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PII: S0168-9452(15)30111-4
DOI: http://dx.doi.org/doi:10.1016/j.plantsci.2015.12.001
Reference: PSL 9333

To appear in: *Plant Science*

Received date: 20-7-2015
Revised date: 19-10-2015
Accepted date: 1-12-2015

Please cite this article as: Maria Antonia Henriquez, Atta Soliman, Genyi Li, Abdelali Hannoufa, Belay T.Ayele, Fouad Daayf, Molecular cloning, functional characterization and expression of potato (Solanum tuberosum) 1-deoxy-D-xylulose 5-phosphate synthase 1 (StDXS1) in response to Phytophthora infestans, Plant Science http://dx.doi.org/10.1016/j.plantsci.2015.12.001

Molecular cloning, functional characterization and expression of potato (*Solanum tuberosum*) 1-deoxy-D-xylulose 5-phosphate synthase 1 (*StDXS1*) in response to *Phytophthora infestans*

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Highlights

1- DXS expression varies in response to late blight
2- Accumulation of isoprenoids is correlated with DXS expression
3- DXS targets the chloroplast envelops
4- DXS expression has a negative effect on ABA and GA

ABSTRACT

1-deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes the initial step of the plastidial 2*C*-methyl-D-erythritol-4-phosphate (DOXP-MEP) pathway involved in isoprenoid biosynthesis. In this study, we cloned the complete cDNA of potato *DXS* gene that was designated *StDXS1*. *StDXS1* cDNA encodes for 719 amino acid residues, with MW of 77.8 kDa, and is present in one copy in the potato genome. Phylogenetic analysis and protein sequence alignments assigned StDXS1 to a group with DXS homologues from closely related species and exhibited homodomain identity with known DXS proteins from other plant species. Late blight symptoms occurred in parallel with a reduction in *StDXS1* transcript levels, which may be associated with the levels of isoprenoids that contribute to plant protection against pathogens. Subcellular localization indicated that StDXS1 targets the chloroplasts where isoprenoids are synthesized. Arabidopsis expressing *StDXS1* showed a higher accumulation of carotenoids and chlorophyll as compared to wild type controls. Lower levels of ABA and GA were detected in the transgenic *DXS* lines as compared to control plants, which reflected on higher germination rates of the transgenic *DXS* lines. No changes were detected in JA or SA contents. Selected downstream genes in the DOXP-MEP pathway, especially *GGPPS* genes, were up-regulated in the transgenic lines.

Keywords: *Solanum*; DXS; *Phytophthora infestans*; DOXP-MEP; jasmonic acid; salicylic acid; abscisic acid; carotenoids; terpenoids; functional analysis.

Introduction

Potato (*Solanum tuberosum L.*) is the most important food crop from the *Solanaceae* family [1] and the fourth after maize, wheat and rice (http://faostat.fao.org), with an annual production of more than 323 million tonnes. Late blight, caused by *Phytophthora infestans* (Mont) de Barry is the most important disease of this crop worldwide [2, 3]. This disease can be initiated either by sporangia or zoospores, and affects leaves, stems, and tubers [4].

Several approaches have been used in disease management of fungal and oomycete pathogens [5, 6]. In the case of late blight, the most practical method would be the use of resistant varieties. However, developing stable resistance to late blight in potato is complex. While specific resistance genes are known, they are rapidly overcome by mutants in local populations of *P. infestans* [7]. Therefore, while awaiting more public acceptance for genetically-modified potatoes, a better understanding of the mechanisms governing the interaction between potato and *P. infestans* remains a requirement for further development of durable resistance to late blight. In this context, the most aggressive strains of *P. infestans* [8] were shown to suppress potato defense mechanisms, through transcriptional inhibition of phenylpropanoid (PAL) and isoprenoid (Ac-MVA) pathways [9, 10], both of which are otherwise powerful defense tools.

Isoprenoids are all derived from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) [11]. Until recently, the mevalonate (Ac-MVA) pathway was considered as the only route for the synthesis of isoprenoid precursors in all organisms [12]. However, the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway, also called the non-mevalonate route or DOXP-MEP pathway was discovered as an alternative terpenoids' biosynthetic route, first in eubacteria [13] and soon after in photosynthetic organisms such as higher plants, algae as well as in cyanobacteria and diatoms [14]. The DOXP-MEP pathway also occurs in the malaria parasite *Plasmodium falciparum* [15] but not in humans, animals or archaebacteria. Therefore, it was suggested as a perfect target to develop herbicides and antibacterial drugs [16].

The DOXP-MEP pathway consists of eight reactions catalyzed by nine enzymes, seven of which have been characterized structurally [17]. Briefly, 1-deoxy-D-xylulose 5-phosphate obtained by

condensation of pyruvate and D-glyceraldehyde 3-phosphate go through a reorganization associated with a reduction step. The resulting 2*C*-methyl-D-erythritol 4-phosphate is transformed into its cyclic diphosphate by the action of three enzymes. The, 2*C*-Methyl-D-erythritol 2,4-diphosphate is transformed into IPP and DMAPP via 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate [18].

The first step of the DOX-MEP pathway is a trans-ketolase decarboxylation of pyruvate and glyceraldehyde 3-phosphate (GA-3P), catalyzed by 1-Deoxy-D-xylulose-5-phosphate synthase (DXS), and produces 1-Deoxy-D-xylulose-5-phosphate (DXP) [19, 20], followed by several enzymatic reactions to produce IPP and DMAPP precursors [21, 22]. In plants, DXS is encoded by a small gene family, unlike most enzymes of the MEP-pathway, which are encoded by single-copy genes [23, 24]. Genes encoding DXS are classified into three groups. The first group represents *DXS1,* with a major housekeeping function, *i.e.*, *AtDXS1* (*CAL1*) gene in Arabidopsis [25]. The second group, which includes *DXS2* genes, was suggested to have a correlation with carotenoid accumulation during mycorrhization [26], *i.e.*, *DXS2* in maize [27] and *Medicago truncatula* (*MtDXS2*) [28]. This group is assumed to be involved in defense responses and secondary metabolites' accumulation [29]. Members of the third group have been identified in some species including maize [27] and rice [29].

Regulation of DXS was reported on both transcriptional and post-transcriptional levels [24]. Different factors control *DXS* response to regulation such as light, development stages and sugars [30], whereas the post-transcriptional level has specific metabolic demands and level of IPP and DMAPP precursors [31, 32]. To date, none of the DOXP-MEP pathway genes have been identified in potato. The presence of the *StDXS1* gene from potato was reported in 2002 by Walter et al. [26]. However, they only used virtual transcripts or tentative consensus-TC of *Solanum tuberosum* originated from the TIGR gene indices to present a tree and alignment data.

In a previous study, we reported the effects of a glucan suppressor from *P. infestans* and eicosapentaenoic acid (EPA) on potato defense responses (33), which included both phenylpropanoid and mevalonate (Ac-MVA) pathways. The application of EPA had an induction effect on two tested potato cultivars in response to inoculation with a genotype US-8 isolate of *P.*

infestans, thereby reducing symptoms of late blight. Such an effect was associated with the expression of *PAL-1* and *PAL-2*. The glucan fraction did not by itself suppress phenylpropanoid genes, but its combination with the pathogen resulted in a down-regulation of *PAL-1*, *PAL-2* and chalcone synthase gene (*CHS*). Expression levels of hydroxymethylglutaryl Co-A reductase (*HMGR*), *HMGR3*, squalene synthase (*SQS*) and sesquiterpene cyclase (*SC*) were not affected in any of the tested treatments, except for *SC* transcripts which were down-regulated in the susceptible variety inoculated with the US-8 isolate and the one that received both inoculation and treatment with the glucan fraction. The specific down-regulation of *SC* in the treatments showing most symptoms confirmed the association of sesquiterpenoid compounds with potato defense to *P. infestans*, and raised questions about the part that the DOXP-MEP pathway may be playing in plant responses to biotic stress.

In order to evaluate the potential association of the DXS gene (key enzyme of the DOXP-MEP pathway for terpenoid biosynthesis) with the interaction between potato and the oomycete *P. infestans*, we (i) cloned and characterized a 2,421 bp cDNA that contains a complete ORF (2,160 bp) from DXS, (ii) analyzed the expression of this gene using real-time qPCR in a potato cultivar inoculated with two strains of *P. infestans* (i.e., weakly vs. highly aggressive), and/or infiltrated with eicosapentanoic acid (EPA) or glucans (GL), and (iii) functionally characterized this gene in *Arabidopsis thaliana*. Based on the phenotypes of transformed *A. thaliana* plants, further tests were done to assess the effects of DXS on chlorophylls, carotenoids, abscisic acid (ABA), gibberellic acid (GA), jasmonic acid (JA), and salicylic acid (SA).

