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Generation and characterization of a double-knockout *Arabidopsis thaliana* line lacking expression of *AOX1a* and *VTC2*

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Abstract. In higher plants, the L-galactose pathway is the main pathway for the biosynthesis of vitamin C (ascorbate, Asc), the final step of which is connected with the functioning of the mitochondrial electron transport chain (ETC). In addition to the main cytochrome pathway, plant ETC includes an alternative pathway (AP) via alternative terminal oxidase (AOX). The engagement of AOX promotes Asc synthesis, and it is hypothesized that AOX suppression under conditions of Asc deficiency may reduce plant viability. The aim of this work was to examine the consequences of simultaneously knocking out two genes in *Arabidopsis thaliana*: *AOX1a*, the most stress-inducible AOX gene, and *VTC2*, encoding a key enzyme of the L-galactose pathway of Asc synthesis. Two lines of *A. thaliana* with T-DNA insertions in the target genes were crossed to generate hybrid lines. Seed characteristics of the first (F_1) and second (F_2) generations were analyzed. F_1 seeds were larger than those of parent lines, possibly due to heterosis. In the F_2 generation, self-pollination of F_1 plants resulted in seeds with significant size variation, including a group of small seeds with degenerative morphological abnormalities. Most of small seeds failed to germinate or died at the seedling stage. PCR genotyping of these seeds revealed the absence of native *AOX1a* and *VTC2* indicating a lethal mutation caused by simultaneous knockout of both genes. One likely genetic cause is the interaction of mutations in non-allelic genes. At the physiological level, irreversible respiratory damage may occur, possibly including the impact of a cryptic mutation in the *vtc2* line. Further studies are necessary to confirm these hypotheses. In general, the results obtained indicate the vital co-functioning of the AP and the L-galactose pathway of Asc biosynthesis and may be useful for the development of genetically engineered techniques for the control of vitamin C synthesis in plants.

Key words: *Arabidopsis thaliana*; alternative oxidase; ascorbate; GDP-L-galactose phosphorylase; *AOX1a* and *VTC2* knockout lines; crossing; genotyping; double mutants; lethal mutation

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Получение и характеристика двойной нокаутной линии *Arabidopsis thaliana*, лишенной экспрессии *AOX1a* и *VTC2*

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Аннотация. У высших растений L-галактозный путь является основным путем биосинтеза витамина С (аскорбата, Аск), последний этап которого связан с функционированием электрон-транспортной цепи митохондрий. Помимо основного цитохромного пути, электрон-транспортная цепь растений содержит альтернативный путь через альтернативную терминальную оксидазу (АОХ). Вовлечение АОХ способствует синтезу Аск, поэтому предполагается, что подавление альтернативного пути в условиях дефицита Аск может привести к снижению жизнеспособности растений. В связи с этим была поставлена цель – рассмотреть последствия нокаутирования в растениях *Arabidopsis thaliana* одновременно двух генов: *AOX1a*, наиболее стресс-индуцибельного гена АОХ, и *VTC2*, гена ключевого фермента L-галактозного пути синтеза Аск. Для этого проведено скрещивание двух линий

A. thaliana с Т-ДНК инсерцией в целевых генах и получены гибридные линии. Изучены характеристики семян первого (F_1) и второго (F_2) поколений. Семена F_1 отличались более крупными размерами по сравнению с родительскими линиями, что могло быть следствием гетерозиса. В поколении F_2 в результате самоопыления растений F_1 сформировались семена, значительно варьирующие по размерам, в том числе группа мелких семян, имеющих дегенеративные морфологические отклонения. Большинство мелких семян не прорастало или погибало на стадии появления проростка. Генотипирование этих семян с помощью ПЦР установило отсутствие нормальных копий *AOX1a* и *VTC2* в геноме, что указывает на появление летальной мутации при одновременном нокауте обоих генов. Обсуждаются причины летального исхода двойного нокаута. Одной из генетических причин, по-видимому, стало взаимодействие мутаций (неаллельных генов). На физиологическом уровне, возможно, возникли необратимые нарушения дыхания, в том числе из-за влияния в линии *vtc2* криптической мутации. Для подтверждения данных гипотез требуются дальнейшие исследования. В целом полученные результаты свидетельствуют о жизненно важном совместном функционировании альтернативного и L-галактозного путей биосинтеза Аск и могут быть полезны для развития генно-инженерных приемов управления синтезом витамина С в растениях.

