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Involvement of Hexokinase1 in Plant Growth Promotion as Mediated by Burkholderia Phytofirmans

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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INVOLVEMENT OF HEXOKINASE1 IN PLANT GROWTH PROMOTION AS
MEDIATED BY *BURKHOLDERIA PHYTOFIRMANS*

(Spine Title: Bacterial affect on plant hexokinase1 and plant growth)

(Thesis format: Monograph)

By

Jae Min Park

Graduate Program in Biology

A Thesis submitted in partial fulfillment of the requirements for the degree of Master
of Science

The School of Graduate and Postdoctoral studies
Western University
London, Ontario, Canada

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Jae Min Park

Entitled:

**INVOLVEMENT OF HEXOKINASE1 IN PLANT GROWTH PROMOTION
AS MEDIATED BY *BURKHOLDERIA PHYTOFIRMANS***

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requirements for the degree of
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Date _____

Chair of the Thesis Examination Board

Abstract

Burkholderia phytofirmans strain PsJN consistently enhanced the growth of potato plants *in vitro*. The role of hexokinase1 in glucose phosphorylation was investigated in plants with PsJN. Increased hexokinase1 activity only in roots of PsJN-treated plants cultivar Kennebec suggests that hexokinase1 is associated with plant root and stem growth.

Plant growth with PsJN was determined when plants were grown with different sugars at various concentrations. PsJN-treated plants expressed diverse forms of growth promotion. When growth promotion did occur, hexokinase1 activity also increased. Growth promotion and hexokinase1 activity appear to be correlated to the enzyme to recognize the substrate for catalytic activity.

Cultivar Yukon Gold showed minimum response to the growth promotion typically induced by PsJN in cultivar Kennebec. Hexokinase1 activity in the roots of PsJN-treated plants was increased at much later stage than found with cultivar Kennebec. This indicates that the mechanism associated with growth promotion by PsJN is different in different potato cultivars.

Keywords: *Burkholderia phytofirmans* strain PsJN, potato plant, Plant hexokinase 1, sucrose, glucose, sugar signaling, Hexokinase1 enzyme activity assay, RT-PCR, Western blot.

Dedication

To my family and my friends

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Table of Contents

Certificate of Examination	ii
Abstract	iii
Dedication	iv
Acknowledgments.....	v
Table of Contents	vi
List of Figures	ix
List of Abbreviations.....	xi
Chapter 1: Introduction and literature review	1
1.1 Plant growth promoting rhizobacteria	1
1.2 Growth promoting modes of PGPR on plants	2
1.2.1 Direct mode of action.....	2
1.2.1.1 Biofertilizers	2
1.2.1.2 Phytostimulators	3
1.2.2 Indirect mode of action: Biological control	4
1.3 <i>Burkholderia phytofirmans</i> strain PsJN.....	5
1.4 Mode of PsJN-induced plant growth promotion.....	8
1.5 The effect of sugar on the induction of plant growth promotion by PsJN.....	8
1.6 Glucose sugar sensor plant hexokinase1.....	9
1.7 Involvement of plant hexokinase1 in plant growth promotion by plant growth promoting rhizobacteria.....	11
1.8 Hypothesis and objectives for thesis.....	13
Chapter 2: Materials and Methods	15
2.1 Generation and maintenance of plant materials.....	15

2.2 Bacterium strain and growth condition, and plant inoculation	15
2.3 Analysis of potato plants physiologies with or without PsJN.....	16
2.4 Determination of hexokinase1 activity	17
2.5 Determination of hexokinases RNA transcript levels	18
2.6 Determination of potato hexokinase1 protein accumulation	19
2.7 Growth promotion test of different potato plant cultivars with PsJN.....	21
2.8 Potato plant cv. Yukon Gold physiologies with or without PsJN and determination of hexokinase1 activity	21
2.9 Statistical analysis	21
Chapter 3: Results	23
3.1 The effect of PsJN treatment on the growth of nodal explants of cv. Kennebec.....	23
3.2 The effect of PsJN treatment on potato hexokinase1 activity.....	28
3.3 Comparison of potato <i>hexokinase1</i> expression in plants with or without PsJN.....	29
3.4 Comparison of potato hexokinase1 protein accumulation with or without PsJN.....	32
3.5 The influence of sugar composition on the growth enhancement by PsJN	36
3.6 The effect of PsJN treatment on hexokinase1 activity in potato stems and roots of plants grown various sugars.....	46
3.7 The effect of PsJN treatment on the plant growth promotion of the potato cv. Yukon Gold.....	49
3.8 The effect of PsJN treatment on hexokinase1 of cv. Yukon Gold.....	51

Chapter 4: Discussion	54
4.1 Alteration of PsJN-treated plants as correlated to induced growth promotion of plants cultured in media containing either 1.5 or 3% sucrose	54
4.2 Indication of changed potato hexokinase1 in PsJN-treated plant, accompanied by growth promotion of plants grown on 1.5 and 3% sucrose	55
4.3 Alteration of PsJN-treated plants as correlated to induced growth promotion of plants cultured in media containing different sugar compositions.....	57
4.4 Indication of changed potato hexokinase1 in PsJN-treated plant, accompanied by growth promotion, grown in different sugar composition.....	59
4.5 Alteration of PsJN-treated plants cv. Yukon Gold as correlated to induced growth promotion of plants cultured in media containing 3% sucrose	61
4.6 Indication of changes in potato hexokinase1 in PsJN-treated plant cv. Yukon Gold, accompanied by growth promotion, grown in 3% sucrose	61
4.7 Thesis conclusion.....	62
References.....	64
Curriculum Vitae.....	71

List of Figures

Figure 1	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 1.5% sucrose for 1 to 4 weeks24
Figure 2	The effect of <i>B. phytofirmans</i> on the growth of potato roots cv. Kennebec grown in medium containing 1.5 and 3% sucrose for 2 weeks25
Figure 3	The effect <i>B. phytofirmans</i> on the growth of potato nodal explants cv. Kennebec when grown in medium containing 1.5 and 3% sucrose for 4 and 6 weeks.....26
Figure 4	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 1 to 4 weeks27
Figure 5	The effect of <i>B. phytofirmans</i> on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 1.5% sucrose for 1 to 4 weeks30
Figure 6	The effect of <i>B. phytofirmans</i> on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 1 to 4 weeks31
Figure 7	Specificity of <i>StHXX1</i> and <i>StHXX2</i> primers to targeted <i>StHXX1</i> and <i>StHXX2</i> region for RT-PCR amplification33
Figure 8	Relative <i>StHXX1</i> and <i>StHXX2</i> mRNA expression in PsJN-treated and uninoculated potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 2 weeks.....34
Figure 9	Relative <i>StHXX1</i> mRNA expression between PsJN-treated and uninoculated potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 2 weeks35
Figure 10	The effect of <i>B. phytofirmans</i> on the hexokinase1 accumulation of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 2 weeks.....37
Figure 11	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 1.5% glucose for 3 and 5 weeks39
Figure 12	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 1.5% fructose for 3 and 5 weeks.....40

Figure 13	The effect <i>B. phytofirmans</i> on the growth of potato nodal explants cv. Kennebec when grown in medium containing 1.5% glucose or fructose, and 3% glucose, fructose, or maltose for 4 and 6 weeks41
Figure 14	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% glucose for 3 and 5 weeks43
Figure 15	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% fructose for 3 and 5 weeks.....44
Figure 16	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% maltose for 3 and 5 weeks45
Figure 17	The effect of <i>B. phytofirmans</i> on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 1.5% glucose or fructose for 3 and 5 weeks47
Figure 18	The effect of <i>B. phytofirmans</i> on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 3% glucose, fructose, and maltose for 3 and 5 weeks48
Figure 19	The effect of bacterial treatment with <i>B. phytofirmans</i> on the growth of potato nodal explants cv. Kennebec and Yukon Gold when grown in medium containing 3% sucrose for 6 weeks.....50
Figure 20	The effect of <i>B. phytofirmans</i> on the growth and chlorophyll content of potato nodal explants cv. Yukon Gold when grown in medium containing 3% sucrose for 3 and 5 weeks.....52
Figure 21	The effect of <i>B. phytofirmans</i> on the hexokinase1 activity of roots of potato nodal explants cv. Yukon Gold when grown in medium containing 3% sucrose for 3 and 5 weeks53

List of Abbreviations

μM	Micromolar
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ADP	Adenosine diphosphate
AtHXK1	<i>Arabidopsis thaliana</i> hexokinase 1
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cv.	Cultivar
EDTA	Ethylenediaminetetraacetic acid
G6P	Glucose 6-phosphate
H41	PsJN mutant
HXK1	Hexokinase 1
IAA	Indole-3-acetic acid
kDA	Kilodalton
mg	Milligram
mL	Milliliter
mM	Millimolar
MS	Murashige and Skoog
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
NB	Nutrient broth
Nod	Nodulation
PGPR	Plant growth promoting rhizobacteria
PVDF	polyvinylidene difluoride
RT-PCR	Reverse transcriptase polymerase chain reaction

SEM	Standard error of mean
TBS-BSA	Tris buffered saline with bovine serum albumin
TBS-T	Tris buffered saline with Tween20
W/V	Weight to volume ratio
V/V	Volume to volume ratio

Chapter 1: Introduction and literature review

1.1 Plant growth promoting rhizobacteria

The rhizosphere is the region of soil adjacent to the plant root that is specifically influenced by chemical constituents produced by the root. The microorganisms that inhabit the rhizosphere can influence plant growth and development in a beneficial, detrimental or neutral manner. The microorganisms that enhance growth are referred to as plant growth-promoting rhizobacteria (PGPR) (20). Growth enhancement has been shown to arise through numerous mechanisms, including nitrogen fixation (43, 58), phosphate solubilization (59), and the production of plant hormones (4, 19, 24, 37, 45). In many cases the growth promotion is indirect that the microorganisms act as biological control agents that minimize the impacts of plant pests and pathogens (20, 31, 66).

The microorganisms that reside in the rhizosphere are nourished by carbon and nitrogen compounds, such as amino acids, sugars, cell wall material, and mucilage released by the root (6). It is these nutrients that support the relatively high population of PGPR in comparison to that found in the surrounding bulk soil. PGPR may colonize either the rhizoplane, the root itself, or the endosphere (6). Those that reside inside the plant tissue are called endophytes and those that reside outside are called epiphytes. Endophytes such as *Rhizobia* are localized in specialized structures (58), whereas epiphytes are thought to enhance plant growth by releasing hormonal compounds to the plant (4, 19, 37, 45). Epiphytes inhabit zones near the root, on the root surface, or in the extracellular spaces of the cortex (20). Some PGPR genera can be both endophytes and epiphytes, such as *Burkholderia* spp. For example, *B. cepacia* acts as an epiphyte to enhance growth of grain plants, while *B. tropica* can act as a biofertilizer, aiding nitrogen fixation in sugarcane and maize (10, 55).

Interactions between plants and PGPR are a potentially useful agriculture tool in the improvement of crop yield and as an alternative to chemical fertilizers and pesticides (33). However, despite the existence of many PGPR with multiple beneficial traits, only a few have been exploited for commercial use in agriculture. The most widely used genera are *Rhizobium* and *Azospirillum* spp., which are sold as biofertilizers, and some *Bacillus* and *Pseudomonas* spp., which are available as biopesticides (69).

1.2 Growth promoting modes of PGPR on plants

There are two modes of action by which PGPR exert a beneficial effect on plant growth. They may act either directly or indirectly.

1.2.1 Direct mode of action

1.2.1.1 Biofertilizers

Nitrogen-fixing bacteria convert nitrogen gas to ammonia which can then be used to make amino acids, or other nitrogen containing compounds (58). Three rhizobial genera, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* produce nodules on legume plant roots, such as soybean, pea, peanut, and alfalfa. It is in these nodules that nitrogen fixation occurs. To establish a successful relationship with a bacterium, plant roots release chemical signaling molecules, such as flavonoids, into the rhizosphere that attract the bacterium to the root hairs, where the first interaction occurs. The flavonoids also act as a signaling molecule to activate transcription of the nitrogen-fixing bacterial nodulation (*nod*) genes in the bacterium. Secondly, nod-encoded enzymes from the bacterium release lipo-oligosaccharides in the plant root tissue to direct a plant organogenic program that initiates nodule formation (58). This

symbiotic relationship benefits both the bacterium and the plant (58).

Phosphorus is an essential nutrient for plant growth and is usually added as a chemical fertilizer to agricultural soils (59). However, in many soil's phosphate is often precipitated by metal-cation complexes and in this insoluble form it cannot be assimilated by the plant. The bacterial genera *Pseudomonas*, *Bacillus*, *Burkholderia*, and *Rhizobium* release organic acids that solubilize complexes of phosphorus or produce acid phosphatases that can solubilize phosphate bound to organic and inorganic matter (59). These phosphate-solubilizing bacteria can significantly increase crop yields (59).

1.2.1.2 Phytostimulators

Beneficial bacteria are also capable of producing plant hormones that can modulate plant hormone levels and increase plant growth. Hormones commonly produced by PGPR include auxin, cytokinin, and gibberellin.

Biosynthesis of auxin, such as indole-3-acetic acid (IAA), is widespread in beneficial bacteria. Five of the six pathways for IAA biosynthesis in bacteria have been shown to involve the IAA precursor tryptophan (47). Plants from canola seeds treated with *Pseudomonas putida* strain GR12-2 had 35-50% longer roots than plants from untreated seeds or plants treated with an IAA-deficient mutant, suggesting that IAA from the bacterium enhanced root growth (31). An IAA-deficient mutant of *Bradyrhizobium elkanii* generated fewer nodules on soybean roots than it did on the wild type, also indicating a role for IAA in N₂ fixation (19). IAA produced by bacteria has numerous effects on plant growth and development. In particular, it has been reported to stimulate seed and tuber germination, increase the rate of xylem and root development, control vegetative growth, and initiate lateral and adventitious roots (16,

79).

The enzyme responsible for cytokinin synthesis was first described in a bacterium, *Agrobacterium tumefaciens* (34), and it was later found that many beneficial bacteria synthesize cytokinin, namely, *Azospirillum*, *Rhizobium*, *Bacillus*, and *Pseudomonas* spp. (20). Cytokinin modulates a wide range of plant physiological processes, including root growth, promotion of root branching, accumulation of chlorophyll, and leaf expansion (16, 73). When lettuce seeds were inoculated with the cytokinin-producing bacteria *Bacillus subtilis*, the accumulation of cytokinin was positively correlated with plant growth (4). Treating *Arabidopsis thaliana* with *B. megaterium* strain UMCV1, a cytokinin producer, caused a three-fold increase in shoot and root fresh weight and more lateral roots compared to uninoculated plants six days after inoculation (45).

Azospirillum brasilense and *Rhizobium japonicum* were the first bacteria shown to be capable of synthesizing gibberellins (75, 78). Gibberellins can influence plant cell division, cell elongation, stem elongation, flowering, and can delay senescence (16). Similar to cytokinin-producing bacteria, plants treated with gibberellin-producing bacteria accumulated higher levels of gibberellins and produced much greater biomass (37).

1.2.2 Indirect mode of action: Biological control

In 2005, the annual worldwide crop loss due to plant diseases was estimated to be 220 billion dollars (2). Use of disease-resistant plants has been the most successful strategy to control crop loss to disease, but resistance genes are not always available for every pathogen. Chemical control with fungicides is widely used but pesticides are now becoming increasingly refused by consumers. For these reasons,

biological control of plant diseases is now being more seriously considered as a means to decrease crop loss (2). Biological control agents protect plants by inducing systemic resistance, producing antibiotics, parasitizing pathogens, or competing with pathogens (20, 66). Most biological agents used today produce antibiotics. For example, *P. fluorescens* strain 2-79 and *P. chlororaphis* strain 30-84 reduced diseases of spring and winter wheat when applied as seed treatments through the production of phenazine-1-carboxylic acid (72). *P. fluorescens* strains WCS374 and WCS365 increased potato tuber yield by the production of hydrogen cyanide under field conditions (21). *B. subtilis* strain RB14 suppressed damping off disease in tomato caused by *Rhizoctonia solani* by producing the antibiotic iturin A and surfactin (5).

