA or methanol as described above (TA test on tuber slices) for 5 days. Area treated with TA or methanol (Fisher Sci.) was cut out of the slices, homogenised in liquid nitrogen and lyophilised. Samples were stored at -80ºC until used. Extraction of phenolic compounds was performed as described in Jin et al., 2018 [25]. Total phenolics determination in samples was done using Folin–Ciocalteu reagent [26].

4.4.9 Chemicals

All chemicals were purchased from Sigma-Aldrich Company unless otherwise indicated.

4.5 Results

In order to determine the effect induced by TA in the scab-resistant variety Russet Burbank and the sensitive variety Yukon Gold, the intensity of browning spots in the TA-treated area was compared. TA was applied on tuber slices at different concentrations. We found that the colored (dark brown) areas on the surface of scab-sensitive YG tuber slices at all TA concentrations were less pronounced than those for Russet Burbank, a potato variety moderately resistant to common scab (Fig. 4.1). These results indicated that the common scab resistance of these potato varieties was not consistent with resistance to TA as suggested before by Wilson et al. [13].

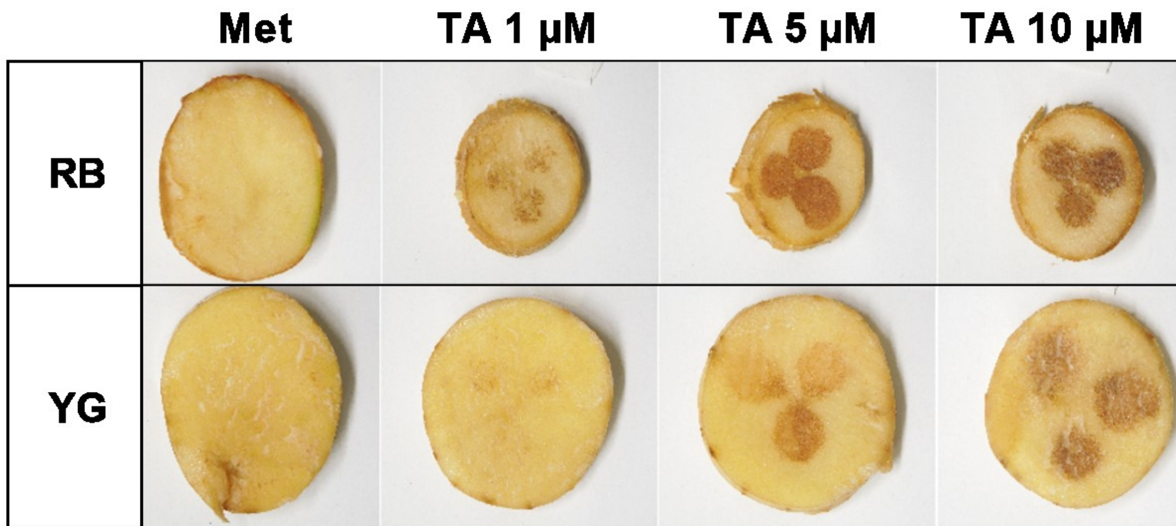

Figure 4.1. Russet Burbank (RB; common scab resistant) and Yukon Gold (YG; common scab sensitive) TA test.

Met – control, tuber slices were treated with filter disks soaked in methanol; TA, tuber slices were treated with filter disks soaked in 1, 5 and 10 µM thaxtomin A. Pictures were taken after 6 days.

Microscopic examination of tuber flesh sites exposed to TA, methanol or untreated helped to clarify the cause of this phenomenon (Fig. 4.2). After seven days either in the control slices or after exposure to methanol, darkening of the cells was observed at the edge of the surfaces of tuber slices. Seven days after the wound, as reported before, a completely formed closing layer was located on the surface of the wound tissue [27]. Changes in cell morphology suggested that the process of formation of the wound periderm had begun (Fig 4.2 A, B and E, F) [28]. After treatment with TA, the slice surface looked much darker than in the control, affecting several layers of cells. In this case, the changes in structure and color were very similar to changes observed during the hypertrophic formation of the wound periderm, as described before [28] (Fig. 4.2).