Ключевые слова: *Arabidopsis thaliana*; альтернативная оксидаза; аскорбат; ГДФ-L-галактозофосфорилаза; линии с нокаутом *AOX1a* и *VTC2*; скрещивание; генотипирование; двойная нокаутная линия; летальная мутация

Introduction

Ascorbate (vitamin C) is an important multifunctional antioxidant that participates in stress resistance and redox signaling (Smirnoff, 2018; Foyer et al., 2020; Matos et al., 2022, and others). Plants replenish their ascorbate pool through several mechanisms, including biosynthetic processes and regeneration via the ascorbate-glutathione cycle. Among these pathways, the L-galactose pathway is the dominant one (Dowdle et al., 2007; Wheeler et al., 2015). In the L-galactose pathway, GDP-D-mannose is converted into GDP-L-galactose, L-galactose-1-phosphate, L-galactose, L-galactono-1,4-lactone, and finally into L-ascorbate (Asc) (Fig. 1). A key step in this pathway is the reaction catalyzed by GDP-L-galactose phosphorylase (GGP) (Yoshimura et al., 2014; Matos et al., 2022). The GGP enzyme is encoded by the paralogous genes *VTC2* and *VTC5* (VITAMIN C), which demonstrate light-dependent expression patterns (Yoshimura et al., 2014; Smirnoff, 2018). Among these, *VTC2* plays the primary role in Asc biosynthesis. Knockout of *VTC2* (unlike its paralog *VTC5*) results in a significant decrease (up to 80 % or more) in Asc levels and plant growth (Dowdle et al., 2007; Lim et al., 2016).

The final stage of the L-galactose pathway is associated with the mitochondrial electron transport chain (ETC), where L-galactono-1,4-lactone dehydrogenase (GLDG) oxidizes L-galactono-1,4-lactone to Asc by transferring electrons between complexes III and IV via the labile electron carrier cytochrome *c* (Fig. 1). Unlike mammalian ETC, plants, in addition to the main energy-conserving cytochrome pathway (CP), possess an alternative pathway for electron transport (AP) through an alternative terminal cyanide-resistant oxidase (AOX) (Garmash, 2022) (Fig. 1). The AP bypasses two sites of proton translocation, complexes III and IV, and therefore represents a non-energy-conserving branch of electron transport (Vanlerberghe et al., 2020). The main function of the AP is to maintain redox balance during mitochondrial electron transport and to reduce reactive oxygen species (ROS) production. This impacts the metabolism of not only mitochondria but also cells and plants as a whole (Del-Saz et al., 2017; Vanlerberghe et al., 2020; Garmash, 2022). Moreover, the AP promotes Asc synthesis by accepting electrons from ubiquinone and maintaining the cytochrome *c* pool in a more oxidized state (Bartoli et al., 2006; Matos et al., 2022).

Studies of mutant lines with *VTC2* suppression reveal a number of defects and metabolic disturbances, especially under stressful conditions (Lim et al., 2016; Matos et al., 2022). Ascorbate deficiency and the stunted phenotype observed in these mutant plants likely result from long-term impact of the mutation on respiratory activity, mitochondrial ETC function, and, in particular, on electron transfer via AOX (Matos et al., 2022).

