JOCLEITA PERUZZO FERRAREZE

EFFECT OF POSTHARVEST JASMONIC ACID TREATMENT ON STORAGE DISEASES, BIOCHEMICAL AND MOLECULAR ANALYSES OF INDUCED DISEASE RESISTANCE IN SUGARBEET (*BETA VULGARIS* L.)

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 16 de Março de 2012.

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Karen Klotz Fugate	Daniela Vieira Chaves
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Fernando Luiz Finger
(Orientador)

Aos meus amados pais Marielcí e Valmor Ferrareze
e ao meu esposo André Brandt

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SUMÁRIO

RESUMO

FERRAREZE, Jocleita Peruzzo, D.Sc., Universidade Federal de Viçosa, março de 2012. **Efeito do tratamento com ácido jasmônico nas doenças pós-colheita e análises bioquímicas e moleculares da resistência induzida em beterraba-açucareira (*Beta vulgaris* L.).** Orientador: Fernando Luiz Finger.

Ácido jasmônico (AJ) e seus derivativos são conhecidos por ativar os mecanismos de defesa das plantas e fornecer proteção contra fungos causadores de podridões em várias espécies de vegetais. No presente trabalho testou-se a eficiência do ácido jasmônico em proteger raízes de beterraba-açucareira (*Beta vulgaris* L.) contra os três principais patógenos causadores de podridões pós-colheita. AJ reduziu a podridão causada por *Botrytis cinerea* e *Phoma betae* em uma média de 51 e 71% respectivamente e em concentrações de 0.01–10 µM AJ reduziu podridão causada por *Penicillium claviforme* em uma média de 44%, enquanto 100 µM reduziu a podridão em 65%. Tendo-se em vista os resultados mostrando a habilidade do AJ em proteger raízes de beterraba-açucareira contra os patógenos citados, os mecanismos responsáveis pela indução da resistência por jasmonatos foram estudados. A atividade de enzimas com função antioxidante como ascorbato peroxidase (APX), catalase (CAT), peroxidase (POD) e superóxido dismutase (SOD), e metabólitos com propriedades antioxidantes foram analisados. A atividade de enzimas relacionadas com a defesa de plantas como β-1,3-glucanase (β-Gluc), quitinase, polifenol oxidase (PPO) e fenilalanina amônia-liase (PAL) também foram estudadas. Constatou-se que o AJ teve pouca ou nenhuma ação sobre a atividade dessas enzimas ou compostos, levando-nos a acreditar que essas enzimas não tem relação com a resistência induzida por jasmonatos em raízes de beterraba-açucareira. Com exceção da PAL todas as enzimas foram afetadas pelo tempo de armazenamento sendo que a maioria teve sua atividade aumentada com o decorrer do armazenamento. Tendo em vista que as defesas das raízes ficam reduzidas com o passar do tempo infere-se que essas enzimas não estejam relacionadas com defesa contra patógenos e doenças em beterraba-açucareira. O efeito do AJ também foi testado em nível de expressão gênica. Utilizando-se a técnica de PCR em tempo real, verificou-se que o AJ teve efeito pequeno e transiente na expressão de genes

relacionados à defesa de plantas. A expressão da fenilalanina amônia-liase (PAL) e peroxidase (POD) foram transientemente induzidas por acido jasmônico. Em contraste, PR1 e quitinase 1 foram transientemente inibidas pelo tratamento e quitinase 3 exibiu uma redução e um aumento nos níveis de transcritos. O transcriptoma global de beterraba açucareira foi caracterizado usando-se a plataforma Illumina paired-end sequencing. Grande quantidade de dados inéditos foram gerados. Os efeitos do AJ no transcriptoma das raízes também foi estudado revelando que 120 e 104 genes tiveram suas expressões alteradas aos 2 e 60 dias após o tratamento respectivamente.

ABSTRACT

FERRAREZE, Jocleita Peruzzo, D.Sc., Universidade Federal de Viçosa, March, 2012. **Effect of postharvest jasmonic acid treatment on storage diseases, biochemical and molecular analyses of induced disease resistance in sugarbeet (*Beta vulgaris* L.).** Adviser: Fernando Luiz Finger.

Jasmonic acid (JA) and its derivatives are known to activate plant defense mechanisms and provide protection against fungi that cause rot in several plant species. In this study we tested the efficiency of JA in protecting sugarbeet roots (Beta vulgaris L.) against three major pathogens causing postharvest rot. JA reduced the decay caused by Botrytis cinerea and Phoma betae in an average of 51 and 71% respectively, at concentrations of 0.01-10 mM AJ reduced decay caused by Penicillium claviforme an average of 44%, and 100 mM reduced the rot at 65%. Bearing in mind the results showing the ability of AJ to protect sugarbeet roots against the mentioned pathogens, the mechanisms responsible for induction of resistance by jasmonates were studied. The activities of ROS-scavenging enzymes such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD), and metabolites with antioxidant properties were analyzed. The activity of defense related enzymes such as β-1 ,3-glucanase (β-Gluc), chitinase, polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were also studied. The results show that JA had little or no effect on the activity of these enzymes or compounds, leading us to believe that these enzymes are not related to jasmonate induced resistance in sugarbeet roots. All the enzymes except PAL were affected by storage time and the majority had their activity increased with the storage time. Considering that the defenses are reduced over time, it appears that these enzymes are not related to defense in sugarbeet. JA effect was also tested on gene expression. Using the qRT-PCR technique, it was found that JA had transient and small effect on the expression of genes related to plant defense. The expression of phenylalanine ammonia-lyase (PAL) and peroxidase (POD) were transiently induced by jasmonic acid. In contrast, a PR1 and chitinase 3 were transiently inhibited by treatment and chitinase 1 exhibited a reduction and an increase of the transcripts levels. The sugarbeet global transcriptome was characterized using the platform Illumina

paired-end sequencing. Large amount of unpublished data were generated. The JA effects on the roots transcriptome was also studied and revealed 120 and 104 genes differentially expressed at 2 and 60 days after treatment, respectively.

1 INTRODUÇÃO GERAL

Patógenos pós-colheita são controlados por técnicas de manejo das pilhas de armazenamento de beterraba-açucareira. Baixas temperaturas reduzem o índice de crescimento de muitos organismos causadores de podridões, diminuindo a temperatura das pilhas e removendo 'hotspots' (locais específicos na pilha onde a temperatura é elevada) e reduz a ocorrência de perdas na pós-colheita. O manejo das pilhas, no entanto, requer condições ambientes favoráveis de armazenamento, monitoramento contínuo e ainda assim são limitados em controlar fungos causadores de podridões. Resistência genética e fungicidas químicos podem ser usados para reduzir as perdas (Miles et al., 1977; Bugbee & Cole, 1979), mas nenhum desses mecanismos de controle é utilizado porque a introdução de características adicionais em programas de melhoramento reduz a rapidez de progresso desses programas em obter outras características desejáveis. Fungicidas geralmente produzem efeitos negativos nas propriedades de armazenamento das beterrabas mesmo sem doenças (Akeson et al., 1979; Campbell, 2005).

Em grande número de produtos hortícolas, aplicações de jasmonatos proporcionam proteção contra fungos pós-colheita (Tripathi and Dubey, 2004; Rohwer and Erwin, 2008). Jasmonatos são hormônios vegetais conhecidos por ativarem os mecanismos de defesas das plantas (Ballaré, 2011). Quando aplicado, jasmonatos reduzem doenças causadas por uma variedade de fungos, bactérias e vírus patogênicos (Thaler et al., 2004; Rohwer and Erwin, 2008; Haggag et al., 2010) por reduzirem a severidade da doença, na maioria dos casos, ou por reduzirem a incidência em outros (Yao and Tian, 2005b; Cao et al., 2008; Zhang et al., 2009).

Botrytis cinerea Pers. ex Fr. (teleomorph: *Botryotinia fuckeliana* [de Bary] Whetz.), *Penicillium claviforme* Bainier e *Phoma betae* Frank (teleomorph: *Pleospora bjorlingii* Byford) são conhecidos patógenos causadores de podridões pós-colheita em beterraba-açucareira (Bugbee, 1986). *B. cinerea* é um organismo agressivo que é caracterizado por massas de esporos acinzentadas, ativo em um grande intervalo de temperatura e distribuído em regiões produtoras de beterraba açucareira em todo o mundo (Gaskill and

Seliskar, 1952; Campbell and Klotz, 2006; Fugate and Campbell, 2009). *P. claviforme* é geralmente menos agressivo que *B. cinerea*, produz corpos de frutificação em formato de haste com massas de esporos verdes e prevalece nas pilhas de armazenamento dos EUA (Bugbee, 1975; Bugbee and Cole, 1976; Fugate and Campbell, 2009). *P. betae* causa uma podridão que inicia no tecido da medula central e se desenvolve para baixo e para fora no interior da beterraba e circulando o tecido da coroa (Bugbee and Cole, 1976). Infecção devido a *P. betae* tipicamente se desenvolve após um período de armazenamento de 80 dias, porém o organismo é capaz de infectar o tecido em qualquer momento na pós-colheita (Bugbee, 1982).

Os mecanismos responsáveis pela indução da resistência em plantas por jasmonatos não são bem estabelecidos. Alterações na atividade de enzimas antioxidantes como ascorbato peroxidase (APX), catalase (CAT), peroxidase (POD) e superóxido dismutase (SOD), e aumentos nas concentrações de metabolitos com propriedades antioxidantes, no entanto, tem sido relacionadas com resistência a doenças induzida por jasmonatos em várias espécies de plantas (Yao and Tian, 2005a; Cao et al., 2008; Wang et al., 2009a). Aumentos nas atividades de enzimas relacionadas com a defesa de plantas como β-1,3-glucanase (β-Gluc), quitinase, polifenol oxidase (PPO) e fenilalanina amônia-liase (PAL) também tem sido associadas com resistências induzidas por jasmonatos em muitas plantas (Yao and Tian, 2005a; Haggag et al., 2010). Essas enzimas presumivelmente aumentam a resistência a doenças pela sua atividade antimicrobiana direta ou seu envolvimento na síntese de compostos antimicrobianos.

Alteração na expressão de genes relacionados à defesa é um elemento chave nos mecanismos de defesa induzida em plantas (Edreva, 2005). Alterações na expressão gênica de fenilalanina amônia-liase (PAL), peroxidase (POD), genes relacionados à patogênese (PR1), e quitinases são comumente relacionadas com defesas induzidas por jasmonatos nas plantas. (Sharan et al., 1998; Curtis et al., 1997; Raymond and Farmer 1998; Ding et al., 2002).

As novas tecnologias de sequenciamento de RNA ou RNA-seq, denominadas de tecnologias de sequenciamento de nova geração, começaram a ser comercializadas em 2005 e estão evoluindo rapidamente. Todas essas tecnologias promovem o sequenciamento em plataformas capazes de gerar

informação sobre milhões de pares de bases em uma única corrida. O grande número de sequencias geradas fornece valiosas informações em nível de transcriptoma para descoberta de novos genes ou investigação de mecanismos moleculares (Wang et al., 2009b; Wei et al., 2011).

No presente trabalho foi estudado o efeito do ácido jasmônico em *Botrytis cinerea, Penicillium claviforme*, e *Phoma betae*, três importantes fungos causadores de podridões na pós-colheita de beterraba açucareira. Estudaram-se também os mecanismos enzimáticos e gênicos da defesa induzida por jasmonatos em raízes daquela espécie.

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Postharvest jasmonic acid treatment of sugarbeet roots reduces rot due to *Botrytis cinerea*, *Penicillium claviforme*, and *Phoma betae*

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ABSTRACT

Although jasmonic acid (JA) and JA derivatives are known to activate plant defense mechanisms and provide protection against postharvest fungal diseases for several horticultural crops, JA's ability to protect sugarbeet (*Beta vulgaris* L.) roots against common causal organisms of storage rot is unknown. To determine the potential of JA to reduce rot due to three common sugarbeet storage pathogens, harvested roots were treated with JA concentrations of 0.01, 0.1, 1, 10, or 100 µM, inoculated with *Botrytis cinerea, Penicillium claviforme*, or *Phoma betae*, and evaluated for the severity of rot symptoms after incubation at 20 °C and 90% relative humidity. JA concentrations of 0.01-100 µM significantly reduced rot due to all three pathogens. All concentrations of JA provided statistically equivalent control against *B. cinerea* and *P. betae*, and reduced the amount of rotted tissue due to these pathogens by an average of 51 and 71%, respectively. Against *P. claviforme*, JA concentrations of 0.01-10 µM were equally effective and reduced rot by an average of 44%, while an increase in JA concentration to 100 µM reduced rot by 65%. Against all three pathogens, JA treatment did not affect the incidence of infection, but reduced rot by reducing the progression of disease symptoms in root storage tissue.

***Keywords*:** *Beta vulgaris*, *Botryotinia fuckeliana* (de Bary) Whetz, Jasmonate, *Pleospora bjorlingii* Byford and Storage rot

1 INTRODUCTION

Storage rots of sugarbeet root consume sucrose and produce sucrose- and cell wall-degrading enzymes whose reaction products impair juice purification and sucrose crystallization operations and decrease sucrose recovery during processing (Buchholz et al., 1998; Tungland et al., 1998; Dutton and Huijbregts, 2006). Storage rots also increase root respiration rate, a process that metabolizes sucrose and generates heat, creating additional storage losses and contributing to the warming of storage piles (Mumford and Wyse, 1976; Kays and Paull, 2004). As storage piles warm, the respiration of both healthy and diseased roots and the incidence and severity of storage rots increase (Campbell and Klotz, 2006). Storage rots, therefore, increase storage and processing losses and initiate events that escalate the rate of storage losses.