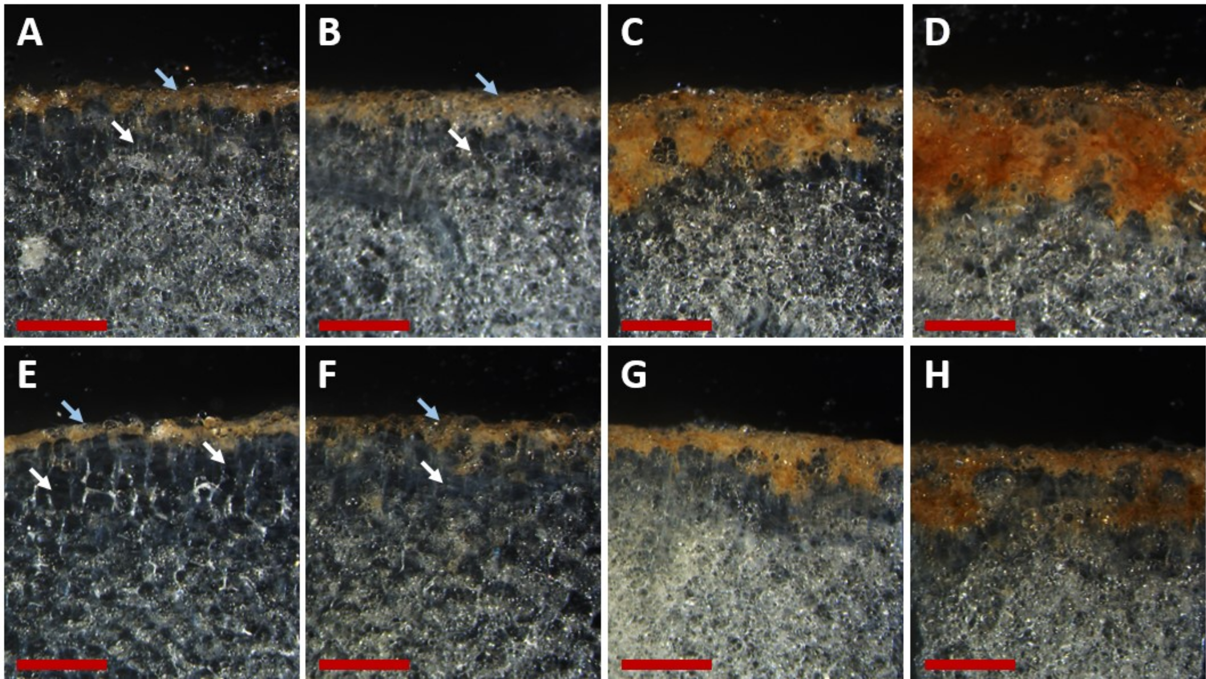

Figure 4.2. TA induces morphological changes in the Russet Burbank and Yukon Gold parenchyma.

Sections were cut perpendicular to the TA treated surface of Russet Burbank (A-D) and Yukon Gold (E-H) tuber slices, 7 days after treatment; A and E: control conditions (no treatment); B and F: treated with methanol; C and G: treated with 5 μM TA; D and H: treated with 7 μM TA; phellogen formation is pointed with white arrows; closing layer marked with blue arrows; scale bar is 0.5 mm.

During the formation of the wound periderm, the cell wall is strengthened, with the accumulation of phenolic compounds. The Toluidine blue O is a polychromatic dye that can stain polyphenolic compounds in plant tissues in green to blue color. When tuber slices were stained with toluidine blue, the area treated with TA was stained in blue, revealing the presence of polyphenolic compounds [29]. Similarly, the surface of the control tuber slice also showed a blue coloration, but to a much lesser extent than that observed on the surface of the slices treated with TA (Fig. 4.3 A-D). Under control

conditions, blue-stained parenchyma cells were located strictly on the surface (Fig. 4.3 A, C), while after exposure to TA, the stained cells were in disordered masses both on the surface and in deeper layers (Fig. 4.3 B, D). The area where blue color was observed completely superposed the area of brown tissues. The reaction to TA was more pronounced in slices of Russet Burbank tubers than in slices from the Yukon Gold variety (Fig. 4.3 B, D). TA treatment also caused changes in the morphology of tuber parenchyma. The shape of the cells located in the blue zone was altered, and some of the cells increased in size (Fig. 4.3 E-H). These results show that the parenchymal cells of potato tubers after treatment with TA accumulated substances that were stained in blue by Toluidine Blue O, suggesting the accumulation of polyphenolic compounds.

To confirm the possible accumulation of phenolic compounds in tissues treated with TA, total phenols were isolated from the TA-treated parenchymal regions of tuber slices, as well as the corresponding regions from control slices. The total phenolic compound content was higher in parenchymal tuber cells after TA treatment when compared to untreated or methanol control (Fig. 4.3 I). A higher level of phenols was found in tissues of the Russet Burbank variety when compared to tissues from the Yukon Gold variety. Treatment with TA caused a significant accumulation of phenols in the parenchyma of Russet Burbank, but there was only a slight increase in the content of phenolic compounds observed in the parenchyma of the Yukon Gold variety compared to the control tissue. Thus, we found that TA stimulated the accumulation of phenolic compounds in the tissues of potato tubers. Moreover, this accumulation was more abundant in the parenchyma of Russet Burbank tubers than in Yukon Gold.

