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Identification and expressional profiling of putative *MAX1* gene in sugar beet (*Beta vulgaris L.*)

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Abstract: Sugar beet is an industrial crop cultivated mostly for sucrose production. Today, modern hybridization applications have raised the sugar content to 20%. Considering the increased demand globally, it is required to develop new yield–increasing application strategies. This requires extensive knowledge of the genetic mechanisms to control plant growth and metabolism. Investigation of phytohormones is promising to increase our knowledge of plant growth and developmental processes. Strigolactones are recently introduced plant hormones. They are responsible for shoot and root architecture, and involved in development, communication, germination, and responses to stress. Four major proteins (AtD27, MAX1, MAX3, MAX4) are responsible for the strigolactone biosynthesis. MAX1 protein is essential for the distinct production of strigolactone molecules. The studies on strigolactones have been carried out mostly using *Arabidopsis thaliana*. There are only few studies on agriculturally important plants. No reports are available for the investigation of strigolactone biosynthesis in sugar beet (*Beta vulgaris*). In this manuscript, we profiled the expression of *MAX1* gene in sugar beet treated with strigolactone hormones (rac-GR24, (±)-strigol and (±)-5-deoxystrigol) and a strigolactone biosynthesis inhibitor (TIS108). Our data suggest that *MAX1* has a conserved biosynthetic and regulatory metabolism in sugar beet compared to previously investigated plant species.

Key words: Sugar beet, *Beta vulgaris*, Strigolactones, *MAX1*, qPCR

1. Introduction

Beta vulgaris L. is a herbaceous and an allogamous dicotyledon plant (Lange et al., 1999). It is classified under the Amaranthaceae family, which covers leaf beet, garden beet, fodder beet, and sugar beet. Sugar beet is considered as a commercially important sucrose–producing plant. In addition to sucrose production, sugar beet pulp and molasses are also extensively demanded as livestock feed. The list of coproducts of sugar beet can be extended to pharmaceuticals, biofuels, and plastics (Finkenstadt, 2013). Therefore, the yield quality of sugar beet is a matter of importance to both farmers and industrialists. There are efforts to increase the quality of sugar beet to meet the demand that has been increasing over the years. In addition to classical breeding programs, in vitro culture techniques including haploid plant production, protoplast culture, somaclonal variation, and in vitro cell selection have been studied (Gürel et al., 2000, 2001, 2002). Today, the sugar content has been increased up to 20% in recently developed commercial cultivars (Gürel et al., 2008). Conventional breeding methods played a major role in these improvements. Phytohormones play key roles in a variety of processes at subcellular and biochemical levels in plants such as regulating gene expression and coordinating signal transduction mechanisms to create a physiological change for plant growth and development (Takatsuka and Umeda, 2014). Therefore, identifying new plant hormones, understanding their functions, biosynthesis, and transduction can elucidate different aspects of plant growth and developmental processes. Understanding the impact of plant hormones and their function at the molecular level in *Beta vulgaris* would provide a new perspective and an advantage to increase the sugar content in sugar beet.

Phytohormones are fundamental for the survival of plants. They can be produced by plants or symbiotic microorganisms to support plant growth, environmental communication, tolerance, and/or resistance to abiotic and biotic stress conditions. The phytohormones are categorized under 9 different groups: auxins, gibberellins (GAs), abscisic acid (ABA), ethylene (ETH), cytokinins (CKs), brassinosteroids (BRs), jasmonates (JAs),

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salicylates (SAs), and strigolactones (SLs) (Su et al., 2017). Strigolactones are relatively the most novel group discovered. Their roles in plant response to abiotic and biotic stresses draw attention to this new group (Gomez-Roldan et al., 2008). Over the years, several strigolactones have been identified from natural sources such as strigol, 5-deoxystrigol, strigyl acetate, orobanchyl acetate, or produced synthetically such as *rac*-GR24. There is evidence that plant architecture aboveground or underground plant architecture can be regulated by SLs in conjunction with other phytohormones (Shinohara et al., 2013). Several studies conducted in different plant species have shown that SLs are considered as carotenoid–derived signaling molecules (Matusova et al., 2005; Lin et al., 2009; Alder et al., 2012; Seto and Yamaguchi, 2014).

Investigation of several genes involved in the biosynthesis of SLs contributed to the understanding of their roles and function in plant metabolism (Al-Babili et al., 2015). Studies showed that SL function is directly related to natural factors affecting grain yield. In a previous study, it was shown that root nodulation, which is required for fixing atmospheric nitrogen, was reduced by 40% in SL–deficient pea plants (Foo and Davis, 2011). In another recent study, it was suggested that strigolactones are required for adaptation to inorganic phosphate deficient conditions in rice (Yamada et al., 2019). It is known that tiller bud formation can result in yield in cereal crops. Studies show that inhibition of tiller bud outgrowth can be regulated by strigolactones in rice and *Arabidopsis* (Umehara et al., 2010; Kohlen et al., 2011). Similarly, tiller bud outgrowth can be inhibited by artificial SL, rac-GR24 in wheat, and the inhibition can be eliminated by an azole–type SLs biosynthesis inhibitor, TIS108 (Zhao et al., 2020).

The *MORE AXILLARY GROWTH (MAX)* gene has been identified with prominent function in the SL biosynthesis and signaling pathway for *Arabidopsis thaliana* and several other plant species (Stirnberg, 2002; Soferan et al., 2003; Booker, 2005; Braun et al., 2012).