1.3 *Burkholderia phytofirmans* strain PsJN

Burkholderia phytofirmans strain PsJN is a gram-negative bacterium that was originally isolated from *Glomus vesiculiferum*-infected onion roots (17). This bacterium is aerobic, rod shaped, non-sporulating, and motile by means of a single polar flagellum. PsJN cells range from 0.5-0.8 μm wide and 0.8-1.8 μm long, grow well at pH 3-9 and at 4-38°C, and can be grown on the sugars D-fructose, D-xylose, and D-glucose (17, 67). The effect of PsJN on plant growth was initially characterized using potato (*Solanum tuberosum*) nodal explants of the cultivar (cv.) Kennebec under *in vitro* conditions (17). PsJN-treated potato plants had greater dry weight, stem length, lignin, chlorophyll, starch, as well as more roots and leaf hairs than uninoculated plants 28 days after inoculation (17). PsJN-treated plants also had larger, more branched root systems with more secondary root structure, taller stems, and achieved pubescence earlier than the uninoculated plants. Furthermore, PsJN-treated plants remained turgid when they were removed from tissue culture conditions

because they had functional stomata that allowed them to regulate water loss, whereas uninoculated plants desiccated rapidly. Most of the bacteria were localized in the roots and the stem xylem tissue (17).

PsJN did not induce the same degree of growth enhancement in all potato cv. tested. Potato cv. Norchip showed similar growth response to cv. Kennebec, but growth was promoted much less in cv. Shepody than in cv. Kennebec. PsJN-treated cv. Chaleur, interestingly, had a 50% decrease in root weight compared to uninoculated plants. The effect of PsJN thus appears to be cultivar specific with respect to its effects on potato. PsJN-treated cv. Kennebec and Norchip plants grown at 10°C, 20°C, and 25°C for 3 weeks showed the greatest growth enhancement at 25°C. But after 6 weeks growth, Kennebec's optimal temperature was 25°C, whereas growth enhancement of Norchip was greatest at 20°C and 25°C (15). This clearly indicates that environmental factors can affect the growth enhancement induced by PsJN.

Plants derived from PsJN-treated potato tubers of the cv. Kennebec grown under greenhouse conditions for 23 days had greater root dry weight, more stolons, earlier tuberization, and higher tuber yield than plants derived from uninoculated tubers (18). However, plants grown from cut or whole seed tubers treated with PsJN and grown under field conditions showed responses that were dependent on location. At two locations, the fresh weight and number of commercial-sized tubers were greater in inoculated plants compared to uninoculated plants by 43.4% and 31.6%, respectively, but there was no effect of inoculation at the other location (18). It has been suggested that soil factors influence root colonization of PsJN (8). Similar results were obtained from field tests carried out in Nova Scotia (28).

Four different tomato cultivars Scotia, Blazer, Mountain Delight, and Celebrity, were inoculated with PsJN and grown under *in vitro* condition similar to the

potato plants described above (51). When compared to uninoculated plants, Scotia had the greatest increase in shoot biomass (61%), followed by Blazer (34%). However, there was no growth promotion in inoculated Mountain Delight and Celebrity plants compared to uninoculated plants of these cultivars (51). The increased shoot biomass of PsJN-treated Blazer plants was due to a significant increase in stem height and the increase in Scotia was attributed to thicker stems. The work with tomato confirms that PsJN can promote the growth of plants other than potato and also that the promotion is cultivar specific (17, 51).

PsJN-treated grapevine plantlets had greater total biomass, shoot mass, leaf mass, and root mass than uninoculated plantlets (7). Root biomass in inoculated plantlets was as much as 12 times that of uninoculated plantlets. At 4°C, biomass of both PsJN-treated and uninoculated plantlets was less than the biomass of uninoculated plantlets grown at 26°C, but the biomass and root weight of the PsJN-treated plantlet was still greater than that of the uninoculated plantlet. The PsJN-treated plantlet at 4°C had secondary vascular structure with wider xylem cells and thicker xylem walls than uninoculated plantlets (7).

It was suggested that resistance to chilling was improved by PsJN. PsJN treatments at both temperatures increased proline accumulation in leaves and stems, and phenolics in leaves. Plant proline increases in response to environmental stress and phenolics, such as flavonoids is the precursor of many antioxidants. PsJN-treated grapevines showed enhanced photosynthesis and increased accumulation of starch in shoots and leaves but not in roots compared to uninoculated plants (7). The author of this study suggested that PsJN initially colonized the root surface, penetrated the tissue and colonized the root interior. It was translocated via stem xylem vessels, and finally was able to colonize even the leaf tissues endophytically (7).

1.4 Mode of PsJN-induced plant growth promotion

The mechanism by which PsJN induces plant growth promotion remains unknown (17, 36, 71). A study using a mutant of PsJN (H41) that is capable of colonizing potato plants but does not promote growth (71) revealed the loss of function to be located at the *nadC* gene encoding quinolinate phosphoribosyltransferase which was found inactivated in the mutant. This enzyme catalyzes the synthesis of nicotinamide adenine mononucleotide from quinolinic acid, in the *de novo* synthesis of nicotinamide adenine dinucleotide. Since the compound is involved in many key regulatory steps of multiple biochemical processes, it is not known whether this is a key step in growth promotion or the loss of this function is one of many. Adding nicotine acid mononucleotide, nicotinamide, nicotinic acid, and nicotinamide mononucleotide to the agar media fully restored the ability of the H41 mutant to promote growth under *in vitro* conditions (71). The inhibition of this pathway likely affects plant growth indirectly rather than directly because of the diverse processes that PsJN may affect. It has also been proposed that 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase is involved in plant growth promotion by PsJN, since PsJN may result in the accumulation of large quantities of this enzyme (44). ACC deaminase catalyzes the immediate precursor of ethylene, ACC, to ammonia and α -ketobutyrate and thereby decreases ethylene synthesis. Since ethylene accumulation may inhibit growth, removal of the precursor allows for better plant growth. ACC deaminase thus induces relative growth promotion (24).

1.5 The effect of sugar on the induction of plant growth promotion by PsJN

Potato plants cv. Kennebec were inoculated with PsJN and then grown *in vitro* for 4 weeks in varied sucrose concentrations, specifically, 0%, 0.1875% (w/v),

0.375% (w/v), 0.75% (w/v), 1.5% (w/v), and 3% (w/v) (85). In the absence of sucrose, there was no growth promotion in treated plants compared to the uninoculated plants. Growth promotion was first detected when PsJN-treated plants were grown in a 0.1875% sucrose concentration. These plants were taller than uninoculated plants but did not have more dry weight. At 0.375% (w/v) sucrose, both height and dry weight were greater in the treated plants compared to uninoculated plants (85). PsJN-treated plants grown at 1.5% (w/v) sucrose were the tallest in the study and had more dry weight than the uninoculated plants. PsJN-treated plants grown at 3% (w/v) sucrose had a shorter stem height but a greater dry weight compared to uninoculated plants (85). This study suggests that the level of sucrose affects growth promotion differently following inoculation with PsJN.

Sucrose is made as the end product of photosynthesis and, in order to be used in subsequent metabolic processes, is transported through the phloem to all cells, where it is hydrolyzed into glucose and fructose by invertase and then metabolized to support the production of leaves, roots, flowers, fruit, and seeds (60). Generally, lower sugar concentrations activate photosynthesis, nutrient mobilization and the export of sugar from leaves. Higher sugar concentrations stimulate plant growth by down regulation of photosynthesis. This is believed to maintain sugar homeostasis in plants and to ensure the most efficient use of this energy resource (60).

In 1994 (26) and 1997 (27), Dr. Jen Sheen's research team provided evidence that plant seedling development is mediated by specific sugar sensing and signaling through plant hexokinase1 (HXK1).

1.6 Glucose sugar sensor plant hexokinase1

Sugar signaling studies have mainly used the model plant *A. thaliana*, as its

whole genome has been sequenced (60, 77). A study of an *A. thaliana* variant grown on 6% (w/v) glucose media (high concentration of glucose), reveal that *A. thaliana* hexokinase 1 (AtHXK1) is the primary glucose sensor that generates the signals involved in plant growth and development (27). On 6% (w/v) glucose media both *A. thaliana* seedlings overexpressing *AtHXK1* mutant with constitutive promoter and wild-type plants showed severe stunting and inhibition in the development of the cotyledons and hypocotyls indicating glucose hypersensitivity. Seedlings of *AtHXK1* knock-out *A. thaliana* mutant when grown on this medium developed normal cotyledons and typical elongation of hypocotyls indicating glucose hyposensitivity. These mutants grown in 6% (w/v) mannitol (a sugar alcohol) or 6% (w/v) 3-O-methylglucose media did not show these effects. Taken together, the results indicated that AtHXK1 acts as a glucose sensor (27). Also, the mutant of *A. thaliana* that shows overexpression of *AtHXK1* when grown in 6% glucose showed greater repression of sugar-repressible target genes coding for the chlorophyll a/b binding protein and ribulose-1,5-bisphosphate carboxylase small subunit when compared to a *AtHXK1* knock out mutant (27). This was interpreted as confirmation that AtHXK1 mediates sugar dependent gene regulation. Although the HXK2-dependent glucose-sensing mechanism is well characterized in the unicellular eukaryotic yeast, *Saccharomyces cerevisiae* (65), it cannot be used to elucidate the role of plant HXK1 as a glucose sensor. While overexpression of the yeast *HXK2* in *A. thaliana* seedlings increased the glucose phosphorylation activity, the seedlings did not exhibit the expected phenotype observed in seedlings overexpressing *AtHXK1* in 6% (w/v) glucose. Despite increasing HXK1 catalytic activity, yeast HXK2 overexpressor did not allow the regulatory function for glucose signaling in the transgenic plants (27). Later studies of AtHXK1 showed evidence that it regulates plant growth and development in response

to nutrients, light, and plant hormone signaling in plants (41, 84). In addition, HXK1 of various plants was shown to be localized in nuclei, cytoplasm, and mitochondria (14).

Eukaryotic HXK was first described in yeast by Otto Meyerhof (39) in 1927 and it was identified as a glycolytic enzyme in yeast and animals in the 1940s (9). Saltman (64) first characterized glucose phosphorylation initiated by HXK as an important step in glycolysis, the first process of carbohydrate breakdown. The product fuels respiration and provides carbon intermediates for anabolic pathways (64).

Three hexokinases (StHXKs) were identified in potato tubers, but significant activity could be detected only for StHXK1 and StHXK2 (57). The enzymes' activities were dependent on the growth stages of the tubers. StHXK1 was most active in sprouting tubers, whereas StHXK2 was most active during tuber growth (56). Both StHXKs had greater affinity for glucose as their substrate (StHXK1 $K_m = 41 \mu\text{M}$, StHXK2 $K_m = 130 \mu\text{M}$), than for fructose (StHXK1 $K_m = 11 \text{mM}$, StHXK2 $K_m = 22 \text{mM}$) (57). Interestingly, StHXK1 and StHXK2 may have functions similar to AtHXK1 because *StHXK1* and *StHXK2* were able to complement the *AtHXK1* knock out mutant grown on 6% glucose to restore glucose-sensitivity from glucose-insensitivity (70). A similar result was reported when rice HXKs (OsHXK1 and OsHXK5) complimented to *AtHXK1* mutant (11). Thus, the role of *AtHXK1* as a glucose sensor could be shared with StHXK1 and StHXK2 in potato plant.

1.7 Involvement of plant hexokinase1 in plant growth promotion by plant growth promoting rhizobacteria

Indication of the involvement of plant HXK1 in plant growth stimulation by PGPR was made by Zhang *et al.* for first time in 2008 (83). This study on the

mechanism of plant growth promotion in *A. thaliana* following treatment with *B. subtilis* strain GB03 showed that increased photosynthesis and down regulation of AtHXK1 in glucose sensing may be involved. Strain GB03 released the volatile compounds 2, 3-butanediol and acetoin and these compounds significantly increased photosystem II photosynthetic efficiency, chlorophyll content, plant growth, and enhanced resistance to the plant pathogen *Erwinia carotovora*, the bacterium that causes soft rot (62, 83). GB03-treated plants grown in media with 4% (w/v) and 6% (w/v) glucose had higher level of hexoses and longer seedling hypocotyls than uninoculated plants grown in the same medium. These latter results suggest repression of AtHXK1 sugar sensing (83). However, AtHXK1 activity, as assessed by glucose phosphorylation, was not measured. Bacterial treatment also reduced the levels of the biosynthetic transcripts for formation of the plant hormone abscisic acid (ABA) (83). Since mutant *A. thaliana* seedlings deficient in ABA synthesis or signaling also showed the same glucose-insensitive phenotype as did the *AtHXK1* mutant of knock out *A. thaliana* seedlings on 6% glucose (3), the authors (83) concluded that decreased ABA biosynthetic transcripts could indicate decreased AtHXK1 glucose sensing in GB03-treated *A. thaliana* as well. ABA has been considered an inhibitor of physiological processes, especially of growth (16). For example, exogenous ABA at even low concentration inhibited root elongation in seedlings of bean, garden cress, and barley (79). Hence, the ABA level may correlate with AtHXK1 in glucose sensing.

PsJN may enhance plant growth by a mechanism similar to GB03. The altered growth promotion observed when potato node explants were grown on varied sucrose concentrations (85) supports this suggestion. An alteration of StHXK1 levels in response to PsJN treatment may be involved in the regulation of plant growth promotion in potato. If such a mechanism is common to PGPR-dependent growth

promotion, it may be possible to use molecular methods to develop more rapid screening methods to identify beneficial bacteria for growth promotion. Having an understanding of the molecular mechanism would greatly facilitate evaluating PGPR efficacy under field conditions (69).

1.8 Hypothesis and objectives for thesis

Plant HXK1 is involved in sugar sensing and signaling in plant growth and development and has been implicated in growth promotion by a beneficial bacterium, GB03 (27, 83). First, it appears that a particular concentration of sucrose is required for optimal growth promotion by PsJN (85). Second, plant HXK1 is well characterized and is a likely candidate for the glucose sensor in PGPR-mediated *A. thaliana* growth promotion (83). Thus, I will examine StHXK1 activity based on glucose phosphorylation, in PsJN-treated plants.

Hypothesis 1

PsJN stimulates the growth of potato plants cv. Kennebec and modulates the activity of StHXK1.

To test this hypothesis I will:

- a. Determine if StHXK1 glucose phosphorylation activity is correlated with PsJN-induced growth promotion of stems and roots.
- b. Determine if StHXK1 activity is correlated with *StHXK1* expression level.

Hypothesis 2

Growth promotion of plants inoculated with PsJN is modulated by the presence of different monosaccharide and disaccharide sugars available in the media.

To test this hypothesis I will:

- a. Determine if growth promotion induced by PsJN is correlated with StHXX1 activity in plants grown in different monosaccharide and disaccharide sugars.

Hypothesis 3

StHXX1 activity is not altered in a potato cultivar that does not show typical growth promotion when treated with PsJN.

To test this hypothesis I will:

- a. Determine if StHXX1 activity in non-responsive plants is altered in a manner that differs from plant showing growth promotion in response to PsJN.

Chapter 2: Materials and Methods

2.1 Generation and maintenance of plant materials

Potato plantlets were grown from nodal explants for five to six weeks on growth media (Murashige Skoog basal minimal salt mixture (MS, (Sigma)) containing 3% sucrose (w/v)) at pH 6. When the plants had reached the top of the test tube (height 15 cm X width 2.5 cm, Sigma) they were cut into nodes under sterile conditions. The apical and basal nodes were discarded and the remainders placed into Magenta boxes containing growth media. New leaf buds developed within one week and these were used for inoculation with PsJN. Plants were grown in a controlled environmental chamber (Power Scientific, Inc.), with light intensity of 100 $\mu\text{mol photon m}^{-2}\text{s}^{-1}$, provided by fluorescent bulbs, and a photoperiod of 16 hours daylight. The temperatures were set at 24°C: 16°C (light: dark). These growth conditions were used for all the experiments unless specified otherwise. The leaf buds generated were dipped for 1 minute into PsJN bacterial suspension (prepared as described in section 2.2). They were then dried in a sterile petri dish, and transferred into test tubes containing MS media at pH 6. Sucrose concentration (w/v) in the media was adjusted to the desired concentrations for each specific experiment. For control plants, the leaf buds were dipped into nutrient broth (NB (Difco™)) alone. Plantlet growth was measured as required over a 4 week period (71, 85).