1. Material and methods

2.1. Plant material

Potato cultivar Russet Burbank was grown in clay pots containing soil-sand-peat-perlite mixture (4:4:4:1) and kept in a growth room at 20°C with photoperiod16/8 h day/night. Arabidopsis (*Arabidopsis thaliana)* plants (ecotype; Columbia) were grown in growth cabinet under controlled conditions at 20°C with photoperiod 16/ 8 hrs day/night and relative humidity was 60%.

2.2. Phytophthora infestans strains and inoculation

Two *Phytophthora infestans* strains were used in this study. The isolate D-03 (lineage US11, weakly aggressive) and D1901 (lineage US8, highly aggressive) were grown on rye agar supplemented with 2% sucrose at 18°C. Potato plants were inoculated with a sporangia suspension (4 X 10^4 sporangia/mL), and with water for non-inoculated control plants following the method described by Wang et al.[9]. This resulted into two inoculation treatments; (A) RB+US8 and (B) RB+US11, , plus control; non-inoculated Russet Burbank (RB+H_2O).

The primary leaflet of the fourth fully-grown potato leaf in each stem of 8-wk-old Russet Burbank plants was treated as reported by Henriquez et al. [33]: 1) 100 µl of 0.2 µM eicosapentanoic acid (EPA) applied on the leaflet 2) 100 µl of glucans from race C (1, 4, 10, 11; Mating type A2) (200 µg in 100 µl of water as emulsion), 6 hours after EPA treatment, 3) Ten 10-µl drops of *P. infestans* US8 spore suspension (4X 10 4 sporangia /ml) 12 hours after EPA application. Control plants were treated with sterilized distilled water. Three replicates per treatment were performed, where each pot containing three potato stems was considered a replicate. The experiment was repeated once.

2.3. Cloning of DXS ORF

A 2421 bp cDNA (GenBank ID: GU936657) that contains a complete *DXS* ORF (2160 bp), was cloned from Russet Burbank using the primer pair DXS1-F (5`-CACCAACACACCCCACTAGA -3`) and DXS1-R (5`-AAGAGGAGGGCTTGAACTCAG -3`) designed through the conserved sequence of the known DXS in *Lycopersicum esculentum* (Genbank ID: AF143812). RNA from Russet Burbank was extracted with RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and genomic DNA was digested in column using RNase-Free DNase set (Qiagen). The cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kits (Fermentas, Thermo Scientific, USA). The PCR was performed using Phusion high fidelity DNA polymerase (Thermo Scientific, USA) according to the manufacturer's instructions. The PCR product was A-tailed using Dream taq DNA polymerase. The nucleotide sequence of *DXS1* was verified by cloning the PCR product into the bacterial plasmid pGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions and propagated in *E. coli* DH5α

strain. The recombinant plasmid was extracted and sequenced using the pGEM-T Easy universal primers, T7 and SP6 (Macrogen, Maryland, USA).

2.4. qRT-PCR and data analyses

For measurement of transcript abundance, five micrograms of cDNA at nine different times (1, 6, 9, 12, 24, 48, 72, 96, 120 hpi) after infection and inoculation with sterile water used as a control, were treated with Deoxyribonuclease I (Invitrogen) in order to preserve the integrity of RNA by degrading any possible residual genomic DNA. The DNase-treated RNA was converted to cDNA in a 50 µl reverse transcription modifying the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations, then diluted 1:3 with sterile water. Quantitative Real time PCR (qRT-PCR) was performed using the IQ SYB Green Supermix (Biorad), in 20 µl containing 2 µl diluted template, 6.5 µL of IQ SYB Green Supermix, and 0.375 µM of each primer dxs-F 5'GCATTTCCTGGGATTTTGAA-3' and dxs-R 5'TTGGCGGTCTCTGTGTGTAG-3' (GenBank ID: GU936657). Gene expression was quantified using a Stratagene Mx3005p cycler, and the thermocycle program included 95°C (2 min), followed by 40 cycles of 95°C (15 s), 50°C (45 s) and 72°C (45 s). Melt-curve analysis was performed to observe primer-dimer formation and amplification of gene-specific products. All PCR reactions were performed from triplicate biological samples. The $2^{\wedge-\Delta\Delta}C_T$ method [34] was used to calculate the fold expression relative to the controls. In addition to non-template (water controls), non-RT controls were used as templates to detect the presence of genomic DNA contamination. The elongation factor 1-alpha gene (EF1α) was used as a reference gene for gene expression normalization using the primers Efactor-F 5'- GATGGTCAGACCCGTGAACAT -3' and Efactor-R 5'-GGGGATTTTGTCAGGGTTGT-3' (180 bp) (Genbank accession number; AB061263).

2.5. Plasmids construction for Arabidopsis transformation

The binary vectors pK2GW7.0 and pEarleyGate101 (Gateway based plasmid) were used to clone the *StDXS1*. The binary vector PK2GW7.0 was used to overexpress the *StDXS1* under the control of 35S promoter and 35S terminator, while pEarleyGate101 expresses *StDXS1* fused with *YFP* in Arabidopsis protoplasts. The *DXS1* gene was amplified from pGEM-T-easy plasmid using gene specific primers flanked by restriction sites for KpnI and XhoI in the forward and reverse

primers, respectively. After PCR purification and digestion, the digested PCR product was cloned into the entry plasmid pENTR-1A (Gateway Tech., Invitrogen, USA), followed by LR clonase reaction (Invitrogen, USA) according to the manufacturer's instructions. The stop codon was eliminated from *DXS1* ORF during amplification for cloning into pEarleyGate101 to produce an ORF contains *DXS1* and *YFP*.

2.6. Bacterial strains and transformation procedures

Two bacterial strains were used to clone *DXS1* in two different constructs for pK2GW7::*DXS1*. These constructs were introduced to *E. coli,* ElectroMax10B strain by electroporation method for propagation and maintenance. After propagation, the recombinant plasmids were isolated using plasmid extraction kit (Qaigen). The binary plasmid was electroporated into *Agrobacterium* GV3101 strain. *Agrobacterium* cells harboring the recombinant plasmid was used for Arabidopsis transformation implementing the floral dip method according to Clough and Bent [35].

2.7. Subcellular localization of StDXS1

Subcellular localization of StDXS1 fused with YFP was transiently expressed in *Arabidopsis thaliana* protoplasts. Arabidopsis protoplasts were transfected by the recombinant plasmid; pEarleyGate101::*DXS1* using PEG-mediated transformation approach according to Sheen' lab protocol [36]. The *DXS1-YFP* expression was examined using confocal scanning laser microscope (Zeiss, Oberkochen, Germany). Excitation wavelengths of 488 nm and broad pass 505-530 nm, and 488 nm and long pass 650 nm were used for DXS1::YFP and chlorophyll, respectively. FITC was detected with excitation of 495 nm and broad pass of 528 nm according to the manufacturer's instructions.

2.8. Chlorophyll content

Chlorophyll content was measured in 2-week old seedlings of the Arabidopsis transgenic and wild type lines. Chlorophyll was extracted from ground leaf tissues (0.5 g) in extraction solvent containing 80% acetone and 0.2M Tris-HCL pH 8.0 for 24 h in dark at 4ºC. Chlorophyll a and b

were measured on spectrophotometer with absorbance of 645 and 662 nm. The calculation was performed according to Inskeep and Bloom [37].

2.9. Quantification of GAs and ABA

The levels of ABA and GA were quantified from mature seeds, leaves and siliques (~300 mg fresh weight) in the Arabidopsis transgenic and wild type lines. Extraction and purification of ABA and GAs, and quantification of their respective levels were performed as described previously [38, 39], except that the final elution of the extracts was performed with 80% acetonitrile containing one percent acetic acid, and the amounts of ABA and GA in the extracts were measured with LC-ESI-MS/MS system (Agilent 1260-6430 system).

2.10. Carotenoids extraction and analysis

Carotenoids were extracted from mature seeds and 2-week old seedlings of the Arabidopsis transgenic and wild type lines. MS-agar grown seedlings (50 mg) and 150 mg mature seeds were used for carotenoids analysis. The procedures for carotenoids extraction and HPLC analysis were conducted according to Yu et al. [40].