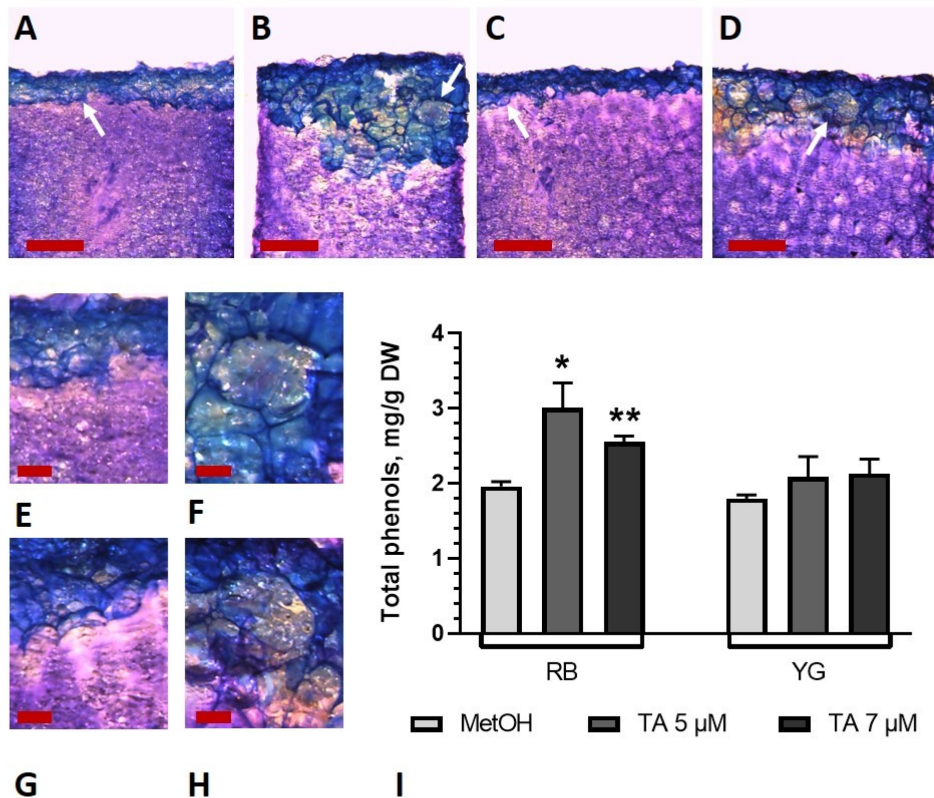

Figure 4.3. TA induces the accumulation of phenolic compounds in tuber parenchyma.

A-H. Toluidine blue O (TBO) staining of methanol (MetOH) and 7 µM of TA treated tuber tissue, 9 days after treatment; A-D. Phenolic deposits on the tuber cut surface after methanol (control) and TA treatment; A. Russet Burbank, methanol; B. Russet Burbank, TA; C. Yukon Gold, methanol; D. Yukon Gold, TA; Scale bar A-D is 0.5 mm; E-H. Changes in cell morphology after exposure to TA. Enlarged from previous photos (2.93 fold), places of magnification are shown by white arrows; E. Russet Burbank, methanol; F. Russet Burbank, TA; G. Yukon Gold, methanol; H. Yukon Gold, TA; Scale bar E-H is 0.1 mm; I. Total phenols extracted from 5 µM and 7 µM TA-treated and control area of Russet Burbank (RB) and Yukon Gold (YG) tuber slices (n=3); chart bars are standard deviation; asterisk signifies significantly different samples according to unpaired *t*-test ($p \leq 0.05$).

One of the main effects of TA exposure on plant cells and tissues is the atypical programmed cell death. Estimation of cell death in the tuber parenchymal tissue caused by TA alowed to unravel whether tissue browning is associated with cell death caused by TA. The occurrence of cell death was evaluated in potato tuber flesh treated with TA, hydrogen peroxide (as a positive control) and in control conditions where the flesh slices were not exposed to any treatment or were treated with methanol. Treated and control slices were incubated with Evans Blue, an azo dye, which specifically accumulates in dead cells leaking through ruptured membrane but excluded by viable cells [24, 30]. After 20 minutes, Evans Blue was extracted from sections of potato slices and determined spectrophotometrically to estimate the amount of Evans Blue that was retained in dead cells (Fig 4.4 A). After 24 hours of exposure to TA in both parenchyma of Russet Burbank tubers and of Yukon Gold tubers, cell death in TA-treated tissue was significantly higher than in samples treated with methanol or untreated controls (Fig 4.4 A, B). TA-induced cell death was slightly lower in the parenchyma cells of Russet Burbank tubers than of Yukon Gold variety, although we did not find a significant difference between the two varieties using Evans Blue dye test. Thus, TA caused a similar level of cell death in the potato tuber parenchymal cells of the Russet Burbank and Yukon Gold varieties.

The biosynthesis of polyphenolic compounds in plants occurs via the metabolic pathway of phenylpropanoids. The key enzyme of the phenylpropanoid pathway is PAL, whose activity can be inhibited by the addition of a competent inhibitor 2-aminoindan-2-phosphonic acid (AIP) [31]. AIP was added to potato slices prior to the treatment with TA. Co-treatment with AIP and TA decreased the browning normally induced in TA-treated regions (Fig 4.5 A, B). These results suggest that TA-induced browning depends on the activation of PAL in the phenylpropanoid pathway.