Botrytis cinerea Pers. ex Fr. (teleomorph: *Botryotinia fuckeliana* [de Bary] Whetz.), *Penicillium claviforme* Bainier and *Phoma betae* Frank (teleomorph: *Pleospora bjorlingii* Byford) are known storage rot pathogens of sugarbeet (Bugbee, 1986). *B. cinerea* is an aggressive rot-causing organism that is characterized by gray spore masses, active over a wide range of temperatures, and widely distributed in sugarbeet growing regions throughout the world (Gaskill and Seliskar, 1952; Campbell and Klotz, 2006; Fugate and Campbell, 2009). *P. claviforme* is generally less aggressive than *B. cinerea*, produces stalked fruiting bodies with green spore masses, and is prevalent in U.S. storage piles (Bugbee, 1975; Bugbee and Cole, 1976; Fugate and Campbell, 2009). *P. betae* causes a rot that begins in the central pith tissue of the root crown and develops downward and outward into the taproot and surrounding crown tissue (Bugbee and Cole, 1976). Rot due to *P. betae* typically develops after roots have been stored in excess of 80 d, although the organism is capable of rotting tissue at any time after harvest (Bugbee, 1982).

Rot of postharvest sugarbeet roots is controlled primarily by maintaining cool temperatures in storage piles since low temperature reduces the growth rate of many rot-causing organisms (Campbell and Klotz, 2006). Roots are harvested in late autumn when temperatures are low, and the large outdoor

piles in which sugarbeet roots are stored are cooled using ambient winter air. The control of storage rots, therefore, is dependent on weather conditions and is limitedly effective against fungal organisms that are capable of growth at low temperatures (Miles et al., 1977). Although chemical fungicides can reduce the incidence and severity of storage rots, fungicides are not used by the industry since they generally have deleterious effects on sugarbeet root storage properties in the absence of disease (Miles et al., 1977; Akeson et al., 1979). Germplasm with genetic resistance against some of the major storage rot-causing fungi has been developed (Bugbee and Cole, 1979), but has not been incorporated into current commercial hybrids.

Jasmonic acid (JA) is an endogenous plant hormone that activates an array of plant defense mechanisms including an induction in the expression of anti-fungal proteins, anti-oxidative enzymes, and enzymes that enhance secondary metabolite production (Creelman and Mullet, 1997). For a number of horticultural products, exogenous application of JA or JA derivatives provides protection against postharvest fungal diseases (Tripathi and Dubey, 2004; Rohwer and Erwin, 2008). The ability of jasmonic acid to protect sugarbeet roots against storage rots, however, is unknown. In research described here, the effect of postharvest jasmonic acid treatment on rot caused by *Botrytis cinerea*, *Penicillium claviforme*, and *Phoma betae* was investigated.

2 MATERIALS AND METHODS

2.1 Plant material and postharvest treatments

Sugarbeet hybrid VDH66156 (SESVanderHave, Tienen, Belgium) was greenhouse grown in Sunshine Mix #1 (Sun Gro Horticultural Products, Seba Beach, Alberta, Canada) in 15-L pots with supplemental light under a 16 h light/8 h dark regime, and watered as needed. Taproots were harvested 16-18 weeks after planting and washed to remove adhering soil. Seed was treated with N-(2,3-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester (Apron-metalaxyl) and tetramethylithiuram disulfide (Thiram) to prevent fungal seedling diseases. No symptoms of root disease were evident at any time during production or at harvest. Jasmonic acid treatments were administered by submerging roots in 0, 0.01, 0.1, 1, 10, or 100 µM JA (Cayman Chemical, Ann Arbor, MI) for 1 h at room temperature, using at least 7 roots per treatment. Roots were incubated at 20 °C and 90% relative humidity for 3 d post-treatment (Yao and Tian, 2005b) to allow for induction of defense mechanisms prior to inoculation with a storage rot-causing pathogen. All experiments were repeated at least once.

2.2 Storage rot resistance assays

Isolates of *Botrytis cinerea*, *Penicillium claviforme*, and *Phoma betae* were obtained from W. Bugbee (USDA-ARS, Fargo, ND, retired). Cultures were initiated by placing mycelial fragments from isolates stored at -80 °C on potato dextrose agar (PDA; Difco, Sparks, MD)-containing petri plates and incubating at 25 °C. Subsequent cultures were obtained by placing a plug taken from the leading edge of this culture on a new PDA plate (100 mm × 15 mm) at its geometric center and incubating at 25 °C until fungal growth covered the agar's surface. Roots were prepared for inoculation by drilling two 12 mm ×10 mm

(diameter × depth) holes into each root with a hand-held drill, with holes located on opposite sides of the root in the region where root girth was greatest. Roots were inoculated by inserting a 10 mm diameter plug obtained from fungal-covered PDA plates into each hole. After inoculation, roots were incubated at 20 °C and 90% relative humidity until severe disease symptoms were visually evident on control roots, approximately 20, 30, and 50 d for *B. cinerea*, *P. claviforme*, and *P. betae*, respectively. Root rot severity was evaluated by excising and weighing the rotted, discolored tissue from each root.

2.3 Statistical analysis

Data were normalized to express the weight of rotted tissue for each treatment as a fraction of the rotted tissue of the control by dividing each data point in an experiment by the average weight for the control. JA concentration and experimental repetition were used as main effects for analyses of variance using a general linear model (Minitab, ver. 16, State College, PA) with $\alpha = 0.05$. Since repetitions of experiments were not significantly different for each disease-causing organism, data from the two experimental repetitions were combined. Treatment differences were determined by Fisher's LSD with $\alpha = 0.05$.

3 RESULTS AND DISCUSSION

Postharvest jasmonic acid treatments of 0.01-100 µM significantly ($P < 0.001$) reduced the severity of rot due to *Botrytis cinerea* in stored sugarbeet roots (Fig. 1A). Roots treated with 0.01, 0.1, 1, 10, and 100 µM JA had 36-62% less rot than water-treated controls after inoculation with the pathogen and incubation under environmental conditions that favor disease development. Although JA treatments varied in concentration by 10,000-fold, all treatments reduced rot severity to a statistically similar extent. This suggests that JA protected sugarbeet roots against *B. cinerea* at an extremely low concentration (0.01 µM) with no further benefit or detriment provided by additional JA. On average, JA reduced the weight of rotted tissue by 51%.

Postharvest jasmonic acid treatments of 0.01 to 100 µM also significantly ($P < 0.001$) reduced the severity of rot due to *Penicillium claviforme* in stored sugarbeet roots (Fig. 1B). Roots treated with 0.01, 0.1, 1, 10, and 100 µM JA had 34-65% less rot than water-treated controls after inoculation with the pathogen and incubation under disease-promoting environmental conditions. JA concentrations of 0.01-10 µM caused a statistically similar reduction of rot due to *P. claviforme* and reduced the weight of rotted tissue by an average of 44%. An increase in JA concentration to 100 µM provided additional protection against *P. claviforme* and reduced the weight of rotted tissue by 65% relative to the water-treated control.

Postharvest jasmonic acid treatments of 0.01-100 µM were also effective ($P < 0.001$) in reducing the severity of rot due to *Phoma betae* (Fig. 1C). Roots treated with 0.01, 0.1, 1, 10, and 100 µM JA had 58-81% less rotted tissue than water-treated controls after inoculation with the pathogen and incubation under disease-promoting conditions. Statistically, all JA treatments provided a similar reduction in rotted tissue relative to the water-treated control. JA, therefore, reduced the severity of rot due to *P. betae* at very low concentrations (0.01 µM), with no benefit or harm caused by increasing JA concentration. On average, JA reduced the weight of rotted tissue by 71%.

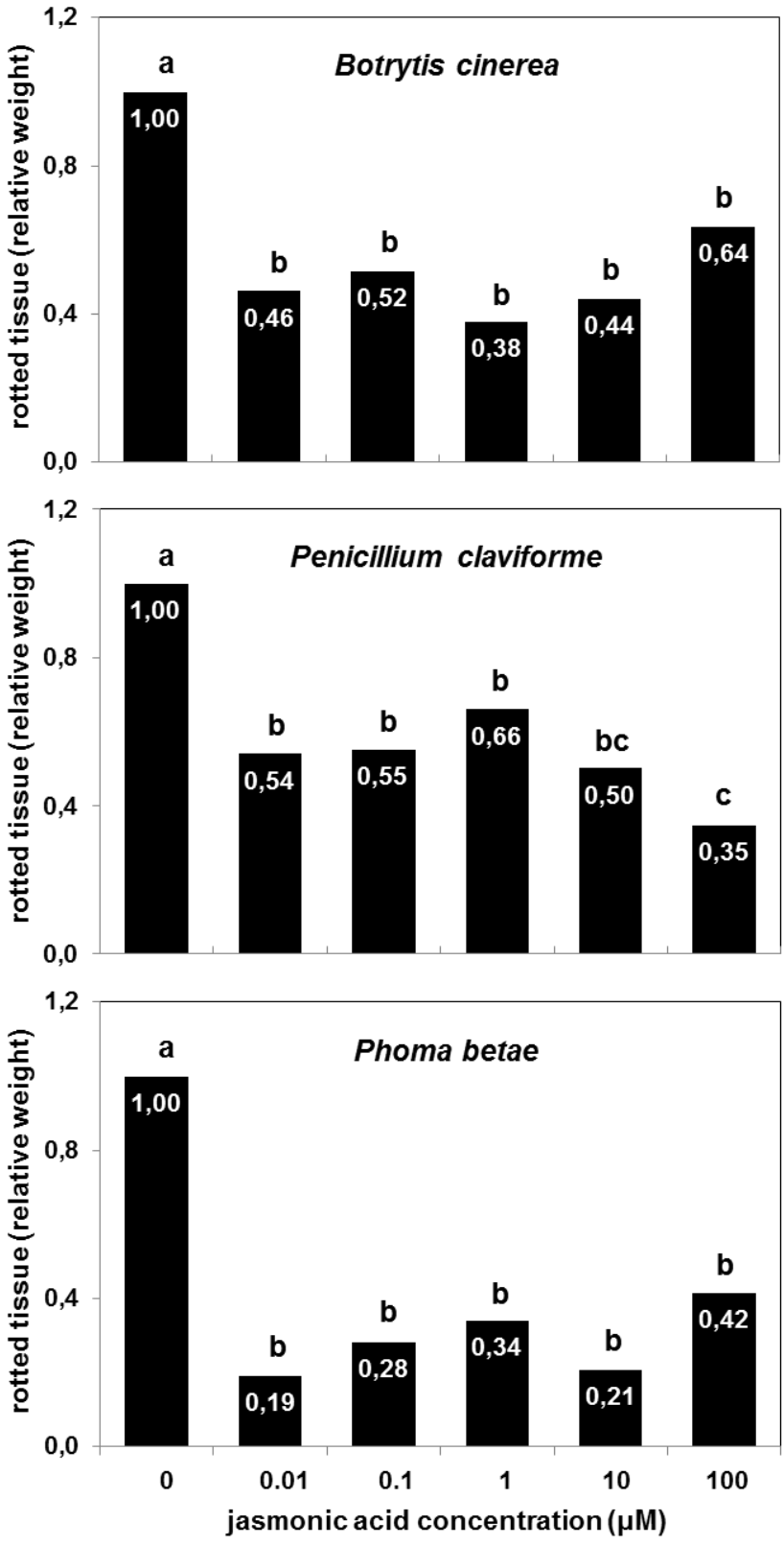

Fig. 1. Relative weight of rotted tissue in jasmonic acid-treated roots after inoculation with *Botrytis cinerea* (A), *Penicillium claviforme* (B), or *Phoma betae* (C) and incubation at 20 °C and 90% relative humidity until severe disease symptoms were evident on control roots (0 µM JA). Harvested roots were treated for 1 h at room temperature and incubated for 3 d at 20 °C and 90% relative humidity prior to inoculation. Weight of rotted tissue is expressed as a fraction of the weight of rotted tissue of the control. Experimental repetitions were not statistically different by ANOVA ($\alpha = 0.05$), and data are the combined results of two experimental repetitions, with at least 7 replicates per repetition. Treatments with different letters are statistically different based on Fisher's LSD ($\alpha = 0.05$).

Exogenous application of jasmonates has previously been shown to protect various plants against *B. cinerea* (Moline et al., 1997; Thomma et al., 1998; Yu et al., 2009) and several species of *Penicillium*, including *P. digitatum*, *P. expansum*, and *P. citrinum* (Yao and Tian, 2005a; Zhang et al., 2009; Wang et al., 2010). To our knowledge, however, this is the first report of jasmonate-associated protection against *P. claviforme*, *P. betae*, or any other *Phoma* species. Although the three pathogens are not related, the application of JA provided protection against all three fungi, suggesting that inducible defense pathways in sugarbeet roots are not pathogen specific, which has been shown in other pathosystems (Bolton, 2009). That sugarbeet root defense mechanisms can protect against more than one disease has previously been demonstrated as germplasm with enhanced *P. betae* resistance often has enhanced resistance against *Rhizoctonia solani* Kühn, a soil-borne fungal organism responsible for a crown and root rot during production (Bugbee and Campbell, 1990).

Sugarbeet roots were unusually sensitive to JA relative to other postharvest products. Other postharvest products for which jasmonate protection against fungal diseases has been reported, however, have predominantly been fruits, while this, to our knowledge, is the first report of jasmonate-afforded disease protection of a root crop. For sugarbeet roots, JA concentrations as low as 0.01 µM provided protection against the three fungal pathogens used in this study. For other plant products, jasmonate concentrations of 10-200 µM (i.e., concentrations 1000-20,000-fold greater) were required to provide protection against fungal pathogens (Yao and Tian, 2005a; Cao et al., 2008; Wang et al., 2009; Zhang et al., 2009). The wide range of concentrations that provided antifungal protection to sugarbeet roots was also atypical. For sugarbeet roots, a 10,000-fold range of concentrations provided nearly equivalent protection against three causal organisms of storage rot. For other postharvest products, a narrow range of jasmonate concentrations, typically of 10-fold variance, has been found to protect against fungal pathogens, with greatly diminished disease suppression with jasmonate concentrations above or below this range (González- Aguilar et al., 2003; Wang et al., 2009; Zhang et al., 2009).