Nuclear genome of *A. thaliana* includes five genes of AOX, with the highest expression in response to various types of stress attributed to the gene *AOX1a* (Del-Saz et al., 2017; Garmash, 2022). *A. thaliana* plants overexpressing *AOX1a* demonstrated increased Asc synthesis under stress conditions (high light) (Garmash et al., 2022; Sweetman et al., 2022), whereas mutant lines with *AOX1a* suppression showed decreased Asc levels (Vishwakarma et al., 2015; Garmash et al., 2022). Additionally, *A. thaliana* plants of the *vtc2* line exhibited AOX activation compared with wild-type plants, and treatment of mutant plants with a specific inhibitor of the AP resulted in a significant (up to 40 %) decrease in ATP concentration (Garmash et al., 2024). Other authors (Talla et al., 2011) studied responses of *A. thaliana* plants with knockout of the GDP-mannose phosphorylase gene catalyzing GDP-D-mannose synthesis (*vtc1* line) on mitochondrial and chloroplast ETC inhibition, demonstrating mutual complementation between Asc and AOX, preventing excess accumulation of ROS and protecting photosynthesis from photooxidation. These data confirm the participation of AOX in maintaining Asc synthesis and optimizing energy balance and indicate that suppression of the AP under conditions of Asc deficiency can result in decreased plant viability. Therefore, we hypothesized that *AOX1a* and *VTC2* play a key role in providing energy and vital functions of the plant organism.

The aim of this paper was to study the consequences of simultaneous knockout of two genes, *AOX1a* and *VTC2*, in *A. thaliana* plants. To this end, mutant lines were crossed, and the characteristics of seeds from the first (F_1) and second (F_2)

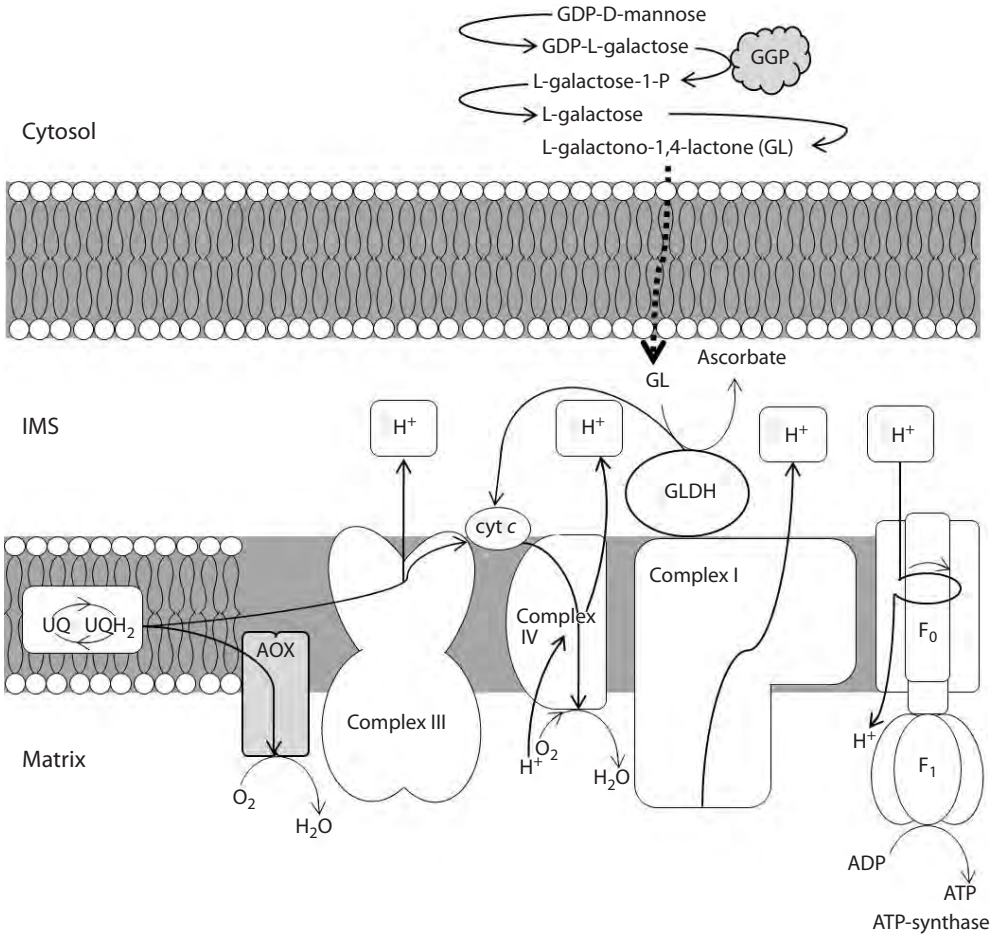

Fig. 1. Schematic representation of the relationship between ascorbate synthesis and the electron transport chain (ETC) of plant mitochondria.