2.2 Bacterium strain and growth condition, and plant inoculation

B. phytofirmans strain PsJN, was used from the collection of Dr. George Lazarovits and was stored with 80% glycerol (Caledon) at -80°C. The bacteria were streaked onto NB agar medium and incubated at 30°C for 48 hours. A single colony growing in NB agar medium was picked with a sterile micropipette tip, transferred

into a test tube (15 cm X 1.5 cm, VWR) containing 5 mL NB medium and incubated overnight at 30°C on a shaker. The PsJN bacterial suspension was used to inoculate newly grown leaf buds under sterile conditions (71, 85). Plants derived from such inoculated buds were used in all studies as described below. The PsJN strain was cultured in the same manner for all studies unless specified otherwise.

2.3 Analysis of potato plants physiologies with or without PsJN

Plants grown for 1, 2, 3, or 4 weeks, with and without PsJN inoculation, were gently removed from the 1.5% (w/v) sucrose MS agar media, their roots rinsed with sterile water for 1 minute, and then blotted free of water with paper towels. Ten plants per treatment at each time point were used to determine total plant fresh biomass, stem mass, root mass, stem height, chlorophyll content, and leaf numbers. Plant biomass was determined using an analytical balance (Electronic balance model FA2004B). Total fresh biomass was measured first, followed by root and stem. Stem height was measured with a ruler. Chlorophyll content was determined using a SPAD 502 chlorophyll meter (Konica, area measured = 2 X 3 mm). The numbers defined by the SPAD 502 chlorophyll meter are values that indicate the relative amount of chlorophyll present in plant leaves. Three leaves were selected from individual plants and random areas of a leaf were taken for chlorophyll determinations. The total numbers of leaves formed were counted. All experiments were repeated at least once unless otherwise indicated. The same protocol for measurements of plant biomass and growth was followed in all experiments with tissue culture using 3% (w/v) sucrose MS agar media and repeated once.

The effect of PsJN on plant growth when grown on MS media containing either 1.5% glucose or fructose (w/v), and 3% glucose, fructose, maltose, mannose, or

galactose (w/v) at pH 6 was determined at 3 and 5 weeks compared to plant without PsJN. The same protocol for measurements of plant biomass and growth was followed in all experiments. This experiment was repeated once for all the sugar compositions used.

2.4 Determination of hexokinase1 activity

Ten cv. Kennebec plants per treatment at each time point were analyzed for StHXX1 enzymatic activity using the glucose phosphorylation assay for each set of experiment using different sugars and different concentrations as plant tissue culture media. Stems were cut below the apical bud and the root was used for protein extraction. The tissue from each plant were transferred into a 2 mL microcentrifuge tube containing protein extraction buffer (50 mM Tris-HCl at pH 7.6 (Sigma), 5 mM MgCl₂ (Sigma), 2 mM EDTA (Caledon), 10 mM KCl (Caledon), 10% (v/v) glycerol (Caledon), 0.1% (v/v) triton X-100 (Sigma), 5 mM 2-mercaptoethanol (Sigma), 0.1% (v/v) protease inhibitor (Sigma)), and 15 to 20 glass beads of 2.5 mm diameter (BioSpec products, Inc) (40). The sample mass to extraction buffer ratio was adjusted in all cases to 1:10 (w/v). Samples were homogenized at a speed of 6.5 meter for 10 seconds using FastPrep bead beater (MPbio) and transferred to an ice bath immediately. Homogenates were centrifuged at 10,000 g for 10 seconds at 4°C to pellet the tissues. The supernatants were transferred into 1.5 mL microcentrifuge tubes and re-centrifuged at 10,000 g for 10 minutes at 4°C. Fifty µL of the supernatant solution was added to 250 µL of reaction mixture containing 50 mM Tris-HCl at pH 7.6 (Sigma), 4 mM MgCl₂ (Sigma), 1 mM glucose (Fisher), 1 mM ATP (Norgen Biotek), 0.2 mM NADP⁺ (Sigma) and 1 unit of glucose-6-phosphate dehydrogenase (Sigma) to measure StHXX1 activity. StHXX1 activity was determined

spectrophotometrically (SpectraMax Plus 384) at 340 nm in 30°C for 2 minutes for glucose phosphorylation measurement (40). This experiment was repeated once.

2.5 Determination of hexokinases RNA transcript levels

Two week old plants cv. Kennebec 3 per treatment grown in 3% (w/v) MS sucrose media were chosen. The mRNA expression of each stem and root sample was homogenized in Trizol (ABICON) and each sample RNA was isolated using an RNA isolation kit (ABICON) following the manufacturer's recommendations. Genomic DNA contamination was removed using the RNase free DNase kit (Qiagen). cDNA was generated from 0.5 µg of total RNA using the QuantiTect® Reverse transcription kit (Qiagen). The following primers were used. Forward 5'gttgagaactcacaaaagc3' and reverse primers 5'ttaccaccagctaattgtcc3' were used for *StHKK1* amplification (NCBI accession: X94302.1). Forward 5'tgttcagaactgacaaaag3' and reverse 5'attggtgaatctaccaccag3' were used for *StHKK2* amplification (NCBI accession: AF106068). Forward 5'gagctatgagcttcccgatg3' and reverse 5'gattccacgtgcttccattc3' were used for the internal standard *ACTIN* (NCBI accession: X55749). RT-PCR was performed in 20 µL of total reaction volume for each sample within a 96-well rotary plate of a Corbett Rotor-gene™ 6000 system using the manufacturer's recommendations. The reaction mixture contained sterile deionized H₂O, PerfeCta® SYBR® Green PCR master mix (Quanta Bioscience), gene specific primers, and each cDNA template. Three technical replicates were run. RT-PCR was carried out with an initial denaturation step at 94°C for 4.5 minutes, followed by denaturation (15 seconds at 94 °C), annealing (20 seconds at 58 °C) and extension (40 seconds at 72 °C). A total of 30 cycles of RT-PCR reaction was performed. A final extension was carried out for 15 seconds starting from 72 °C to 95 °C. The threshold

cycle (C_T values) of the target genes and *ACTIN* in each sample were obtained after RT-PCR. C_T refers to the specific cycle number where each sample template started to increase exponentially and calculated from the Corbett Rotor-gene™ 6000 system software. Negative controls were included to confirm that there was no DNA contamination. For each negative control, reverse transcriptase was omitted during RNA to cDNA synthesis and subjected to RT-PCR with *StHXK1*, *StHXK2*, and *ACTIN* primer. The primer specificities were confirmed by sequencing the RT-PCR products. The products were purified using a PCR purification kit (Qiagen) and sent to Robarts research institute of Western University (London, Ontario, Canada) for sequencing. Relative expression levels of all the samples were calculated and analyzed with Corbett Rotor-gene™ 6000 system software.

StHXK1 and *StHXK2* C_T values were subtracted from *ACTIN* C_T value, housekeeping gene, from each PsJN-treated and uninoculated sample. For each sample, subtracted C_T values were indicated as delta (dC_T) value. These numbers were known as relative expression to *ACTIN* and the smaller dC_T indicate the higher gene expression (32, 35, 42). dC_T from *StHXK1* and *StHXK2* was compared for each sample. This experiment was repeated once.

StHXK1 C_T value from PsJN-treated and uninoculated samples were subtracted from *ACTIN* C_T value from PSJN-treated and uninoculated samples, respectively. dC_T from *StHXK1* was compared between PsJN-treated and uninoculated plants. This experiment was repeated once.

2.6 Determination of potato hexokinase1 protein accumulation

Three two week old PsJN-treated and uninoculated plants grown on medium containing 3% (w/v) sucrose were used for the extraction of total plant protein as

previously described in section 2.4. Gels were cast to yield a final acrylamide monomer concentration of 10% (w/v) in the separating gel and 4% (w/v) in the stacking gel. Fifty μg (determined by BSA standard curve) of protein from each sample were incubated at 95°C to denature the proteins and this was then added to the stacking gel and the gel run at 120 V for 90 minutes at room temperature. A polyvinylidene difluoride (PVDF) membrane was washed with 100% methanol (Caledon) for 10 seconds to activate just prior to completion of polyacrylamide gel electrophoresis. After gel electrophoresis, the gel was immersed in transfer buffer (25 mM Tris base, 192 mM glycine, 10% (v/v) methanol) for 15 minutes. The gel was placed on top of the PVDF membrane and this was then placed in a Bio-Rad Semi-Dry transfer Cell. Protein transfer to the PVDF membrane was carried out at 25 V for 40 minutes. After transfer, the PVDF membrane was incubated in blocking solution (Tris-buffered saline, Tween20 (TBS-T) with 5% (w/v) carnation skim milk powder) for 1 hour at room temperature. The primary antibody, polyclonal anti-HK IgG, was obtained from Dr. Jean Rioval from University of Montreal. Anti-HK IgG solution, 15 mL, in Tris buffered saline with bovine serum albumin (TBS-BSA) was poured on the PVDF membrane and incubated overnight at 4°C . After incubation, the PVDF membrane was washed with TBS-T twice and washed again with 0.5% (w/v) blocking solution two times. The secondary antibody used was anti-rabbit conjugated to horseradish peroxidase, and this was applied with 0.5% (w/v) blocking solution to the PVDF membrane and incubated for 1 hour. After incubation the PVDF membrane was washed with TBS-T four times for 10 minutes each time. The PVDF membrane was transferred to a dark room and enhanced chemiluminescence reagent was poured onto the PVDF membrane. After 1 minute, the PVDF membrane was dried on a paper towel and then an X-ray film was placed over it and sealed into a cassette. The X-ray

film was exposed for 20 minutes and the signal produced by the secondary antibody was detected using an X-ray film developer (40). This experiment was repeated once and this experiment was carried out in Agriculture and Agri Food Canada, London, Ontario station.

2.7 Growth promotion test of different potato plant cultivars with PsJN

Nodes of potato plants cv. Yukon Gold, Shepody, and Superior were prepared and inoculated with PsJN, as previously described in section 2.1 and 2.2, and grown for 6 weeks. Yukon Gold was selected for further study on the potential role of StHXX1 activity in growth promotion by PsJN because Yukon Gold had dissimilar root growth compared to PsJN-treated cv. Kennebec. This experiment was repeated once.

2.8 Potato plant cv. Yukon Gold physiologies with or without PsJN and determination of hexokinase1 activity

Nodes of 6 week old potato of plantlets cv. Yukon Gold grown on the growth media were used to generate leaf buds. The plants generated from these were treated with PsJN or left uninoculated and grown as previously described in section 2.1 and 2.2. Ten Yukon Gold plants of both PsJN inoculated and uninoculated were grown in medium containing 3% (w/v) sucrose for 3 and 5 weeks. They were analyzed for changes in phenotype and tested for StHXX1 activity as previously described in section 2.3 and 2.4, respectively. This experiment was repeated once.

2.9 Statistical analysis

Analysis of variance (One way-ANOVA) was carried out to analyze the data

collected between treatments for biomass, stem mass, root mass, stem height, leaf number, chlorophyll contents, StHKK1 activity, and *StHKK1* and *StHKK2* mRNA expression. Group statistics were expressed as mean \pm standard error of mean (SEM). All statistical analysis was performed in Microsoft Excel 2007. The significant differences are denoted by asterisk (*) where the difference were significant at $P \leq 0.05$.

CHAPTER 3: Results

3.1 The effect of PsJN treatment on the growth of nodal explants of cv. Kennebec

Once plants were inoculated with PsJN, PsJN-treated and uninoculated plant growth was monitored for 1 to 4 weeks. Significant differences in growth promotion between PsJN-treated plants and uninoculated plants first became apparent as increased root mass and chlorophyll content after 2 weeks of growth on 1.5% (w/v) sucrose (Figure 1). At this stage there was a slight increase in formation of secondary root structures with PsJN-treated plants compared to uninoculated plants (Figure 2). By week 3 growth, total biomass and stem mass was significantly different between PsJN-treated and uninoculated plants, and by week 4 stem height also differed (Figure 1). Plants treated with PsJN and grown for 4 weeks in 1.5% (w/v) sucrose when compared to uninoculated plants showed significant increases in total biomass of 32%, in stem mass of 32%, root mass of 57%, stem height of 33%, and chlorophyll content of 21% (Figure 1). No difference was seen in leaf number between treatments. The differences in growth characteristics between PsJN-treated and uninoculated plants became even more apparent after 6 weeks inoculation (Figure 3).

Plants grown in 3% (w/v) sucrose showed significant increases in chlorophyll content by week 2, root mass by week 3, and total biomass by week 4 (Figure 4). More formations of secondary roots were observed with PsJN-treated plants than uninoculated plants within 2 weeks of growth (Figure 2). Yet, lower total root mass was found with PsJN-treated plants compared to uninoculated plants after 2 weeks perhaps due to generation of more secondary roots rather than root elongation (Figure 4). PsJN-treated plants grown in 3% (w/v) sucrose showed a significant growth increase in total biomass by 16%, in root mass by 49%, and in chlorophyll content by 29% after 4

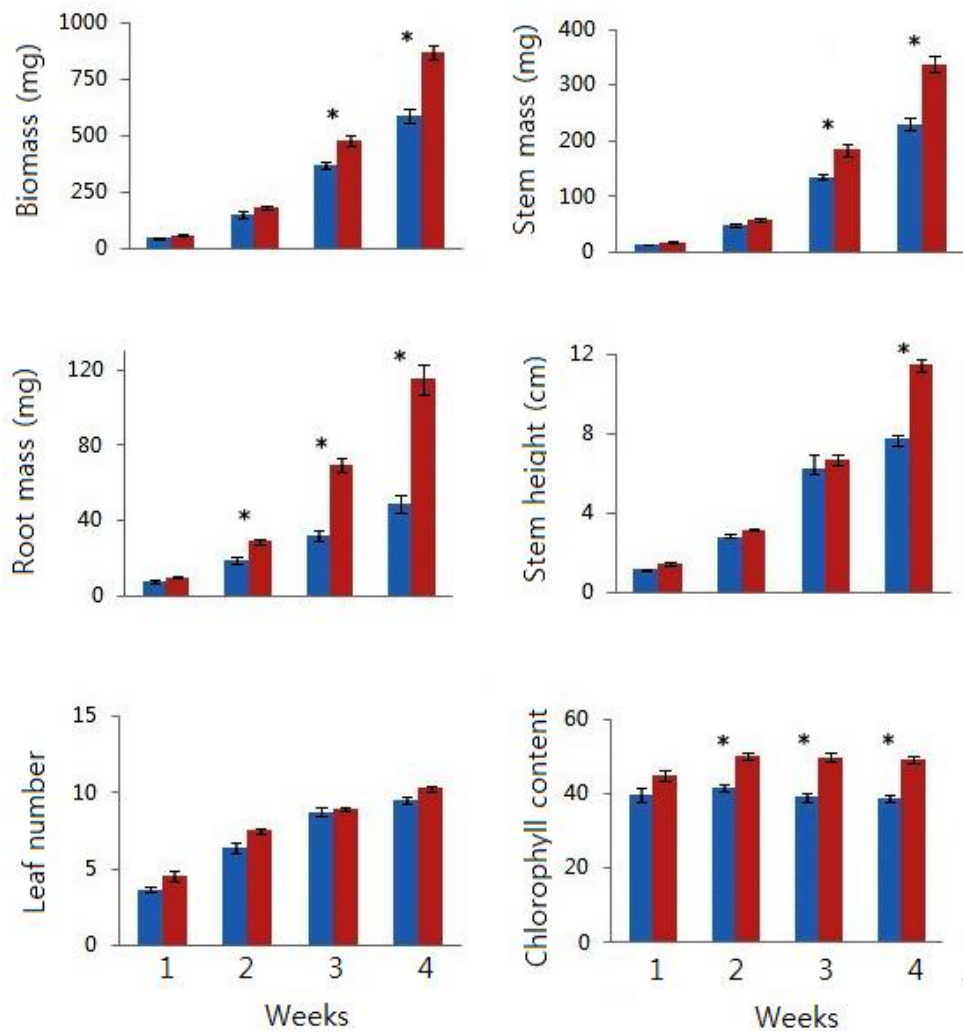


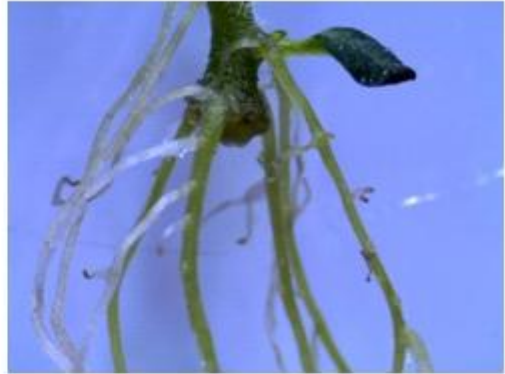
Figure 1. The effect of *B. phytofirmans* on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 1.5% sucrose for 1 to 4 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

Plants grown at 1.5% sucrose



Plants grown at 3% sucrose



Uninoculated

PsJN treated

Figure 2. The effect of *B. phytofirmans* on the growth of potato roots cv. Kennebec grown in medium containing 1.5 and 3% sucrose for 2 weeks.