Visual evaluation of roots treated with JA and inoculated with *B. cinerea*, *P. claviforme*, or *P. betae* indicated that JA reduced the progression of disease symptoms in the storage tissue of sugarbeet roots, but did not prevent infection (Fig. 2). Signs of infection were evident at all inoculation sites regardless of the treatment applied (Fig. 2A). However, the progression of disease symptoms, as evidenced by the depth and breadth of necrotized tissue extending from the infection site, was reduced in JA-treated roots relative to water-treated controls (Fig. 2B). Zhang et al. (2009) similarly found that jasmonate treatment reduced infection area but not incidence of infection for *P. expansum* on pears. This contrasts with other studies that have found a reduction in both incidence and size of infection after jasmonate treatment (Yao and Tian, 2005b; Cao et al., 2008; Wang et al., 2010).

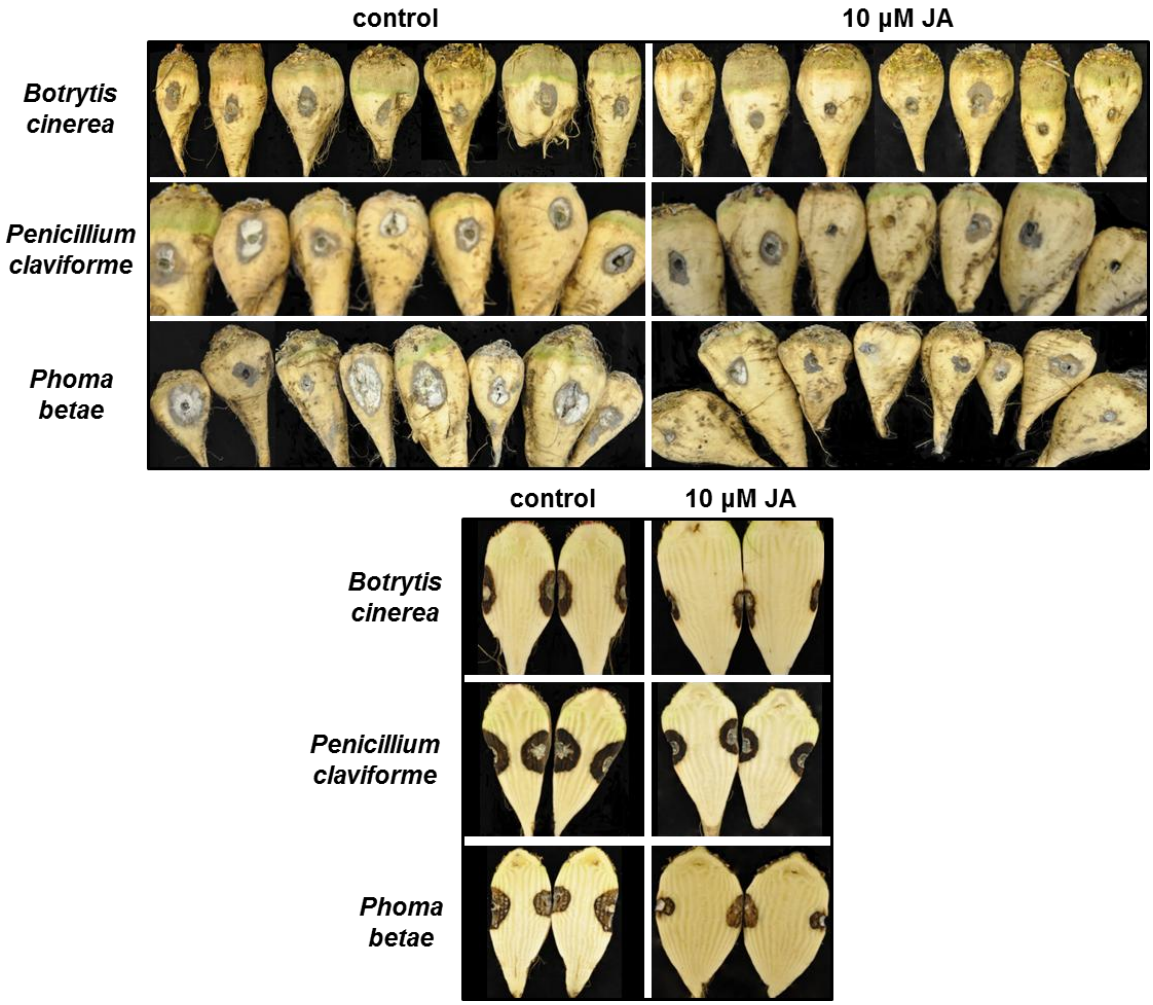

Fig. 2. Surface symptoms (A) and internal symptoms (B) of storage rot on water- (control) and jasmonic acid- (JA) treated roots after inoculation with *Botrytis cinerea*, *Penicillium claviforme*, or *Phoma betae* and incubation at 20 °C and 90% relative humidity until severe disease symptoms were evident on control roots (0 µM JA). Rot symptoms for a single JA concentration are shown since all JA treatments, generally, provided the same level of control. Longitudinal sections are shown for representative roots from each treatment using a root that contained near average weights of rot for the treatment.

4 CONCLUSIONS

Postharvest JA treatment reduced rot due to *B. cinerea*, *P. claviforme*, and *P. betae*, three common storage pathogens of sugarbeet root. JA concentrations of 0.01-100 µM reduced rot due to *B. cinerea* and *P. betae* by an average of 51 and 71%, respectively. JA concentrations of 0.01-10 µM reduced rot due to *P. claviforme* by an average of 44%, while 100 µM JA reduced rot due to *P. claviforme* by 65%. JA treatment reduced rot by reducing the progression of disease symptoms in root storage tissue, but it had no effect on the incidence of infection. Relative to other postharvest products, sugarbeet roots were unusually sensitive to jasmonate treatment and were protected against fungal pathogens over a wide range of JA concentrations.

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4 ARTIGO 2

Jasmonic acid does not increase oxidative defense mechanisms or common defense-related enzymes in postharvest sugarbeet roots

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ABSTRACT

Jasmonic acid (JA) treatment significantly reduces rot due to several sugarbeet (*Beta vulgaris* L.) storage pathogens. The mechanisms by which JA protects postharvest sugarbeet roots from disease, however, are unknown. In other plant species and organs, alterations in antioxidant defense mechanisms and elevations in common pathogen-related defense enzymes have been implicated in jasmonate-induced disease resistance. To investigate whether these mechanisms are involved in JA-induced disease resistance in stored sugarbeet roots, the activities of several reactive oxygen species (ROS)-scavenging and pathogen-related defense enzymes and the total concentration of antioxidant compounds were determined in harvested sugarbeet roots in the 60 d following treatment with JA. ROS-scavenging and pathogen-related defense enzymes and the concentration of antioxidant compounds were largely unaffected by JA as JA-treated roots exhibited small declines in superoxide dismutase (SOD) and chitinase activities, and were generally unaltered in ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), β-1,3-glucanase (β-Gluc), polyphenol oxidase (PPO) and phenylalanine ammonia-lyase (PAL) activities or antioxidant compounds concentration. The lack of increase in enzyme activities or metabolites related to defense against oxidative stress or pathogens suggests that JA-induced disease resistance in postharvest sugarbeet roots does not arise from a direct increase in any of the ROS-scavenging enzymes, defense-related enzymes, or the concentration of total antioxidant compounds that were examined in this study. However, ROS-scavenging enzymes and pathogen-related defense enzymes were affected by storage duration with POD, SOD, β-Gluc, chitinase, and PPO activities elevated and APX and CAT activities reduced in roots stored for 10 d or more. Storage-related changes in activities of ROS-scavenging enzymes and defense-related enzymes provide further evidence that these enzymes are uninvolved in sugarbeet root disease resistance since many of these enzymes increased in activity with prolonged storage when disease resistance generally declines.

Keywords: *Beta vulgaris*; disease resistance; induced resistance; jasmonate; reactive oxygen species; storage duration

1 INTRODUCTION

Jasmonates are endogenous plant hormones that activate native plant defense mechanisms (Ballaré, 2011). When applied exogenously, jasmonates reduce disease caused by a variety of fungal, bacterial and viral pathogens (Thaler et al., 2004; Rohwer and Erwin, 2008; Haggag et al., 2010) by reducing disease severity in most cases and disease incidence in others (Yao and Tian, 2005b; Cao et al., 2008; Zhang et al., 2009). Although some studies have suggested that jasmonate treatment reduces disease directly through antimicrobial activities that affect spore germination, germ tube elongation or mycelial growth (Yao and Tian, 2005a; Cao et al., 2008), exogenous jasmonate treatment is widely believed to protect plants by systemically inducing the same plant defense responses that are activated by endogenous jasmonates (Tripathi and Dubey, 2004; Pozo et al., 2005).

The defense mechanisms responsible for jasmonate-induced disease resistance are not well established. However, alterations in the activities of enzymes that scavenge reactive oxygen species (ROS), such as ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD), and increases in the concentration of metabolites with antioxidant properties have been correlated with jasmonate-induced disease resistance in multiple plant species (Yao and Tian, 2005a; Cao et al., 2008; Wang et al., 2009). Modifications in antioxidative enzymes and metabolites are thought to promote disease resistance by altering the internal concentrations of ROS such as superoxide anion (O_2^-) and hydrogen peroxide. These compounds have direct antimicrobial activity and act as signals that activate plant defense pathways (Lamb and Dixon, 1997; Mittler, 2002). Although beneficial for plant defense, ROS also damage membranes and other cellular components, necessitating control of their accumulation (Mittler, 2002). Because of both beneficial and detrimental effects of ROS, both increases and decreases in ROS-scavenging enzymes have been observed after jasmonate treatment as plants presumably balance protection against pathogens with protection from oxidative stress. Increases in the activities of common defense-related enzymes such as β-1,3-glucanase (β-Gluc), chitinase, polyphenol

oxidase (PPO) and phenylalanine ammonia-lyase (PAL) also have been associated with jasmonate-induced disease resistance in many plant species (Yao and Tian, 2005a; Haggag et al., 2010). These enzymes presumably increase disease resistance by their direct antimicrobial activity or their involvement in the synthesis of antimicrobial compounds. Although ROS-scavenging and pathogen-related defense enzymes are commonly altered after jasmonate treatment, the combination of enzymes affected differ between plant species (Rohwer and Erwin, 2008) and even plant organs (Parra-Lobato et al., 2009).

In sugarbeet (*Beta vulgaris* L.), exogenous jasmonate treatment reduced foliar symptoms due to Beet Mosaic Virus and root rot symptoms due to postharvest infections of *Botrytis cinerea* Pers. ex Fr., *Penicillium claviforme* Bainier, and *Phoma betae* Frank (Haggag et al., 2010; Fugate et al., 2012). In sugarbeet leaves, jasmonate treatment caused elevations in POD, chitinase, and PPO activities and the concentration of phenolic compounds that are thought to act as antioxidants (Haggag et al., 2010). In sugarbeet roots, no information is available on the effect of jasmonates on these or other ROS-scavenging or defense-related enzymes.

In research described here, the effect of exogenous jasmonate treatment on the ROS-scavenging enzymes, defense-related enzymes, and the concentration of antioxidant compounds, which have been implicated in jasmonate-induced disease resistance in other plant species and organs, was investigated in postharvest sugarbeet roots. Roots were subjected to a jasmonic acid (JA) treatment that reduced disease symptoms caused by three storage rot-causing organisms (Fugate et al., 2012), and enzyme activities and metabolite concentrations were determined in root peripheral and internal tissues in the 60 d following treatment. Analysis of the two root tissues allowed localized and systemic effects of JA to be evaluated; analysis throughout the 60 d after treatment allowed short-term and long-term effects of JA to be evaluated. The purpose of this research was to determine the potential of plant oxidative defense mechanisms and common pathogen defense mechanisms to contribute to jasmonate-induced disease resistance in stored sugarbeet roots.

2 MATERIAL AND METHODS

2.1 Plant material and JA treatment

Sugarbeet hybrid VDH66156 (SESVanderHave, Tienen, Belgium) was greenhouse grown in Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, BC, Canada) in 15-L pots with supplemental light under a 16 h light/8 h dark regime. Taproots were harvested 16 to 18 weeks after planting, all leaf and petiole material was removed, and roots were gently washed to remove potting media. Roots were submerged in 0 or 10 µM JA (Cayman Chemical Co., Ann Arbor, MI, USA) for 1 h at room temperature, then incubated at 20 °C and 90% relative humidity for up to 60 d in a controlled environment chamber (Model MTR30, Conviron, Winnipeg, MB, Canada). Peripheral and internal tissues were collected at 0, 1, 2, 3, 10, 30, and 60 d post-treatment. Peripheral tissue contained the epidermis and approximately 2 mm of underlying tissue; internal tissue contained all remaining tissue. Tissue was collected from the main portion of the root, free of tissue from the above-ground root crown or lower tail region where root girth was less than 2 cm. Samples were flash frozen in liquid N_2, lyophilized, ground to a powder, and stored at -80 °C until analysis. Individual roots were the experimental unit with 8 replicates per treatment per time point.

2.2 Protein extraction and enzyme activity assays

Protein extracts were prepared by adding 8 to 10 volumes (w/v) of an assay-specific extraction buffer to tissue. The resulting suspension was vortexed for 30 s, sonicated for 10 min (Model 4.6, Mettler Electronics, Anaheim, CA, USA), and centrifuged at 21,000 × *g* for 20 min, with all operations conducted at 4 °C. Extraction buffers contained 0.1 M sodium acetate, pH 5.0 for β-Gluc assays, 14 mM β-mercaptoethanol and 0.1 M sodium

acetate, pH 6.4 for chitinase assays, 2 mM EDTA, 5 mM β-mercaptoethanol, 10 mM sodium sulfite and 0.1 M sodium borate, pH 8.8 for PAL assays, and 0.1 M potassium phosphate, pH 7.5 for APX, CAT, POD, PPO, and SOD assays.