Several reports suggest that MAX1 (cytochrome p450 monooxygenase), MAX3 and MAX4 proteins are required for the biosynthesis of branch inhibiting signals, while MAX2 protein is more likely related to signal perception and transduction (Booker et al., 2005; Stirnberg et al., 2002). During the process of biosynthesis of active SLs, MAX3 and MAX4 proteins are involved in converting carotenoids into carlactone molecule, which functions as the precursor of tricyclic lactone–containing SLs (Seto and Yamaguchi, 2014). In *Arabidopsis*, *MAX1* is a class III cytochrome P450 protein-encoding gene, and it has been demonstrated to catalyze the oxidation of carlactone (CL) into active SL molecules like carlactonic acid (CLA) or (±)-5-deoxystrigol molecules in the cytosol (Booker et al., 2005; Pulido et al., 2012; Alder et al., 2012; Kramna et al., 2019). Thus, *MAX1* is the most prominent gene for the biosynthesis of functional SLs. With the help of RNA-Seq analysis, several *MAX1* orthologs have been proposed in diverse plants including alga[1]. However, investigations of the *MAX1* function in nonmodel plants is limited, especially in agricultural crops such as sugar beet.

Understanding the molecular nature of strigolactones in sugar beet will enhance our perspective in the development of alternative strategies to increase the root and sugar yield in the future. In this study, we aimed to evaluate an ortholog of *MORE AXILLARY GROWTH1* (*MAX1*) gene in sugar beet (*Beta vulgaris L.)* by profiling *MAX1* expression patterns after exposing to different SL hormones (rac-GR24, (±)-strigol, or (±)-5-deoxystrigol) and 1 SL inhibitor (TIS108). (±)-strigol, or (±)-5-deoxystrigol are well-known hormones that were shown to be related to yield production in several plant species (reviewed in Xie, 2016). Our work is the first study to show the expressional regulation of *MAX1* gene by strigolactone hormones in sugar beet.

2. Materials and methods

2.1 Plant culture and sampling

A commercial variety of sugar beet seeds (cv. Serenada obtained from KWS, Germany) was used in this study. Water soaked seeds were planted in 400 g of commercial soil. Six seeds were planted at 2 cm depth under the soil in each pot. Pots were placed in a temperature– and humidity–controlled room. They were incubated at 24 ± 1 °C following a 16 h light (50 μ mol^{-2} S^{-1}) and an 8 h dark period. The relative humidity level was maintained at 50–60%. Five days after germination, 1 seedling in each pot with similar seedling size across the other pots was kept, and the rest were discarded to sustain homogeneity among the treatment groups. After 3 days, 50 mL of water was poured into the pots every day.

2.2 Hormone treatments

To test the impact of SLs on *MAX1* gene expressions in sugar beet, 3 SL hormones (rac-GR24, (±)-strigol, and (±)-5-deoxystrigol) and 1 SL inhibitor (TIS108) were applied separately to each seedling after 10 days of germination. Ten-day-old *Beta vulgaris* seedlings were used for treatments of 3 SL hormones in question within every 2 days, in total 7 times over 14 days. A 5 mL aqueous solution of each hormonal treatment was carried out at different concentrations of 0 μM (Control), 2.5 μM, 5.0 μM and 7.5 μM) (Figure 1). Five seedlings in separate pods were used for each treatment. All treatments were carried out using 3 biological replicates (total of 15 seedlings).