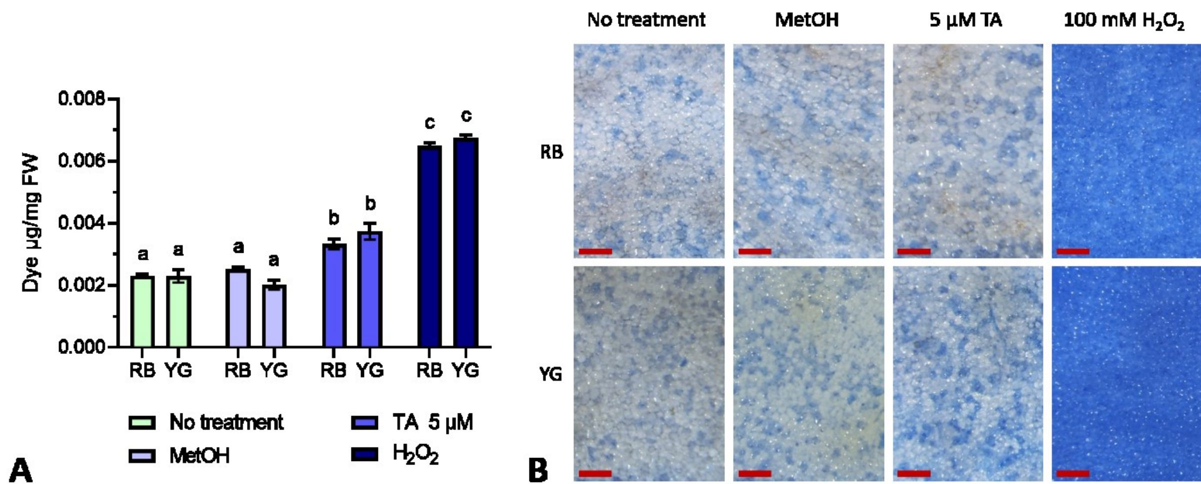

Figure 4.4. TA induces cell death in parenchymal cells of Russet Burbank (RB) and Yukon Gold (YG) tubers.

A. Evans Blue concentration in tuber parenchymal sections after treatments with methanol (MetOH), TA (5 µM), H_2O_2 (100 mM) or in untreated sections after 24 h. B. Parenchyma sections stained with Evans Blue after being treated with methanol (MetOH), 5 µM TA or H_2O_2 (100 mM) for 24 h; Scale bar=0.5 mm. Data is presented as a mean of 4 biological replications, chart bars are standard deviation; different letters signify significantly different samples according to unpaired *t*-test ($p \leq 0.05$).

4.6 Discussion

Thaxtomin A treatment of potato tuber slices induced the formation of dark brown lesions that have been generally accepted as being the result of necrosis [13, 17, 18, 19, 20]. In this work, we showed that tissue browning in response to TA was correlated with the accumulation of phenolic compounds and not with TA induced cell death. The intensity of browning of tissues and the size of the induced spots did not correlate with the resistance of potatoes to common scab.

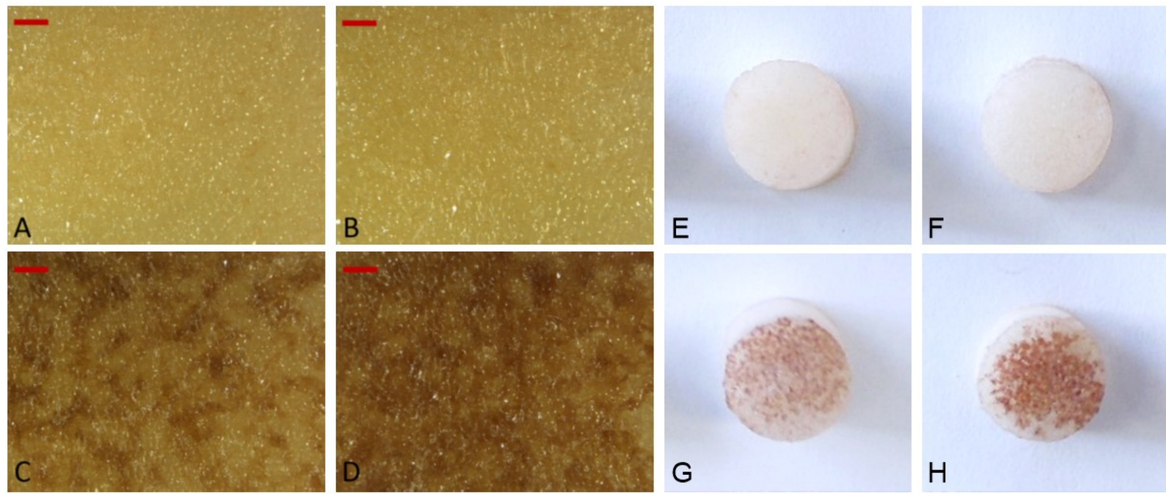

Figure 4.5. Application of AIP inhibits tuber tissue browning.

A. Application of AIP (10 μM); B. Application of Methanol. C. Application of TA (5 μM) in combination with AIP (10 μM). D. Application of TA (5 μM). Pictures were taken 5 d after TA treatment. Red scale bar is equal to 0.5 mm.

Histological examination of tuber parenchyma tissue showed that the effect caused by TA was the induction of browning of the tuber parenchyma cells underlying the cut surface area (Fig. 4.1 and Fig. 4.2 C, D, G, H). Cell browning correlated with the synthesis and accumulation of phenolic compounds, as detected by Toluidine blue O and phenol quantification (Fig. 4.3). The deposition of phenolic substances in parenchyma cells also correlated with the TA concentration applied to tuber slices. However, the accumulation of phenols was different in the two potato varieties. TA induced a higher accumulation of total phenolic compounds in Russet Burbank tuber slices than in Yukon Gold tuber slices. This could indicate that phenolic compounds biosynthesis was more strongly activated by TA in Russet Burbank cells. The same or lower amount of phenolic compounds was extracted from the darker tuber flesh areas treated with the 7 μM of TA than from the lighter areas treated with 5 μM of TA (Fig. 4.1 and 4.3 I). This may be due to the synthesis and transformation of phenolic compounds in a plant cell, which comprise the production of free soluble phenols and further the

formation of insoluble-bound phenolic compounds attached to the cell wall matrix [32]. Probably a higher concentration of TA leads to accelerated formation of insoluble phenolic compounds.