Enzyme activities were determined using established, end-point spectrophotometric assays modified for use with a microplate reader (SpectraMAX Plus, Molecular Devices Corp., Sunnyvale, CA, USA). β-Gluc activity was determined by measuring glucose formation at 37 °C in solutions containing 5 g L^{-1} laminarin and 0.1 M sodium acetate, pH 5.2 (Kauffmann et al., 1987). Controls assayed for glucose concentration in the absence of laminarin. Chitinase activity was determined by absorbance changes at 550 nm and 37 °C in solutions containing 0.5 g L^{-1} carboxymethyl-chitin-remazol brilliant violet (CM-Chitin-RBV; LOEWE Biochemica GmbH, Sauerlach, Germany) and 50 mM sodium acetate, pH 6.4 using the substrate manufacturer's protocol. PAL activity was determined by absorbance changes at 290 nm and 37 °C in solutions containing 0.2 M phenylalanine and 0.1 M sodium borate, pH 8.8, relative to controls that lacked phenylalanine (Jiang and Fu, 1999). APX, CAT, and POD activities were determined by absorbance changes at 25 °C and 290, 240 and 460 nm, respectively, using the methods of Rao et al. (1996). APX activity assays contained 2.3 mM ascorbate, 29 mM hydrogen peroxide, and 90 mM potassium phosphate, pH 7.5. CAT activity assays contained 9.7 mM hydrogen peroxide and 90 mM potassium phosphate, pH 7.0. POD activity assays contained 15 mM guaiacol, 5.9 mM hydrogen peroxide, and 0.1 M sodium phosphate, pH 6.5. PPO activity was determined by absorbance changes at 495 nm and 25 °C in solutions containing 0.3 M catechol and 0.1 M sodium phosphate buffer, pH 7.0 (Tarrad et al. 1993). SOD activity was determined by absorbance changes at 560 nm of solutions containing 16 mM methionine, 6 mM EDTA, 175 µM nitroblue tetrazolium, 16 µM riboflavin, and 50 mM potassium phosphate, pH 7.8, after 10 min irradiance at room temperature by two 15 W fluorescent bulbs placed 0.3 m above the surface of the solution (Giannopolitis and Ries, 1977). Control reactions contained all reactants but were kept in the dark. All enzyme activities were expressed as a function of total soluble protein concentration. Total soluble protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.3 Total phenolic compounds concentration and antioxidant capacity

Freeze-dried tissue was extracted using the protocol of Policegoudra and Aradhya (2007) with modification. Tissue was suspended in 5 volumes (w/v) of 80% methanol by rapid vortexing. The suspension was sonicated for 30 min, centrifuged at 21,000 × g for 20 min, and the supernatant was removed, with all operations performed at 4 °C. Total phenolic compounds (TPC) concentration was determined with Folin-Ciocalteu reagent (Sigma-Aldrich Corp., St. Louis, MO, USA) by the method of Magalhães et al. (2010) and expressed in gallic acid equivalents per dry weight. Antioxidant capacity (AOC) was measured as DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity by the method of Brand-Williams et al. (1995) and expressed as Trolox (EMD Chemicals, Inc., San Diego, CA, USA) equivalents per dry weight.

2.4 Statistical analysis

Data were normalized by dividing each data value by the average of all values for day 0. Analyses of variance using treatment and time after harvest as main effects were used to detect significant differences. Tukey's test was used to determine differences between groups. Pairwise comparisons between treatments for a given day were made using t-tests. For all analyses, α was equal to 0.05. Statistical analyses were conducted with Minitab, ver. 16 (State College, PA, USA).

3 RESULTS

3.1 ROS-scavenging enzyme activities

Enzymes that scavenge for ROS were generally unchanged or reduced in activity by postharvest JA treatment of sugarbeet roots throughout the 60 d following treatment (Fig. 1). APX and POD activities were unaltered by JA treatment in root internal and peripheral tissues. CAT activity was unaltered by JA treatment in root peripheral tissue and in root internal tissue for the first 10 d after treatment, but exhibited a small decline and a small increase in activity in internal tissues 30 and 60 d after treatment, respectively. JA treatment reduced SOD activity in both internal and peripheral tissues over the 60 d of the experiment. Overall, SOD activity was reduced in internal and peripheral tissue by 27% and 19%, respectively. No differences between localized and systemic effects of JA on ROS-scavenging enzymes were apparent in sugarbeet roots in this study, since internal and peripheral tissues responded similarly to JA treatment for all enzymes examined.

Activities of ROS-scavenging enzymes, however, were altered by storage duration, with all changes occurring after 10 d or more in storage except by cat peripheral tissue (Fig. 1). APX and CAT activities decreased with prolonged storage. Relative to time of harvest, APX activities declined in both internal and peripheral tissues between 10 to 60 d after harvest. CAT activity declined between 30 to 60 d in internal tissues and between 3 to 60 d in peripheral tissues. At 60 d after harvest, APX activity was 58 and 49% lower in internal and peripheral tissues, respectively, relative to its value at time of harvest, while CAT activity in these same tissues was reduced by 36 and 46%. POD and SOD activities, in contrast to APX and CAT activities, generally increased after prolonged storage. POD activity was elevated in internal and peripheral tissues 30 to 60 d after harvest and increased 43 and 64% at 60 d, relative to its value at time of harvest. SOD activity was elevated between 10 to 60 d after harvest in internal tissue and 30 d after harvest in peripheral tissue. Maximum SOD activity was observed 30 d after harvest at which time activity

increased 250 and 90% for internal and peripheral tissues, respectively, relative to activity at time of harvest.

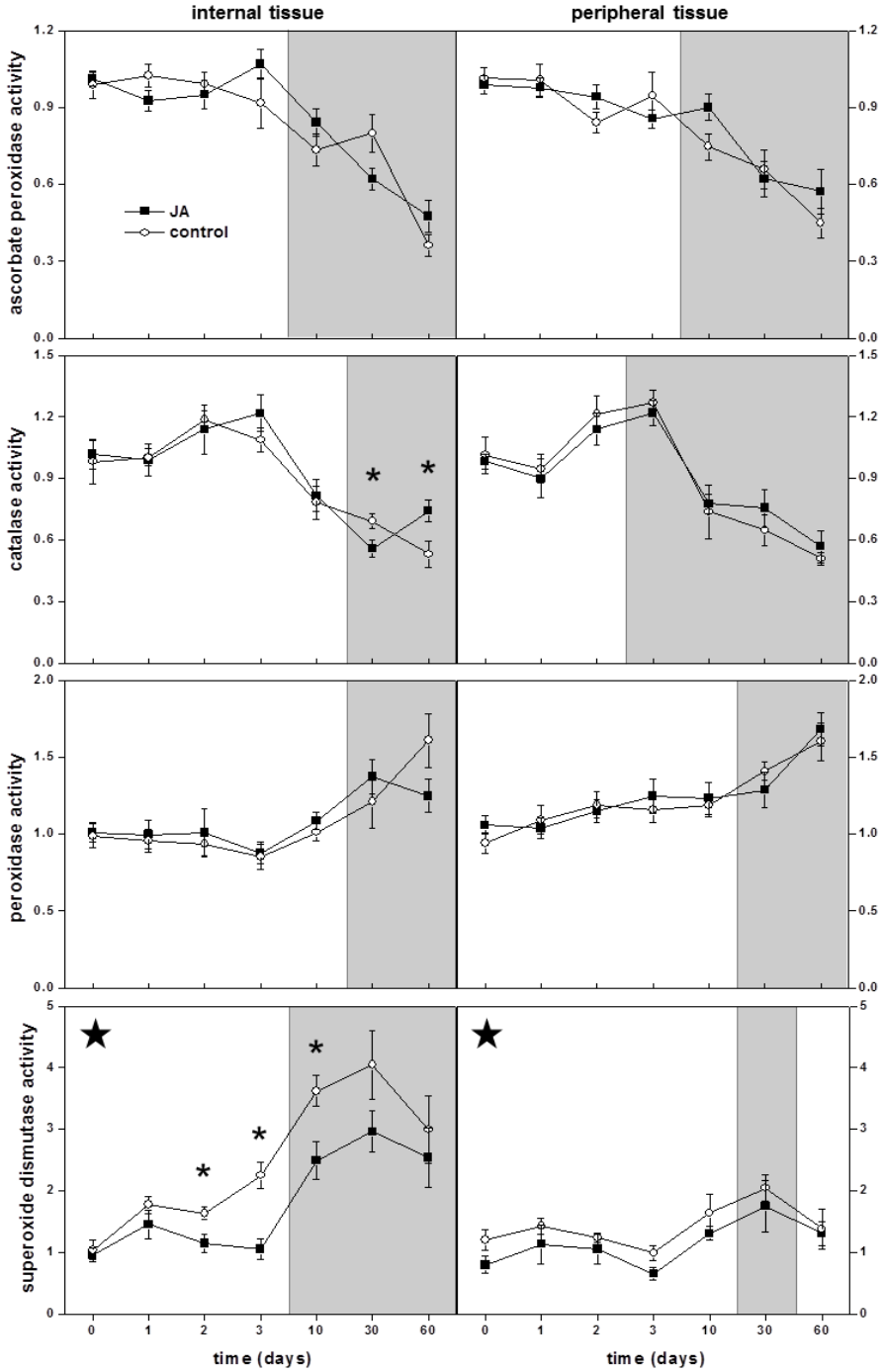

Fig. 1. Relative activities of reactive oxygen species-scavenging enzymes after jasmonic acid (JA) treatment in internal and peripheral tissues of sugarbeet roots. Roots were treated with 10 µM JA or water (control) following harvest and incubated at 20 °C and 90% relative humidity for up to 60 d. Enzyme activities are expressed as a function of total soluble protein concentration and normalized to express data as a function of the activity at day 0. Enzyme activities that were significantly altered by JA treatment, as determined by ANOVA, are identified by a star (★) in the upper left portion of the graph; time points (days) at which enzyme activities were significantly altered from the activity at harvest (day 0) are identified by a shaded box filling the length of the graph. Individual days where activities between treatments were statistically different, as determined by a t-test, are identified by asterisks (*). Data are mean ± SE, where *n* = 8. For all statistical analyses, α = 0.05.

3.2 Pathogen-related defense enzyme activities

Postharvest JA treatment did not increase the activity of common pathogen-related defense enzymes during the 60 d following treatment (Fig. 2). β-Gluc and PPO activities were unaltered by JA treatment in root internal and peripheral tissues. PAL activity, which was barely detectable in sugarbeet roots (Supplementary Anexo A), was also unaltered by JA treatment except for a transient decrease in activity occurring 10 d after harvest in peripheral tissue. JA treatment reduced chitinase activity in both internal and peripheral tissues, with the greatest suppression of activity occurring 30 to 60 d after treatment. During this period, chitinase activity was reduced in internal and peripheral tissues by 24 and 27%, respectively. Localized and systemic effects of JA were similar for the defense-related enzymes examined in this study, since enzymes in internal and peripheral tissues responded similarly to JA treatment.

Long-term storage increased the activity of several pathogen-related defense enzymes (Fig. 2). β-Gluc, chitinase, and PPO activities were elevated 30 to 60 d after harvest, relative to their activities at harvest, in both internal and peripheral tissues. By 60 d after harvest in internal and peripheral tissues, respectively, β-Gluc activity increased 510 and 210%, chitinase activity increased 110 and 135%, and PPO activity increased 52 and 67%, relative to their activities at time of harvest. PAL activity was unaltered by storage duration.

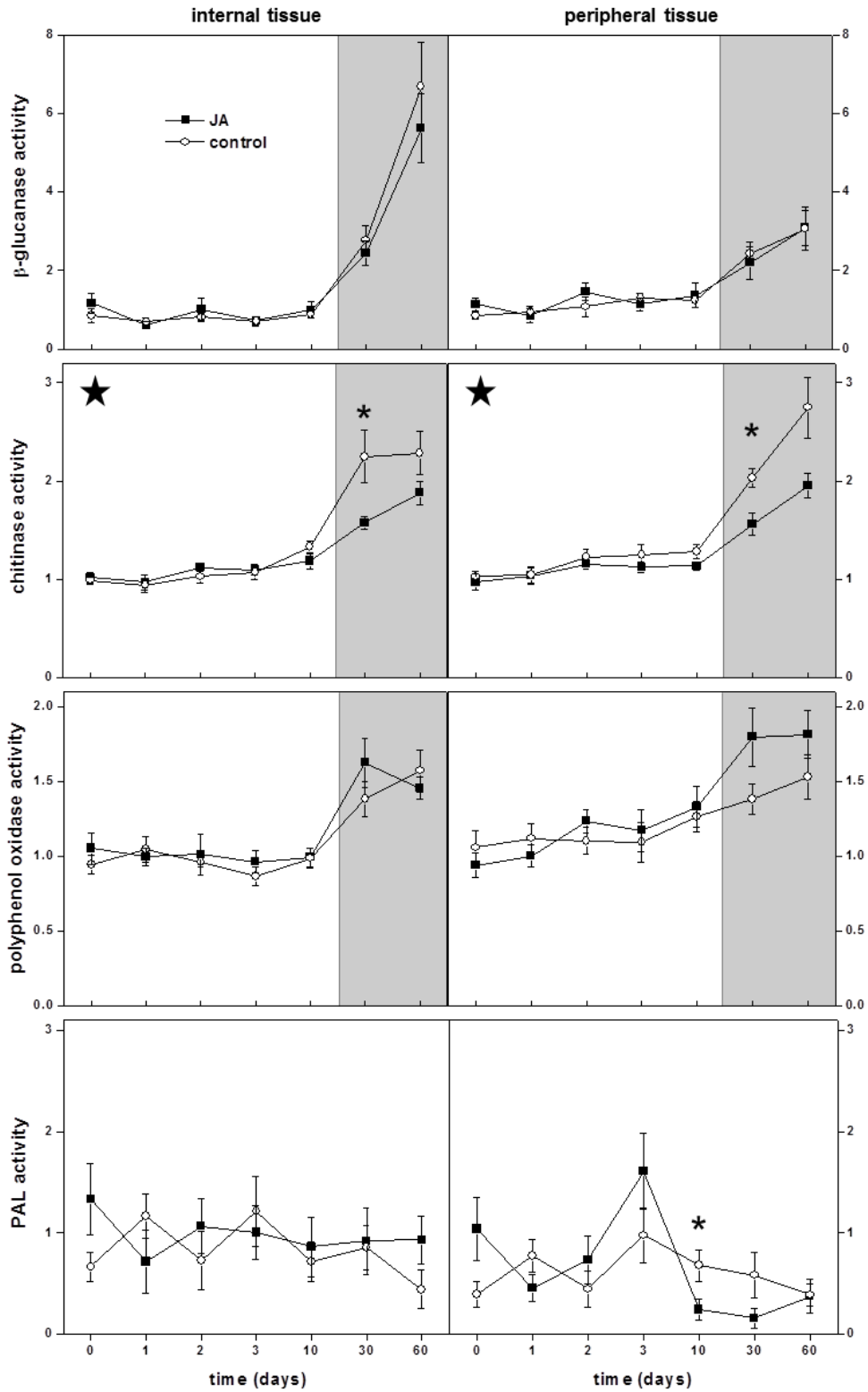

Fig. 2. Relative activities of pathogen-related defense enzymes after jasmonic acid (JA) treatment in internal and peripheral tissues of sugarbeet roots. Roots were treated with 10 µM JA or water (control) following harvest and incubated at 20 °C and 90% relative humidity for up to 60 d. Enzyme activities are expressed as a function of total soluble protein concentration and normalized to express data as a function of the activity at day 0. Enzyme activities that were significantly altered by JA treatment, as determined by ANOVA, are identified by a star (★) in the upper left portion of the graph; time points (days) at which enzyme activities were significantly altered from the activity at harvest (day 0) are identified by a shaded box filling the length of the graph. Individual days where activities between treatments were statistically different, as determined by a t-test, are identified by asterisks (*). Data are mean ± SE, where *n* = 8. For all statistical analyses, α = 0.05.