Plants grown at 1.5% sucrose



Plants grown at 3% sucrose



U

P

U

P

4 weeks

6 weeks

Figure 3. The effect of *B. phytofirmans* on the growth of potato nodal explants cv. Kennebec when grown in medium containing 1.5 and 3% sucrose for 4 and 6 weeks.

U: Uninoculated

P: PsJN-treated

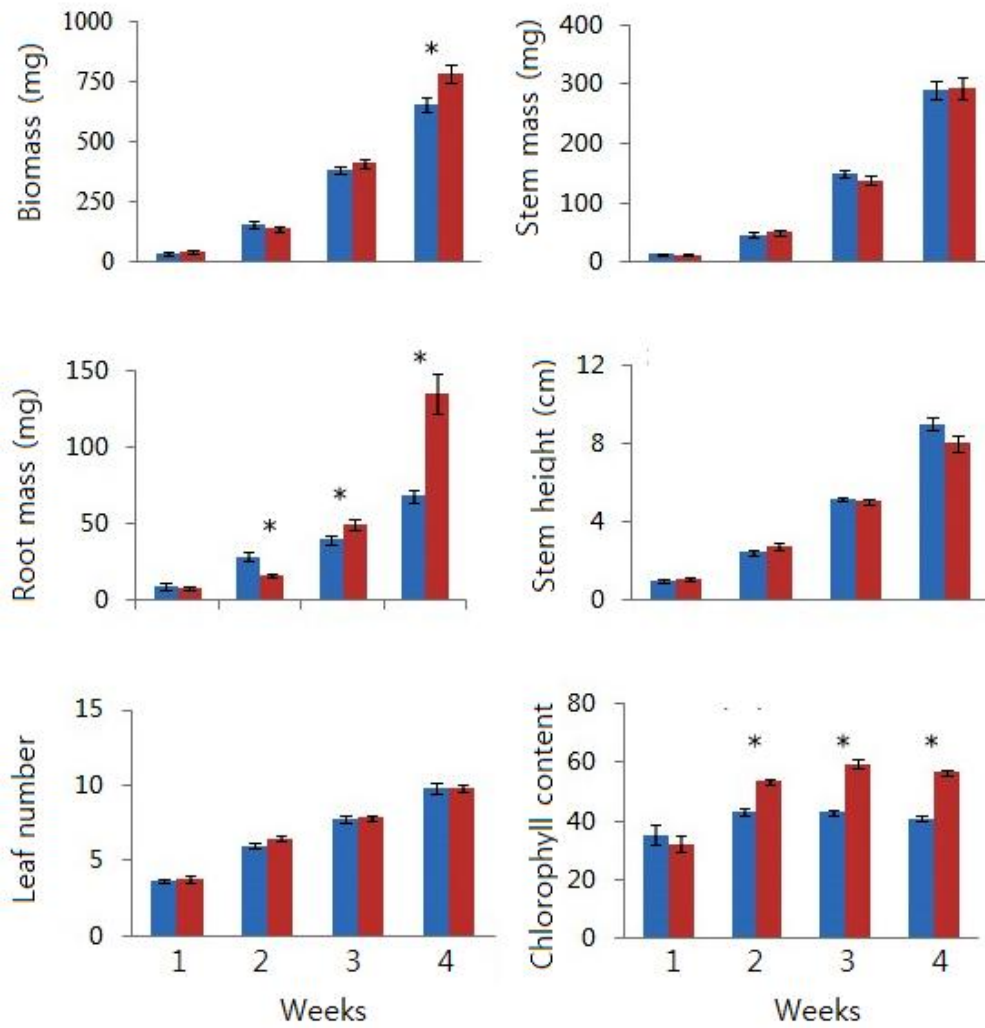


Figure 4. The effect of *B. phytofirmans* on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 1 to 4 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

weeks post-inoculation compared to uninoculated plants (Figure 4). No difference in stem mass, stem height, and leaf numbers were found between the two treatments. By week 4, PsJN-treated plants had slightly shorter stem heights and more enhanced root system than the uninoculated plants (Figure 4). The root growth promotion was significantly more pronounced than that found with PsJN-treated plants grown in 1.5% sucrose. At 6 weeks, the height of PsJN-treated plants remained much shorter, but these plants had more massive root systems than uninoculated plants (Figure 3).

PsJN stimulated growth promotion in potato plants, but the type of growth promotion that occurred was greatly influenced by the sucrose concentration in the medium. A significant increase in chlorophyll content in PsJN-treated plants found at week 2 from plants grown on both 1.5 and 3% (w/v) sugar concentrations indicated that the altered regulation of chlorophyll accumulation is one of the primary effects and a major feature of enhanced growth promotion (Figure 1 and 4).

3.2 The effect of PsJN treatment on potato hexokinase1 activity

After completing the analysis of growth and chlorophyll levels, the plants were immediately used for measurement of StHKK1 activity. StHKK1 activities were determined for both PsJN-treated and uninoculated plants in extracts from stems and roots of plants grown for 1, 2, 3, and 4 weeks in MS agar medium containing either 1.5% (w/v) or 3% (w/v) sucrose. Stems of plants grown in 1.5% (w/v) sucrose had similar StHKK1 activities to that found in extracts from PsJN-treated and uninoculated tissues at all times measured, except at week 2 when PsJN-treated plants exhibited a significant increase of 20% in activity (Figure 5). In root samples, StHKK1 activity was similar at weeks 1 and 2 between the two treatments. StHKK1 activities had increased significantly by 54% and 28% in weeks 3 and 4, respectively,

in PsJN-treated plants compared to uninoculated plants (Figure 5).

With plants grown on 3% (w/v) sucrose there was little difference in stem StHXX1 activities through weeks 1 to 4, except again at week 2 when PsJN-treated plants showed a 19% increase StHXX1 activity, a value that was significant compared to uninoculated plants (Figure 6). In roots activity of StHXX1 was similar in PsJN-treated and uninoculated plants at week 1 but by week 2 StHXX1 activity in PsJN-treated plants had increased significantly by 42% as compared to uninoculated plants (Figure 6). At this time the only visible differences between PsJN-treated and uninoculated plants were the increased number of secondary roots and the greener colour of the PsJN-treated plants (Figure 4). Activity of StHXX1 in roots of PsJN-treated plants was found to be significantly lower than those of uninoculated plant roots by week 4 (Figure 6).

3.3 Comparison of potato *hexokinase1* expression in plants with or without PsJN

Three 2 week old PsJN-treated and uninoculated plants, grown in 3% (w/v) sucrose, were used to determine *StHXX1* gene expression. Plants of this age were selected because differences in StHXX1 stem and root activities were found at this age between PsJN-treated and uninoculated plants grown on 3% (w/v) sucrose containing medium. A study on potato tubers showed that StHXX1 is much more active in glucose phosphorylation than StHXX2 (57). However, it would be of relevance to confirm that this also occurred in this model system. The mRNA expression of each *StHXX1* and *StHXX2* gene was analyzed by RT-PCR.

To be sure that the mRNAs for the two enzymes (*StHXX1* and *StHXX2*) which share 82% similarity, were differentiated, the specificity of the two pairs primers used was tested. Sequenced RT-PCR products of *StHXX1* and *StHXX2*

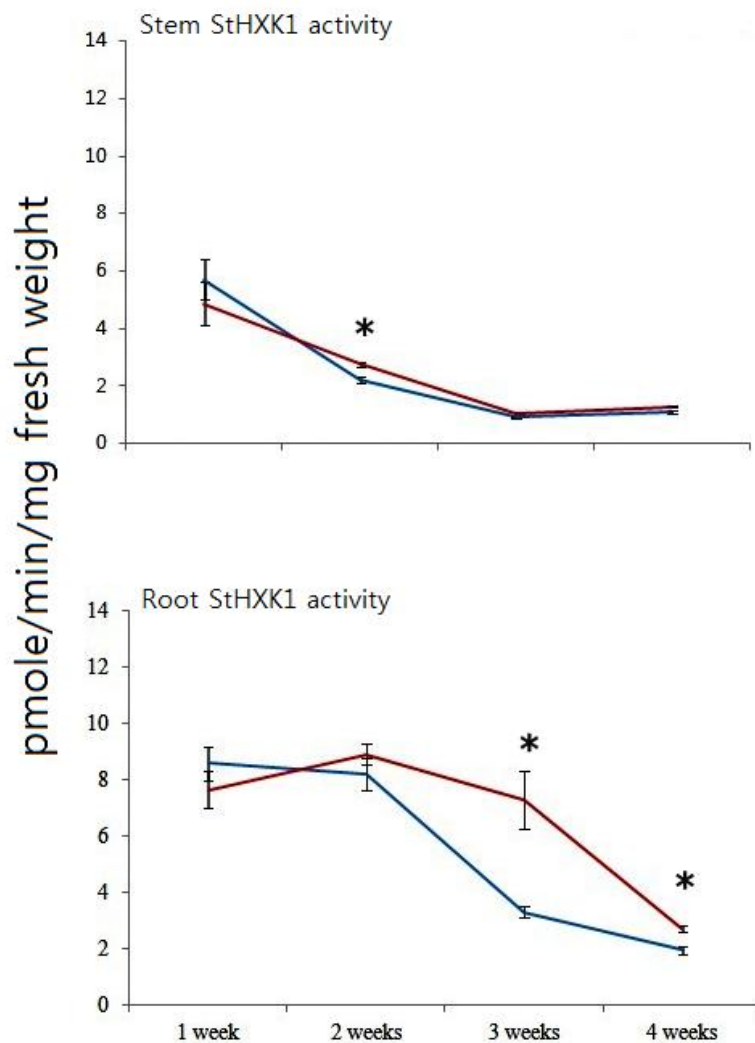


Figure 5. The effect of *B. phytofirmans* on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 1.5% sucrose for 1 to 4 weeks.

The data shows amounts of picomoles of glucose converted into glucose-6-phosphate in one minute by StHXK1 in PsJN-treated and uninoculated plants. Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue line: Uninoculated

Red line: PsJN-treated

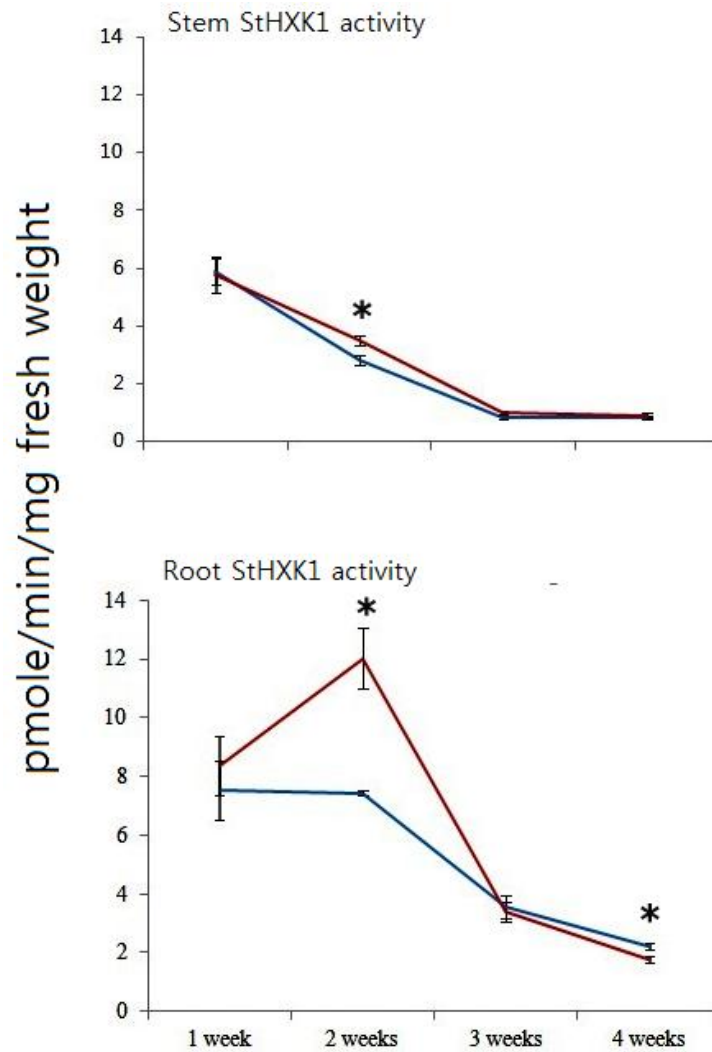


Figure 6. The effect of *B. phytofirmans* on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 1 to 4 weeks.

The data shows amounts of picomoles of glucose converted into glucose-6-phosphate in one minute by StHXX1 in PsJN-treated and uninoculated plants. Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue line: Uninoculated

Red line: PsJN-treated

directly after RT-PCR, and showed that *StHXX1* and *StHXX2* primers only amplified the target region of *StHXX1* and *StHXX2* (Figure 7). In addition, the melting curve from RT-PCR for each product was also examined to ensure specificity of each product (data not shown).

The relative mRNA expression levels between *StHXX1* and *StHXX2* was compared and showed *StHXX1* was expressed in greater levels than *StHXX2* in stems and roots of 2 week old PsJN-treated and uninoculated plants when grown on 3% (w/v) sucrose (Figure 8). This indicates that in the enzyme assays used in this study for measuring glucose phosphorylation activity, it is *StHXX1* that is most likely being measured.

The relative mRNA expression levels between *StHXX1* from PsJN-treated and uninoculated plants appears to be no different in the degree of mRNA in treated plant stems and roots in 2 weeks old (Figure 9). It suggests that *StHXX1* mRNA levels can be induced earlier than 2 weeks.