[1] NCBI. Website www.ncbi.nlm.nih.gov

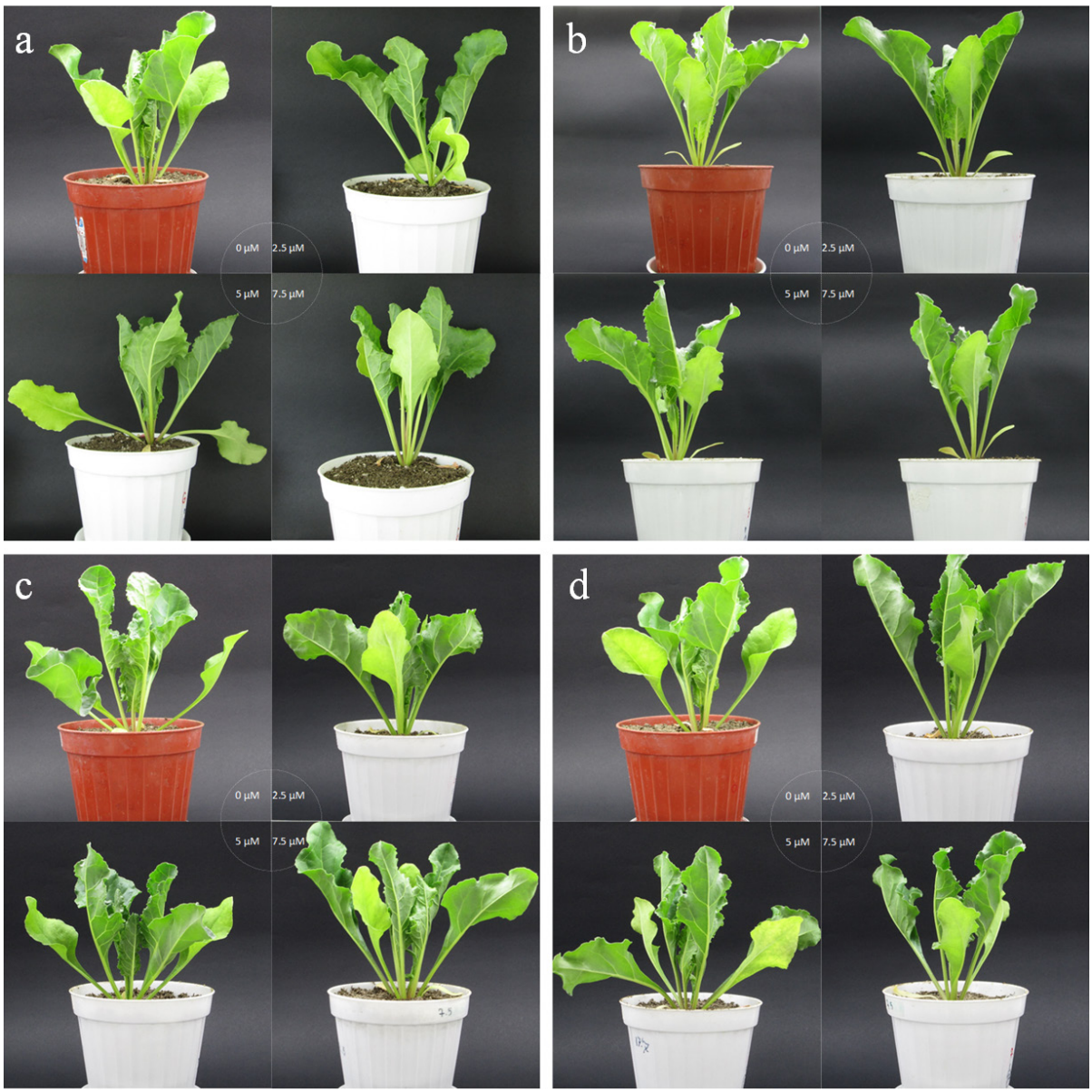

Figure 1. The appearance of the *Beta vulgaris* plants after 7 times treatment of rac-GR24 (a), (±)-strigol (b), (±)-5-deoxystrigol (c), and TIS108 (d) hormones with increasing concentrations. Ten-day-old *B. vulgaris* seedlings were treated 3 SL hormones and 1 triazole–type strigolactone biosynthesis inhibitor within every 2 days over 14 days. A 5 mL aqueous solution of each hormonal treatment was applied at increasing concentrations of 0 µM (Control), 2.5 µM, 5.0 µM and 7.5 µM.

2.3 Identification of *MAX1* gene

To identify putative *MAX1* ortholog in *B. vulgaris*, we used Blast Search (Altschul et al., 1997; Johnson et al., 2006) to parse any sequence in the genome of *B. vulgaris* similar to genes encoding the MAX1 protein in *A. thaliana* (NCBI Accession ID: NP_565617.2).

2.3.1 Phylogenetic analysis of *MAX1* gene

A few *MAX1* orthologs have been reported in different plant species under rosids, asterids and caryophyllenes, and their amino acid sequences are available in the gene bank (Supplementary Table 1). Multiple sequence alignments were constructed using the T-Coffee tool (Notredame et al., 2000). Maximum likelihood analysis was performed in RAxML GUI (Version 2.0) with 1000 rapid bootstrap replicates and under the PROTGAMMA substitution model.

2.4. Evaluation of *MAX1* expression levels

2.4.1 RNA isolation and cDNA library preparation

Leaf tissue of sugar beet (control and treated samples) was grounded to fine powder in liquid nitrogen using a mortar

and pestle. 50 mg of powder was transferred to a DNase/RNase-Free 2 mL tube. NucleoZOL reagent (Macherey-Nagel, Germany) was used for the isolation of RNA. To assess the integrity of total RNA, an aliquot of the RNA samples was run on a denaturing agarose gel stained with ethidium bromide (EtBr). Based on sharp and clear 28S and 18S rRNA bands and the ratio of 28S:18S, which is approximately twice in the visualized gel, it was indicated that total RNA was intact (Supplementary Figure 1a). For the cDNA synthesis, a ProtoScript First Strand cDNA Synthesis Kit (NEB, USA) was used according to the manufacturer's instructions. RNA isolation and cDNA library preparations were carried out for the control and treated samples with 3 biological replicates.

2.4.2 qPCR experiment

For the amplification of the *MAX1* gene, CAGCAGTTGAGATAGGAGGTTAC (forward) and TCGGGTTCTGGAAAGTTCTTC (reverse) primers were used to amplify 100nt length template. β-actin gene was used as the internal control based on the reference studies (Mashiguchi et al., 2009, Tumer et al., 2018). For the amplification of the β-actin gene, TCAATGTGCCTGCTATGTATGT (forward) and GTGACTAACACCATCACCAGAG (reverse) primers were used. In a total of 20 µL mix, 10 µL of SYBR Green (BioRad, USA) mix was combined with 100 ng of cDNA, 10 µM of each forward and reverse primers. The PCR temperature cycling protocol was set as follows: initial 95 °C for 10 min, then 39 cycles of 95 °C for 30 s, 58 °C for 20 s, 72 °C for 30 s followed by 95 °C for 10 s, 65 °C for 5 s, and 95 °C for 5 s using a BioRad CFX connect real–time PCR system (BioRad, USA). SYBR Green (BioRad, USA) fluorescence was used for monitoring the reactions. Supplementary Figure 1b-c represents the curve data for qPCR experiments. Quantifying gene expression levels was carried out using 2−ΔΔCT formula. One-way analysis of variance (ANOVA) was used to assess the variances, and mean comparisons among the samples were carried out using Tukey's test.

3. Results and discussion

3.1 Identification of *MAX1* gene

Blast analysis of the *A. thaliana* MAX1 protein sequence (NP_565617.2) against the *Beta vulgaris* peptide sequences (Dohm et al., 2014) showed high sequence similarity (71.98% identity with 96% query coverage) with the sequence (XP_010669378.1) deposited in NCBI database.

Since there is limited knowledge about the *MAX1* sequences for nonmodel organisms, we carried out phylogenetic analysis to briefly evaluate the evolutionary state of *MAX1* for *Beta vulgaris* (Figure 2). In a previous report (Dohm et al., 2014), the whole genome of *Beta vulgaris* was sequenced. In the study, scientists applied a multigene phylogenetic analysis approach to obtain a reliable evolutionary model for *Beta vulgaris*, and they demonstrated that branching off for *Beta vulgaris* was before the separation of asterids and rosids (Dohm et al., 2014). In this study, the phylogenetic position of *Beta vulgaris* was reflected by the highest fraction of species-specific genes. Separation of caryophyllales is associated with gene family expansions and losses throughout the evolution. The changes resulted in several phenotypical changes including an increased number of stamen whorls, pollen shape, and/or various carpel structures until the rise of a wide range of species under rosids (Taylor et al., 2009; Ferrándiz et al., 2010). Caryophyllales are proposed as the most basal eudicot clade, and separation of the clade cannot be assessed with a lineage–specific, whole genome duplication event in *Beta vulgaris* (Dohm et al., 2014). When we compare the phylogenetic tree (Figure 2) to this model, branching off for *MAX1* follows the similar path as presented in a previous study suggesting that the *MAX1* gene exists in plant genomes since the early stages of plant evolution and independent from gene duplication event (Dohm et al., 2014).