The ETC components in the inner mitochondrial membrane are shown: from the ubiquinone pool (UQ/UQH_2) electrons are transferred via the alternative pathway (AP) catalyzed by alternative oxidase (AOX) and via the cytochrome pathway through complex III, cytochrome *c*, and complex IV. GGP denotes GDP-L-galactose phosphorylase. L-galactono-1,4-lactone (GL), synthesized through the L-galactose pathway and transported from the cytosol into the mitochondrial intermembrane space (IMS), is oxidized to ascorbate by L-galactono-1,4-lactone dehydrogenase (GLDG), which transfers electrons to cytochrome *c*. Electron transport along the ETC is coupled with proton pumping into the intermembrane space and the "return" of protons into the matrix via ATP synthase, driving ATP production.

generations were analyzed. Double knockouts were confirmed by T-DNA insertion localization using primers specific to the target genes.

Material and methods

Mutant lines with T-DNA insertion into *AOX1a* – SALK_084897 and into *VTC2* – SALK_146824C were used for hybridization (Fig. 2).

Seeds of mutant lines were obtained from the Arabidopsis Biological Resources Centre (ABRC, Ohio University, USA). The line with knockout of *AOX1a* (*aox1a*) contains a T-DNA insertion in one copy of the *AOX1a* gene, making the line *aox1a* heterozygous. This line also has an insertion in the *EFS* gene, which encodes the flowering inhibitor histone-lysine N-methyltransferase. The knockout line for *VTC2* (*vtc2*) has insertions in both copies of the gene and is homozygous. The wild-type line Columbia-0 (Col-0) was used to control for relative transcript content. Seeds of Col-0 were obtained from the Nottingham Arabidopsis Stock Centre (NASC, UK) and kindly provided by O.I. Grabelnykh and V.I. Tarasenko (SIPPB SB RAS).

Seeds were planted, stratified, and germinated in 200 cm^3 pots containing a mixture of perlite, vermiculite, and soil in a 1 : 1 : 2 ratio. Seedlings were grown under controlled conditions at 22 °C with a light intensity of 90 μmol quanta/($m^2 \cdot s$) and a 16-hour photoperiod until the budding phase (stage 5.1; Boyes et al., 2000). Lighting was provided by luminescent lamps (TL-D 30W and TL-D 30WAquarelle, Philips, Netherlands).

Hybridization was performed by transferring pollen from the line SALK_146824C (*vtc2*) onto the line SALK_084897 (*aox1a*) under microscopic control. For this purpose, unblown buds (one day before blooming) on the maternal plant were carefully opened and dissected to remove all stamens with

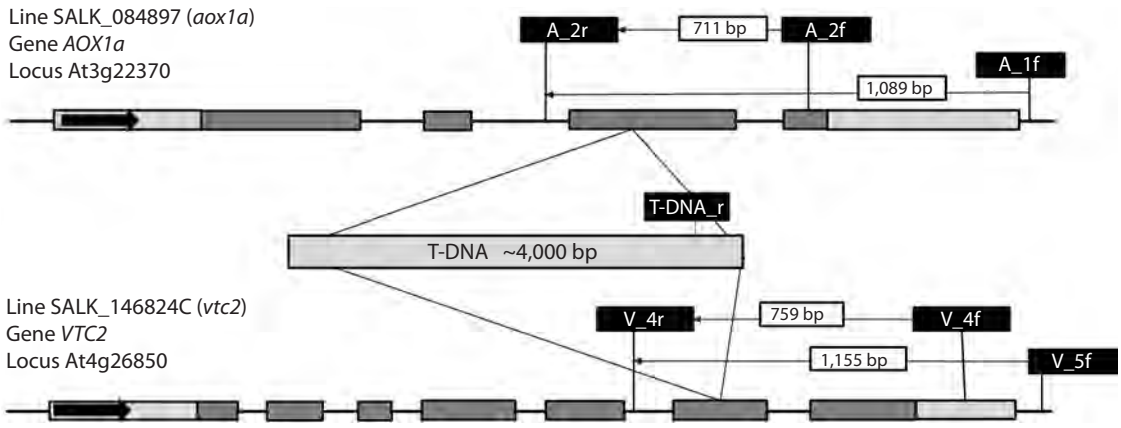

Fig. 2. Scheme of primer locations, PCR product lengths, and T-DNA insertion sites in the *AOX1a* gene from line SALK_084897 (*aox1a*) and *VTC2* from line SALK_146824C (*vtc2*).