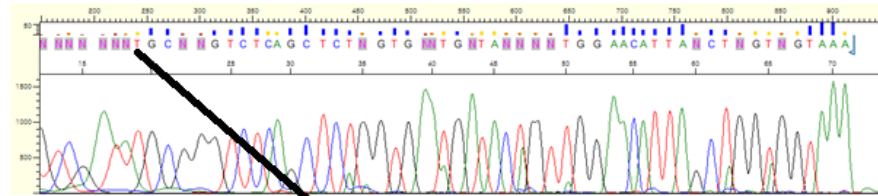
3.4 Comparison of potato hexokinase1 protein accumulation with or without PsJN

The antibody that was used to quantify *StHXX1* was anti-HK IgG (40). This anti-HK IgG is a specific anti-hexokinase polyclonal anti-serum that was generated from New Zealand white rabbits and the antibody used for a wild relative of the cultivated potato *Solanum chacoense* hexokinase 2 (*ScHK2*) (40). This *ScHK2* amino acid sequence shares 76% and 100% similarities to *StHXX1* and *StHXX2*, respectively (NCBI). Moisan and Rioval used anti-HK IgGs to quantify *StHXX1* after purifying *StHXX1* from potato tuber using column chromatography and detected one distinct band, about 51 kDA, on a western blot (40). Mass spectrometry sequencing

A. Amplified region of *StHXX1*

TTGGAGAACTCACAAAAGCTATGAAAGAAAAGGTGCTCGATA
TGCGGGTCTCA
GCTCTGGTGAATGATACTGTTGGAACATTAGCTGGTGGTAA

Sequenced amplified region of *StHXX1*

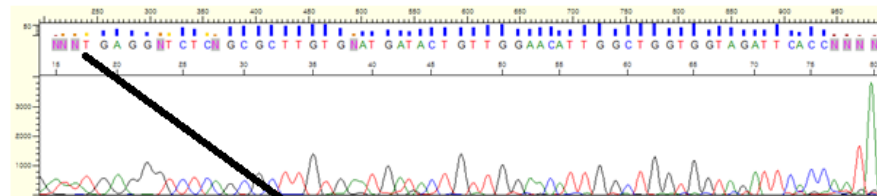


NNNNNNNNNNNNNNNNNN**TGCNNGTCTCAGCTCTNGTGNNTGNTANNNTGGA**
ACATTANCTNGTNGTAAA

B. Amplified region of *StHXX2*

TGTTCGAGAACTGACAAAAGCAATGCAAAAACGAGAAATTGATA
TGAGGGTCT
CAGCGCTTGTGAATGATACTGTTGGAACATTGGCTGGTGGTAGATTACCAAT

Sequenced amplified region of *StHXX2*



NNNNNNNNNNNNNNNN**TGAGGNTCTCNGCGCTTGTGNATGATACTGTTGGAAC**
ATTGGCTGGTGGTAGATTACCANNNNNNNNNGNTNNNNNNNN

Figure 7. Specificity of *StHXX1* and *StHXX2* primers to targeted *StHXX1* and *StHXX2* region for RT-PCR amplification.

Bolded and underlined letters indicate the region where *StHXX1* and *StHXX2* primers annealed to amplify the selected region for *StHXX1* and *StHXX2* RT-PCR amplification. Matched sequences were highlighted in yellow.

A) Sequences of cDNA of *StHXX1* PCR product as compared with targeted region of *StHXX1*.

B) Sequences of cDNA of *StHXX2* PCR product as compared with targeted region of *StHXX2*.

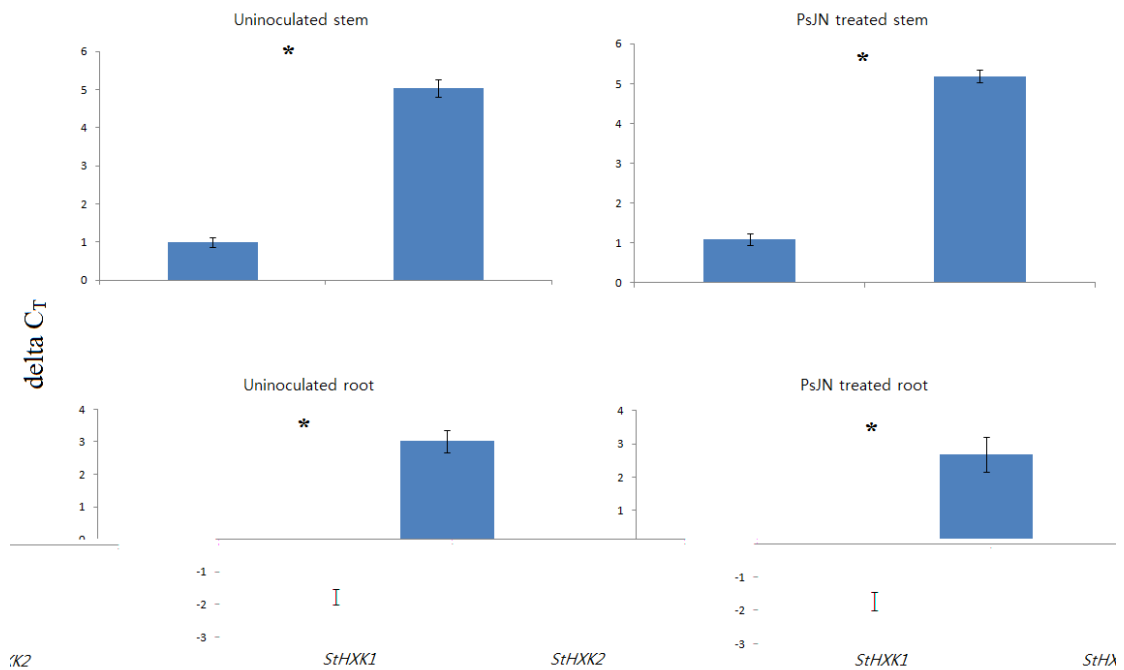


Figure 8. Relative *StHXX1* and *StHXX2* mRNA expression in PsJN-treated and uninoculated potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 2 weeks.

Data are presented as the mean \pm SEM, n=3. One way ANOVA was used to determine significant differences. dC_T was calculated by subtracting the C_T of the *StHXX1* and *StHXX2* from the C_T of the *ACTIN* (the smaller the dC_T , the higher the gene expression).

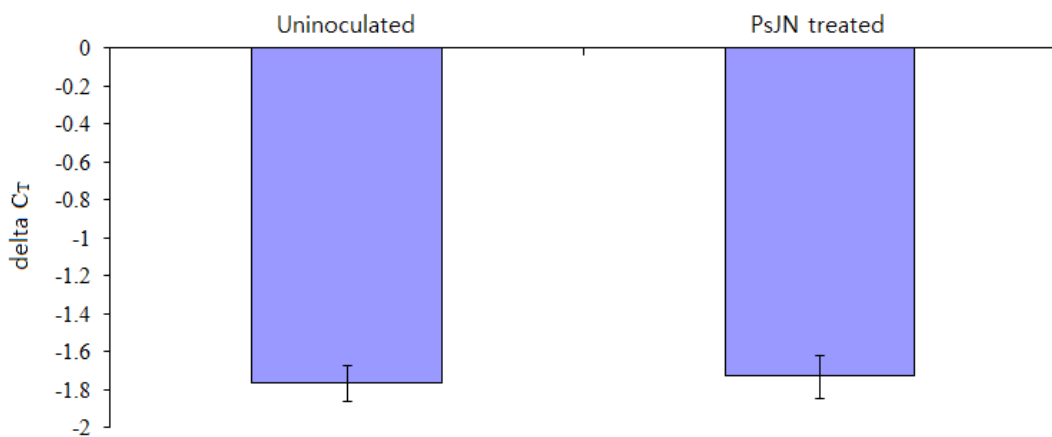


Figure 9. Relative *StHXK1* mRNA expression between PsJN-treated and uninoculated potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 2 weeks.

Data are presented as the mean \pm SEM, n=3. One way ANOVA was used to determine significant differences. dC_T was calculated by subtracting the C_T of the *StHXK1* from the C_T of the *ACTIN* (the smaller the dC_T , the higher the gene expression).

showed that this band was most likely StHXX1 because it matched the published (NCBI) StHXX1 amino acid sequence data base. Therefore, anti-HK IgGs could be used for my study to compare StHXX1 expression between PsJN-treated and uninoculated plants.

There were two bands present in my western blot, tentatively identified as StHXX1 and StHXX2. The top band with the stronger signal may be the StHXX2, since anti-HK IgGs was used for the antibody. In addition, the molecular weight of StHXX2 suggested being between 54 to 66 kDA (57, 71). Thus, the band spotted near 60 kDA is speculated to be StHXX2, even though molecular weight calculator predicted 53.73 kDA (Science Gateway). Since the antibody I used was polyclonal antibody, it could have been less sensitive to StHXX2 and possibly react with hexokinase-like proteins. The weaker band spotted near 50 kDA could be StHXX1 expression (Figure 10). Although, molecular weight calculator predicted 54.14 kDA (Science Gateway), it is close agreement with that I observed for StHXX1 band.

StHXX1 protein expression in stem and root of 2 week old PsJN-treated and uninoculated plants were found to be almost identical using the Western Blot procedure (Figure 10). As there was no observable increase in *StHXX1* gene expression resulting from PsJN treatment it infers that PsJN may influence StHXX1 activity by posttranslational modification.

3.5 The influence of sugar composition on the growth enhancement by PsJN

In the second experiment, different monosaccharide and disaccharide sugars at 1.5 and 3% (w/v) were used in place of sucrose in the tissue culture media in order to reveal how different sugars can influence PsJN-treated plant growth.

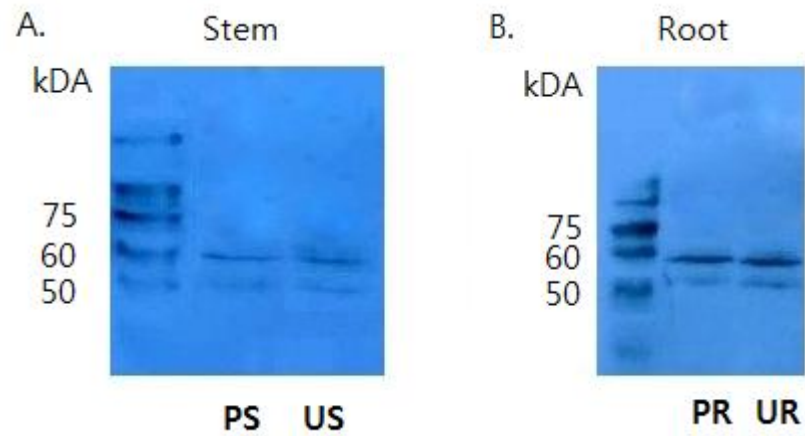


Figure 10. The effect of *B. phytofirmans* on the hexokinase1 accumulation of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 3% sucrose for 2 weeks.

A. Stem StHXK1 and StHXK2 protein levels

B. Root StHXK1 and StHXK2 protein levels

PS: PsJN-treated plant stem

US: Uninoculated plant stem

PR: PsJN-treated plant root

UR: Uninoculated plant root

PsJN-treated plants grown for 3 weeks in MS agar having 1.5% (w/v) glucose showed increases in total biomass by 30%, of stem mass by 41%, and in stem height by 33% compared to uninoculated plants. After 5 weeks of growth, PsJN-plants showed increases in biomass of 56%, in stem mass of 67%, root mass of 28%, stem height of 58%, and leaf number of 22% compared to uninoculated plants (Figure 11).

Similarly, PsJN-treated plants grown in 1.5% (w/v) fructose also showed significant increases in biomass of 52%, stem mass of 57%, and stem height of 45% by week 3, and by 5 weeks increases in biomass of 52% , stem mass of 60% , root mass of 23%, stem height of 45%, and leaf numbers of 11% (Figure 12). Growth promotion was first evident with PsJN-treated plants on both sugars as stem height followed by increased stem mass, root mass, and leaf number. At 6 weeks, PsJN-treated plants had much higher shoot heights than uninoculated plants whether they were grown in 1.5% (w/v) glucose or fructose (Figure 13).

Plants inoculated in the presence of mannose and galactose did not grow at all and the plants eventually died (data not shown). This indicates that plants are highly sensitive to 3% (w/v) mannose and galactose. Therefore, plants may have different sensitivity to different sugars and different sugars may affect plant growth differently.

PsJN-treated plants grown in MS agar media containing 3% (w/v) glucose showed significant increases in biomass of 17%, in stem mass of 22%, root mass of 38%, and stem height of 50% by 3 weeks of growth compared to uninoculated plants. By week 5, even greater increases in growth were found when compared to uninoculated plants such that the biomass had increased by 35%, stem mass by 53%, root mass by 35%, stem height by 48%, and leaf number by 29% (Figure 14).

PsJN-treated plants grown in 3% (w/v) fructose also showed significant increases in biomass of 36%, stem mass of 49%, root mass of 24%, and stem height

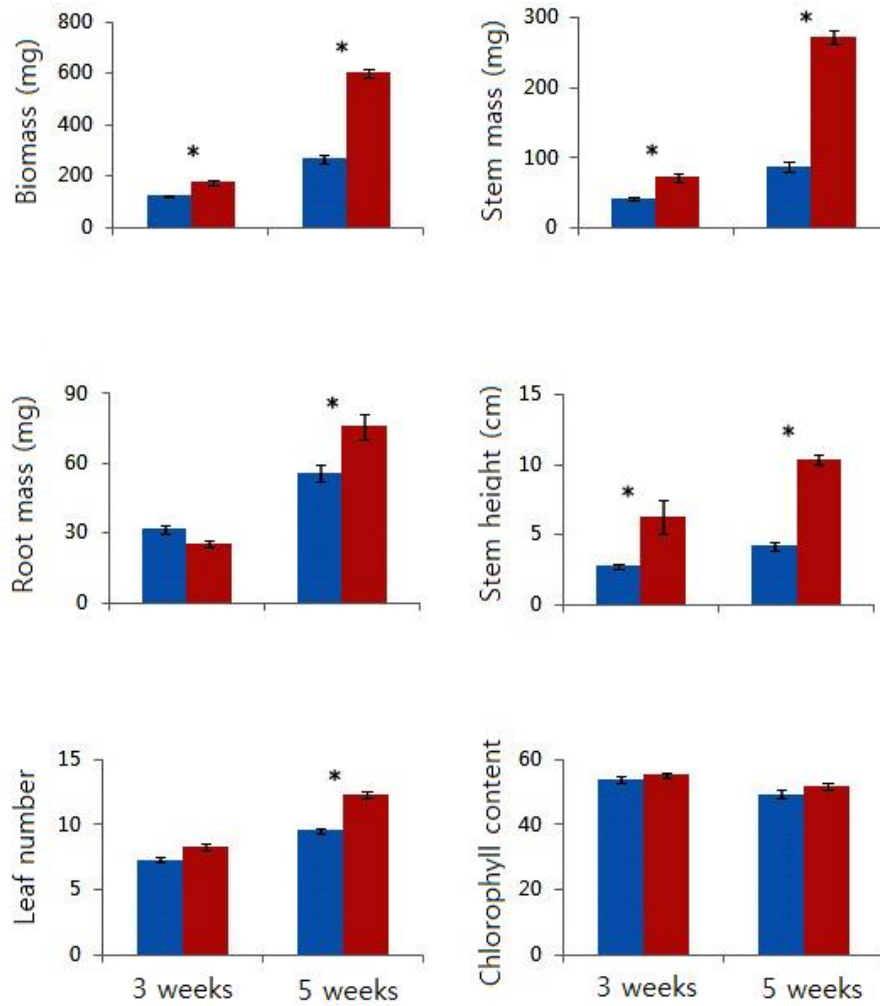


Figure 11. The effect of *B. phytofirmans* on the growth and chlorophyll contents of potato nodal explants cv. Kennebec when grown in medium containing 1.5% glucose for 3 and 5 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

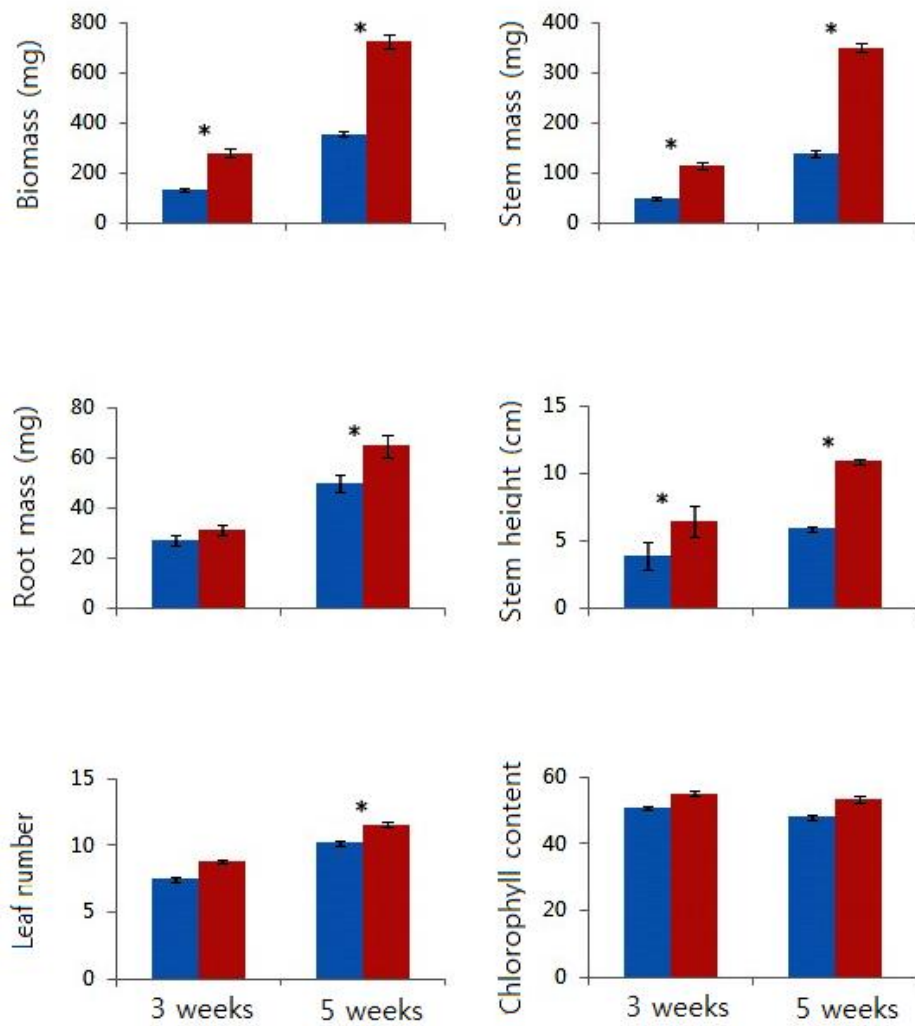


Figure 12. The effect of *B. phytofirmans* on the growth and chlorophyll contents of potato nodal explants cv. Kennebec when grown in medium containing 1.5% fructose for 3 and 5 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