3.2 *MAX1* expression analysis

3.2.1 Effect of hormonal treatments

The effect of rac-GR24 hormone treatment is presented in Figure 3. Data shows that the rac-GR24 application significantly decreased the expression of *MAX1* through the increasing concentration of the hormone. When compared the relative expressions the data obtained from 2.5 and 5 µM rac-GR24 treated samples are not statistically significant. However, there is a significant decrease in *MAX1* expression when the data compared against the control samples that there were 3.33-fold and 5.26-fold decrease, respectively. After the application of 7.5 µM MAX1 levels were almost diminished (62.50-fold decrease). It is obvious that the application of rac-GR24 negatively affects the *MAX1* gene expression and the level of the impact is concentration-sensitive. It was previously reported that the application of rac-GR24 mimics the *MAX1* mutants of *A. thaliana* (Lantzouni et al., 2017; Soundappan et al., 2015). Our results suggest that a similar mechanism is involved in *B. vulgaris* as well.

(±)-strigol and (±)-5-deoxystrigol are natural SL hormones when compared to rac-GR24. Our findings suggest that the application of natural hormones ((±)-strigol and (±)-5-deoxystrigol) results with a similar decrease pattern compared to the application of rac-GR24 (Figures 3–5). Statistically significant decrease in *MAX1* expression was detected for 5µM and 7.5 application of either (±)-strigol (Figure 4) or (±)-5-deoxystrigol (Figure 5) hormones. When compared to (±)-5-deoxystrigol treatment, (±)-strigol treatment shows a more dramatic decrease. The decrease levels were 6.66 (5 µM (±)-strigol)

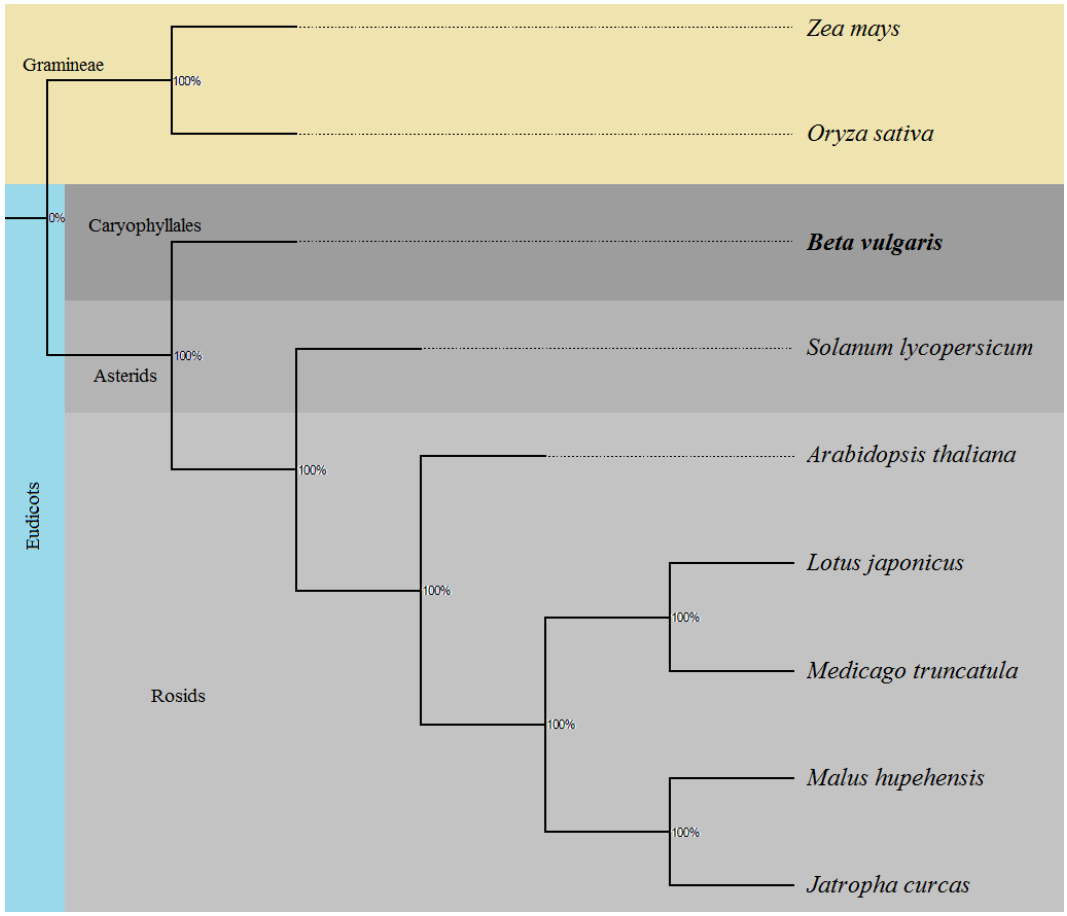

Figure 2. Phylogenetic analysis of the *MAX1* peptide sequence among diverse plant species. The tree is based on maximum-likelihood analysis of *MAX1* orthologs from different species. The phylogenetic tree was obtained by 1000 rapid bootstrap replicates under the PROTGAMMA substitution model.

and 7.69 (7.5 µM (±)-strigol) folds, and the differences for (±)-5-deoxystrigol treatments were 2.22 and 3.85, respectively. Unlike rac-GR24, the impact of natural hormones was dose–specific. The application of lower (2.5µM) or higher (7.5 µM) concentration did not alter the expressional decrease level significantly. The data is in parallel with previous reports (Lantzouni et al., 2017; Soundappan et al., 2015). The overall evaluation shows that a more drastic decrease in *MAX1* gene expression was observed in rac-GR24 treated samples, which is related to the synthetic nature of the hormone. It was previously reported that synthetic hormones show more dramatic effects when compared to their natural counterparts (Paciorek et al., 2005; Jasik et al., 2016).