Primer pairs V_4f-V_4r and V_5f-V_4r, as well as A_2f-A_2r and A_1f-A_2r, allow identification of the wild type *VTC2* or *AOX1a* genes, respectively. Primer pairs V_4f-T-DNA_r and V_5f-T-DNA_r, as well as A_2f-T-DNA_r and A_1f-T-DNA_r, allow identification of the corresponding mutant genes.

Table 1. Primers for genotyping and checking for T-DNA insertions in the *VTC2* gene (V) of line SALK_146824C (*vtc2*), and the *AOX1a* gene (A) of line SALK_084897 (*aox1a*)

Name	Sequence
T-DNA_r	CTTCCTTTCTCGCCACGTTC
V_4f	ACTGAAGGACAAGGCACTCG
V_4r	ACCTTTTCACCCTTGCTCATCT
V_5f	TCATAACCAGAGCTTGTGGTGG
A_1f	GATCCACATCCATTGGGTCCT
A_2r	CACTTCGTTAGTGCTTTGGATTCTTGG
A_2f	GCTTCCTTTAGTTCACGACCTTGG

pollen with tweezers. Pollen from the paternal plant was transferred onto the stigma of the maternal plant's pistil. The fertilized flower was marked, and fertilization was repeated for three consecutive days. All surrounding flowers were regularly removed during the budding phase. The plant was isolated with a transparent plastic cylinder until seed maturation.

Seed size from the F_1 and F_2 generations was measured (n = 111 and 496 for F_1 and F_2, respectively). The length (major semi-axis of the ellipse, a, mm) and width (minor semi-axis of the ellipse, b, mm) of the seeds were measured under a microscope. The ellipse area (S, mm^2) was calculated as $S = a \cdot b \cdot \pi$. F_2 seeds were divided according to size into three groups: large, medium, and small (n = 49, 365, and 82 for each group, respectively).

To check for embryo presence, some seeds from each group, swollen after soaking in water, were dissected under a microscope.

To analyze the relative levels of gene transcripts, genotyping and qPCR were performed on plant samples at growth stage 1.14 (Boyes et al., 2000) and on seeds.

To genotype the parental lines (*aox1a*, *vtc2*) and crossbred lines, DNA from the corresponding samples was used along with primers (Fig. 2, Table 1). DNA was extracted from 50–100 mg of air-dried leaves or dry seeds using the Sorb-GMO-B kit (Sintol, Russia) according to the manufacturer's instructions. The PCR reaction mixture contained 4 μL of Screen Mix (Evrogen, Russia), 4 μL of each corresponding forward and reverse primer (0.3 μM) (Sintol, Russia), 7 μL of ddH_2O (PanEco, Russia), and 1 μL of DNA. Amplification consisted of a preliminary denaturation for 5 minutes at 95 °C, followed by 35 cycles of denaturation (15 s) at 95 °C, primer annealing (30 s) at 55 °C, and elongation (60 s) at 72 °C, with a final elongation for 2 minutes at 72 °C. PCR products were analyzed by electrophoresis on a 1 % agarose gel in Tris-Acetate buffer. DNA fragments were stained with Safe Green Stain (APGENA, Russia) and visualized with blue light using the SuperRay Maxi system (APGENA, Russia). Fragment lengths were determined using a 100+ bp DNA ladder (100–1,500 bp) (Evrogen, Russia).