Deposition of phenolic compounds induced by TA did not correlate with the cell death caused by TA. According to our results, TA caused a similar level of cell death in both Yukon Gold and Russet Burbank parenchyma cells. Thus, the tuber tissue browning caused by TA was not essentially due to cell death caused by the toxin, but to the accumulation of phenolics. Consequently, the tuber slices test should not be used as a test for resistance to TA, since it does not reflect the mechanism of action of the toxin (i.e., cell death), but rather the intensity of the reaction of potato tuber to the stimulus in the form of phenolic compounds synthesis.

Microscopical examination of TA treated tuber tissues has revealed the accumulation of brown compounds and cell morphological changes reminiscent of the production of a closing layer that is formed in response to wounding. At the first stage of closing layer formation, suberin polyphenolics are synthesised and integrated in the cell walls of the cells at the wound surface. Later on, this process is followed by the synthesis of suberin polyaliphatic biopolymers which are laminated over the suberin phenolics [27].

Although TA was not shown to stimulate suberin synthesis, it was found to cause the deposition of lignin and scopoletin [10, 9]. These compounds, like suberin, are synthesized through the phenylpropanoid metabolic pathway [33]. Moreover, TA was shown to induce gene expression of phenylalanine-ammonia lyase *PAL* in Arabidopsis cell suspensions and to modify lignin and flavonoid biosynthesis genes expression in poplar cell suspensions habituated to TA [6, 12]. In the first steps of the phenylpropanoid pathway, phenylalanine is converted to various intermediates, such as cinnamic, coumaric, caffeic and ferulic acid using the following enzymes: PAL, C4H (cinnamate 4-hydroxylase), C4L (4 coumarate:CoA ligase), HCT (Hydroxycinnamoyl-

coenzyme A shikimate:quinate hydroxycinnamoyl-transferase) [33]. At the initial stages, some derivatives of phenylpropanoic acids may be responsible for the development of yellow to brown color [34]. It is possible that some of these compounds are synthesized in response to thaxtomin A, which may explain the observed darkening of tuber tissues detected after TA treatment.

These results indicate that the TA-induced darkening in tuber tissue primarily reflects the ability of a given potato variety to accumulate phenolic substances in response to TA, and not cell death as previously thought. Hence, the application of TA on potato tubers slices is not a good indicator of common scab resistance. In our opinion, the physiological role of TA induced tissue browning is that potato cells most probably respond to TA by the synthesis of phenolics that may have a protective role against pathogen infection by forming the protective barrier against pathogenic organisms. They may also be precursors for the synthesis of suberin on the tuber surface [35].

4.7 Conclusions

Flesh tissue browning of potato tubers induced by TA was reported earlier as a TA and common scab resistance test. However, this test did not reflect the level of resistance to common scab in Russet Burbank and Yukon Gold varieties. We showed that tissue browning induced by the application of TA on tuber parenchyma was mainly due to the accumulation of phenolic compounds while necrosis of plant tissues had no impact on the observed response. In our experiments, TA induced accumulation of phenolics in Russet Burbank and Yukon gold tuber flesh did not correlate with the level of the cell death induced by TA. As a result, this test is not reliable for the selection of potato germplasm with increased resistance to common scab. Fundamentally, our results shed light on one of the mechanisms of potato tuber response to *S. scabies*, which may help to understand the phenomena of potato resistance to common scab.

4.8 References

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CHAPTER 5
Discussion and General Conclusions

5.1 Habituation to thaxtomin A enhances common scab resistance in potato varieties Kennebec, Envol, Belle d'août and Chieftain.

The use of TA as an inducing agent for the direct selection of potato cells and adaptation of potato tissues has been shown to be effective in inducing increased long-term resistance of potato varieties Iwa and Russet Burbank to common scab (Wilson et al., 2009; Wilson et al., 2010; Tegg et al., 2013).

Our first research hypothesis was that TA-adaptation of undifferentiated tissue of any variety of potato would lead to an improvement in common scab resistance. We showed that TA-adaptation of varieties exhibiting different scab resistance level frequently led to a subsequent improvement in resistance in somaclonal variants. We successfully induced callus formation and adjusted cultural conditions for the regeneration of selected potato varieties. Despite the fact that different potatoes had different needs in terms of hormonal and nutritional composition, we were able to regenerate plants adapted to TA from calli of all studied varieties. We noted that thaxtomin A had a slight inhibitory effect on callus regeneration. Both red potato varieties Chieftain and Rubiconde had very low morphogenic ability that could be due to faster tissue aging, associated with high anthocyanin content (Bailey et al., 1994; Benson, 2000).

In order to improve the screening of plants with increased scab resistance among the somaclonal variants, we developed a new method based on the infection of potato mini-tubers formed in the axillary buds of stem nodal segments. This method has several advantages over testing the resistance to scab in the classical way in the field. Infection

of axillary buds does not require a large amount of plant material and vast planting areas. It implies the synchronized development of tubers, which increases the accuracy of the method.