Sugar beet is a heterozygous plant. The heterozygosity throughout the genome would have an impact on the strigolactone pathway. However, considering the statistically significant results, the effects of strigolactones on *MAX1* regulation remains conserved.

The dose–dependent downregulation of *MAX1* after the application of SLs suggests that the regulation of the *MAX1* gene is controlled by feedback regulatory system in sugar beet as previously suggested for *Arabidopsis* (Mashiguchi et al., 2009).

3.2.2 Effect of SL inhibitor treatments

We also analyzed to see if the expression of the *MAX1* gene (*B. vulgaris*) is affected by the inhibition of the biosynthetic pathway of SLs by TIS108, which is a triazole–type strigolactone biosynthesis inhibitor. Changes in *MAX1* gene expression depending on 2.5, 5 and 7.5 µM of TIS108 concentrations are presented in Figure 6. Results show the complete opposite impact of TIS108 treatment on *MAX1* expression compared to SL ((±)-strigol and (±)-5-deoxystrigol and rac-GR24) treatments. Previous reports indicated that the impact of TIS108 or *MAX1* deletion could be diminished by the application of SL hormones (Roldan et al., 2008; Umehara et al., 2008; Ito et al., 2011).

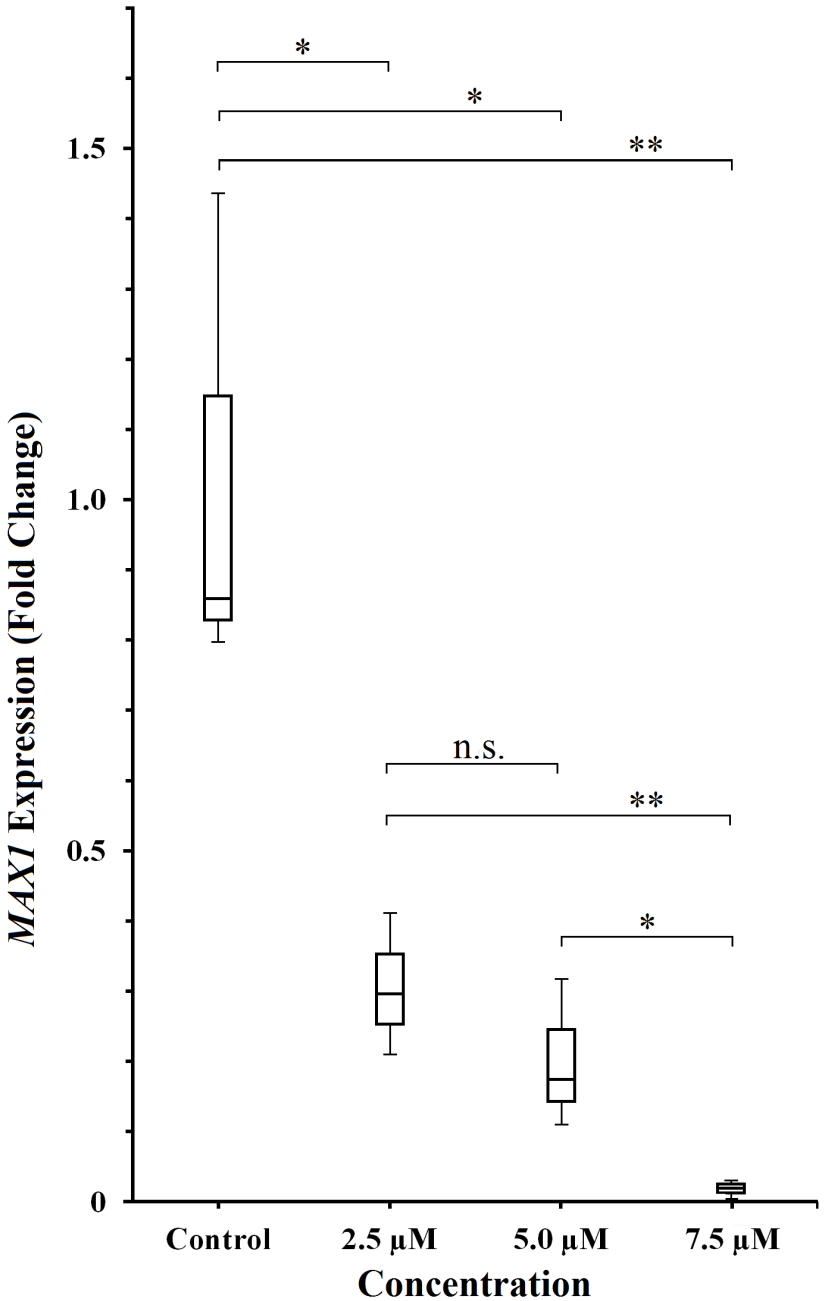

Figure 3. Relative expression of the *MAX1* gene in rac-GR24 treated *B. vulgaris*. *MAX1* gene expressions were normalized using the β-actin expressions. The data are mean ± SD of 15 plants from 3 biological replicates. Star sign indicates a significant difference between conditions (* $P < 0.05$, ** $P < 0.01$) and n.s. indicates nonsignificant changes.

Our data shows a significant increase in *MAX1* expression after 5 µM treatment. The increase levels in *MAX1* expression were 2.89-fold, 6.77-fold, and 16.10-fold higher to the control group for 2.5 µM, 5.0 µM, and 7.5 µM TIS108 treated samples respectively. Our data on *MAX1* gene expression show parallel results with the studies reported which focused on the *MAX3/4* expression in TIS108 treated *Arabidopsis* samples. It was previously proposed that the target for TIS108 would be *MAX1* (Ito et al., 2013). Our data support this hypothesis considering the influence of TIS108 on *MAX1* gene expression. The upregulation of *MAX1* gene expression after the inhibitor treatment can also be explained by the feedback regulation of the *MAX1* gene in sugar beet.