2.11. Jasmonic and salicylic acids extraction and quantification

Jasmonic and salicylic acids were analyzed using modified protocols by Pan et al. [41] and Nawrath and Métraux [42], respectively. Arabidopsis ground tissues (500 mg) from 2-weeks old seedlings were used for quantifying JA and SA in the transgenic StDXS1 (T3) and wild type control. Extraction solvent (5mL) containing; 2-Propanol/H_2O/HCL (2:1:0.002) was added to the tissues, and 9,10 dihydrojasmonic acid (DHJA) /o-Anisic acid (OAA) (1 µg/mL) was added as internal standard. The samples were shaken at 4ºC for 30 min followed by addition of Dichloromethane (7mL). The samples were centrifuged at 2500 rpm for 5 min, and the lower phase was transferred to a new tube and dried under N_2 on ice. The sediments were dissolved in 500 µL 50% Methanol, and then filtered and stored at -20ºC. Bound Salicylic acid was hydrolyzed by adding equal volume of 8N HCL to the upper phase and then heated at 100°C for 2 h. The samples were extracted by equal volume of Ethyl acetate (2X), transferred to a new tube and dried under N_2 stream and re-suspended in 500 µL 50% Methanol. All samples were filtered through 0.2µm PTFE prior to injection. Standard Jasmonic acid (Sigma J2500) and Salicylic acid (Sigma 247588) were prepared with the internal standards to generate a calibration curves and

response factors. The samples were run on UPLC-MS/MS (Waters Acquity UPLC with Micromass Quattro micro API in ESI mode) (Waters Limited Mississauga, Ontario). The separation of compounds was achieved by injecting 10µl of sample and standards onto a Kinetex XB-C18 column (100 x 2.1 mm, 1.7µm, Phenomenex, Torrance, CA) held at 30°C and eluted at a rate of 0.22 $ml.min^{-1}$ with a solvent gradient consisting of water with modifier methanol both of which contained 0.1% formic acid as follows: Time in min / % Methanol: 0/30, 2/30, 12/100, 13/100, 15/30. These compounds were monitored by Multiple-Reaction-Monitoring (MRM) with settings is shown in Table 1.

2.12. Quantitative expression analysis in A. thaliana using qRT-PCR

Total RNA was extracted from 3-week old Arabidopsis seedlings of transgenic and wild type plants. The tissues were immediately frozen in liquid nitrogen and ground to a fine powder and then RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The genomic DNA was digested in columns using RNase-Free DNase I Kit (Qiagen). The cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kits (Fermentas, Thermo Scientific, USA). Quantitative Real-Time RT-PCR was performed using C1000 Thermal Cycler (Bio-Rad, CA, USA) and SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. The expression analysis was performed using primer sets shown in Table 2. The results were analyzed using $2^{\wedge-\Delta\Delta}C_T$ method [34]. The elongation factor 1-alpha gene (EF1α) was used as a reference gene for gene expression normalization using the primers E factor-F 5'- GATGGTCAGACCCGTGAACAT -3' and E factor-R 5'- GGGGATTTTGTCAGGGTTGT-3' (180 bp) (Genbank ID: AB061263). Before using the $2^{-\Delta\Delta}C_T$ method, we confirmed that both target (y = -3.7247 + 44.4; R^2= 0.9793) and reference (y = -3.7261 + 45.231; R^2= 0.9909) genes amplified with efficiencies within 5% of each other.

2.13. Germination assay of Arabidopsis expressing StDXS1

Arabidopsis seeds were stratified in water at 4ºC for 2 days, and then surface sterilized with 20% commercial bleach with a few drops of tween-20 for 10 min. Seeds were rinsed several times with autoclaved water and plated onto MS medium (1x basal salt mixture (Invitrogen, USA), 15 g/L sucrose, 0.5 g/L MES, and 3.5 g/L phytagel, pH 5.8). Plates were sealed with parafilm and incubated at room temperature (23ºC) under photoperiod 16 h light / 8 h darkness, and monitored

for germination daily. The germinated seeds were scored against the total seeds overtime. The graph represented the mean values ±SE (n=3).

2.14. Bioinformatics analysis

DXS protein sequences were collected using the BLASTp program [43] and were aligned using the ClustalX program [44]. The phylogenetic tree was generated using the MEGA 6 program [45].

2.15. Statistical analysis

The statistical analyses were performed with the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC; release 9.1 for Windows). One-way ANOVA analysis was performed using PROC GLM. Treatment means were separated using the Tukey's Studentized Range test. A $p<0.05$ was considered to indicate significant differences.

2. Results

3.1. Cloning of StDXS1

Using cDNA from the potato cultivar Russet Burbank as template and primer pairs flanking the ORF region from the conserved sequence of the *DXS* gene from *Lycopersicum esculentum* (GenBank ID: AF143812), a band of size 2,421-bp was produced, and named *StDXS1*. The nucleotide sequence of *StDXS1* was submitted to the GenBank database (GenBank ID: GU936657). The cloned fragment encoding for a putative DXS1 had an open reading frame of 2,160-bp starting with an initiation codon ATG at the position 133 and ending with a stop codon TAA at position 2,289. The protein encoded by this cDNA has 719 amino acid residues with a predicted molecular mass of 77.8 kDa and a deduced isoelectric point of 6.3.. The amino acids sequence alignment revealed that StDXS1 had high similarity with other published DXS proteins from higher plants *Lycopersicon esculentum* (LeDXS, AF143812, 99% identity), *Nicotiana tabacum* (NtDXS, FN429979, 93% identity), *Medicago truncatula* (MtDXS1, AJ430047, 83% identity, and MtDXS2, AJ430048, 66% identity), *Ricinus communis* (RcDXS, XM_002516797, 85% identity), *Hevea brasiliensis* (HbDXS, AY502939, 84% identity), *Zea mays* (ZmDXS, EF507248, 81% identity), *Oryza sativa* (OsDXS, NM_001062059, 81% identity), *Catharanthus*

roseus (CrDXS, AJ011840, 67% identity) and *Mentha piperita* (MpDXS, AF019383, 64% identity). The blast search and multiple alignments strongly suggested that StDXS1 has high homology with other DXSs and probably a functional plant DXS protein. In addition, plant DXS proteins contain an N-terminal domain which is not present in bacteria and feature a plastid targeting sequence [20]. Our *StDXS1* clone is predicted to encode a DXS enzyme, based on the heterologous functional expression and the very high score of 99% identity of a 719 amino-acid sequence from *Lycopersicon esculentum* (LeDXS; AF143812). LeDXS had demonstrated *in vivo* activity and targeted plastids [20]. *Solanum tuberosum* genome has a single copy of *DXS1* based on the potato genome database (http://potato.plantbiology.msu.edu).

3.2. Phylogenetic analysis of DXS amino acid sequences

The phylogenetic analysis of several DXS amino acid sequences (Fig. 1) of *Solanum tuberosum* (Genbank ID: NP-001275130.1) and other plant species was performed to estimate the evolutionary distances between StDXS1 and other orthologs. Neighbor-Joining tree was produced using Mega6 program. The phylogenetic tree classified the DXS homologues into two groups. The first group was dedicated to DXS1 homologues, while the second group included both DXS2 and DXS3 homologues. StDXS1 was assigned to the first group with closely related species such as *S. lycopersicum* and other DXS1 homologues from mono- and dicotyledonous species. Some DXS members have been functionally characterized in different species, such as *S. lycopersicum* (SlDXS1) [20], *Zea mays* ZmDXS1 [27], and the well-characterized DXS1/CLA1 in Arabidopsis [46]. In addition, DXS2 and DXS3 homologues have been identified and characterized from the second group such as, maize DXS2 and DXS3 [27], and SlDXS2 in tomato [47]. It has been reported that some DXS2 members were involved in particular secondary metabolites synthesis such as apocarotenoids, which have a correlation with mycorrhizal fungi colonization in roots [28].

3.3. Expression of StDXS1 under pathogen inoculation in potato

No symptoms were visible within the first 48 hpi on Russet Burbank inoculated with US-8 and only small lesions became noticeable at 72 hpi. In Russet Burbank inoculated with US-11, late blight symptoms were visible only at 120 hpi. After the initial symptoms, the US-8 isolate

caused spreading disease lesions and extensive tissue damage in Russet Burbank, whereas the US-11 isolate only caused small lesions (Fig. 2).

QRT-PCR analysis of *StDXS1* was performed on cDNA from potato leaf RNA samples extracted at nine time-points post-inoculation from RB+US8 and RB+US11. In order to eliminate possible variations due to experimental conditions, inoculation, plant development, or other factors, a parallel inoculation with sterile water was also evaluated as control for each time-point from non-inoculated Russet Burbank (RB+H_2O). *StDXS1* transcripts in RB+US8 were induced until 48 hpi (Fig.2), and decreased drastically thereafter compared to non-inoculated potato plants. The highest induction of this transcript occurred at 12 hpi. Similar transcript levels were induced in RB+US11, with a highest induction at 12 hpi, decreased thereafter and remained relatively constant from 24 to 120 hpi.