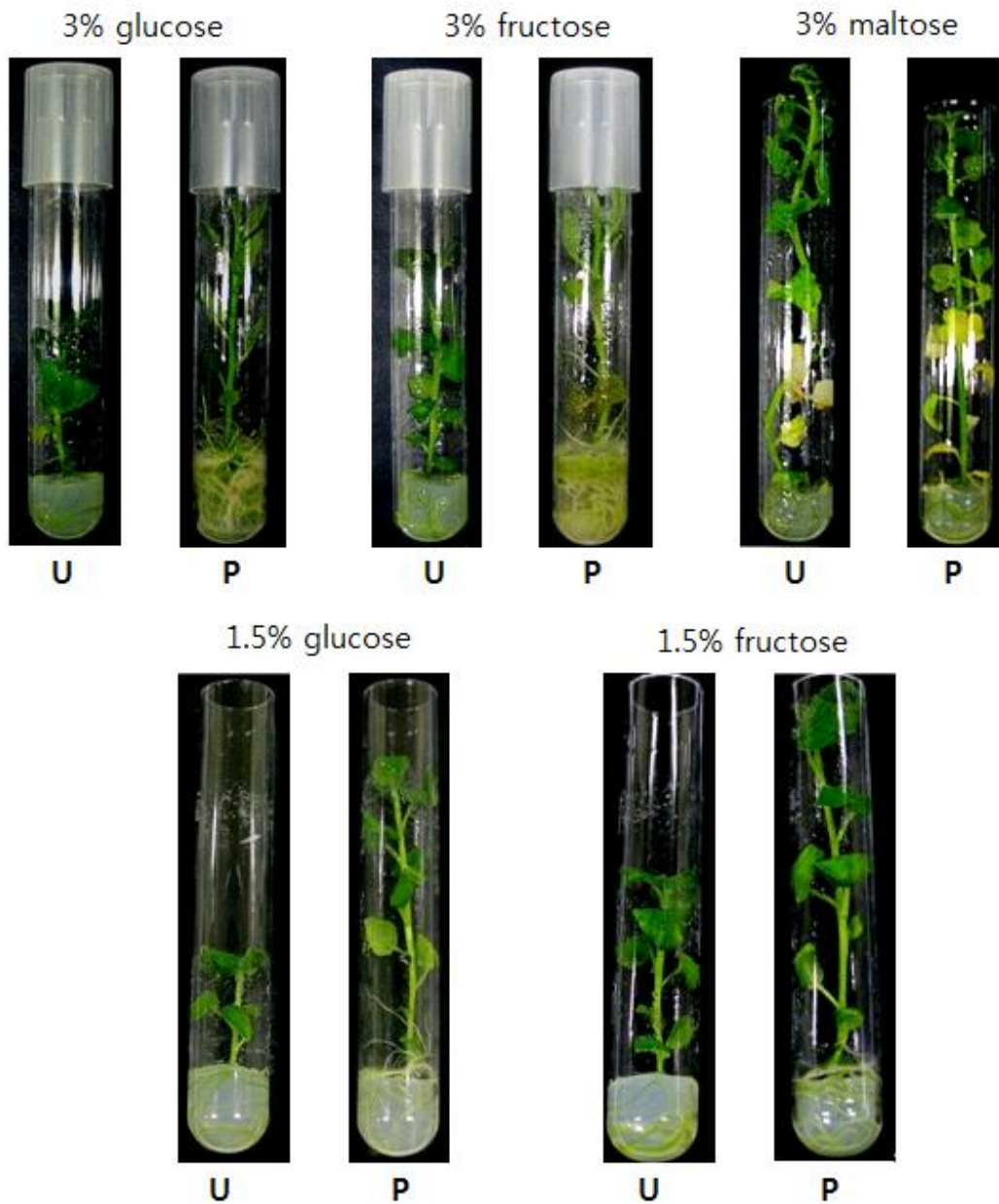


Figure 13. The effect *B. phytofirmans* on the growth of potato nodal explants cv. Kennebec when grown in medium containing 1.5% glucose or fructose, and 3% glucose, fructose, or maltose for 4 and 6 weeks.

U: Uninoculated

P: PsJN-treated

of 43% after 3 weeks of growth. After 5 weeks, the growth stimulation was expressed as 41% increase in biomass, 51% increase in stem mass, 15% increase in root mass, 44% increase in stem height, and 18% increase in leaf number production (Figure 15). In most previous experiments, PsJN-treated plants grown in 3% sucrose did not show any growth promotion in stem mass and stem height, and generally shoot height was much shorter than found with uninoculated plants (Figure 3). At 6 weeks, PsJN-treated plants had taller shoots and more massive root systems as compared to uninoculated plants whether they were grown in 3% (w/v) glucose or fructose (Figure 13). The growth promotion induced by PsJN on plants grown on glucose and fructose media was similar to that seen with PsJN-treated Kennebec plants grown in 3% sucrose except for the increased heights of stems found with PsJN treatment.

Interestingly, there was no increase in chlorophyll content as a result of PsJN treatment with plants grown in 1.5 or 3% (w/v) glucose and fructose compared to that seen with PsJN-treated plants grown on 1.5% and 3% (w/v) sucrose. In the presence of glucose and fructose instead of sucrose we observed increases in leaf number production (Figure 1, 4, 11, 12, 14, 15). This again confirms that the growth promotion induced by PsJN is significantly regulated by the presence of not only the concentrations of sugar, but also by nature of sugar used.

Most surprising was the finding that plants grown in the presence of 3% (w/v) maltose grew faster and were taller than plants grown on the other sugars (Figure 13), but typical growth promotion was not observed with PsJN-treated plants either at week 3 or week 5 (Figure 16). However, root mass was significantly increased in PsJN-treated plants and this is because the roots of treated plants were swollen and thicker than those found with uninoculated plant roots (data not shown). The presence of PsJN in maltose containing medium indicates that maltose is still suitable for

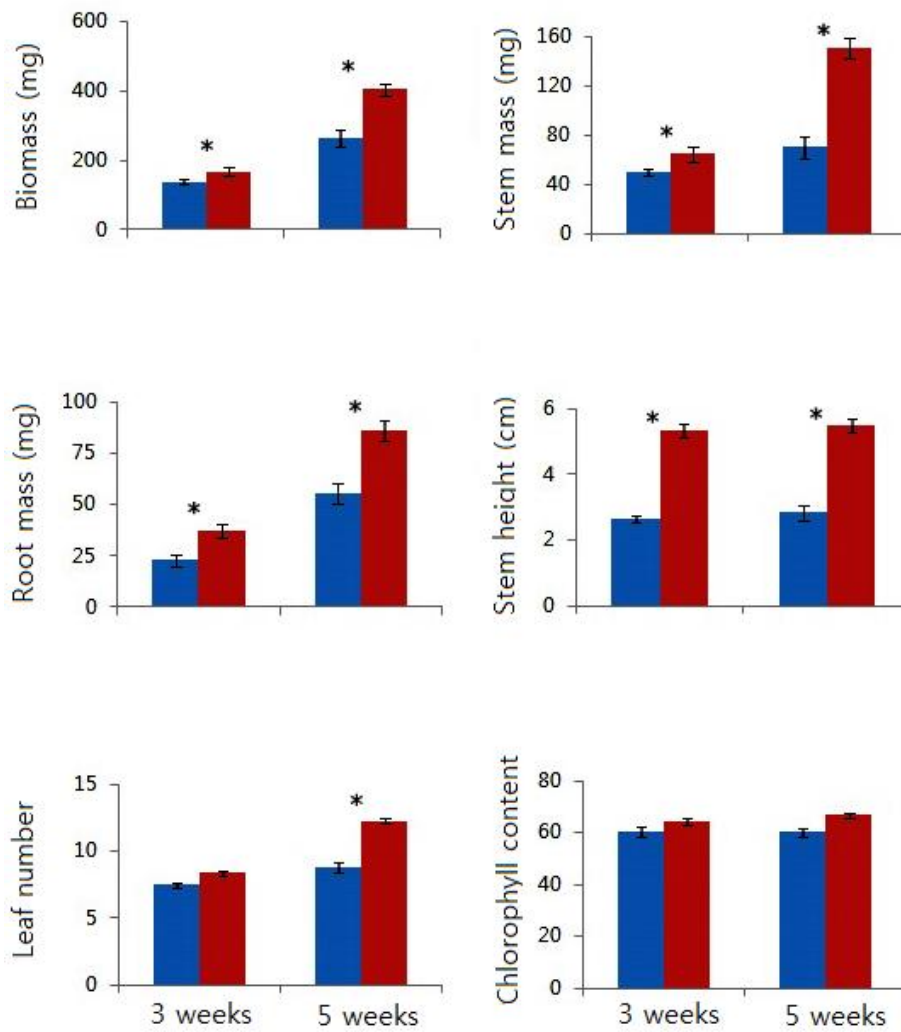


Figure 14. The effect of *B. phytofirmans* on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% glucose for 3 and 5 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

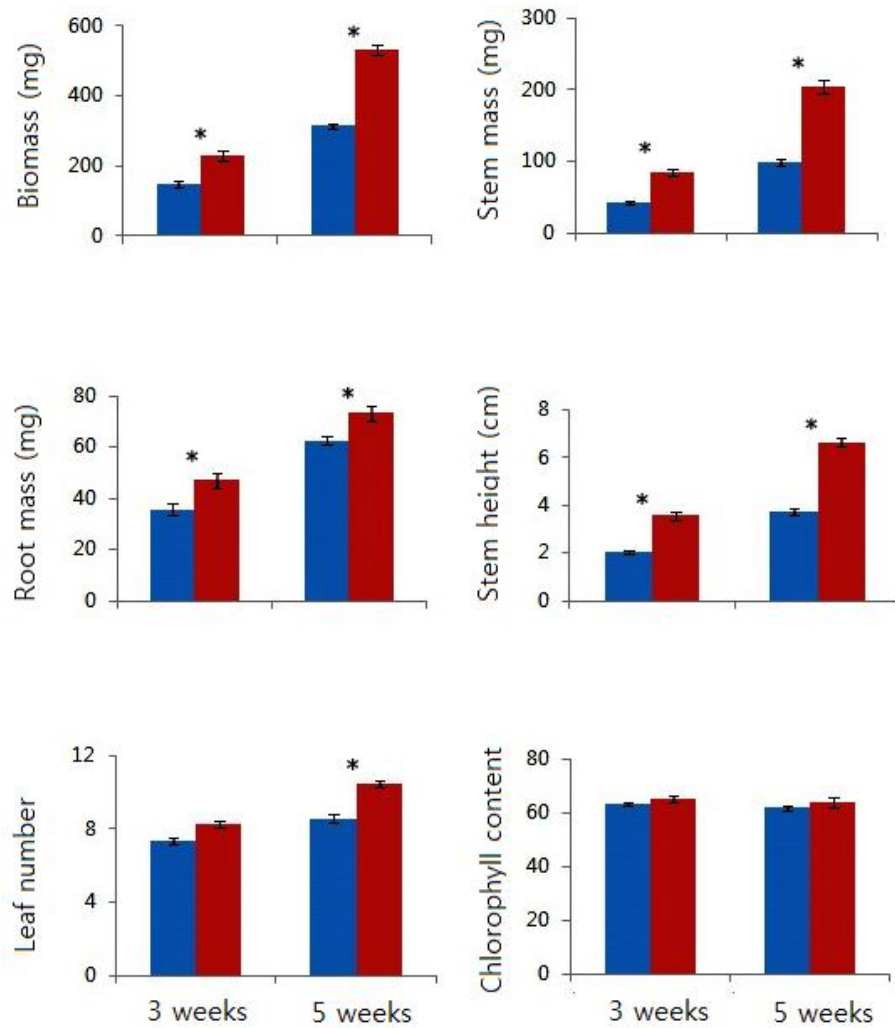


Figure 15. The effect of *B. phytofirmans* on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% fructose for 3 and 5 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

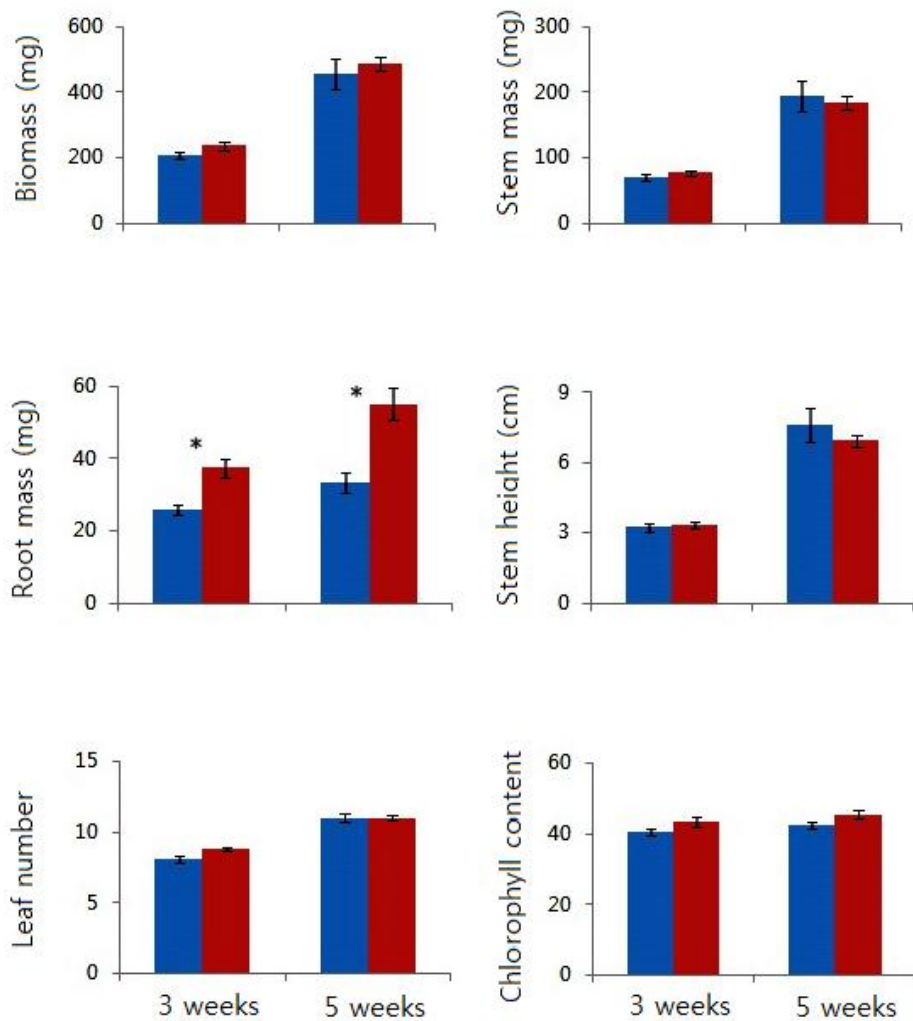


Figure 16. The effect of *B. phytofirmans* on the growth and chlorophyll content of potato nodal explants cv. Kennebec when grown in medium containing 3% maltose for 3 and 5 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

PsJN to grow (data not shown). Maltose does not induce PsJN-mediated plant growth promotion.