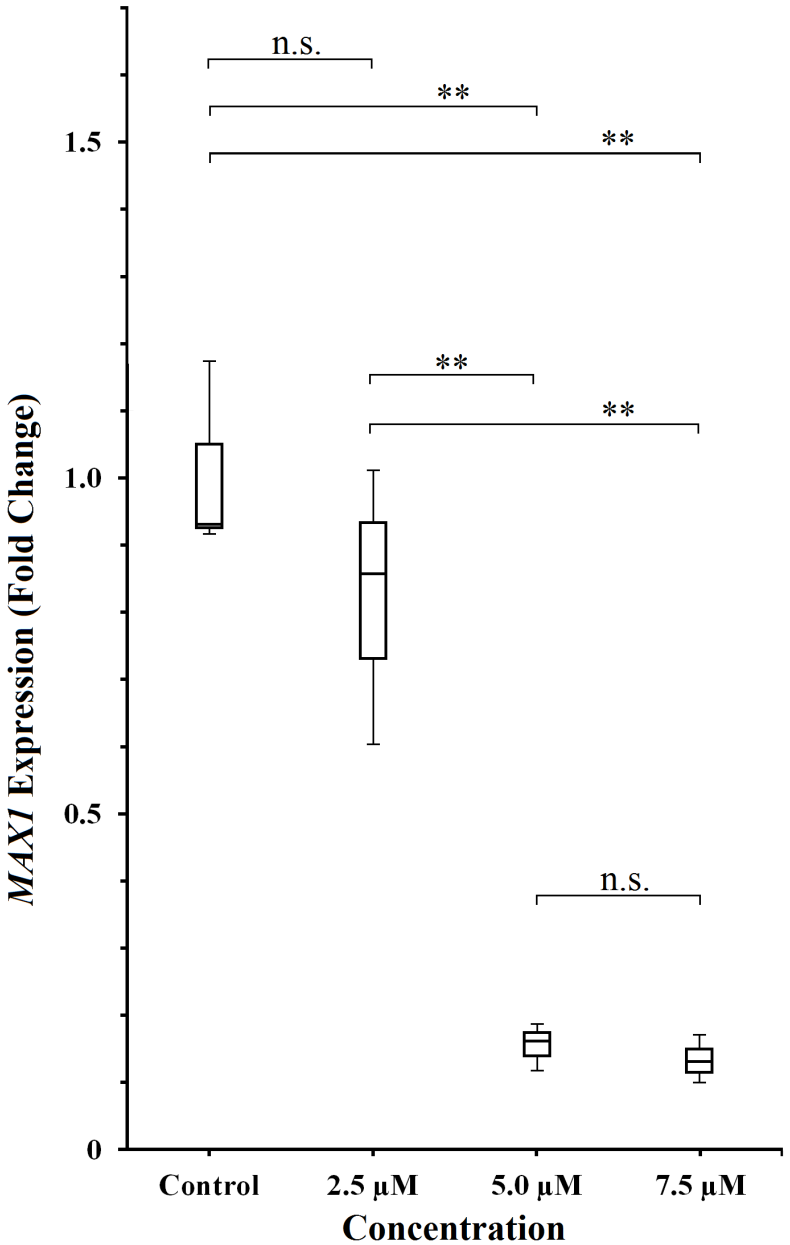

Figure 4. Relative expression of the *MAX1* gene in (±)-strigol treated *B. vulgaris* samples. *MAX1* gene expressions were normalized using the β-actin expressions. The data are mean ± SD of 15 plants from 3 biological replicates. Star sign indicates a significant difference between conditions (* $P < 0.05$, ** $P < 0.01$) and n.s. indicates nonsignificant changes.

Studies in *Arabidopsis* shows that *MAX* mutant phenotypes (e.g., increase shoot branching, repressed root hair elongation) can be rescued by application of SL hormones. The mutant related phenotypes are also observed in TIS108 treated *Arabidopsis* samples (Ito et al., 2013). Based on the evidence, it can be speculated that similar phenotypes are likely possible for *Beta vulgaris*. Indeed, there should be more comprehensive studies to show which phenotypes can be manipulated by the regulation of the SL pathway in sugar beet.

4. Conclusions

In this study, we demonstrate that the sequence deposited in the NCBI database with NM_128175.3 accession number encodes the *MAX1* gene in *Beta vulgaris*. Phylogenetic analysis shows that the evolution of the *MAX1* gene is in