The potato cultivar Russet Burbank inoculated with US-8 (RB+US8) showed typical late blight lesions (Fig. 3), as well as potato plants pretreated with EPA and then inoculated with US-8 (RB+EPA+US8), plants treated with the glucan extract and then inoculated with US-8 (RB+GL+US8), and RB+EPA+GL+US8. The latest showed 80% of the leaf area affected by late blight disease. Fig. 4 shows a down-regulation of *StDXS1* transcripts in all treatments, except RB+GL, when compared with the RB+H_2O control.

3.4. Germination assays of A. thaliana overexpressing StDXS1

The transgenic seeds showed higher germination rate as compared to the wild type control on MS medium. The transgenic seeds reached full germination in two days, while it took three days for the wild type control seeds. The difference in the growth rate continued up to four weeks (Fig.4).

3.5. Quantitative Real-Time PCR analysis of StDXS1 overexpression in A. thaliana

The successful transformation of *StDXS1* was indicated by high expression of the transgene in Arabidopsis seedlings using qRT-PCR (Fig.5). Different genes in the DOXP-MEP pathway, downstream *DXS1* were tested using qRT-PCR. Our results showed that the expression of some genes increased in the transgenic lines as compared to the wild type control. The transcripts level

of geranylgeranyl diphosphate synthase 1 (*GGPPS1*), geranylgeranyl diphosphate synthase 2 (*GGPPS2*), *AtDXS1* and phytoene synthase (*PSY*), lycopene β-cyclase (*bLYC*), and lutein deficient 1 (*LUT1*) was significantly higher in the transgenic lines as compared to wild type control, while the transcripts level of zeaxanthin epoxidase (*ZEP*), phytoene desaturase (*PDS*) and β-carotene hydroxylase1 (*BCH1*) was not significantly different. The transcripts level of putative ζ-carotene desaturase (*ZDS*) and β-carotene hydroxylase 2 (*BCH2*) was significantly lower in the transgenic lines as compared to the wild type control (Fig.6).

3.6.Subcellular localization of StDXS1 in protoplasts of A. thaliana

Subcellular localization of the DXS1::YFP fusion protein showed that DXS1 protein targeted the chloroplast (Fig.7A). This result was validated by immunolocalization of DXS1::YFP; where the fluorescence of FITC was detected in the chloroplast, but not in any other cellular compartments (Fig.7B).

3.7.Chlorophyll and carotenoids content in transformed and wild type A. thaliana

Total chlorophyll was higher in the transgenic lines as compared to wild type control. Total chlorophyll was represented of both chlorophyll a and b, where chlorophyll a scored 10% increase, while chlorophyll b increased by 80%. Total chlorophyll increased by 23% as compared to wild type control (Fig. 8).

β-carotene and lutein contents in the seedlings of the transgenic lines were significantly higher as compared to wild type control (Fig.9). While, in the mature seeds, carotenoids content in the transgenic line seeds was significantly lower as compared to wild type control (Fig.10).

3.8. Jasmonic acid and salicylic acid content in transformed and wild type A. thaliana

Jasmonic acid in the seedlings of Arabidopsis transgenic lines and wild type control showed no significant differences (Fig.11). On the other hand, free salicylic acid was significantly higher in the wild type compared to transgenic lines, while there was no difference in the bound-salicylic acid among the tested lines (Fig.11).

3.9. Abscisic acid (ABA) and gibberellins (GA) contents in transformed and wild type A. thaliana

ABA content in the seedlings showed no significant differences between wild type and *DXS1* lines, while the ABA content in the siliques and mature seeds in the *DXS1* lines was significantly lower as compared to the wild type. The highest ABA content was observed in the siliques (Fig.12A). Moreover, GA content in the transgenic seedlings was significantly lower than the wild type (Fig.12C).

4. Discussion

The DOXP-MEP pathway is an alternative isoprenoid biosynthetic route in plants [48]. We isolated an open reading frame of 2,160-bp in length, from potato, encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which catalyzes the first step in the pathway. The first report of DOXP-MEP pathway regulation in a plant-pathogen interaction was reported by Walter et al. [49]. They reported root colonization of wheat, maize, rice and barley mycorrhizal fungi to involve the induction of DXS and DXR (1-deoxy-D-xylulose 5-phosphate reductoisomerase). Subsequently, Walter et al. [26] studied the expression of *MtDXS1* and *MtDXS2* in *Medicago truncatula* upon colonization of arbuscular mycorrhizas and Gil et al. [50] analyzed resistance of the Arabidopsis *cbs3* mutant to biotrophic pathogens. The presence of the *StDXS1* gene from potato was reported by Walter et al. [26]. However, they only used virtual transcripts or tentative consensus-TC of *S. tuberosum* as originated from the TIGR gene indices for a tree and alignment data. Therefore, the expression of those virtual transcripts were never confirmed or cloned in potato.

The phylogenetic analysis of the deduced DXS amino acid sequences from *Solanum tuberosum* and other plant species (Fig.1) indicated that DXS1 homologues are highly conserved among species, and share common conserved domains. Potato *DXS* was grouped with closely related homologues from different species such as *SlDXS1* from *S. Lycopersicon*. In Arabidopsis, the At4g15560 gene has been demonstrated to encode a functional DXS enzyme [46]. Also two additional genes with high homology to DXS sequences were identified in Arabidopsis [51]. Three paralogs for Maize *DXS* were identified [26, 52] and the recently discovered paralog *DXS3* in maize is also present in sorghum and rice species [52]. In comparison, a single copy of *DXS1* gene was detected in the potato genome.

Suppression of non-mevalonate or DOXP-MEP pathway genes in Russet Burbank, might lead to reduction in the accumulation of isoprenoids that participate in essential plant processes such as respiration, photosynthesis, regulation of growth and development as well as plant protection against pathogens [20]. In this research, late blight symptoms correlated with a decrease in *StDXS1* transcript levels. Initially, *StDXS1* transcripts in RB+US8 were increasingly induced until 12 hpi, with no visible disease symptoms, but decreased drastically thereafter, with a down regulation after 48 hpi, compared with the non-inoculated potato plants. The fact that late blight symptoms became noticeable at 72 hpi and increased drastically thereafter, causing extensive tissue damage, are in line with the ability of the US-8 genotype isolates to suppress terpenoid genes' transcription as shown with *HMG2* [10]. On the other hand, late blight symptoms were visible in Russet Burbank inoculated with the US-11 isolate only at 120 hpi. This late appearance of symptoms could be associated with the absence of downregulation of *StDXS1* in RB+US11, as compared with RB+US8, and is comparable to results with another isolate from the A1 mating type of *P. infestans* [9, 10].

The high up-regulation of *StDXS1* transcripts in RB+US8 occurred at 12 hpi, which coincides with appressoria formation, as previously reported in RB+US8 [53]. Appressorium maturation in *Colletotrichum lindemuthianum* was sufficient to induce plant defense responses such as superoxide ion production and strong induction of pathogenesis-related proteins [54]. However, the up-regulation of *StDXS1* in Russet Burbank did not seem to effectively help the plant stop the infection process. Similar results were obtained in Russet Burbank inoculated with the highly aggressive isolate US-8, where the high accumulation of the terpenoid T1 occurred at 12 hpi, but did not help the plant stop the infection process [53].

We previously reported that the mevalonate (Ac-MVA) pathway genes *HMGR*, *HMGR3* and squalene synthase (*SQS*) were not affected in potato in response to *P. infestans*, its tested effectors (EPA and glucans) or the combination of effectors and inoculum. However, sesquiterpene cyclase (*SC*) was down-regulated in RB+US8 and RB+GL+US8 [33]. In the present study, *StDXS1* was down-regulated in RB+US8 and RB+GL+US8, as well as in RB+EPA+US8 and RB+EPA+GL+US8, but not in RB+GL. Similarly, we have previously reported that the glucan fraction did not by itself suppress phenylpropanoid genes, but its

combination with the pathogen resulted in a down-regulation of *PAL-1*, *PAL-2* and *CHS* [33]. A reason for these observations could be that *P. infestans* might have endoglucanase inhibitor proteins that bind to endoglucanase isoforms and suppresses glucan elicitor-mediated defense responses. This kind of suppression was reported with *P. sojae* [54]. These results could also mean that late blight symptoms could be associated with a suppression of *StDXS1* that leads to a reduction in the accumulation of isoprenoids such as rishitinol, rishitin, lubimin, phytuberin, α-solanine, α-chaconine, solanidine, abscisic acid, cedrol and/or farnesol, which contribute to plant protection against pathogens [55, 56].