3.3 Total phenolic compounds (TPC) concentration and antioxidant capacity (AOC)

The concentration of antioxidants in sugarbeet roots, as measured by total phenolic compounds concentration and antioxidant capacity, was generally unaltered by postharvest JA treatment of sugarbeet roots during the 60 d following treatment (Fig. 3). TPC concentration and AOC in root internal and peripheral tissues were unaltered by JA treatment, except for a 24% decrease in AOC occurring 60 d after treatment in internal tissue. However, antioxidant concentrations were affected by storage duration in both internal and peripheral tissues. One day after harvest, there was a transient increase in TPC concentration by 33 and 28% and in AOC by 33 and 43%, in internal and peripheral tissues, respectively, relative to their values at harvest. Additional elevations in TPC concentration occurred 10 d after harvest in internal tissue and 30 d after harvest in peripheral tissue, while AOC was additionally elevated in peripheral tissue 3 to 30 d after harvest.

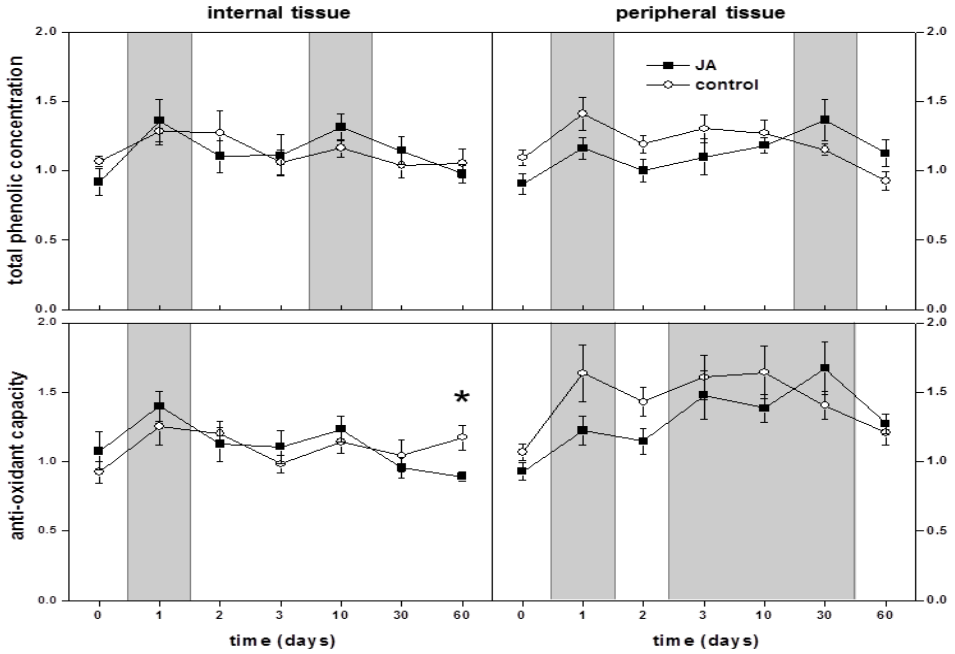

Fig. 3. Relative concentration of antioxidants, as measured by total phenolic compounds (TPC) concentration and antioxidant capacity (AOC), after jasmonic acid (JA) treatment in internal and peripheral tissues of sugarbeet roots. Roots were treated with 10 µM JA or water (control) following harvest and incubated at 20 °C and 90% relative humidity for up to 60 d. TPC concentration and AOC are expressed as a function of dry weight and normalized to express data as a function of the average value at day 0. Time points (days) at which data were significantly altered from values at harvest (day 0) are identified by a shaded box filling the length of the graph. Individual days where data between treatments were statistically different, as determined by a t-test, are identified with an asterisk (*). Data are mean ± SE, where n = 8. For all statistical analyses, $\alpha = 0.05$.

4 DISCUSSION

Enzymes that scavenge for reactive oxygen species such as APX, CAT, POD and SOD, and metabolites that act as antioxidants have been implicated in jasmonate-induced disease resistance in many plant species and organs (Seema et al., 2003; Yao and Tian, 2005b; Ali et al., 2006; Haggag et al., 2010; Chen et al., 2011). In harvested sugarbeet roots, however, JA treatment generally had no effect on APX, CAT, and POD activities or the concentration of antioxidants in the 60 d following treatment, indicating that these enzymes and metabolites are not directly involved in JA-induced resistance in this crop. SOD activity, in contrast, was reduced by JA treatment. In root internal and peripheral tissues, JA treatment decreased SOD activity an average of 27 and 19%, respectively. Jasmonate-induced reductions in SOD activity have been reported in other plant species and organs (Seema et al., 2003; Ali et al., 2006), and it has been suggested that these reductions contribute to disease resistance by allowing accumulation of O_2^-, a ROS implicated in defense against pathogens (Lamb and Dixon, 1997). Whether the relatively minor reduction in SOD activity that was observed in sugarbeet roots could effectively alter O_2^- accumulation upon pathogen attack and contribute to plant defense is unknown. Certainly, however, the lack of increase in any ROS-scavenging enzyme or total antioxidants indicates that JA-induced resistance in sugarbeet roots is not due to enhanced protection against oxidative stresses.

Pathogen-related defense enzymes have also been implicated in jasmonate-induced disease resistance with several studies finding increases in β-1,3-glucanase, chitinase, polyphenol oxidase or PAL activities after jasmonate treatment (Yao and Tian, 2005b; El-Khallal, 2007; Haggag et al., 2010). In harvested sugarbeet roots, however, JA treatment reduced chitinase activity in the 60 d following treatment and generally had no effect on β-Gluc, PPO, or PAL activities. The reduction in chitinase activity may reflect antagonism between JA and salicylic acid (SA) signaling pathways (Niki et al., 1998), since chitinase is commonly induced by SA (Schneider-Müller et al., 1994). However, if the reduction in chitinase activity was due to JA antagonism of SA responses, β-Gluc would also be expected to be suppressed since this enzyme is

commonly induced by SA (Ohme-Takagi and Shinshi, 1990). Nevertheless, the lack of increase in β-Gluc, chitinase, PPO, and PAL activities indicates that these pathogen-related defense enzymes do not contribute to JA-induced disease resistance.

Storage duration significantly altered the activities of all ROS-scavenging enzymes examined in this study, with all changes occurring after 10 d storage. With prolonged storage, activities of APX and CAT declined and activities of POD and SOD increased. Similar storage-related changes in ROS-scavenging enzymes have been observed in other postharvest products. For example, APX activity declined in peach and loquat fruits, CAT activity or gene expression declined in peach, loquat, tomato and kiwi fruits, POD activity increased in loquat, sweet cherry, and kiwi fruits, and SOD activity increased in peach and kiwi fruits during storage (Ding et al., 2002; Yao and Tian, 2005b; Cao et al., 2008; Zhu et al., 2008; Jin et al., 2009a; Cai et al., 2011). The significance of these storage-related changes in ROS-scavenging enzymes is unknown. However, it is evident that the ability to detoxify O_2^- and H_2O_2 and mount ROS-mediated defense mechanisms and signals is altered in sugarbeet root by prolonged storage.

Storage duration also affected the activity of several defense-related enzymes. After 30 and 60 d in storage, β-Gluc, chitinase, and PPO activities increased in both peripheral and internal root tissues. Similarly, β-Gluc and chitinase gene expression increased in tomato fruit, chitinase activity increased in banana fruit, and PPO activity increased in loquat and peach fruits during storage (Ding et al., 2002; Cao et al., 2008; Jin et al., 2009b; Tang et al., 2010). The induction of defense-related enzyme activities in stored plant products, however, is unrelated to improved disease resistance since disease resistance declines with storage duration in sugarbeet root and other postharvest products (Bugbee, 1979; Tang et al., 2010).

Although JA treatment improves disease resistance in stored sugarbeet roots (Fugate et al., 2012), JA-induced resistance does not arise from a direct increase in ROS-scavenging ability or induction of common pathogen-related defense enzymes. This contrasts with studies that demonstrated elevations in these enzymes or metabolites in fruits or leaves after jasmonate treatment (Ding et al., 2002; Hung and Kao, 2004; Yao and Tian, 2005a; El-Khallal, 2007;

Jin et al., 2009a) and a study that reported increased activities of POD, chitinase, and PPO and total phenolic compounds concentration in jasmonate-treated sugarbeet leaves (Haggag et al., 2010). It is unknown whether the disparities between this and other studies arise from differences between plant species, plant organs, tissue age, or other unidentified factor. However, differential responses to jasmonates among plant species are common (Rohwer and Erwin, 2008) and can occur between plant organs (Parra-Lobato et al., 2009). In sugarbeet root, alterations in the activities of ROS-scavenging enzymes and several pathogen-related defense enzymes were related to prolonged storage, when disease resistance is reduced. The inverse association between these activities and disease resistance further suggests that these enzymes are unlikely to promote disease resistance in postharvest sugarbeet root.

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5 ARTIGO 3

Characterization of the sugarbeet (*Beta vulgaris* L.) transcriptome, RNA-sequencing of jasmonic acid differentially expressed genes and JA effect on expression of defense-related genes.

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ABSTRACT

Although jasmonic acid (JA) protects sugarbeet roots (*Beta vulgaris* L.) against three common storage pathogens, the mechanisms involved in jasmonate induced-resistance remain unknown. Here, we studied the effect of JA on the expression of well-known defense related genes using real time quantitative PCR and examined JA-differentially expressed genes using RNA sequencing (RNA-seq). A global transcriptome of sugarbeet was also generated using next generation, paired-end sequencing technology to provide a reference for the RNA-seq data. Phenylalanine ammonia-lyase (PAL), pathogenesis-related protein 1 (PR1), chitinase 1 (CHI 1), chitinase 3 (CHI 3) and a cell wall peroxidase (POD) exhibited small and transient changes in expression in response to JA treatment. PAL and POD transcript levels were transiently induced by JA treatment, while PR1 and CHI 1 were transiently reduced by JA treatment and CHI 3 exhibited both a reduction and increase in transcript levels associated with JA treatment at different times after treatment. The sugarbeet reference transcriptome generated from cleaned raw reads from sequencing a composite leaf and root RNA sample contained 82404 unigenes. From this total 17,191 unigenes were assigned gene ontology (GO) terms and 15,201 unigenes were assigned to clusters of gene ontology (COG) classifications. JA's effect on sugarbeet expressed genes was analyzed at 2 and 60 d after treatment. At 2d after treatment, 120 unigenes were altered in expression by JA (P<0.01 and logFC >2). Among these unigenes 71 or 59.2% were up-regulated and 49 or 40.8% were down-regulated by JA. At 60 d post-treatments 104 unigenes were affected by JA. Among these unigenes, 62 or 59.6% were up-regulated and 42 or 40.4% were down-regulated by JA. A BLASTnr search (E-value < 0.00001) revealed that at 2 d after treatment 3 of the up-regulated unigenes were identified as putative peroxidases and 1 up-regulated unigenes was homologous with an acidic chitinase. Among the 60 d after treatment differentially expressed known sequences, two putative chalcone synthase unigenes were up-regulated by JA, and a putative peroxidase gene and a defensin-like gene were down-regulated by JA treatment. Genes altered in expression by JA may contribute to JA-induced

disease resistance. Identification of genes altered by JA treatment in these studies provides targets for future research into the mechanisms by which JA protects postharvest sugarbeet roots against pathogens.

Keywords: *Beta vulgaris*; disease resistance; induced resistance; jasmonate; transcriptome; RNA-seq

1 INTRODUCTION

Jasmonates are endogenous plant hormones that activate native plant defense mechanisms (Ballaré, 2011). When applied exogenously, jasmonates have been found to reduce disease caused by a variety of fungal, bacterial and viral pathogens (Thaler et al., 2004; Rohwer and Erwin, 2008; Haggag et al., 2010) by reducing disease severity in most cases and reducing disease incidence in others (Yao and Tian, 2005b; Cao et al., 2008; Zhang et al., 2009). Although some studies have suggested that jasmonate treatment reduces disease by directly limiting antimicrobial activity, and the ability of jasmonates to reduce spore germination, germ tube elongation and mycelial growth has been demonstrated (Yao and Tian, 2005a; Cao et al., 2008), jasmonate treatment is widely believed to protect plants by systemically inducing the same plant defense responses that are activated by endogenous jasmonates (Tripathi and Dubey, 2004; Pozo et al., 2005).

Changes in expression of defense-related genes is a key element in inducible defense mechanisms in plants (Ryder et al., 1984; Edreva, 2005). Changes in expression of phenylalanine ammonia-lyase (PAL), peroxidase (POD), pathogenesis related 1 (PR 1), and chitinases genes are commonly related to jasmonates-induced defense in several plants (Sharan et al., 1998; Curtis et al., 1997; Reymond and Farmer 1998; Ding et al., 2002).

Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease (Wang et al., 2009). High-throughput RNA sequencing (RNA-seq) is an efficient and reliable platform for transcriptomic analysis in non-model organisms. The large number of sequences generated provides valuable sequence information at the transcriptomic level for novel gene discovery, or for the investigation molecular mechanisms (Wang et al., 2009; Wei et al., 2011).

In sugarbeet (*Beta vulgaris* L.), exogenous jasmonate treatment has been shown to reduce foliar symptoms due to Beet Mosaic Virus and root rot symptoms due to postharvest infections of *Botrytis cinerea* Pers. ex Fr., *Penicillium claviforme* Bainier, and *Phoma betae* Frank (Haggag et al., 2010;

Fugate et al., 2012). In sugarbeet roots, there is no information available on the effect of jasmonic acid (JA) on the expression of defense-related genes.

The effect of an exogenous jasmonate treatment on the gene expression of known defense-related genes that have been implicated in jasmonate-induced disease resistance in other plant species and organs was studied using quantitative real time PCR. Analyses were performed using a jasmonic acid (JA) treatment previously demonstrated to significantly reduce disease symptoms due to three storage rot-causing organisms (Fugate et al., 2012). In the research described here, we also generated a sugarbeet transcriptome reference library and used it to identify other genes whose expression was altered by JA treatment using RNA sequencing. Quantitative PCR evaluated transcriptional changes throughout 60 d storage, and RNA-seq of JA and water-treated control roots was conducted using samples 2 and 60 d after JA treatment. This allowed evaluation of JA short-term and long-term effects. The purpose of this research was to identify genes potentially involved in JA-induced disease resistance in postharvest sugarbeet roots.

2 MATERIAL AND METHODS

2.1 Plant material and JA treatment

Sugarbeet hybrid VDH66156 (SESVanderHave, Tienen, Belgium) was greenhouse grown in Sunshine Mix #1 (Sun Gro Horticulture, Vancouver, BC, Canada) in 15-L pots with supplemental light under a 16 h light/8 h dark regime. Taproots were harvested 16 - 18 weeks after planting, all leaf and petiole material was removed, and roots were gently washed to remove potting media. Roots were submerged in 0 or 10 µM JA (Cayman Chemical, Ann Arbor, MI) for 1 hr at room temperature, then incubated at 20 °C and 90 % relative humidity for up to 60 d in a controlled environment chamber (Conviron, model MTR30, Winnipeg, MB, Canada). Root samples were collected at 0, 1, 2, 3, 10, 30 and 60 d post-treatment by collecting tissue from the main portion of the root, free of crown or tail tissue, with the epidermis and approximately 2 mm of subepidermal tissue excluded. Samples were flash frozen in liquid N_2, lyophilized, ground to a powder, and stored at -80 °C until analysis. Individual roots were the experimental unit with 4 replicates per treatment per time point. Experiment was repeated three times.

2.2 RNA extraction and qRT-PCR

Total RNA was extracted using a PureLink RNA Mini Kit (Ambion, Foster, CA, USA) or RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. RNA quality was confirmed with an Agilent Technologies (Palo Alto, CA, USA) 2100 Bioanalyzer. High quality RNA has clearly visible 18S/28S rRNA peaks. cDNA was synthesized using SuperScript III (Invitrogen, Foster, CA, USA) or RETROscript (Ambion) reverse

transcriptases. Quantitative real-time PCR (qPCR) was used to determine JA-related changes in transcript levels of genes for a chitinase 1 (CHI 1), a chitinase 3 (CHI 3), a phenylalanine ammonia-lyase (PAL), a cell wall peroxidase (POD), a pathogenesis-related protein 1 (PR1), using an actin (ACT) gene as a reference (Table 1). qPCR was performed on a MJ Research (Watertown, MA, USA) PTC-200 thermal cycler, equipped with a Chromo 4 real-time detector (Bio-Rad Life Science Hercules, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA, USA) and the primer pairs listed in Table 1. For qPCR, samples were denatured for 2 min at 95°C and amplified during 39 cycles of 15 s at 95°C and 45 s at 60°C. Melting curves were used to confirm that single products were amplified. Primer efficiency (E) of each primer pair was determined by analyzing standard curves of Ct values generated by PCR amplification of 5-fold serial dilutions of the cDNA synthesized from each treatment. Primer efficiency was calculated according to the equation $E = (10^{-1/slope}-1)$. The relative quantification of real-time PCR results was performed using the mathematical model of Pfaffl (Pfaffl, 2001).

Table 1. Sugarbeet genes quantified by qPCR analysis, their accession numbers and primer pairs used to effect their amplification. Genes analyzed include those encoding a chitinase 1 (CHI 1), a chitinase 3 (CHI 3), a phenylalanine ammonia-lyase (PAL), a cell wall peroxidase (POD), a pathogenesis-related protein 1 (PR1) and an actin (ACT). Actin served as a reference gene in these studies.

Gene	Accession no.	Forward	Reverse
Actin	DQ866829	GATTTGGCACCACACCTTCT	TCTTTTCCCTGTTTGCCTTG
CHI 1	A23786	TCATAACTGGGCAATGGACA	CCGCATTCTAAAGCACCATT
CHI 3	S66038	CATCAAAACATGCCAACAGG	CTGGACTGACCCCCAAGATA
PAL	AJ810175	AGACAGCTGAGGCAGTGGAT	CCTTCTGCACACCTGACTGA
POD	AF067188	GGAACGTGAAGTTGGGAAGA	GCACCAGATAAAGCGACCAT
PR1	AM932128	TGGCAAAGTGTGTGGACATT	ACAAAGTTGCCTGGTGGAT

2.3 Reference transcriptome generation

A reference transcriptome for sugarbeet was generated by high-throughput, paired-end RNA sequencing using a pooled RNA sample. The pooled sample contained equivalent weights of RNA extracted from young newly developed leaf tissue, fully expanded leaf tissue, root tissue 5 weeks after planting, root tissue 16 weeks after planting, root tissue after 60 d postharvest storage, root tissue 2 d after a 10 μM JA postharvest treatment, and root tissue 2d after a postharvest 1 mM salicylic acid treatment. Roots subjected to storage, JA treatment or SA treatment were harvested 16 weeks after planting and maintained at 20 ^{o}C and 90% relative humidity after harvest. Sequencing was performed on an Illumina, Inc. (San Diego, CA, USA) HiSeq 2000 system by BGI Americas (Cambridge, MA, USA). Transcriptome assembly was carried out using SOAP*denovo* assembly software (Li et al., 2009). BLASTx alignment (e-value < 0.00001) of unigenes with protein sequences available in NCBI's nr, Swiss-Prot, KEGG and COG databases was used for function, biological pathway, cellular location, and biological process annotation.

When a Unigene happened to be unaligned to none of the above databases, a software named ESTScan (Iseli et al., 1999) was introduced to decide its sequence direction.

2.4 Unigene Annotation

With nr annotation, we use Blast2GO program (Conesa et al., 2005) to get GO annotation of Unigenes. After getting GO annotation for every Unigene, we use WEGO software (Ye et al., 2006) to do GO functional classification for all Unigenes and to understand the distribution of gene functions of the species from the macro level.

2.5 RNA-sequencing of JA treated samples

RNA sequencing was performed on roots treated with 0 and 10 µM JA and collected at 2 and 60 days after treatment. Roots were harvested 16 weeks after planting and maintained at 20 ^{o}C and 90% relative humidity after harvest. Sequencing was performed on an Illumina, Inc. (San Diego, CA, USA) HiSeq 2000 system by BGI Americas (Cambridge, MA, USA).

Clean reads were mapped to reference sequences using SOAPaligner/soap2 (Li et al., 2009). Mismatches no more than 2 bases were allowed in the alignment. Statistical analysis was conducted to summarize the number of clean reads that align to the reference genome/genes, which provides the general information of the project. During the RNA-Seq experiment, mRNA are firstly broken into short segments by chemical methods and then sequenced. If the randomness is poor, reads preference from specific gene region will directly affect subsequent bioinformatics analysis. We use the distribution of reads locating on the genes to evaluate the randomness. Since reference genes have different lengths, the reads location on gene is standardized to a relative position (which is calculated as the ratio between reads location on the gene and gene length), and then the number of reads in each position is counted. If the randomness is good, the reads in every position would be evenly distributed. The gene expression is calculated by the numbers of reads mapped to the reference sequence and every gene. The gene expression level is calculated by using RPKM (Mortazavi et al., 2008) method (Reads Per kb per Million reads). The RPKM method is able to eliminate the influence of different gene length and sequencing discrepancy on the calculation of gene expression. Therefore, the calculated gene expression can be directly used for comparing the difference of gene expression among samples. If there is more than one transcript for a gene, the longest one is used to calculate its expression level and coverage.

Screening of differentially expressed genes (DEGs) analysis was used to find genes that have different expression levels among samples, and then GO function analysis and KEGG pathway analysis was carried out.

3 RESULTS

3.1 Defense-related genes expression

A postharvest jasmonic acid treatment altered expression of five common defense-related genes in sugarbeet roots in the 60 d following treatment (Fig. 1). Relative to expression of an actin gene which served as an internal control, steady state transcript levels of genes encoding PAL, a cell wall peroxidase, PR1, chitinase 1 and chitinase 3 were significantly altered by JA treatment, where significance was defined as expression level changes of two-fold or greater. PAL and POD transcript levels were transiently induced by JA treatment. PAL transcript levels were induced 3.5-fold 2 d after treatment, and POD transcript levels were induced 3-fold 1, 2 d after treatment and 2-fold 30 d after treatment. In contrast, PR1 and chitinase 1 were transiently reduced by JA treatment. PR1 transcript levels were reduced 2-fold at 3 and 10 d after treatment and 5-fold 60 d after treatment; chitinase 1 transcript levels were reduced 2-fold and 3-fold 3 and 10 d after treatment, respectively. Chitinase 3 exhibited both a reduction and increase in transcript levels associated with JA treatment. One day after treatment, chitinase 3 transcript levels were reduced 3-fold. Sixty days after treatment, chitinase 3 transcript levels increased 2-fold.

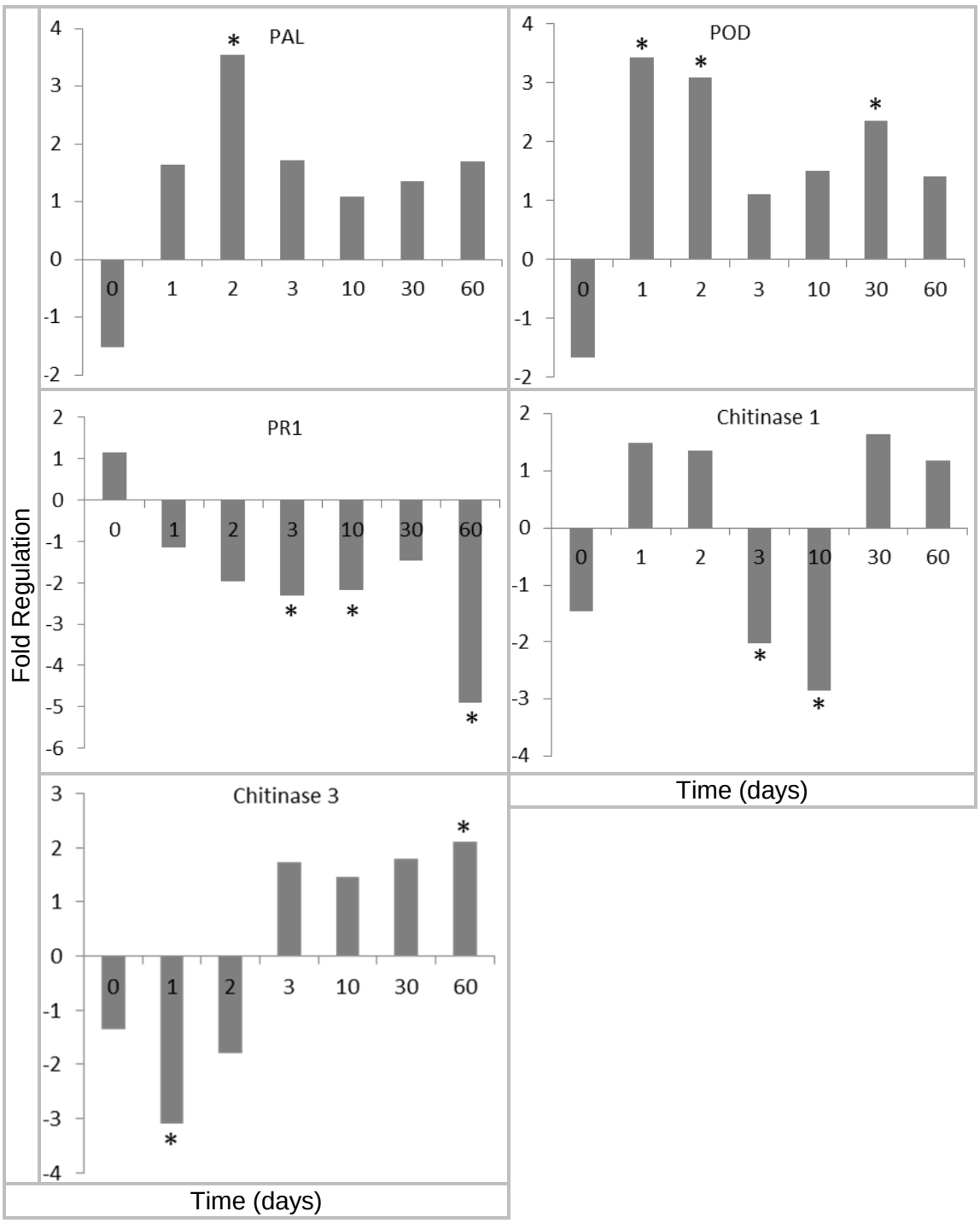

Fig. 1. Effect of jasmonic acid (JA) treatment on expression of five common defense-related genes in sugarbeet roots. Roots were treated with 10 µM JA or water (control) following harvest and incubated at 20°C and 90% relative humidity for up to 60 d. Transcripts levels are presented as fold change differences in relation to controls, with actin used as a reference gene. Alterations in Transcript levels greater than 2.0 were considered significant in relation to water treated controls. Time points (days) at which transcript levels were changed by JA treatment are indicated by asterisks (*).