In our initial screening for scab resistance, which we performed by leaf bud infection, we found three of the six TA-adapted Belle d'août somaclones, three of five TA-adapted Kennebec, both somaclones of Envol and the single somaclone produced from Chieftain to be more resistant to common scab. In subsequent experiments where infection with *S. scabies* was carried out in pots, somaclonal variants selected by leaf bud infection method exhibited reduced common scab symptoms when compared to those observed in the initial variety.

Despite the fact that scab resistance in some TA-adapted somaclones increased, somaclones with absolute resistance were not found among the tested plants. This could have happened for several reasons. Firstly, the pathogen is not only armed with thaxtomin A toxin, but also produces other substances that contribute to colonization of the host and the development of infection (Li et al., 2019). Secondly, TA induces different physiological reactions depending on plant tissue, as well as on the phase of the cell life cycle (Bischoff et al., 2009; Brochu et al., 2010; Duval et al., 2005; Errakhi et al., 2008; Fry and Loria, 2002; Leiner et al., 1996; Lerat et al., 2009a; Meimoun et al., 2009; Scheible et al., 2003; Tegg et al., 2005). This suggests that the reaction caused by TA in rapidly growing and dividing cells of calli may differ from the effect when exposed to differentiated cells of the periderm and parenchyma of potato tubers. Therefore, the mechanism necessary for the survival of undifferentiated callus cells may turn out to be different from that necessary to prevent the development of disease symptoms. However, the fact that among the somaclones adapted to TA there are plants with increased resistance to the pathogen suggests that one of the possible pathways activated by TA is involved in the resistance of potato to common scab.

In general, the results of this test were confirmed by tests on the resistance of potted plants. Slight differences were observed in some cases, that could be explained by the fact that plant resistance to common scab probably includes not only the response of the developing tuber to infection, but also an adaptation of the plant's general metabolism to stress cues induced by the pathogen (Rojas et al., 2014). Overall, our test may be relevant in practice for screening new breeding varieties for common scab resistance.

It will be interesting, then, to establish whether resistance activated by the toxin was determined by a similar metabolic process for various somaclones or if there were different metabolic pathways involved. To this purpose, we analyzed the abundance of patatins, lipoxygenases and protease inhibitors in tubers of somaclones adapted to TA that also showed improved resistance to common scab as well as tuber periderm morphology. An interesting alternative would also have been to analyze major proteins (patatins, lipoxygenase, Kunitz-protease inhibitors) or to perform a proteomic analysis of somaclonal variants adapted to TA that had not acquired resistance to the pathogen. Thus, changes that have occurred as a result of adaptation to TA would be distinguished from changes that may be important for defense against *S. scabies*.

5.2 Thaxtomin A adaptation induced proteomic and periderm changes associated with common scab resistance in Russet Burbank somaclone RB9

The adaptation of Russet Burbank variety to TA improved common scab resistance in somaclone RB9. We hypothesized that an increase in somaclone resistance is associated with specific metabolic changes that can be detected at the proteome level in RB9 tubers. Changes in the RB9 proteome were apparently related to a certain decrease in metabolic activity and cellular energy charge in the somaclone tubers. At the same time, RB9 metabolism switched to synthetic processes involved in stress responses. The most significant changes were associated with the accumulation in

RB9 tubers of patatins, 9S-lipoxygenases and serine-type Kunitz protease inhibitors (KTI). Other differentially accumulated proteins in RB9 tubers belonged to various groups including calcium signalling, cell wall processing and oxidative stress-related proteins. Changes in the occurrence of these proteins may be associated with increased scab resistance of somaclone RB9, shown earlier to be involved in the pathogen response (Racusen et al., 1986; La Camera et al., 2004; Vellosillo et al., 2007; Li et al., 2008; Hwang and Hwang, 2010; Perla et al., 2014; Sharma et al., 2004; Bártová et al., 2019).

We propose that lipoxygenases played a key role in somaclone RB9 proteome rearrangements. Lipoxygenases are involved in modeling the response to pathogen infection. The accumulation of lipoxygenases in potato correlated with the resistance of potato varieties to powdery scab (Perla et al., 2014) and transient expression of lipoxygenase in pepper plants induced cell death and defense responses (Hwang and Hwang, 2010). It was shown that transposon-insertion mutation in 9-LOX gene in maize increased susceptibility to *Fusarium verticillioides* (Christensen et al., 2014). The increase of 9-Lox abundance in RB9 somaclone, as well as the induction of 9-Lox accumulation by *S. scabies* in RB9 tubers, possibly activated the metabolism of oxylipins. Since most described oxylipins have antimicrobial properties, increased synthesis of these metabolites can potentially suppress infection with *S. scabies* (Prost et al., 2005). The production of fatty acid hydroperoxides mediated by lipoxygenase is also involved in the oxidative stress leading to cell death (Rustérucci et al., 1999). Thus, we can suggest an interplay between the accumulation of lipoxygenases and a change in the abundance of proteins involved in oxidative stress in somaclone tubers.