In the current study, Arabidopsis lines expressing potato DXS homolog were generated to investigate the effects of this gene on isoprenoid biosynthesis and further effects on the host's defense genes. The high level of potato DXS transcripts were confirmed in the transgenic lines by qRT-PCR as shown in lines DXS1-2, DXS1-3, DXS1-4, and DXS1-5 when compared to the wildtype (Fig. 5). Previous studies reported that DXS is a limiting factor in isoprenoid biosynthesis and leads to increased carotenoid content in the plastids and fruits of Arabidopsis and tomato plants, respectively [57 58]. Using subcellular localization, we provided evidence that StDXS protein targets the chloroplasts. Similar results were observed using DXS1-GFP in maize [27]. Up-regulation of GGPPS1 and GGPPS2 in the transgenic lines resulted into increased levels of the intermediate precursor GGPP. GGPPS is the first key gene in the carotenoids biosynthesis by producing the main precursor of this process [59]. Also, increasing the expression of Phytoene synthase (PSY) gene resulted in an increase of lutein and B-carotene in the Arabidopsis transgenic seedlings. The direct injection of 1-deoxy-d-xylulose 5-phosphate precursor into tomato fruits resulted in increased Phytoene synthase transcripts [20]. Similar results were obtained with overexpression of bacterial DXS in potato plants [60]. Interestingly, lutein and carotene content in the transgenic seeds was lower than the wild type control, which led to low ABA accumulation in the transgenic seeds, which might reflect on the increase in germination rates of the DXS transgenic seeds. Opposite results were obtained by Lindgren et al. [61], where overexpression of the endogenous PSY resulted in delaying germination by accumulation of high levels of ABA and B-carotene in the transgenic lines. On the other hand, there was no apparent effect of the expression of DXS gene on gibberellins' content in the transgenic lines. However, this may be a result of limitations in GA measurement. Our results

showed no significant impact of the expression of DXS gene on the JA or SA accumulation in both transgenic and wild type control.

Increase in plant carotenoids is associated with an increase in ABA and chlorophyll. Transgenic plants showed significantly higher germination rates as they accumulated less carotenoids, and subsequently low levels of ABA. This result is in a line with the results from the expression of Phyteone synthase (PSY) in Arabidopsis, where the transgenic seeds had delayed germination as ABA and carotenoids increased in the seeds [62]. ABA levels are associated with carotenoid levels in plants, where altering the carotenoid levels affected those of ABA in Arabidopsis cdc mutant [63]. Decreasing the carotenoids content in the transgenic seeds of Arabidopsis was associated with low ABA content, which translated into higher germination rates in the transgenic seeds. Carotenoids are intermediate precursors in the biosynthesis of the plant hormone ABA [64]. Expressing DXS in Arabidopsis resulted in higher chlorophyll contents in the transgenic seedlings. Carotenoids co-accumulate with chlorophyll in a pigment-binding protein complex embedded in the thylakoid membranes of the chloroplasts [65]. Arabidopsis carotenoids biosynthesis-deficient mutant cdc exhibits low carotenoids and chlorophyll contents due to developmental defects in the chloroplasts [63]. Similar results were observed in the Arabidopsis cla1 mutant that possesses a disrupted DXS gene, where it showed defects in the chloroplast development and accumulated low levels of carotenoids and chlorophyll [58, 66].

This work opens up new perspectives to direct further research into (i) novel functions of the DOXP-MEP pathway in plants in response to pathogens, (ii) DOXP-MEP regulation of defense signalling, and (iii) the involvement extent of this pathway, versus the mevalonic pathway, in terpenoid-related mechanisms of defense. Transgenic plants expressing DXS and the other DOXP-MEP genes should provide further evidence to determine the role of each enzyme, and their combination as a pathway, in plant-pathogen interactions.

Acknowledgements

Dr. Daayf's work on late blight has been supported by grants from a Discovery grant from NSERC, and from research grants from ARDI Manitoba, and industry partners, including McCain Foods, Peak of the Market and KPPA.

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Figure legends

Fig. 1 Phylogenetic analysis of amino acid sequence of DXS
The evolutionary history of the amino acid sequences of DXS1 was inferred using the Neighbor-Joining method (Saitou et al. 1987).The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the JTT matrix-based method (Jones et al. 1992) and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 36 amino acid sequences. All positions in both analysis containing gaps and missing dates were eliminated. Evolutionary analysis was conducted in Mega5 (Tamura et al. 2011)

Fig. 2 *StDXS1* transcripts in RB+US8 and RB+US11 and infection of potato by *Phytophthora infestans*. A: RB+US8: Russet Burbank inoculated with US8; B: RB+US11: Russet Burbank inoculated with US11. Grey striped bars represent the control. All qRT-PCR reactions were performed from triplicate biological samples. The 2-ΔΔC(T) method [35] was used to calculate the fold expression relative to the control. The elongation factor gene was used to normalize small differences in template amounts. Mean of three replicates ±1 standard error are shown.

Fig. 3 StDXS1 transcripts profiles. RB: Russet Burbank, H20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, high aggressiveness), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, low aggressiveness), EPA: eicosapentanoic acid (EPA), GL: Glucans race C. RB+US8: Russet Burbank inoculated with US8; RB+US11: Russet Burbank inoculated with US11. All qRT-PCR reactions were performed from triplicate biological samples. The 2-ΔΔC(T) method [35] was used to calculate the fold expression relative to the control (RB+H_2O). The elongation factor gene was used to normalize small differences in template amounts. Means with the same letter are not significant different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

Fig. 4 Germination rate of Arabidopsis *DXS1* line and wild type. A: germination rate of the wild type (WT) and DXS1 line. WT is shown by red line, while *DXS1* line is shown by black line. The mean values ± SE were plotted in the graph with 12 h intervals. **B**: Stereoscopic analysis of germinated seeds at 2, 3, and 4 days on MS media and 4 weeks in soil.

Fig. 5 Expression level of *StDXS1* in Arabidopsis seedlings. The level of StDXS1 transcripts were measured by qRT-PCR in Wild type and *DXS1* lines. The wild type did not give signal as the *StDXS1* primers are specific to *StDXS1*.

Fig. 6 Expression analysis of MEP-pathway genes in Arabidopsis. A: represents level of transcripts of *GGPPS1, GGPPS2, DXS1, PSY* and *ZEP*. **B:** represents level of transcripts of *PDS, ZDS, bLYC, BCH1, BCH2* and *LUT1*. The wild type (WT) is represented by black bars, while DXS1 lines are presented by light and dark gray. The mean values (n=3) denoted with the same letter are not significantly differ at $P \leq 0.05$.

Fig. 7 Subcellular localization of StDXS1. The signal was visualized by Zeiss confocal laser scanning microscope. **A:** Transient expression of StDXS1::YFP in living protoplasts; chlorophyll autoflorescence (red color), YFP fluorescence (yellow color) and merged image (orange color). **B:** Immunolocalization of StDXS1::YFP was detected using anti-YFP antibody and visualized by the fluorescence of FITC-conjugated antibody. FITC fluorescence (blue color), bright filed and merged image which show the localization of StDXS1::YFP on the chloroplasts membranes.

Fig. 8 Chlorophyll content in Arabidopsis DXS1 plants. Chlorophyll a (Chla), chlorophyll b (Chlb) and total chlorophyll are shown by blue, brown and green colors. Y-axis represents the chlorophyll content (µg/g FW) and X-axis represents the wild type (WT) and two lines *DXS1* *DXS1*-3 and *DXS1*-4. The graph represents the mean values (n=3) ± SE.

Fig. 9 Carotenoids content in Arabidopsis seedlings. β-carotene and Lutein were measured in 2 week old seedlings of wilt type and five transgenic *DXS1* lines. Lutein is represented in the graph by yellow bars, while B-carotene is represented by orange bars. The graph represents the mean values (n=3) ± SE.

Fig. 10 Carotenoids content in Arabidopsis mature seeds. β-carotene and Lutein were measured in mature seeds of wilt type and five transgenic *DXS1* lines. Lutein is represented in the graph by yellow bars, while B-carotene is represented by orange bars.

Fig. 11 Salicylic acid (SA) and Jasmonic acid (JA) content in Arabidopsis seedlings. Salicylic acid (SA) and Jasmonic acid (JA) were measured in 2 week old Arabidopsis seedlings. **A**: Free SA content, **B**: Bound-SA content, and **C**: JA content. Wild type (WT) is represented y black bars, while *DXS1* lines are represented by brown bars. The graph represents the mean values (n=3) ± SE. Asterisk star shows significance at $P \leq 0.05$.

Fig. 12 Abscisic acid and gibberellins contents. ABA content was measured in 2 week old seedlings, siliques and mature seeds, while GA4 was measured in seedlings. **A**: ABA content in siliques and mature seeds, **B**: ABA content in seedlings, **C**: GA4 content in seedlings. Wild type (WT) is shown by black bars, while *DXS1* line is shown by orange bars. The mean values (n=3) ± SE is plotted in the graph. Asterisk star shows significance difference at $P \leq 0.05$.