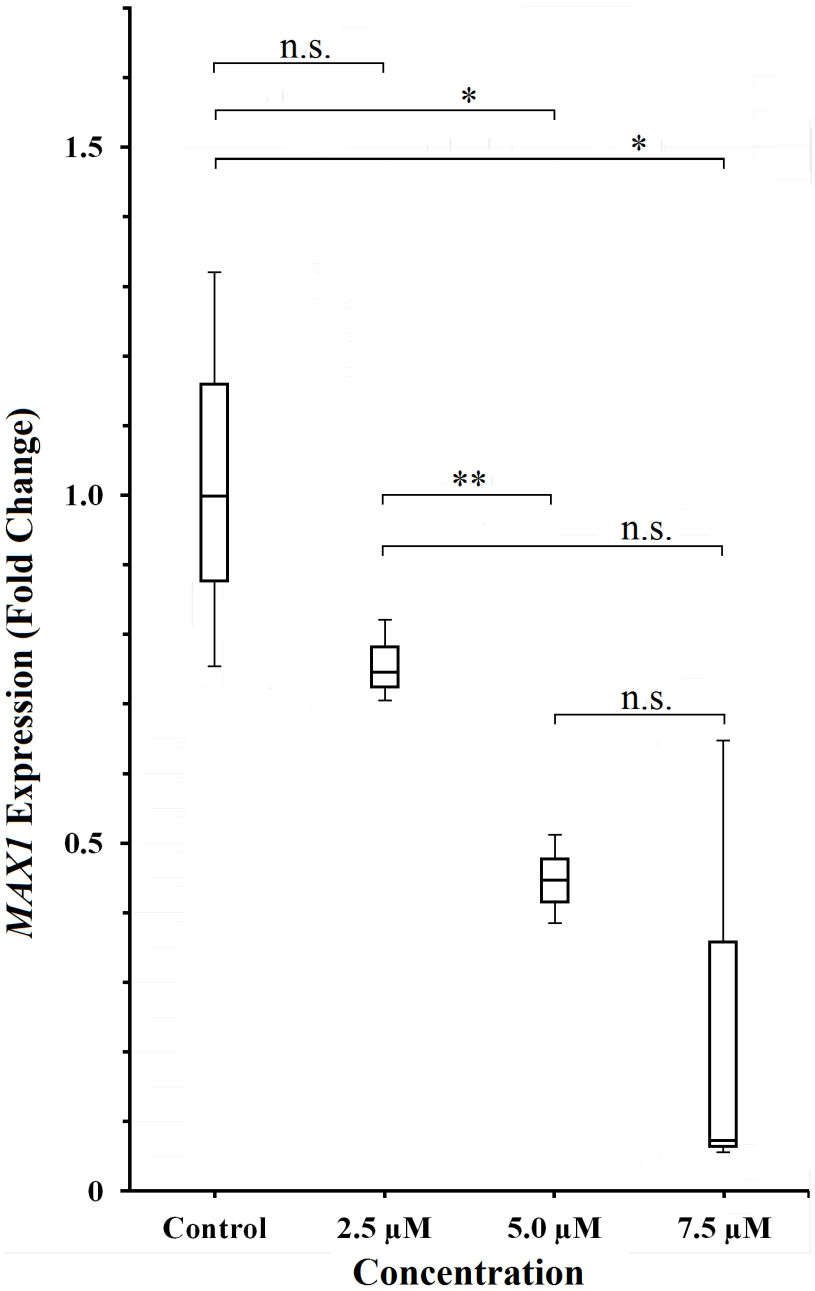

Figure 5. Relative expression of the *MAX1* gene in (±)-5-deoxystrigol treated *B. vulgaris*. *MAX1* gene expressions were normalized using the β-actin expressions. The data are mean ± SD of 15 plants from 3 biological replicates. Star sign indicates a significant difference between conditions (* $P < 0.05$, ** $P < 0.01$) and n.s. indicates nonsignificant changes.

parallel with the evolution of *Beta vulgaris* itself. *MAX1* gene encodes a cytochrome P450 protein which acts just upstream of MAX2 protein and responsible for the biosynthesis of various SLs in the pathway which makes *MAX1* a critical gene for studying the function of SL. *MAX1* is considered one of the key regulators for the control of SL biosynthesis through feedback regulation which is controlled by the *MAX2* gene (Mashiguchi et al., 2009). Our data provide evidence that *MAX1* is under negative regulatory feedback through the SL signaling pathway in *Beta vulgaris*.

Most of the studies on SL biosynthesis have been focused on *Arabidopsis*. The data presented in this manuscript prove that the general function of *MAX1* is conserved for

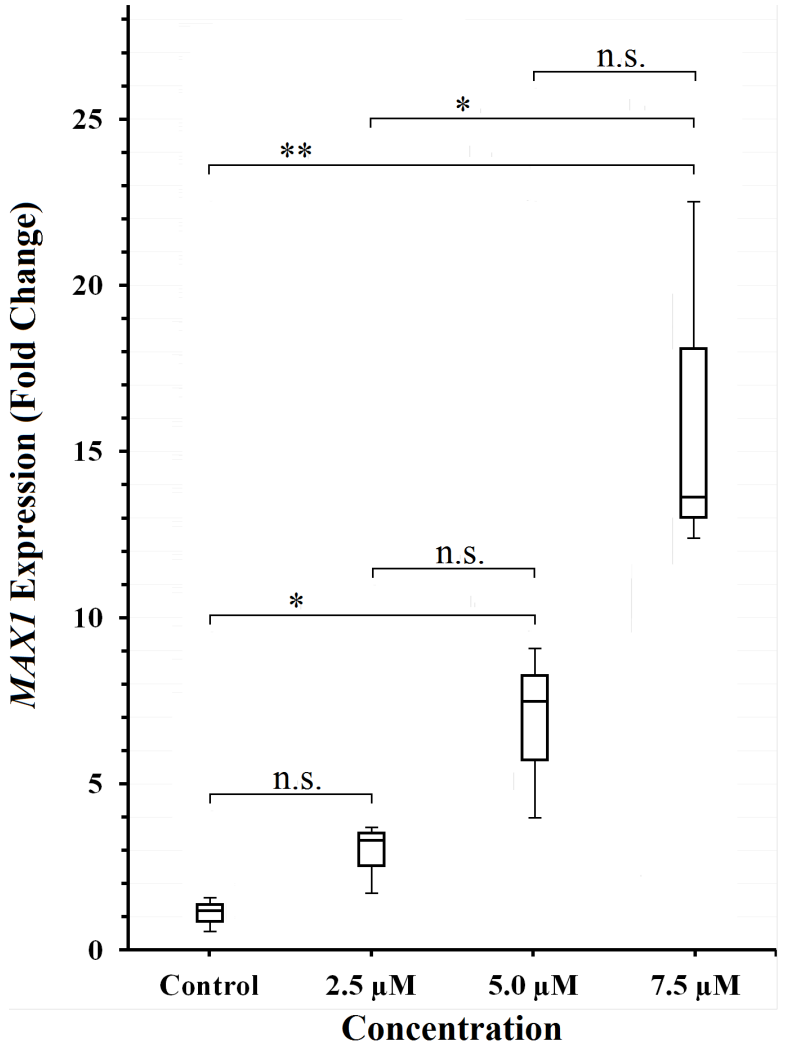

Figure 6. Relative expression of the *MAX1* gene in TIS108 treated *B. vulgaris*. *MAX1* gene expressions were normalized using the β-actin expressions. The data are mean ± SD of 15 plants from 3 biological replicates. Star sign indicates a significant difference between conditions (* $P < 0.05$, ** $P < 0.01$) and n.s. indicates nonsignificant changes.