Relative levels of gene transcripts were analyzed by qPCR using the CFX96 system (Bio-Rad, USA). Rosette leaves were frozen in liquid nitrogen and stored at –80 °C prior to analysis. RNA was extracted using the HiPure Plant RNA Kit (Magen, China) according to the manufacturer's instructions. RNA concentration was measured using Qubit (Thermo Fisher Scientific, USA) with the Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific, USA). For cDNA synthesis, the MMLV RT kit with oligo(dT) primers (Evrogen, Russia) was used. qPCR reactions were performed with "qPCRmix-HS SYBR" (Evrogen, Russia) and the corresponding primers (Table 2). Target gene expression was normalized to the geometric mean of the *AT2G28390* and *AT4G34270* reference genes (Czechowski et al., 2005). Relative transcript levels were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak, Schmittgen, 2001). Analyses were performed on three biological replicates, including leaves from one crossbred plant or 3–5 individual plants of the original lines, with two technical replicates for each sample.

Table 2. The list of primers for qPCR in *A. thaliana*

Gene	Locus	Name (by NCBI)	Forward primer (5′–3′) Reverse primer (5′–3′)	PCR product length, bp
AOX1a	At3g22370	Alternative oxidase 1A	ATGATAACTCGCGGTGGAGC TCGTCGGAGCTCTAGTCCAT	166
VTC2	At4g26850	GDP-L-galactose phosphorylase 1	GGACCTCCATGGGTTCTGTT GAGGTTACTGCTCTCGCCTT	131
*AT2G28390**	At2g28390	SAND family protein	AACTCTATGCAGCATTTGATCCACT TGATTGCATATCTTTATCGCCATC	61
*AT4G34270**	At4g34270	TIP41-like family protein	GTGAAAACTGTTGGAGAGAAGCAA TCAACTGGATACCCTTTCGCA	61

Note. Primers were designed using Primer-BLAST (Ye et al., 2012). * – reference genes (from Czechowski et al., 2005).

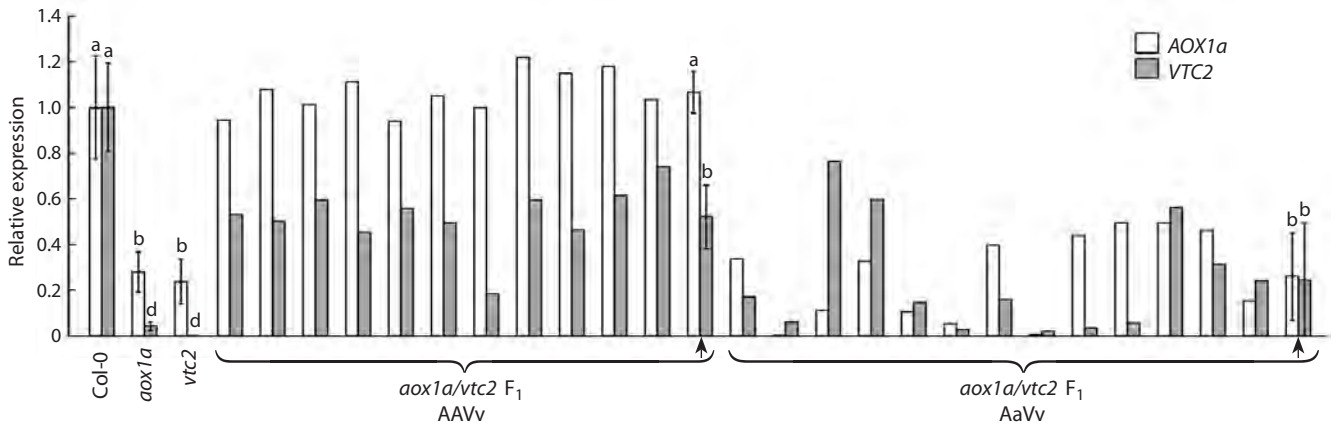

Fig. 3. Relative levels of *AOX1a* and *VTC2* transcripts in leaves of *A. thaliana* plants from the wild-type line (Col-0), parental lines (*aox1a* and *vtc2*), and 24F_1 hybrids (*aox1a/vtc2*).

Arrows indicate mean values and their standard errors for the presumed genotypes (AAVv and AaVv). Statistical significance of differences in gene expression levels is indicated by different letters (ANOVA, Duncan's test, $p \leq 0.05$).