Changes in the content of lipoxygenases may have a metabolic link with the accumulation of patatin observed in RB9 tubers (La Camera et al., 2004). Patatin is a key storage protein in potato tuber that is also involved in resistance to certain diseases. Patatin was shown to inhibit germination in *P. infestans* spores (Sharma et al., 2004)

and the β-1,3-glucanase activity of patatin was proposed to play a role in plant defense against fungal pathogens by digesting β-1,3-glycans in hyphal cell walls (Shewry and Lucas 1997; van Loon and van Strien 1999). It was shown that the induction of LAH-coding patatin genes occurs simultaneously with the induction of oxylipin biosynthesis genes and subsequent accumulation of 13-Lox and 9-Lox derivatives in tobacco. As a result, it was suggested that the LAH activity of patatin is involved in the liberation of fatty acids from plasma membrane and supply of fatty acid precursors for various pathways of oxylipin biosynthesis (La Camera et al., 2004). However, it was shown that total protein levels of potato tuber patatin do not play a role in tuber powdery scab resistance (Perla et al., 2014). It is plausible that the accumulation of patatin in tubers of scab-resistant somaclone, as well as the induction of patatin by *S. scabies* infection in tubers of both RB and RB9, was necessary to provide substrates for their subsequent conversion with lipoxygenases.

KTI might also have contributed to the increased resistance of somaclone RB9. KTI belong to the main proteins of potato tubers and have functions in protecting against pathogens (Fisher et al., 2015; Grosse-Holz and van der Hoorn, 2016). It has been shown that KTI can inhibit insect midgut proteases (Schlüter et al., 2010; Mendonça et al., 2019) and intracellular proteases of the host plant in case of bacteria damage (Kumar et al., 2015). They also have been shown to inhibit cell death caused by the bacterial pathogen *Pseudomonas syringae* and to enhance resistance to the nectrotroph bacterium *Erwinia carotovora* (Li et al., 2008). Moreover, the increase in the content of KTI in RB9 may also depend on the increase in lipoxygenase activity. It has been shown that the expression of KTI genes, as well as KTI activity in the host plant, is induced by lipid hydroperoxides and oxylipins (Yamagishi et al., 1993; da Silva Fortunato et al., 2007). Among other functions of KTI, inhibition of PCD and regulation of intra and extracellular protease activity during infection may be involved in increasing common scab resistance.

In addition to changes in protein content, we observed morphological changes in the tuber periderm of TA-adapted somaclone RB9, including an increase in the number of suberized cell layers and a visual change in the shape of suberin-containing cells. The periderm of potato tubers serves as the main barrier layer against diseases, and disease resistance, including scab, can depend on this structure. Perturbations in the structure of the periderm may be associated to some extent with the induction of lipoxygenases since it was suggested that some of the intermediates, derived from the lipid hydroperoxides of the Lox pathway, are involved in synthesizing the polyaliphatic domain of suberin. The accumulation of lipoxygenases could indicate the prevalence of the aliphatic domain of suberin in the periderm of somaclone RB9 tubers compared to the original variety. The physiological levels of the Lox protein have been shown to regulate the suberin-mediated pathway in potatoes, which underlies the resistance to powdery scab in russet tuber genotypes (Perla et al., 2014).

In conclusion, our results support the hypothesis that TA adaptation of Russet Burbank variety leads to the improvement of resistance in the adapted somaclone RB9, associated with changes in tuber protein content and periderm morphology.

Confirming the involvement of patatins, lipoxygenases and protease inhibitors in TA-induced scab resistance will now be necessary. The involvement of Kunitz protease inhibitors can be determined using knockdown, as well as overexpression of the locus-candidate *DrTI*. Since these inhibitors are involved in the regulation of protein content in potato tubers during infection, it would be interesting to analyze the proteome of tubers of mutant plants (Kumar et al., 2015). It would be intriguing as well to determine whether DrTI could directly inhibit proteases secreted by *S. scabies.* For this purpose, protease activity in the presence of DrTI protein could be investigated using fluorescein-labeled casein isothiocyanate (FITC-casein) as a substrate (Cupp-Enyard, 2009).

Since lipoxygenases are essential for the development of potato tubers, inducing mutations that affect the expression of genes coding for these proteins does not seem to be a viable option (Kolomiets et al., 2001). A gas chromatography/mass spectrometry approach which would determine oxylipin metabolites profile induced by common scab infection is more likely to provide information on the implication of lipoxygenase (Mueller et al., 2006). According to the branches of oxylipin biosynthesis caused by *S. scabies*, potato plants can be produced by an impairment of genes located downstream of lipoxygenases (Vellosillo et al., 2007). Analysis of common scab resistance of the mutants will help determine whether oxylipins could be implicated during *S. scabies* infection.

Patatins are the most abundant proteins in potato tubers, forming a multi-member family encompassing highly homologous isoforms, and introducing mutations into individual loci could be a difficult task. Using RNA interference could possibly reduce the overall patatin content in potato tubers (Kim et al., 2008). Plants with reduced patatin content could be further tested for common scab resistance.

5.3 Thaxtomin A induces the production of phenolic compounds in tuber slices

The TA toxin, among other effects on plant tissues and cells, causes ectopic deposition of lignin in Arabidopsis seedlings, changes in the expression of genes involved in lignin biosynthesis and synthesis of phytoalexin scopoletin (Bischoff et al., 2009; Lerat et al., 2009; Brochu et al., 2010). These changes suggest that plant cells exposure to TA stimulates the induction of the phenylpropanoid pathway.