3.2 Gene ontology and clusters of orthologous groups of the RNA library

RNA sequencing was used to generate a library of sugarbeet expressed genes that could be used as a reference for determining genes altered by JA treatment. The library was generated from RNA that was pooled from RNA extracts from immature leaf tissue, mature leaf tissue, root tissue 5 weeks after planting, root tissue 16 weeks after planting, root tissue 2 days after JA treatment, root tissue 2 days after SA treatment, and root tissue from a root that had been stored for 60 days. Multiple tissues were used to maximize the number of expressed genes in the library. RNA sequencing yielded a sugarbeet RNA library of 82,404 unigenes. From this total, only a small percentage had matches when compared to gene databases. 17,191 unigenes were assigned gene ontology (GO) terms. Unigenes were classified according to biological processes, cellular components, and molecular functions clusters and were distributed across 44 categories. In the biological processes category, metabolic processes (the chemical reactions and pathways, including anabolism and catabolism, by which living organisms transform chemical substances) (24.47%) was the most dominant group, followed by cellular processes (any process that is carried out at the cellular level, but not necessarily restricted to a single cell) (23.49%). In regards to the assignment of unigenes to cellular component, 34.38% of the unigenes were assigned to cell (all components within and including the plasma membrane and any external encapsulating structures, such as the cell wall), followed by cell part (any constituent part of a cell) (31.18%). Among the molecular function category, binding (the selective, non-covalent, often stoichiometric, interaction of a molecule with one or more specific sites on another molecule) (46.65%) and catalytic activity (catalysis of a biochemical reaction at physiological temperatures) (43.82%) were the dominant groups (Fig. 2). Unigenes were also subjected to a search against the Cluster of Orthologous Groups (COG) database for functional prediction and classification. A total of 15,201 unigenes were assigned to COG classifications. COG-annotated putative proteins were functionally classified into 24 clusters.

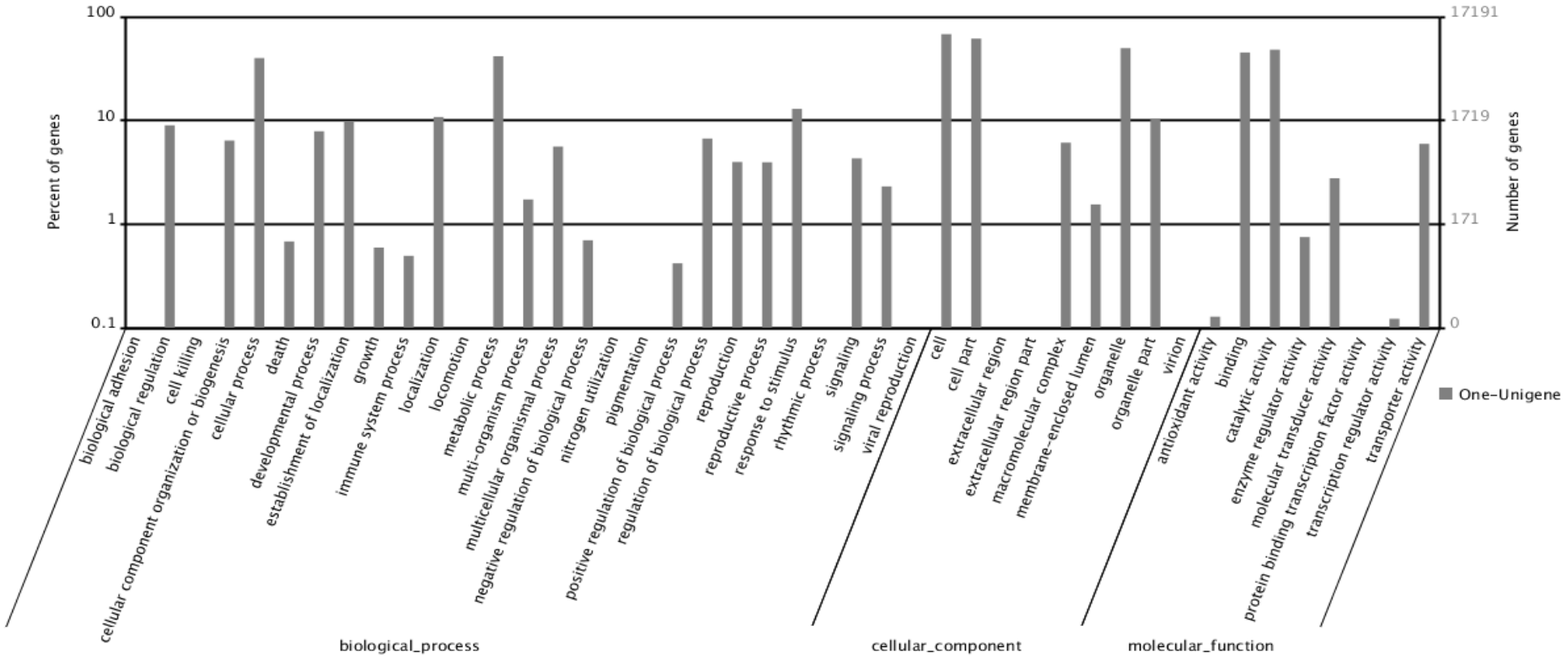

Fig. 2. Gene ontology classification of assembled unigenes of sugarbeet transcriptome. The unigenes were annotated to three categories: biological processes, cellular components, and molecular functions. Using BLASTnr matches, 17191 unigenes were assigned to gene ontologies.

The cluster for general function prediction (2,450; 16.12%) represented the largest group this is a class for which only a general functional prediction (e.g., that of biochemical activity) was feasible, followed by replication, recombination and repair (1665; 10.95%), transcription (1273; 8.37%), posttranslational modification, protein turnover and chaperones (1037; 6.82%). Only 5 unigenes were assigned to nuclear structure (Fig 3).

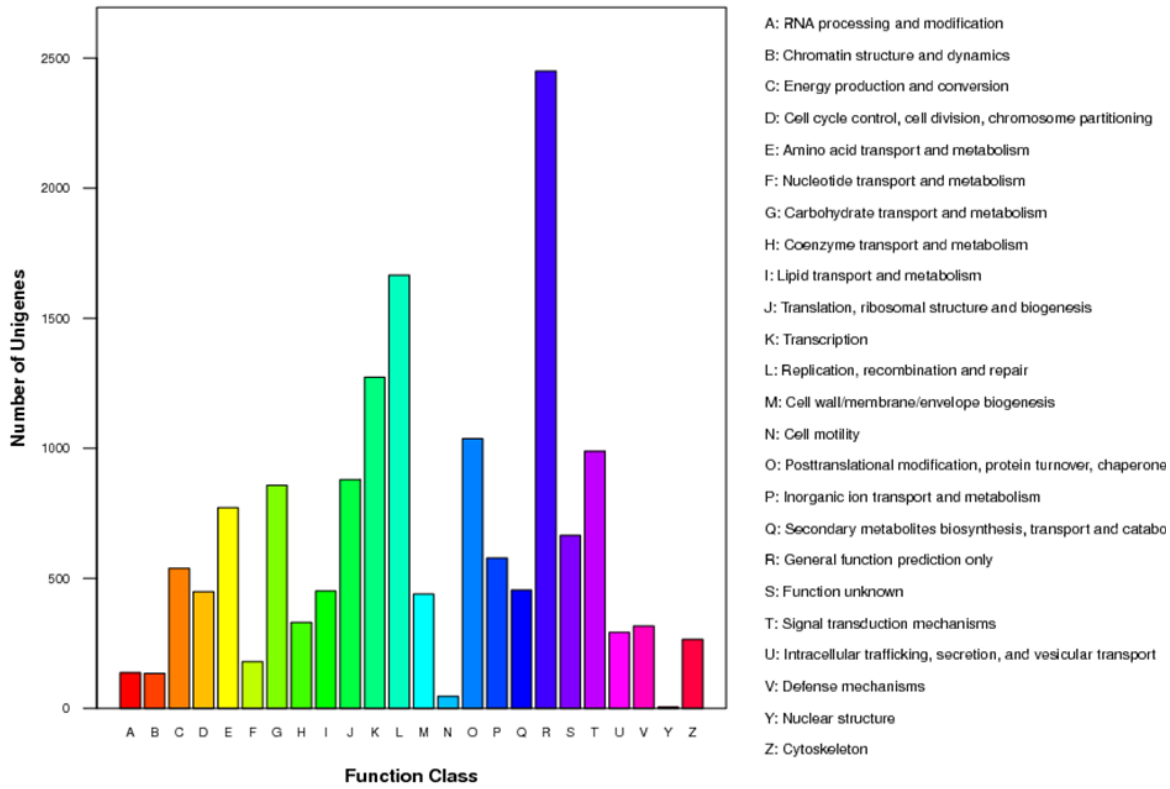

Fig. 3. Clusters of orthologous groups (COG) classification of the sugarbeet transcriptome. A total of 15,201 sequences with nr hits were grouped into 24 COG classifications.

3.3 JA effect on sugarbeet transcriptome

The effect of JA on the sugarbeet transcriptome was analyzed at 2 and 60 d after treatment. At 2 d after treatment, 120 unigenes were affected by JA (P < 0.01 and logFC > 2). Among these unigenes, 71 or 59.2% were up-regulated and 49 or 40.8% were down-regulated by JA (Fig 4A). At 60 d post-treatment, 104 unigenes were affected by JA (P<0.01 and logFC >2). Among these unigenes 62 or 59.6% were up-regulated and 42 or 40.4% were down-regulated by JA (Fig 4B). BLASTnr alignment (E-value < 0.00001) was performed. Among the 120 differentially expressed unigenes at 2 d after

treatment, 83 (69.2%) revealed no hit while another 23 (19.2%) matched unknown targets and only 14 (11.7%) matched known sequences (Fig. 5A). Among the 104 differentially expressed unigenes at 60 d after treatment, 60 (57.7%) revealed no hit while another 24 (23.1%) matched known sequences and 20 (19.2%) matched unknown targets (Fig. 5B). From the known sequences at 2 d after treatment (Table 2), three up-regulated unigenes were homologous with peroxidases and one up-regulated gene was homologous with an acidic chitinase. Among the 60 d post-treatment differentially expressed known sequences (Table 3), one down-regulated unigene was homologue with a peroxidase gene.

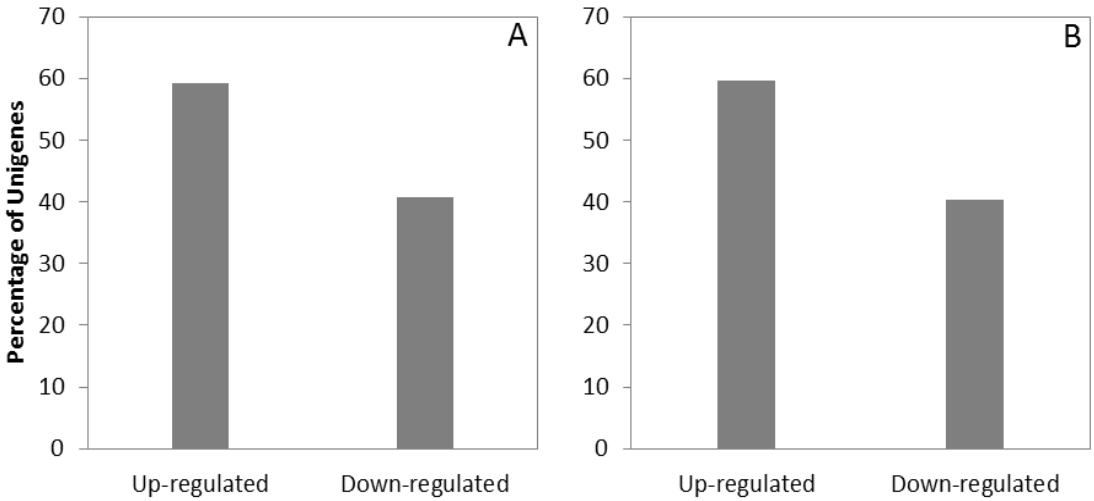

Fig. 4. Percentage of unigenes up-regulated or down-regulated by JA treatment. Sugarbeet roots were treated with 0 or 10 µM JA following harvest and incubated at 20°C and 90% relative humidity for 2 d (A) and 60 d (B).

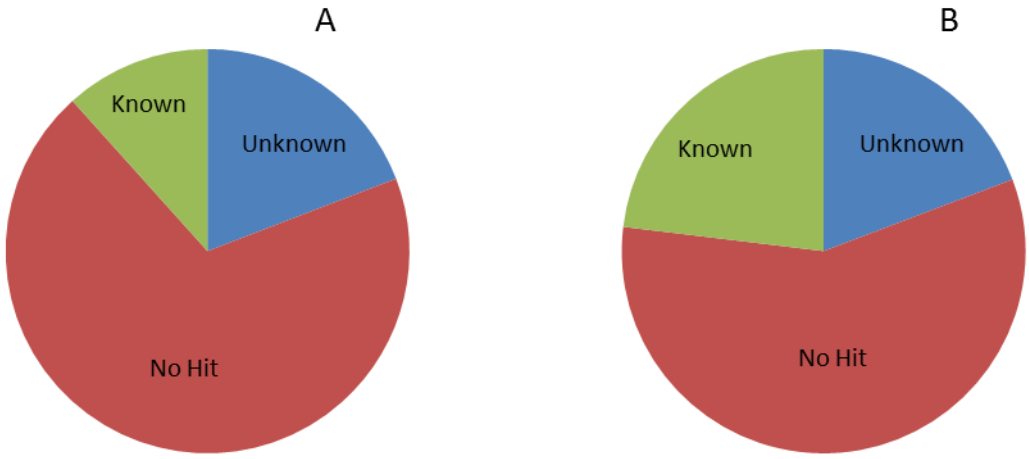

Fig. 5. Classification of JA expression-altered unigenes into no hit, unknown and known sequences. Unigenes were subjected to BLASTnr alignment (E-value < 0.00001). Sugarbeet roots were treated with 0 or 10 µM JA following harvest and incubated at 20°C and 90% relative humidity for 2 d (A) and 60 d (B).