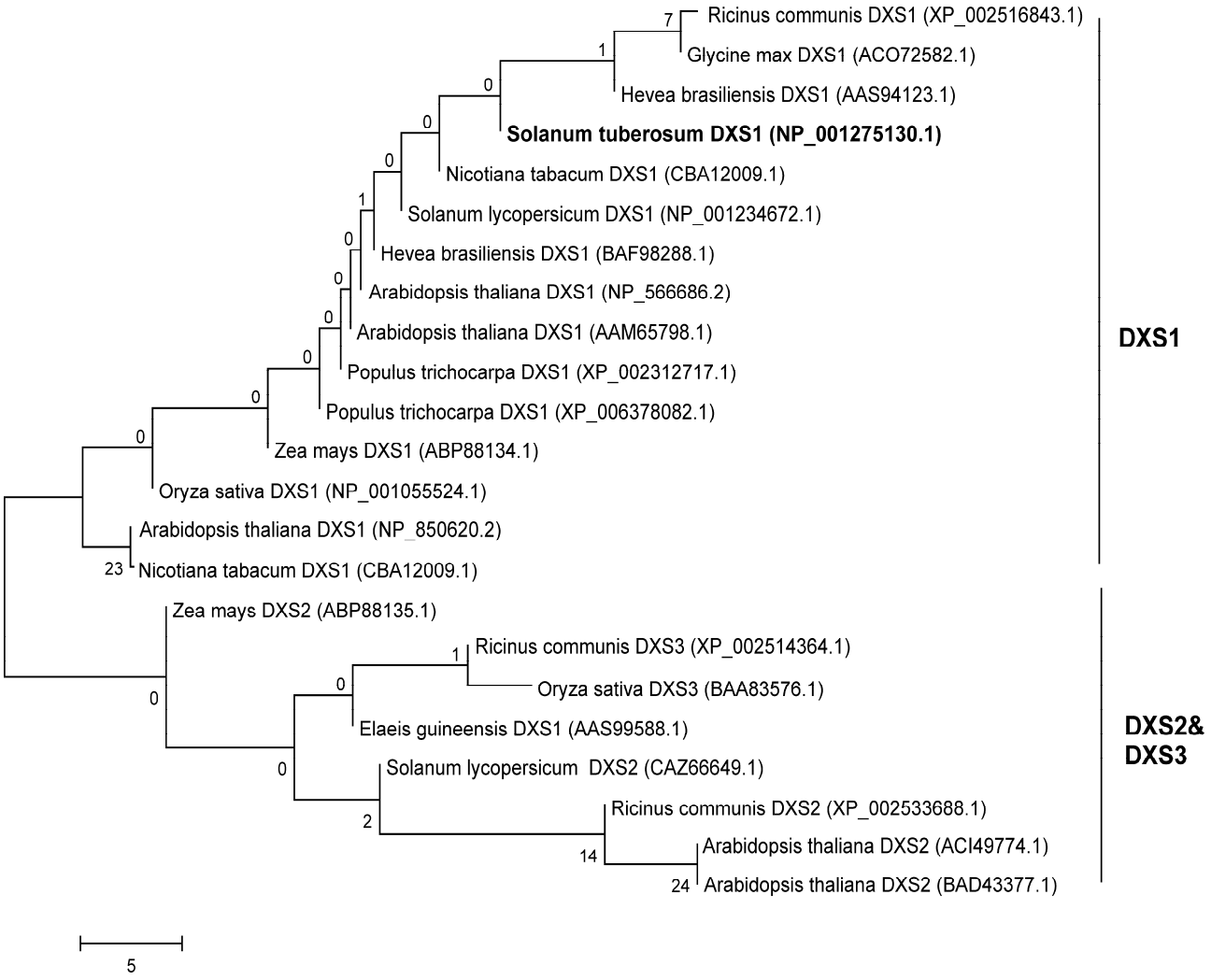


Figure 1

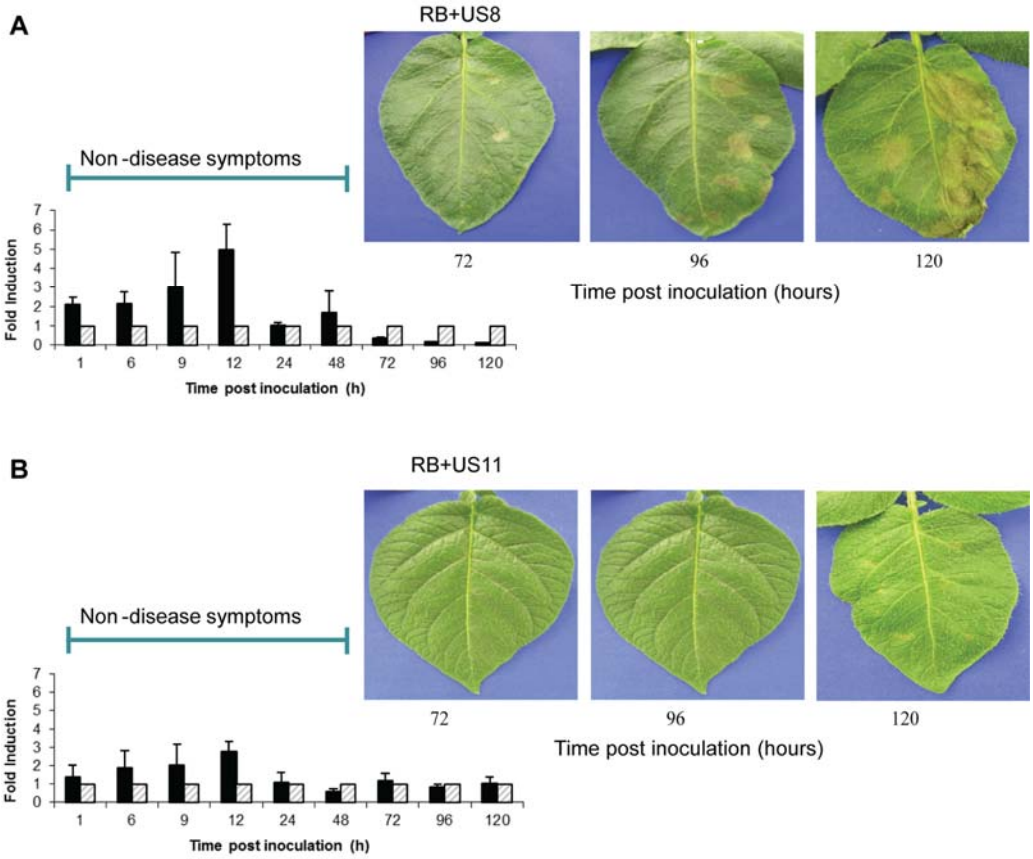
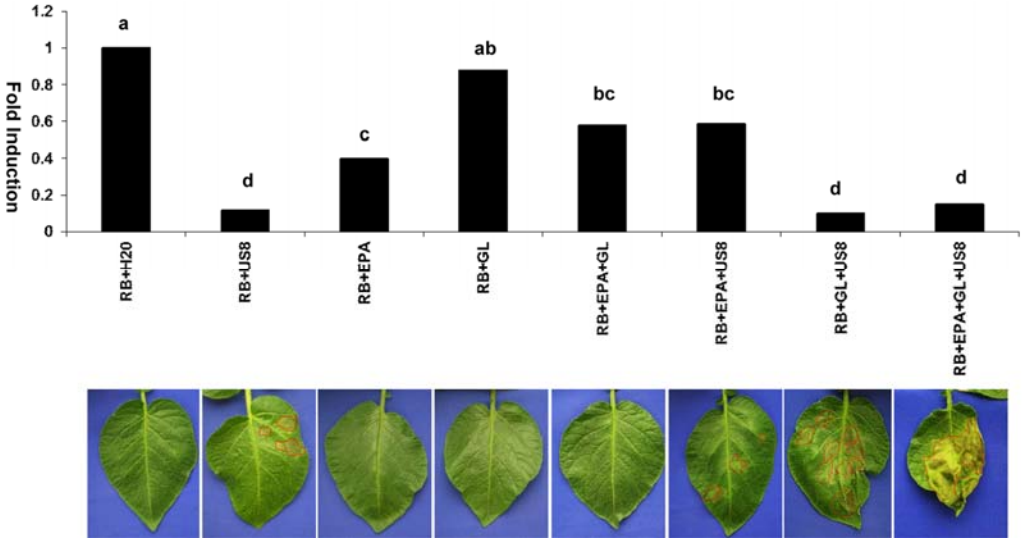