3.6 The effect of PsJN treatment on hexokinase1 activity in potato stems and roots of plants grown on various sugars

Extracts from roots of PsJN-treated plants that were grown in MS agar medium containing 1.5% (w/v) glucose had 17% and 37% significantly higher glucose phosphorylation activities in their roots than found in roots of uninoculated plants after 3 and 5 weeks of growth, respectively (Figure 17). Similarly, roots of PsJN-treated plants grown in 1.5% (w/v) fructose also had 26% and 32% significantly higher StHXX1 activities than uninoculated plants after 3 and 5 weeks, respectively (Figure 17). However, there were no differences in StHXX1 stem activity between PsJN-treated and uninoculated plants when grown in 1.5% (w/v) glucose and fructose.

PsJN-treated plants grown in MS agar medium containing 3% (w/v) glucose had 38% and 28% significantly higher StHXX1 activities at 3 and 5 weeks of growth, respectively (Figure 18). Similarly, PsJN-treated plants grown in media supplemented with 3% fructose had 34% and 30% higher StHXX1 root activities by weeks 3 and 5, respectively (Figure 18). However, stems activity of StHXX1 was similar for PsJN-treated and uninoculated plants grown in the presence of 3% (w/v) glucose and fructose. The results again indicate that increases in StHXX1 root activity appear to be a good indicator of plant growth promotion.

PsJN-treated plants grown in MS agar medium having 3% (w/v) maltose did not exhibit growth promotion and StHXX1 in stem and root activity was found to be similar in PsJN-treated and uninoculated plants after 3 and 5 weeks of growth. In fact,

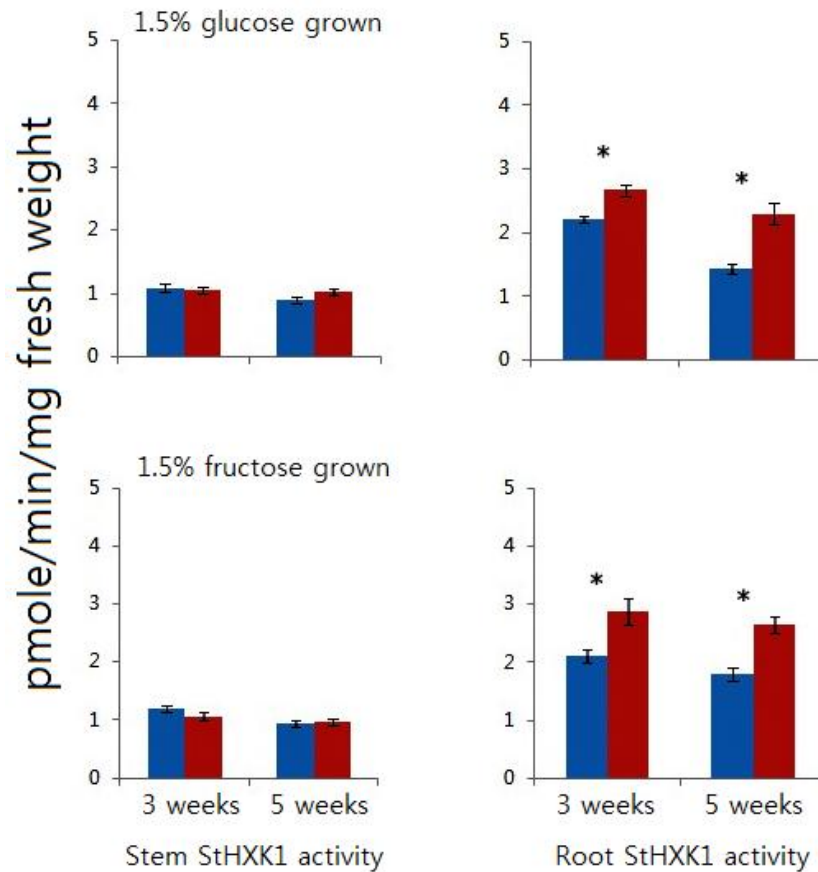


Figure 17. The effect of *B. phytofirmans* on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 1.5% glucose or fructose for 3 and 5 weeks.

The data shows amounts of picomoles of glucose converted into glucose-6-phosphate in one minute by StHXK1 in PsJN-treated and uninoculated plants. Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

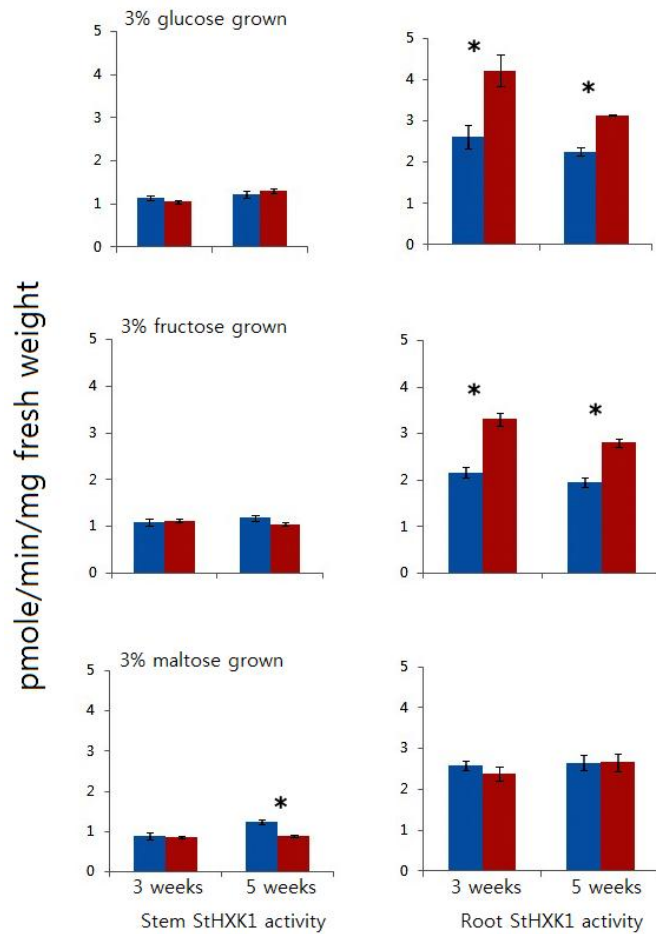


Figure 18. The effect of *B. phytofirmans* on the hexokinase1 activity of stems and roots of potato nodal explants cv. Kennebec when grown in medium containing 3% glucose, fructose, and maltose for 3 and 5 weeks.

The data shows amounts of picomoles of glucose converted into glucose-6-phosphate in one minute by StHXK1 in PsJN-treated and uninoculated plants. Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

after 5 weeks of growth StHXX1 activity was significantly higher in uninoculated plant stems than in stems of PsJN-treated plants (Figure 18). Since uninoculated plants exhibited slightly taller stem height and mass than PsJN-treated plants, this may be due to higher StHXX1 stem activity from uninoculated plants (Figure 13 and 16).

3.7 The effect of PsJN treatment on plant growth promotion of the potato cv. Yukon Gold

Visual monitoring indicated that Shepody and Superior explants showed similar growth promotion to that of Kennebec in root development when grown on MS agar medium containing 3% (w/v) sucrose (Data not shown). However, the cv. Yukon Gold showed very minimal root growth when compared to PsJN-treated Kennebec and the uninoculated Yukon Gold plant. PsJN-treated Yukon Gold plants, however, were slightly taller and had visible greener leaves than their uninoculated counterparts on MS agar medium containing 3% (w/v) sucrose (Figure 19). As the growth promotion was not what we typically see with PsJN inoculated potato explants, we selected Yukon Gold for further study on the potential role of StHXX1 in growth promotion.

Yukon Gold explants inoculated with PsJN showed increases in stem height and chlorophyll content of 19% and 23%, respectively after 3 weeks of growth, compared to uninoculated plants when grown on 3% (w/v) sucrose (Figure 20). Although PsJN-treated plants were taller and had greener leaves there were no significant differences in total plant biomass, stem mass, root mass, and leaf number production compared to uninoculated plants at week 3 (Figure 20). By week 5 however, compared to uninoculated plants, PsJN-treated plants did have greater biomass, stem mass, stem height, and chlorophyll content of 21%, 28%, 25%, and 40%, respectively.

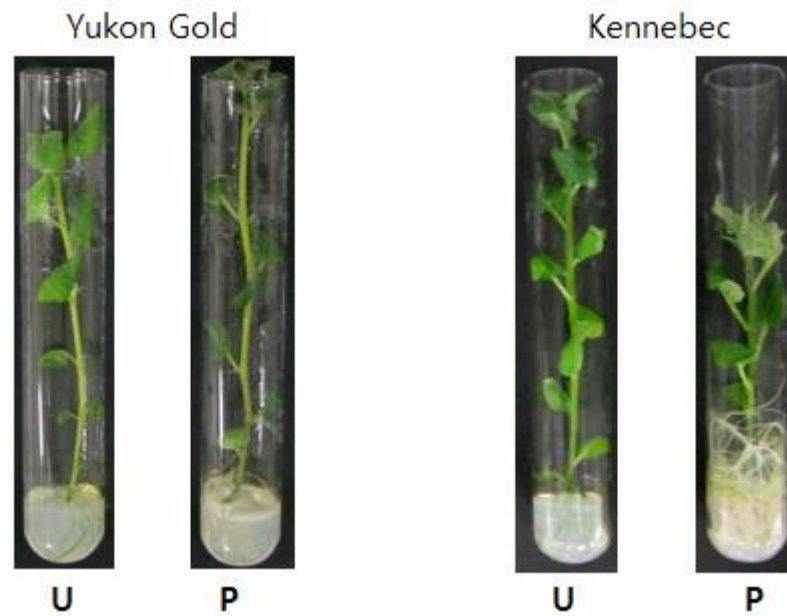


Figure 19. The effect of bacterial treatment with *B. phytofirmans* on the growth of potato nodal explants cv. Kennebec and Yukon Gold when grown in medium containing 3% sucrose for 6 weeks.

U: Uninoculated

P: PsJN-treated

With this cultivar however, there was no increase in root mass and leaf number in PsJN-treated over uninoculated plants. Rather, the increase in biomass and stem mass resulted from the increased stem height seen with PsJN-treated plants (Figure 20) after 6 weeks of growth. PsJN-treated plants were taller and greener than uninoculated plants (Figure 20). The root mass with PsJN-treated Yukon Gold plants were slightly less than that of uninoculated plants at weeks 3 and 5. Previous study showed that PsJN-treated potato plant cv. Chaleur also had decreased root mass by 50% compared to uninoculated plants (15). This indicated that with some potato cultivars root production can be inhibited by PsJN treatment.

3.8 The effect of PsJN treatment on hexokinase1 of potato cv. Yukon Gold

Activity of StHXK1 in roots of PsJN-treated plants was approximately 16% higher than in roots of uninoculated plants, although this was not statistically significant in week 3(Figure 21). After 5 weeks of growth, StHXK1 activity of PsJN-treated roots was 37% higher than found with roots of uninoculated plants and this was statistically significant. The increased StHXK1 activity in roots may be related to the increased levels of chlorophyll and stem height found in PsJN-treated Yukon Gold plants but since there was no increase in root mass it is unlikely to be involved in the activation of root biomass.

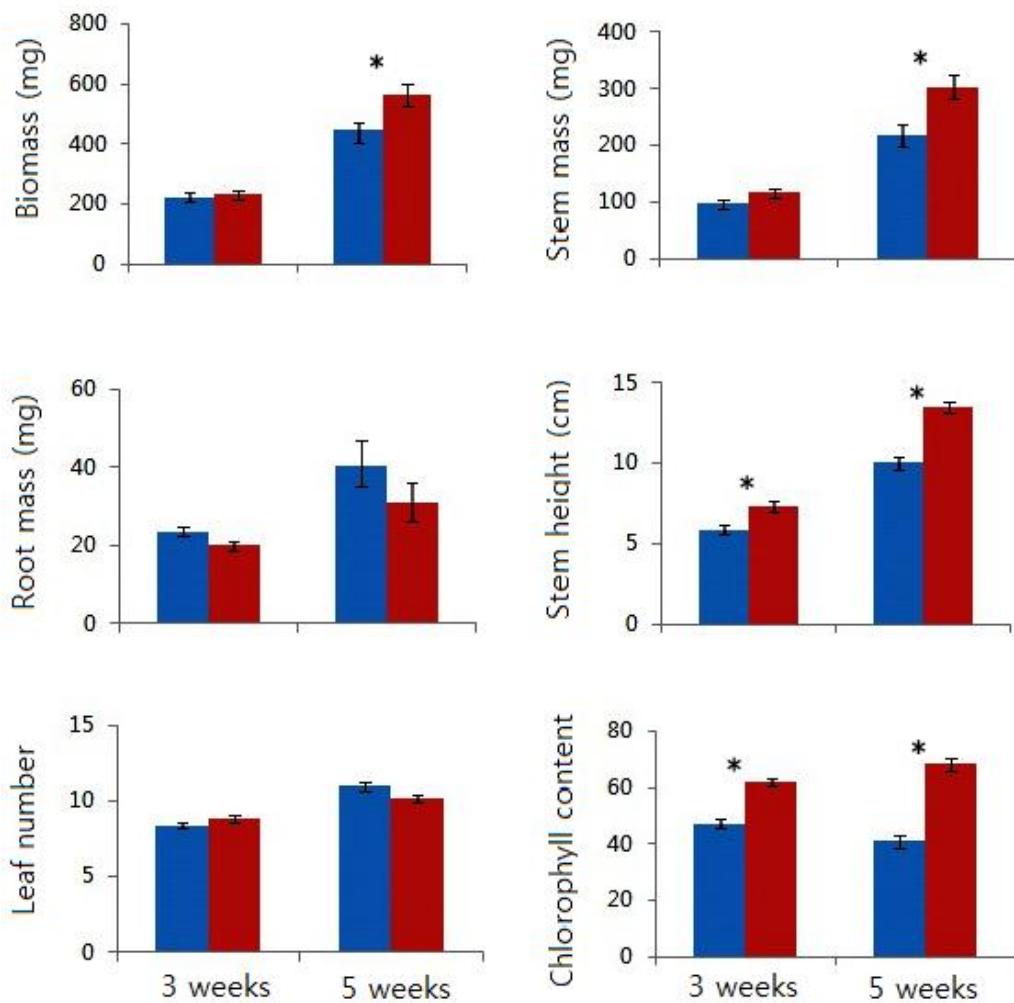


Figure 20. The effect of *B. phytofirmans* on the growth and chlorophyll content of potato nodal explants cv. Yukon Gold when grown in medium containing 3% sucrose for 3 and 5 weeks.

Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

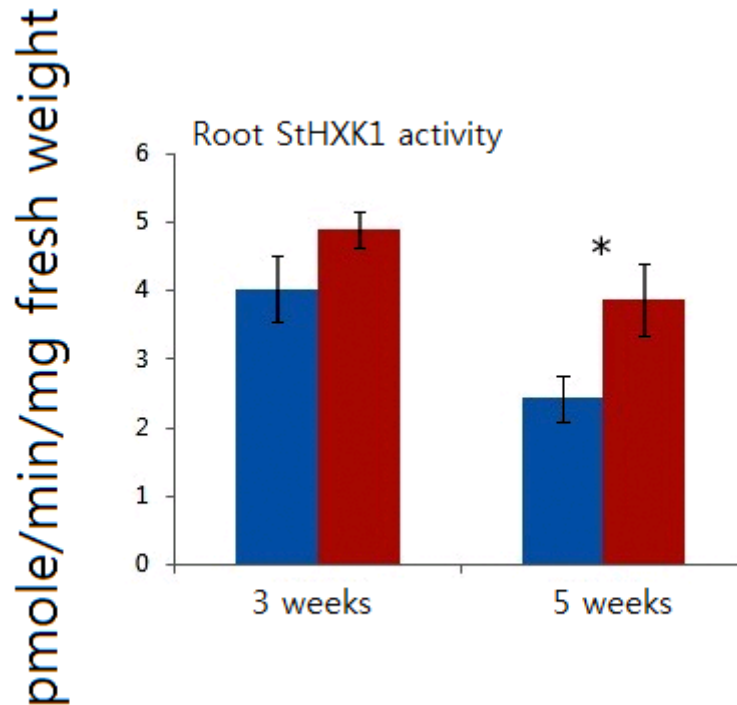


Figure 21. The effect *B. phytofirmans* on the hexokinase1 activity of roots of potato nodal explants cv. Yukon Gold when grown in medium containing 3% sucrose for 3 and 5 weeks.