Table 2. Predicted functions of 13 genes differentially expressed in sugarbeet roots treated with JA 10 µM in the comparison of water treated controls at 2 days post treatment

Unigene ID	Log Fold Change	P value	E value	Predicted function
Unigene1219	3.58	2.26E-17	5.80E-19	Retroelement pol polyprotein-like [*Arabidopsis thaliana*]
Unigene71046	3.32	7.41E-12	1.01E-30	Acidic chitinase [*Elaeagnus umbellata*]
Unigene34661	2.72	3.63E-13	6.66E-30	Peroxidase [*Spinacia oleracea*]
Unigene29351	2.59	1.64E-15	1.45E-28	Nbs-lrr resistance protein [*Populus trichocarpa*]
Unigene77250	2.22	7.13E-11	9.66E-35	Nbs-lrr resistance protein [*Populus trichocarpa*]
Unigene66692	2.12	1.93E-09	5.81E-50	Peroxidase [*Spinacia oleracea*]
Unigene42562	2.09	5.00E-08	1.12E-24	Peroxidase [*Spinacia oleracea*]
Unigene61024	2.02	6.94E-07	7.31E-37	ALDH3F1 [*Arabidopsis lyrata subsp. lyrata*]
Unigene47752	-2.01	0.001184	2.99E-22	Copia LTR rider [*Solanum lycopersicum*]
Unigene10322	-2.34	0.00047	1.46E-13	ECP63 protein [*Daucus carota*]
Unigene69209	-2.43	2.52E-06	1.58E-15	Polypepetide with reverse transcriptase and RNaseH domains [*Petunia x hybrida*]
Unigene55354	-2.92	1.05E-07	7.92E-07	Putative gag-pol polyprotein, identical [*Solanum demissum*]
Unigene57265	-2.97	3.89E-09	1.52E-26	RecName: Full=Alpha-glucosidase; AltName: Full=Maltase; Flags: Precursor

Table 3. Predicted functions of 24 genes differentially expressed in sugarbeet roots treated with JA 10 µM in the comparison of water treated controls at 60 days post treatment

Unigene ID	Log Fold Change	P value	E value	Predicted function
Unigene41641	31.32	1.04E-12	1.67E-17	Orf764 [*Beta vulgaris* subsp*. vulgaris*]
Unigene40247	5.65	9.64E-11	5.03E-22	Orf764 [*Beta vulgaris* subsp. *vulgaris*]
Unigene18654	5.39	5.56E-19	2.26E-49	Orf764 [*Beta vulgaris* subsp*. vulgaris*]
Unigene357	5.38	7.96E-25	7.76E-35	Orf764 [*Beta vulgaris* subsp. *vulgaris*]
Unigene996	5.28	4.01E-14	6.74E-43	Pol-polyprotein [*Silene latifolia*]
Unigene6711	5.09	2.48E-16	9.62E-13	Pol-polyprotein [*Silene latifolia*]
Unigene28794	4.82	3.46E-26	1.10E-82	Orf764 [*Beta vulgaris* subsp*. vulgaris*]
Unigene4931	2.69	7.30E-07	1.02E-14	Chalcone synthase-like protein [*Pinus strobus*]
Unigene4459	2.48	8.66E-06	1.78E-19	RecName: Full=Albumin-2; AltName: Full=PA2
Unigene60876	2.35	7.72E-06	2.86E-07	Retrotransposon protein, putative, unclassified [*Oryza sativa* Japonica Group]
Unigene22838	2.32	1.36E-12	1.50E-41	CBF4a [*Populus hopeiensis*]
Unigene12865	2.18	4.44E-05	3.26E-13	Flavonol 4'-sulfotransferase, putative [*Ricinus communis*]
Unigene59479	2.09	3.32E-06	6.46E-49	Xyloglucan endotransglucosylase [*Beta vulgaris* subsp*. vulgaris*]
Unigene45666	2.07	7.47E-07	4.96E-25	Chalcone synthase [*Paeonia suffruticosa*]
Unigene11290	-2.11	2.34E-05	4.00E-11	Glycosyltransferase [*Panax notoginseng*]
Unigene81204	-2.12	9.30E-12	1.19E-139	Sulfate transporter, putative [*Ricinus communis*]
Unigene8409	-2.22	8.46E-09	2.61E-84	Peroxidase [*Beta vulgaris*]
Unigene48268	-2.35	7.72E-06	4.16E-24	RecName: Full=NAD(P)H:quinone oxidoreductase; Short=NAD(P)H:QR
Unigene5082	-2.38	9.14E-15	4.15E-08	RecName: Full=Defensin-like protein AX2; AltName: Full=Antifungal protein AX2
Unigene48703	-2.38	1.31E-08	1.87E-24	high affinity sulfate transporter SAT-1, Sulfate/bicarbonate/oxalate exchanger SLC26 family protein [*Populus trichocarpa*]
Unigene20294	-2.63	1.32E-15	3.68E-41	O-methyltransferase [*Vitis vinifera*]
Unigene69228	-2.67	9.36E-06	5.77E-34	Serine/threonine-protein kinase, putative [*Ricinus communis*]
Unigene28329	-2.88	4.68E-12	2.40E-52	FAD-binding domain-containing protein [*Arabidopsis lyrata* subsp*. lyrata*]
Unigene77351	-4.12	2.96E-28	6.29E-58	CTV.20 [*Citrus trifoliata*]

4 DISCUSSION

Changes in expression of defense-related genes have been frequently associated with exogenous applications of jasmonates in many plant species and organs (Yaqoob et al., 2012; Belhadj et al., 2007; Baileya et al., 2005). In sugarbeet roots, the effect of treatment with 10 µM JA on the expression of PAL, PR1, CHI 1, CHI 3 and POD genes was relatively small and transient with the time. PAL and POD expression were induced by JA. PAL was induced 3.5-fold compared to the control at 2 d after treatment and did not exceed two times the control for any other treatment time point. Belhadj et al. (2007) also observed a transient increase in PAL expression as an effect of jasmonate treatment in grapevine cell cultures. PAL transcriptional changes, however, did not cause detectable increases in PAL enzyme activity (Chapter 2). Transcriptional changes, however, are often unrelated to protein or activity changes. Cell wall peroxidase expression was induced at 1, 2 and 30 d after treatment. Moore et al. (2003) postulate that JA acts as a signalling molecule in a long-distance pathway responsible for inducing resistance to future attack through increases in cell wall bound peroxidase activity leading to cell wall toughening. Cell wall peroxidases catalyze lignin and suberin synthesis (Espelie et al., 1986; McDougall, 1991) and are believed to be involved in plant defense by strengthening the cell wall and restricting pathogen ingress (Kawano, 2003). PR1 expression was inhibited by JA at 3, 10 and 60 d after treatment. Salicylic acid induces PR1 gene expression (Durner et al., 1997), and inhibition of PR1 expression in sugarbeet roots may be due to antagonism between SA and JA pathways (Niki et al., 1998). Chitinases are up-regulated by a variety of stress conditions and phytohormones including ethylene, jasmonic acid, and salicylic acid (Busam et al., 1997). Contrasting to this, in sugarbeet roots, chitinase 1 transcript levels were inhibited by JA treatment at 3 and 10 d after treatment and chitinase 3 expression was inhibited at 1d after treatment and induced at 60 d after JA treatment. JA treatment also had an inhibitory effect in sugarbeet roots chitinase activity.

We also used RNA sequencing to identify additional genes that were altered in expression by JA and may contribute to induced disease resistance.

Since the genome of sugarbeet is not publically available, a transcriptome reference library was prepared from RNA extracted from different tissues at different developmental stages, and after JA or SA treatment to maximize the diversity of expressed genes. A total of 82404 unigenes with 32889791 nucleotides were assembled. Many of the sugarbeet unigenes were assigned to GO categories and COG classifications, although most unigenes were not assigned an identity by a Blastnr search. The large number of sequences generated in this study provides valuable sequence information at the transcriptomic level for novel gene discovery, This reference transcriptome was then used to identify genes that were altered in expression by JA treatment using samples collected 2 and 60 d after treatment. At 2 and 60 d after treatment, 120 and 104 unigenes were differentially expressed between roots treated with JA or water controls. From these sequences, only a small percentage had matches when compared to gene databases. The majority of the unigenes presented no hits or where matched to unknown sequences. JA led to both up-regulation and down-regulation of genes. Among the 2 d known sequences, three up-regulated unigenes were identified as peroxidases and 1 up-regulated gene was identified as an acidic chitinase. These genes are different from the POD, CH1 and CH3 studied with the qRT-PCR technique. Peroxidases and chitinases have been implicated in jasmonate-induced disease resistance in many plant species and organs (Yaqoob et al., 2012; Belhadj et al., 2008). Distinct to the RNA-seq results, *Beta vulgaris* acidic class III chitinase (chitinase 3) expression studied using qRT-PCR technique was not affected significantly by JA at 2d after treatment. Among the 60 d known sequences, two chalcone synthase sequences were up-regulated by JA. Chalcone synthase is a key enzyme for production of flavonoids and has been related to jasmonates induced defense in plants (Ryder et al., 1984; Campos et al., 2003; Richard et al., 2000). One down-regulated unigene was identified as a peroxidase that is differente from the gene studied with q-PCR technique, and a defensin-like gene was also down-regulated by JA at 60 d. Clearly JA affects gene expression in sugarbeet root for a relatively long time after treatment.

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6 CONCLUSÃO GERAL

Tratamento com ácido AJ reduziu o grau de podridões causadas por *Botrytis cinerea, Penicillium claviforme* e *Phoma betae*, três patógenos comuns na pós-colheita de raízes de beterraba açucareira. Ácido jasmônico nas concentrações de 0.01-100 µM reduziu a podridão causada por *B. cinerea* e *P. betae* em média de 51 e 71% respectivamente. Concentrações de 0.01–10 µM reduziram a podridão causada por *P. claviforme* em uma média de 44%, enquanto 100 µM reduziu a podridão em 65%. O tratamento com ácido jasmônico reduziu a podridão por diminuir a progressão dos sintomas em raízes de beterraba armazenadas, mas não teve efeito na incidência da infecção. Relativamente a outros produtos pós-colheita, raízes de beterraba-açúcareira mostraram sensibilidade incomum ao tratamento com jasmonatos, que as protegeram contra ataque de fungos patogênicos em uma vasta gama de concentrações.

Mecanismos de defesa antioxidante e enzimas relacionadas à defesa contra patógenos se mostraram de maneira geral pouco afetados por AJ. Raízes tratadas com ácido jasmônico apresentaram pequenos declínios nas atividades da superóxido dismutase (SOD) e quitinase e, de maneira geral, não afetaram as atividades da ascorbato peroxidase (APX), catalase (CAT), peroxidase (POD), β-Glucanase (β-Gluc), polifenoloxidase (PPO) e fenilalanina amônia-liase (PAL) ou a concentração de compostos antioxidantes. De forma geral, pode-se dizer que todas as enzimas com excessão da PAL foram afetadas pelo tempo de armazenamento. Atividades da POD, SOD, β-Gluc, quitinase, e PPO foram aumentadas e APX CAT tiveram suas atividades inibidas em raízes armazenadas por 10 dias ou mais. A não indução nas atividades das enzimas ou metabolitos relacionados à defesa contra estresse oxidativo ou relacionados a defesa contra patógenos sugere que a resistência induzida por ácido jasmônico em raízes de beterraba açucareira não provém de um aumento direto nas enzimas ou compostos estudados. As mudanças na atividade das enzimas relacionadas com o tempo de armazenamento sugerem também que essas enzimas não estão envolvidas na resistência a patógenos

porque muitas dessas enzimas aumentaram em atividade com armazenamento prolongado quando a resistência a patógenos geralmente declina.

A expressão de genes relacionados à defesa de plantas foi afetada de maneira transiente com pequenas alterações. A expressão da fenilalanina amônia-liase (PAL) e peroxidase (POD) foram transientemente induzidos por AJ. Em contraste, PR1 e quitinase 1 foram transientemente inibidas pelo tratamento e a quitinase 3 exibiu uma redução e um aumento nos níveis de transcritos.

As novas tecnologias de sequenciamento de RNA ou RNA-seq, denominadas de tecnologias de sequenciamento de nova geração se mostraram uma ferramenta interessante no estudo do transcriptoma da beterraba açucareira, gerando uma grande quantidade de dados inéditos que poderão também ser usados em estudos futuros.

ANEXO A

Minimum (min), maximum (max) and average (avg) of ascorbate peroxidase (APX), catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), β-glucanase (β-Gluc), chitinase (CHI), polyphenol oxidase (PPO) and phenylalanine ammonium-lyase (PAL) activities in the internal and peripheral tissues of sugarbeet roots, during 60 d incubation at 20 °C and 90% relative humidity. Values were obtained from averages of each time point per treatment.

enzyme	tissue	activity		
		min	max	avg
APX (mmol min^{-1} μg^{-1} protein)	internal	0.18	0.55	0.43
	peripheral	0.19	0.47	0.35
CAT (µmol min^{-1} μg^{-1} protein)	internal	2.9	6.5	4.9
	peripheral	5.1	13	9.0
POD (mmol min^{-1} μg^{-1} protein)	internal	0.23	0.40	0.28
	peripheral	0.30	0.55	0.40
SOD (U min^{-1} mg^{-1} protein)	internal	6.9	32	15
	peripheral	7.3	18	13
β-Gluc (nmol h^{-1} μg^{-1} protein)	internal	4.0	48	13
	peripheral	5.1	19	10
CHI (Abs min^{-1} mg^{-1} protein)	internal	0.11	0.26	0.15
	peripheral	0.080	0.24	0.12
PPO (ΔAbs h^{-1} μg^{-1} protein)	internal	0.24	0.45	0.31
	peripheral	0.20	0.42	0.28
PAL (nmol h^{-1} μg^{-1} protein)	internal	0.013	0.035	0.025
	peripheral	0.0040	0.044	0.021