Figure 2



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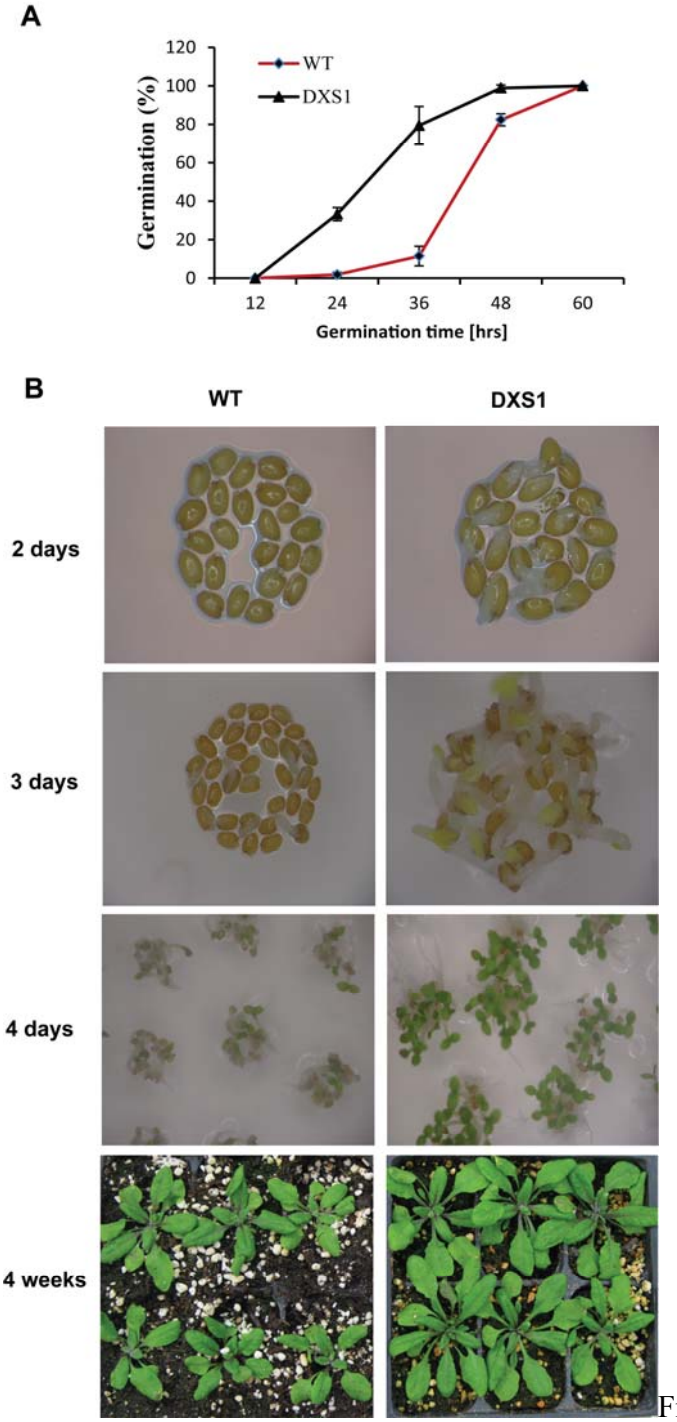

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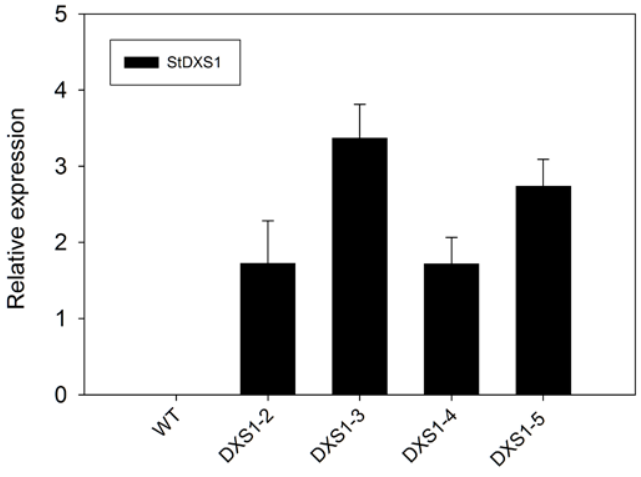

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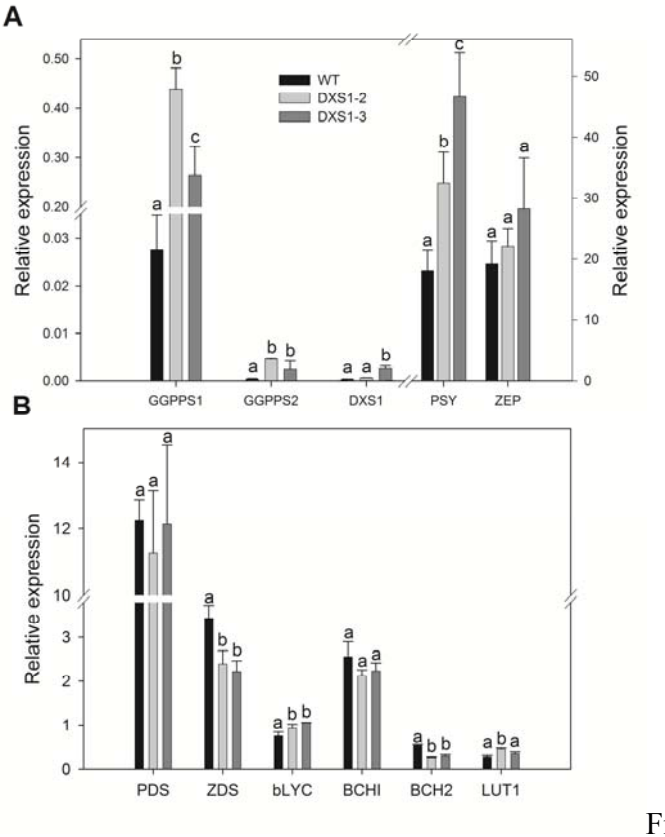

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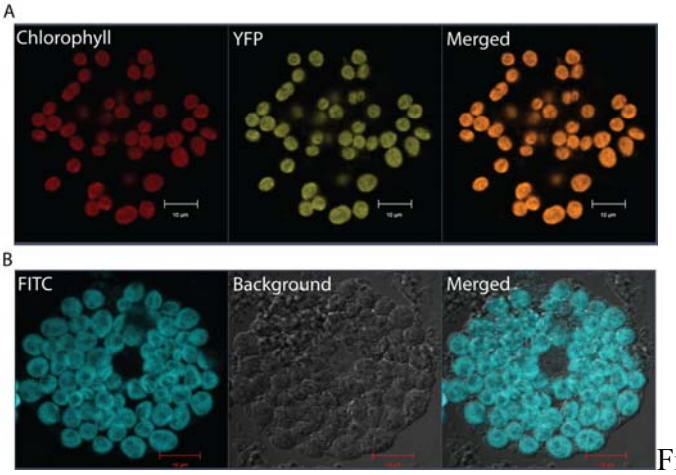

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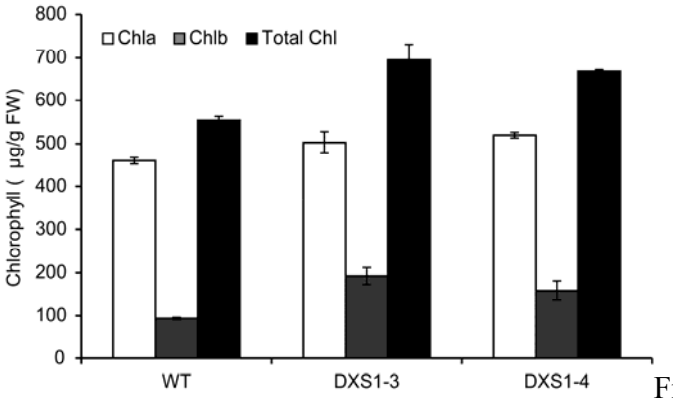

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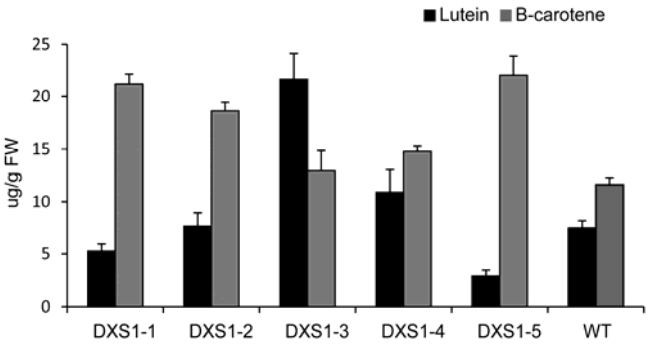