The data shows amounts of picomoles of glucose converted into glucose-6-phosphate in one minute by StHXK1 in PsJN-treated and uninoculated plants. Data are presented as the mean \pm SEM, n=20 (n=10/repeat). One way ANOVA was used to determine significant differences.

Blue bar: Uninoculated

Red bar: PsJN-treated

CHAPTER 4: Discussion

4.1 Alteration of PsJN-treated plants as correlated to induced growth promotion of plants cultured in media containing either 1.5 or 3% sucrose

Growth promotion of PsJN-treated potato plants compared to uninoculated plants was evident after 2 weeks of growth on medium containing both 1.5 and 3% (w/v) sucrose concentrations. These plants also showed significant increases in chlorophyll content and formation of secondary roots (Figures 1, 2, 4). By week 6 of growth the growth characteristics of treated plants differed substantially on the two sugar concentrations as previously found by Li (85) (Figure 3). There were, however, also similarities in growth stimulation including increases in total fresh biomass, in chlorophyll content, and in root mass. In rare cases, PsJN-treated plants grown on medium with 3% sucrose were similarly tall as those grown on 1.5% (w/v) sucrose but in the most cases they were even shorter than uninoculated plants. The basis for such outlier response could not be predicted or explained. Li (85) also found similar responses in her work.

The causes behind the change in the phenotypes of the PsJN-treated plants after 6 weeks of growth on different sucrose concentrations remain unknown. At the level of 3% (w/v) sucrose, for whatever reason, the plant diverts more resources toward the generation of roots whereas, with plants grown on 1.5% (w/v) sucrose, the energy resources are used to enhance shoot height. Determination of whether nutrients are shunted to increasing shoot height or root growth appear to depend on the concentrations of sugar provided to PsJN-treated plants and this obviously has an impact on the type of growth that occurs after several weeks of incubation. Sugar concentration may trigger different mechanisms of plant growth with PsJN and possibly different sugar sensing and signaling (12, 27, 29). These differences could

explain in the shift in plant growth from shoot elongation to root mass enlargement.

4.2 Indication of changed potato hexokinase1 in PsJN-treated plant, accompanied by growth promotion of plants grown on 1.5 and 3% sucrose

Li (85) showed that sugar concentration was positively correlated with plant growth and that increasing the sucrose concentration not only increased growth of uninoculated potato plants but increased the growth of PsJN-treated plants to an even greater degree. Hida *et al.* (82) reported that increasing sugar concentration increased cell division in plants. In this study uninoculated plants grown in medium with 3% (w/v) sucrose concentration were generally taller than uninoculated plants grown on 1.5% (w/v) sucrose (Figure 3) validating studies showing that 3% sucrose is an optimal concentration for culturing potato plants *in vitro* (15, 17, 54, 85). Inoculation with PsJN produced much faster growth and much higher biomass on media with both sucrose concentrations at the early stages. This suggests that PsJN inoculation may be enhancing sucrose uptake and perhaps its metabolism thereby affecting the growth of stems and roots. The most likely mechanism for this would be the possibility that PsJN is either altering or producing some plant hormone that enhance sucrose uptake. Such a role has been identified for ABA (63).

The results from my experiments show that significant increases in StHXK1 activity were induced in roots of PsJN-treated plants at 2 weeks after inoculation in plants grown on 3% (w/v) sucrose and at 3 weeks with roots of plants grown on 1.5% (w/v) sucrose as compared to StHXK1 levels found in uninoculated plants cultured on the respective sugar concentrations. Although other statistically significant differences in StHXK1 activity were also found in the time course measurements in treated roots and stems, the differences were relatively minor compared to that in uninoculated

plants and hence they likely do not have much biological significance (Figure 5 and 6). Since sampling was done on a weekly basis it is not possible ascertain for how long elevated levels of StH XK1 persisted in treated plants. As growth promotion was induced very early in plant development increased sucrose uptake is likely involved in the responses to PsJN. If so, then increased StH XK1 activity would be expected to occur since its primary role is phosphorylation of glucose destined for the glycolysis pathway (52). Plant glycolysis is required for actively growing plant tissue as sugars, primarily glucose and fructose, are used to generate numerous compounds such as secondary metabolites, isoprenoids, amino acids, nucleic acids, and fatty acids for plant growth (52). The results suggest that the elevated levels of StH XK1 coincide with the first signs of growth stimulation. Greater uptake and conversion to energy would be required for growth stimulation (52). Also, the presence of greater levels of sucrose in plant tissues would be harmful to cells as it would alter homeostasis if PsJN increased sucrose uptake. Increases in StH XK1 in root activity can modulate sugar concentration and re-establish appropriate osmotic conditions (60).

The direct role of StH XK1 in inducing growth promotion cannot be assigned from this series of experiments. Measurement of StH XK1 activity from PsJN-treated plants grown with 0% sucrose, where no growth promotion occurs (85), could have added further insight as to the mode of PsJN in sugar uptake (85). Elevated StH XK1 in root activity found as a result of PsJN treatment may be a consequence of growth promotion as there have been numerous physiological changes reported (7, 17, 51)

RT-PCR was employed to determine if the altered StH XK1 activity in roots was due to increased *StH XK1* transcripts. RT-PCR revealed that there were no detectable differences in *StH XK1* expression between PsJN-treated and uninoculated plants. This finding is consistent with findings by Zhang *et al.* (83) that there was no

change in *AtHXK1* expression in GB03 treated and uninoculated *A. thaliana*. Western blot analysis was carried out to determine if expression of the StHXK1 protein in roots differed between PsJN-treated and uninoculated plants. Here again no differences in StHXK1 expression were found between these two groups of plants (Figure 10).

Thus, StHXK1 activity in roots may be altered in PsJN-treated plants after the enzyme has been synthesized, perhaps by a regulatory activator, such as additional sugar. Since I used crude extracts for StHXK1 activity measurement, anything that is present in crude extracts could have increase StHXK1 activity in PsJN-treated plants. The exact mode of how plant HXK1 can be regulated for glucose phosphorylation activity remains to be addressed, but there are effectors that can influence plant HXK1 activity such as ADP, G6P, glucosamine, *N*-acetylglucosamine, and mannoheptulose (14). These effectors can down-regulate plant HXK1 activity. A study on potato tuber StHXK1 showed that StHXK1 activity was dependent on Mg^{2+} and pH (57). Thus, possibly amounts of mentioned effectors could have been different in PsJN-treated and un-inoculated crude extracts and influence StHXK1 stability and activation. Therefore, elevated StHXK1 in root activity found as a result of PsJN treatment may be a consequence of growth promotion as a diversity of other changes occur in plants treated with PsJN. Also, it is possible that potato plants could have hexokinase-like proteins and glucokinase that have detectable glucose phosphorylation activity in my assay (60).

4.3 Alteration of PsJN-treated plants as correlated to induced growth promotion of plants cultured in media containing different sugar compositions

A second series of experiments was performed to determine the effects of

different sugars on growth promotion by PsJN and possible relationship to StHXX1 activity. PsJN-treated and uninoculated plants were grown in 1.5% (w/v) glucose and fructose, and 3% (w/v) glucose, fructose, maltose, mannose, and galactose. Plant HXX1 has a high affinity for glucose, fructose, mannose, and galactose, but not for maltose (26, 27). If PsJN increases sugar uptake, plant growth promotion would be expected only when the plants were grown with sugars which are metabolized via plant StHXX1 activation. Thus results from the experiments with the various diverse sugars should provide some clues as to the mechanism of plant growth promotion with PsJN and the roles of HXX1.

Plants placed on medium containing 3% mannose and galactose completely failed to grow and eventually died, indicating a hypersensitivity to these sugars (27). It is unlikely that these plants died due to a lack of carbon source from these sugars because plants could still able to grow in the MS medium without sucrose (85).

PsJN-treated plants grown in 1.5% (w/v) glucose and fructose were much taller than uninoculated plants and plants grown in 3% (w/v) glucose and fructose had massive root structures. This was very similar to PsJN-treated plant grown 1.5 and 3% (w/v) sucrose. Plants grown in media with these sugars exhibited extremely slow growth suggesting that the metabolism of these sugars was not as effective as 3% sucrose. It would have been interesting in retrospect to try growing plants on a combination of 1.5% of a mixture of the two sugars.

PsJN-treated plants grown in 1.5 and 3% (w/v) sucrose always produced more chlorophyll than uninoculated plants. However, PsJN-treated plants grown in 1.5 and 3% (w/v) glucose and fructose had more leaves, but they did not produce elevated levels of chlorophyll compared to uninoculated plants. Thus, the effect of monosaccharides and disaccharides is different in PsJN-treated plants, thereby

indicating different roles for sugars in regulation of plant growth (12, 13, 23, 26, 27, 30, 41, 60, 61, 68, 74, 76, 80).

The expected typical growth promotion of PsJN-treated plants did not occur with plants grown on medium containing 3% (w/v) maltose but maltose did provide excellent growth of the plants. There was some limited increase in root mass due to the formation of lateral roots but not the massive root structures found with treated plants grown on in 3% (w/v) glucose, fructose, or sucrose. PsJN was present on/in the maltose medium and associated with treated plants indicating that maltose does not inhibit its growth (data not shown). Maltose however, does not appear to be an adequate sugar medium in which PsJN can activate plant growth promotion. Therefore, PsJN can induce plant growth promotion differently based on different sugars used for tissue culture media.

4.4 Indication of changed potato hexokinase1 in PsJN-treated plant, accompanied by growth promotion, grown in different sugar composition

The very slow growth of plants on glucose and fructose containing media precluded StHXX1 assays at week 2 as there was not enough plant biomass for such measurements. PsJN-treated plant grown in 1.5 and 3% (w/v) of glucose or fructose were found to have significantly increased StHXX1 activity in their roots at 3 and 5 weeks after inoculation, but not in stem tissues. Glucose and fructose are products of the hydrolysis of sucrose (52) and plant HXX1 has a very high affinity for glucose and is a confirmed glucose sensor (27). StHXX1 is also likely involved in the metabolism of fructose after glucose because fructose is the next preferred sugar (57). Supplementing tissue culture medium solely with glucose and fructose should indicate whether growth promotion is related to increased metabolism of either sugar or to

both in increased root StHXX1 activity. The results obtained however, did not show preferential growth promotion on either sugar as the levels of plant development were quite similar with both. Although, I used a glucose-based assay for measuring StHXX1 activity and most phosphorylated fructose is generated by the activity of fructokinase (57), increased StHXX1 in root activity found in PsJN-treated plants grown in fructose medium can indicate increased StHXX1 activity in fructose. This is because if PsJN increased sugar uptake, fructose could be mostly present in plants grown fructose. Plants growing *in vitro* are considered to be heterotrophic, since the sugar for growth is supplied in the medium and often it inhibits photosynthesis (48, 49, 50).

Increased levels of StHXX1 activity in the roots of inoculated plants grown in media containing these sugars persisted for a much longer period than seen with plants grown on sucrose. This may be partly due to the fact that the plants grown on these sugars grew much slower than on 1.5 and 3% sucrose. The induction of StHXX1 appears to be correlated to induction of growth promotion seen with the plants grown on these sugars.

There was no growth promotion of PsJN-treated plants when grown in medium containing 3% (w/v) maltose. There was also no alteration in StHXX1 activity in PsJN-treated plants grown on this sugar as compared to that found in uninoculated plants. It would suggest that for growth promotion to occur following bacterial treatment there must be a sugar present that can be metabolized by StHXX1. Maltose is a disaccharide that is not acted on by StHXX1 as HXX reacts only with hexoses, such as glucose, fructose, galactose, and mannose (27, 57). Maltose could be hydrolyzed into glucose by invertase in PsJN-treated plants (60), but may not be accumulated sufficiently to increase StHXX1 root activity if increased StHXX1

activity was due to sugar accumulation.

This second experiment showed that where plant growth promotion could occur, there was increased root StHXXK1 activity. Importantly, growth promotion was only achieved when PsJN-treated plants were grown in sugars that StHXXK1 can recognize as its substrate. Therefore, there appears to be a correlation between StHXXK1 root activity and the sugar used for PsJN-treated plant growth promotion.

4.5 Alterations of PsJN-treated plants cv. Yukon Gold as correlated to induced growth promotion of plants cultured in media containing 3% sucrose

PsJN's growth promotion is known to be specific for potato and tomato plant cultivars (15, 51). In my third set of experiments I tested a number of potato cultivars for their response to inoculation with PsJN when grown in medium with 3% sucrose. PsJN-treated cv. Yukon Gold did not show any of the distinct signs of growth promotion found with cv. Kennebec. However, when detailed measurements were taken the stem length of inoculated Yukon Gold plants were slightly taller and their leaves slightly greener than found with uninoculated plants after 3 weeks of growth. Measuring the potential role of HXXK1 in such plants was expected to provide further insight as to how PsJN mediates growth promotion and its influence on StHXXK1 activity.

4.6 Indication of changes in potato hexokinase1 in PsJN-treated plant cv. Yukon Gold, accompanied by growth promotion, grown in 3% sucrose

StHXXK1 activity in roots of PsJN-treated cv. Yukon Gold plants was greater than in the uninoculated plants at 5 weeks of growth but not at 3 weeks. There was only a minor level of growth promotion seen with treated cv. Yukon Gold plants and

this may be associated with a delayed induction of StHXX1 activity in roots. This suggests that PsJN treatment could influence subtle changes in the plants' physiology through not to the extent seen with the cv. Kennebec. The regulatory factors thought to be PsJN-treated cv. Kennebec plants that resulted in increased StHXX1 root activity may be missing in cv. Yukon Gold. Differences in the genetic background of cultivars and intimacy with bacteria (1, 22, 25, 38, 46, 53, 81) could explain the different growth and developmental responses to PsJN.

4.7 Thesis conclusion

The data generated in this study provide clear evidence that PsJN treatment results in increased levels of root StHXX1 activity within at least two weeks after inoculation. It is at this time that root enlargement becomes obvious in treated plants. Larger root systems would obviously allow plants to increase their sugar uptake from the culture media. The excess sugar would then have to be somehow regulated in order for the plant to maintain cellular homeostasis. An obvious way to regulate the sugar content is to catalyze it by increasing the StHXX1 activity. With the increases in energy uptake the plants gain a pool of sugars that feed numerous metabolic processes and lead to enhanced plant growth. The results observed however, do not provide any new information as to what factors stimulate the increases in root structure. The evidence gained suggests more that increases in hexokinase activity¹ are a consequence of sugar uptake.

PsJN induced plant growth promotion was only achieved when plants were grown sugar media where StHXX1 was able to recognize and metabolize the specific sugar. This does indicate that sugar sensing has a major role in growth promotion and perhaps activation of the growth stimulatory activity in PsJN. It will be interesting to

determine how PsJN stimulates plant growth. Plant hormone substances are likely candidates for initiation of the complex of reactions required to start the process. Which hormone however, remains to be discovered? Finding that key biological factor that influences the bacteria to activate plant growth promotion will be essential if we are to use such agents for improving agricultural production.

Yukon Gold did not exhibit the typical growth promotion when inoculated with PsJN as observed with the cultivar Kennebec. Increases in stem height and root StHXX1 activity were found but were much delayed in time. Nevertheless the increased root StHXX1 activity found with Yukon Gold indicates that this enzyme is crucial for the steps leading to growth promotion. More research focusing HXX activity in plants inoculated with other beneficial bacteria may help to identify whether this enzyme is a useful indicator of microorganisms that can act as plant beneficial bacteria.

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