Beta vulgaris and most likely in other plants. It is important to carry out comprehensive transcriptome-based studies to understand the unique roles of *MAX1* genes in *Beta vulgaris*.

The study of genes responsible for the synthesis of strigolactone in sugar beet has the potential to select genotypes with high yield and quality and to produce industrial plants with increased product quality. This study can be used as a reference to investigate sugar beet strigolactones along with genes involved in the hormonal regulations.

Sugar beet shows a wide range of genetic variations due to its heterozygous nature. Thus, interplant variations can be expected even among phenotypically similar individuals (Gürel, 1997). This would be a challenging problem for the development of alternative strategies to increase the yield. Our results show that the impacts of strigolactone applications at the molecular level are conserved among the individuals. In addition, the impact of strigolactones on *MAX1* regulation is similar to species showing quite low heterozygosity (e.g., *A. thaliana*). Thus, using strigolactones in the process of development of new strategies is an alternative that can be applied to a wide range of cultivars.

Acknowledgments

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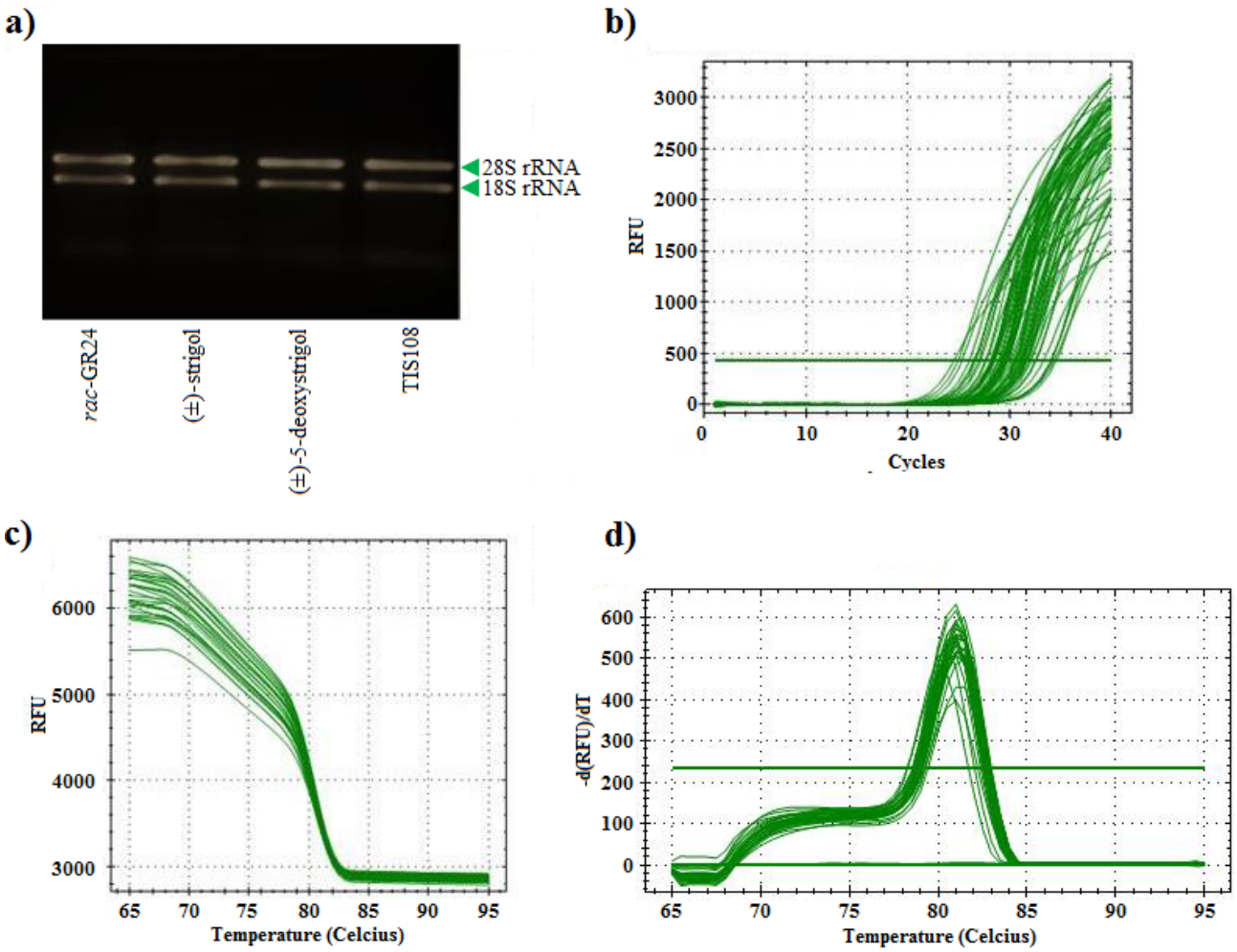
Supplementary Table 1. List of peptide sequences used for *MAX1* phylogenetic tree analysis.

NCBI Accession	Description	Species
XP_010669378.1	PREDICTED: cytochrome P450 711A1 isoform X2	*Beta vulgaris* subsp. *vulgaris*
AKM06059.1	*MAX1*	*Jatropha curcas*
AWB09331.1	*MAX1*	*Solanum lycopersicum*
OAP07831.1	*MAX1*	*Arabidopsis thaliana*
AKC54671.1	MAX1a protein	*Malus hupehensis*
AGI65361.1	cytochrome p450 family CYP711A member	*Medicago truncatula*
AGI60164.1	SLB1	*Oryza sativa Japonica Group*
BBM90835.1	cytochrome P450 CYP711A	*Lotus japonicus*
BBA85739.1	cytochrome P450 CYP711A	*Zea mays*



Supplementary Figure 1. Assessment of total RNA integrity (a) and quality of the qPCR experiment. Gel image (a) represents total RNA extracts of 7.5 µM hormone or inhibitor–treated samples. Amplification (b), melt (c), and melt peak (d) curves retrieved from the device during qPCR experiments