One of TA's effects on plant cells is the induction of cell death (Duval et al., 2005; Meimoun et al., 2009), which could be necessary during the infection of potato tubers. Until recently, the main test available to determine the resistance of a particular variety to common scab was based on the application of TA on potato slices (Wilson et al., 2009; 2010). The TA-induced browning of potato tuber slices was considered to be due to necrosis (Loria et al., 1995), but it has been found that TA's effect on potato tuber slices did not correlate with the level of common scab resistance (Tegg and Wilson, 2010). This apparent discrepancy was investigated in this work. We hypothesized that the observed tissue browning occurs as a result of phenolic compounds accumulation.

In this part of the work, we showed that the addition of TA to slices of potato tubers, in addition to cell death, caused the accumulation of phenolic compounds in potato parenchyma. When comparing the response to TA in tubers from a moderately resistant variety (i.e., Russet Burbank) and a sensitive variety (i.e., Yukon Gold), a stronger development of tissue browning was observed in tuber slices of Russet Burbank than in Yukon Gold, associated in fact with a significantly higher accumulation of phenolic compounds in tuber slices of the former variety. TA also caused cell death by affecting the tuber parenchyma cells. The fact that inhibition of PAL, the first key enzyme of the phenylpropanoid pathway, with a competitive inhibitor reduced the development of brown color caused by TA suggests that a *de novo* synthesis of phenolic compounds occurred in response to TA.

The physiological significance of phenolic compounds accumulation could be that these compounds are involved in protection against pathogenic organisms. It has been shown that some phenolic compounds exhibit antifungal properties, helping the host plant suppress pathogenic fungi (Lattanzio et al., 1994). Phenolic compounds constitute a part of the suberin biopolymer, which is the primary barrier of potato tubers to infection (Graça, 2015).The accumulation of phenolic compounds in tubers could play an important role in protecting against *S. scabies*. It was noted that the high content of chlorogenic acid in potato tubers correlates with the resistance of the varieties to common scab (Johnson and Schaal, 1952). It has also been shown recently that the phenolic acid content in the peel of potato tubers has a positive correlation with common scab resistance (Singhai et al., 2011). It is possible that application of TA to tuber tissues activates the production of polyphenols to form a protective layer against infection (Hammerschmidt, 1984). It is possible also that the accumulated phenolic compounds have toxic properties against *S. scabies* (Nicholson and Hammerschmidt, 1992).

According to our data, phenolic compounds accumulation in the tuber parenchyma would depend on each variety's ability to synthesize these substances and not depend on the level of cellular death induced by the toxin. We conclude that this test is not adequate for the analysis of potato resistance to common scab. When using this test for any other purpose, it should be considered that the observed browning is not necrosis, but deposition of phenolic compounds, and the intensity of this process does not depend on the level of cell death caused by TA.

We now propose to study at the molecular level the induction of the phenylpropanoid pathway in potato tubers caused by TA. In order to understand the possible function of synthesized phenolic compounds, it would be interesting to determine the type of phenolic compounds that make up the deposits caused by TA. This could be done by a HPLC-DAD analysis with extraction methods for phenolic compounds that allow for

their classification as free, soluble ester and insoluble-bound phenolics (Dvořáková et al., 2008; Shahidi and Yeo, 2016).

It would also be interesting to investigate how TA affects the biosynthetic pathway of phenylpropanoids in the process of TA-adaptation of potato calli, and whether this has an effect on the resistance to common scab in the somaclones described in this study. For this purpose, we propose histochemical staining of potato callus tissue during adaptation to thaxtomin A to detect phenolic compounds (toluidine blue O and phloroglucinol). The analysis of phenolic compounds in root, stem, leaf and tuber tissues of somaclones already adapted to TA, together with testing their resistance to common scab, could clarify this issue.

5.4 General conclusion.

In this work, we showed the habituation of potato calli to a key pathogenicity factor of *S. scabies*, to successfully lead to the production of plants with improved resistance to common scab. Improved resistance of somaclone RB9 to common scab as induced by adaptation to TA was associated with an increse in the abundance of patatin isoforms, 9S-lipoxygenases and KTI's in tuber flesh. We suggest that patatin and 9S-lipoxygenases accumulation contribute to the activation of fatty acid metabolism, with subsequent synthesis of hydroperoxide products, oxylipins (La Camera et al., 2004 and 2009). Changes in the metabolism of fatty acids possibly caused an increase in the abundance of KTI and changes in the content of proteins involved in the oxidative stress response (Yamagishi et al., 1993; Rustérucci et al., 1999; da Silva Fortunato et al., 2007).

We also showed TA to induce the accumulation of phenolic compounds and flesh browning in potato tuber parenchyma. Accumulation of phenolic compounds in tuber cells did not correlate with cell death caused by TA, thus suggesting that the darkening

of the tuber parenchyma as a result of exposure to the toxin depended on the ability of each given variety to accumulate phenolic compounds.

Globally, these findings contribute to a better understanding of the plant-pathogen interaction of *S. scabies* and potato tubers, while also shedding light on a possible mechanism for potato resistance to common scab. Our data clarify and supplement previously reported information on the response of plant tissues to TA, the main pathogenicity factor of *S. scabies*. Our findings also allow for a better understanding of the physisological response of potato tuber to *S. scabies* infection.

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