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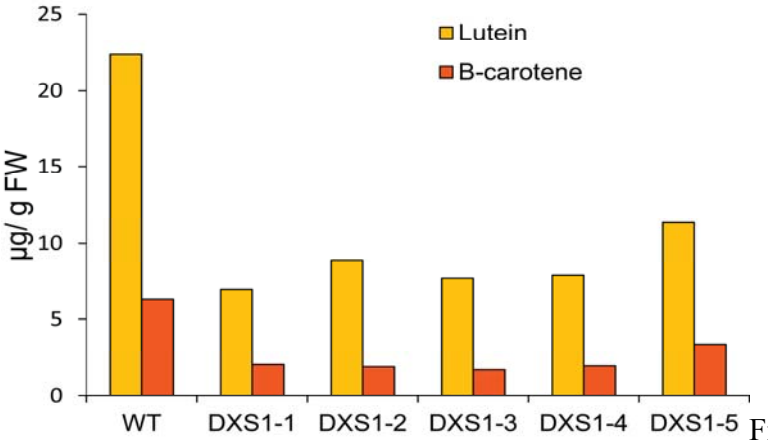

Figure 10

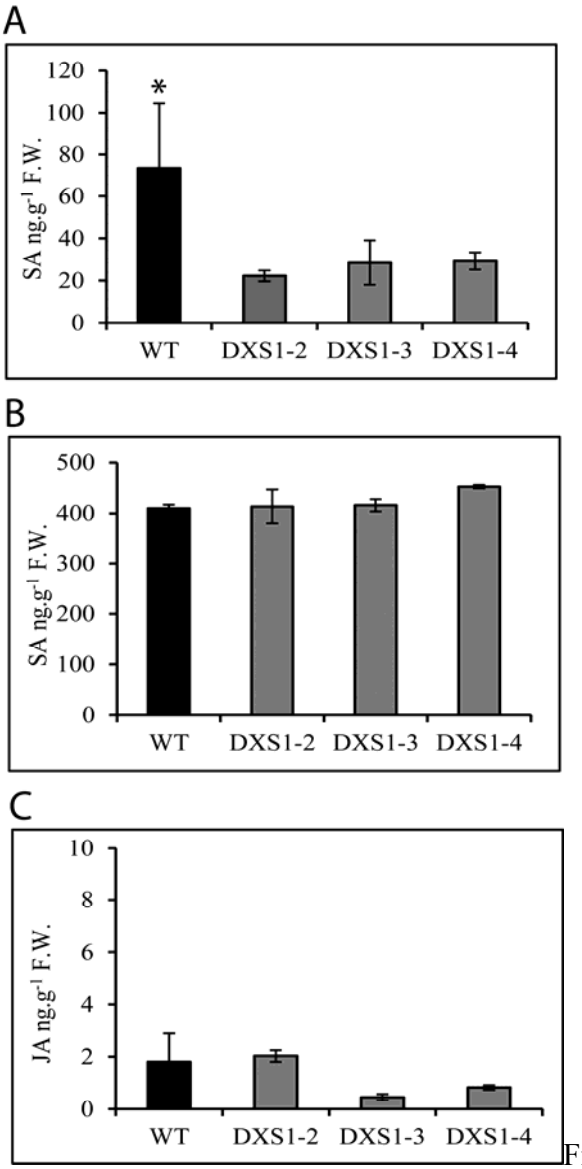

Figure 11

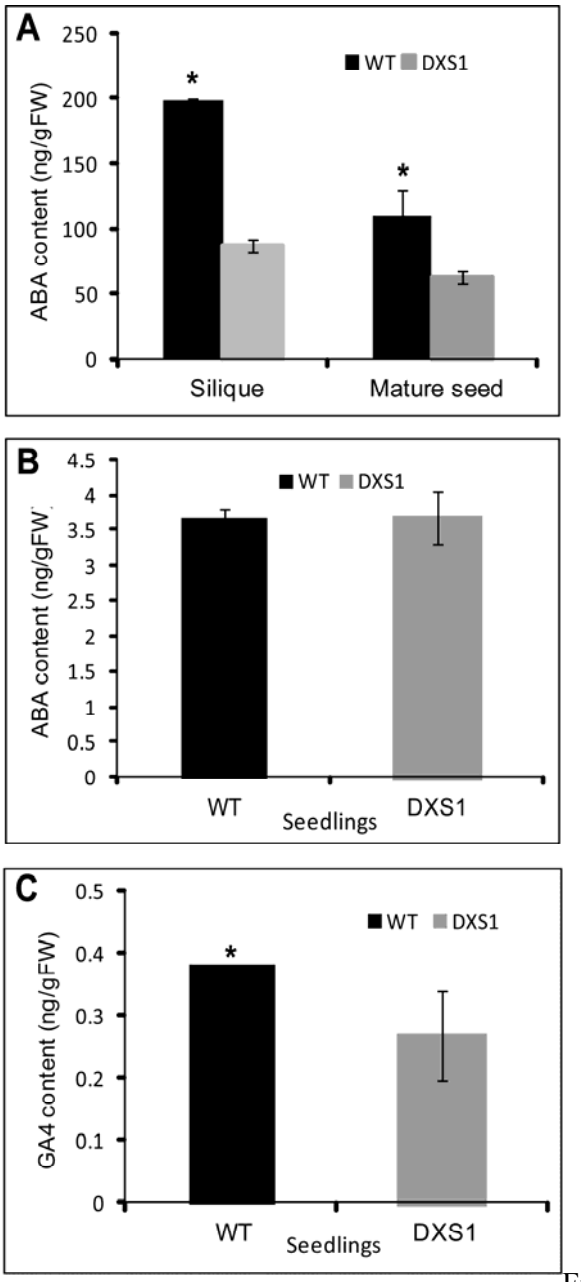

Figure 12

Table 1. Multiple reaction monitoring (MRM) settings.

Analyte	**Ionization mode**	**Cone voltage**	**Q1 (precursor ion)**	**Q3 (product ion)**	**Q2 (collision energy) (V)**	**Retention time (min)**
Jasmonic acid	-ve	30	209	58.9	-23	7.5
Dihydrojasmonic acid	-ve	30	211.1	58.8	-23	8.3
Salicylic acid	-ve	29	136.70	92.9	-24	6.5
***o*-anisic acid**	+ve	20	152.9	134.8	+14	4.2

Table 2. Primer sequences used for qRT-PCR.

Gene	**Forward (5'-3')**	**Reverse (5'-3')**	**NCBI**
For Arabidopsis (*Arabidopsis thaliana*)			
GGPPS1	ATTCAGCTGTTCCTCTCCGT	GCATAGCGGTTGATTCTTCA	NM119845
GGPPS2	ACATGACCACCGCTGAAATA	ATGCTCTAATCCGACCTCGT	NM127943
PSY	TGCGGTGAAGTTTGCGCTGA	TGAAGCATTTGGCCCATCCA	AT5G17230*
ZEP	ATGACCGGCTTCGAGAGTGG	TTCCGACGATGCAAGGTTGA	AT5G67030*
PDS	GTCGGTCACGCGCTCAGGTA	CGAGATGCTGACATGGCCAGA	AT4G14210*
ZDS	CCATCGTCACGAGGCCTAGAA	TGTGTATGAACCGGCGAGGA	AT3G04870*
bLYC	TGGTAGCGCTGCTCTTTTGGA	ACCAGCAGGACCACCACCAA	AT3G10230*
BCH1	ACGCTTCTCTATGGAATATGCATGA	TCCATAAGAGAGGAGACCAATCGCT	AT4G25700*
BCH2	GGAGTTTTGGGCAAGATGGGCTCAT	GAACCGCGTTTGTTATAGCAAACACA	AT5G52570*
LUT1	CGAAATCCCAATCATGGGTCA	GCACCTCCGAGGAGATCAGC	AT3G53130*
UBC	ACTGCGACTCAGGGAATCTT	GCGAGGCGTGTATACATTTG	
For potato (*Solanum tuberosum*)			
DXS1	TACCAACACACCCCACTAGA	AAGAGGAGGGCTTGAACTCAG	GU936657
DXR	CCATCCTGATGCTGTCACTG	CAAGCCTTGTATGCACTGGA	AF331705
MCT	CTTTCTCCAGGATGCCTCAG	TCTTGCAGAGTCATGGATGC	EF636807

CMK	TGCCTACTGGAGCTGGTCTT	CCTGAACAACCTCACCCCTA	AF263101
MDS	TCTGGGGCTTCCTGATATTG	GCACCAAGCAGCTTACACAA	AK246318
HDS	CAGCATTTGAGTTTGCCAGA	CCGATTGCAGACTTCATCCT	AF435086
E-factor	GATGGTCAGACCCGTGAACAT	GGGGATTTTGTCAGGGTTGT	AB061263

*****Yu et al. (2012)