Results

Genes *AOX1a* (At3g22370), *VTC2* (At4g26850), and *EFS* (At1g77300) are located on chromosomes 3, 4, and 1 of *A. thaliana*, respectively. The offspring of heterozygotes obtained by crossing lines SALK_084897 and SALK_146824C, according to unlinked inheritance and Mendel's third law, allow obtaining all possible combinations of variants in the F_1 generation, and homozygous offspring for both target mutations in the F_2 generation.

Relative transcript levels of *AOX1a* obtained by qPCR in the *aox1a* line were significantly lower than in the Col-0 line, and the level of *VTC2* mRNA in the *vtc*2 line was nearly undetectable (Fig. 3). Seed size of the *aox1a* line also varied notably compared to the *vtc2* line (Fig. 4). These data indirectly confirm the heterozygosity of *aox1a* and the homozygosity of *vtc2* mutant alleles, which was further verified by the presence of T-DNA insertions in the corresponding lines (Fig. 5). Bands corresponding to PCR products with primer pairs V_4f-V_4r and V_5f-V_4r, specific to wild-type genes, were absent, while bands with primer pairs V_4f-T-DNA_r and V_5f-T-DNA_r, confirming T-DNA insertion, were present in the electrophoregram of the *vtc2* line. The T-DNA insertion was located precisely at position 13,499,579 in the sixth exon of the gene At4g26850 in the *vtc2* line (Belykh et al., 2024). The presence of a band with primer pair A_2f-A_2r and the absence of one with A_2f-T-DNA_r indicated the wild-type status of the *AOX1a* gene.

The *aox1a* line, in contrast, is characterized by a band corresponding to the PCR product of the V_4f-V_4r primer pair and the absence of a band corresponding to V_4f-T-DNA_r, confirming the presence of a wild-type copy of the *VTC2* gene (Fig. 5). Bands corresponding to both primer pairs for the *AOX1a* gene (A_2f-A_2r and A_2f-T-DNA_r; A_1f-A_2r and A_1f-T-DNA_r) indicate the coexistence of a wild-type copy together with a T-DNA insertion-disrupted copy, characteristic of a heterozygous line.

To predict segregation in the F_1 generation, a scheme based on a Punnett square was drawn up to determine possible genotype variants of the offspring based on parental genotypes and their gametes, calculated by dividing the number of cells of a given genotype by the total number of cells. According to the scheme, segregation by genotype was 1 : 1 : 1 : 1 (four

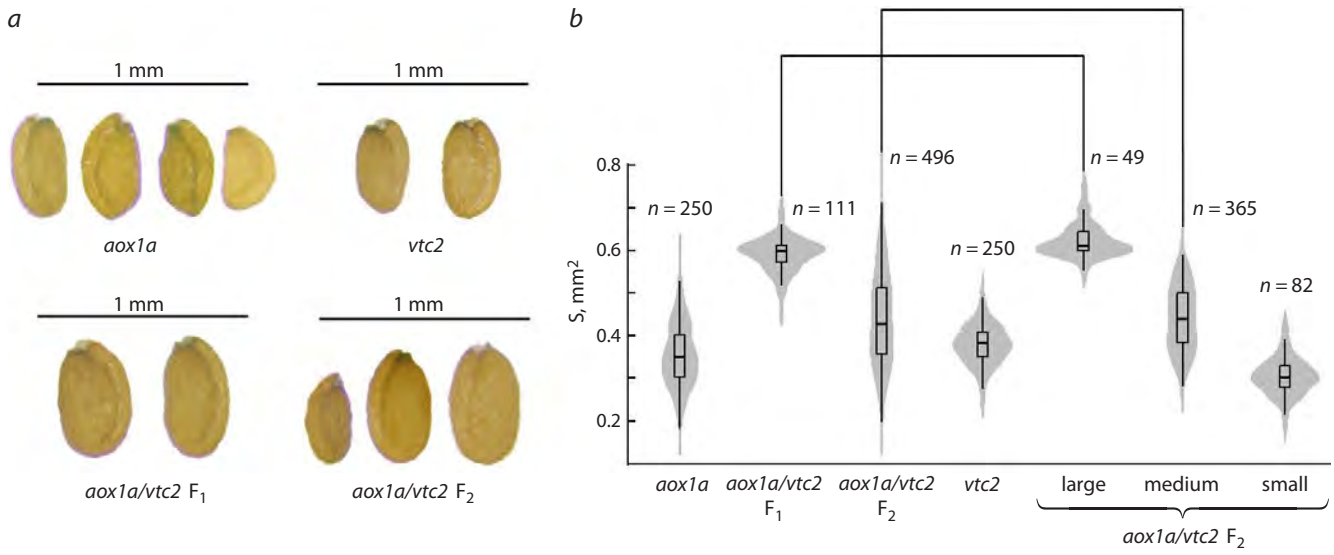

Fig. 4. Appearance (*a*) and seed size (*S*) (*b*) of parental and crossbred (*aox1a/vtc2*) *A. thaliana* lines from the F_1 and F_2 generations.

Panel (*b*) shows seed size distribution presented as violin plots combined with boxplots, including arithmetic mean values and standard errors. *n* indicates sample size. Differences between paired parameters not marked with a square bracket are statistically significant at $p < 0.01$ (Dunn's test with the Bonferroni correction).

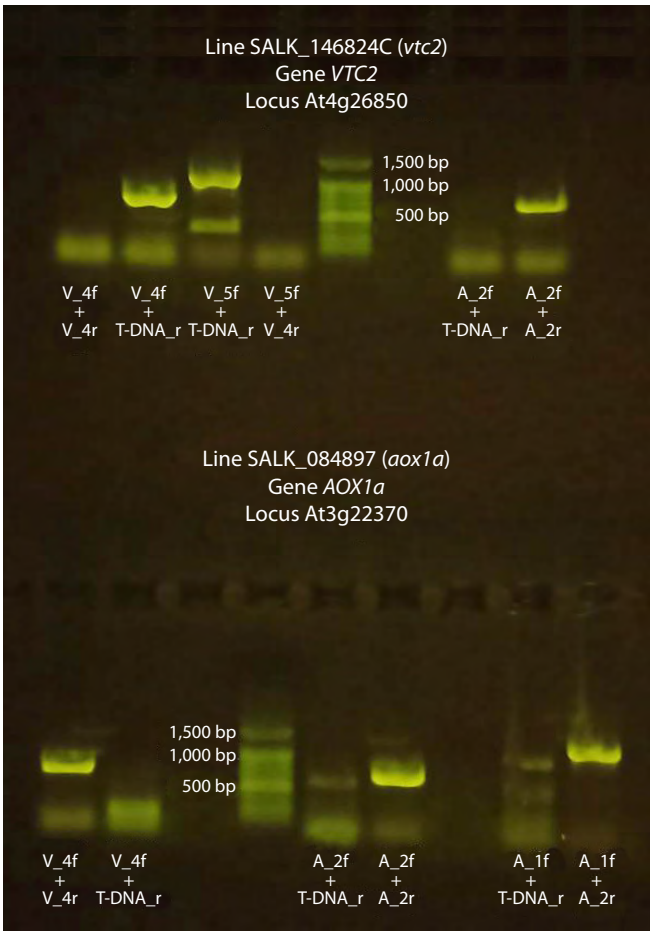

Fig. 5. Electrophoregram of DNA amplicons from *A. thaliana* plants of the *vtc2* and *aox1a* lines, demonstrating the presence of T-DNA insertions in the *VTC2* or *AOX1a* genes correspondently.

Primer descriptions are provided in Table 1.

phenotypes in equal proportions) (Table 3). In other words, 25 % of F_1 seeds belonged to the mutant line AaVvEE, deficient in *VTC2* and *AOX1a*; 25 % had genotype AaVvEe, deficient in all genes (*AOX1a*, *VTC2*, *EFS*); the remaining seeds were represented by genotypes deficient in *VTC2* or in *VTC2* and *EFS*.

F_1 seeds obtained from the cross were significantly larger than those from the parental lines (Fig. 4). This likely results from heterosis, manifested as increased hybrid viability due to the inheritance of diverse alleles from genetically distinct parents.

Plants grown from 24 F_1 seeds exhibited decreased *VTC2* transcript levels (Fig. 3). More than half (54 %) also showed decreased *AOX1a* transcript levels. The genome of the remaining 46 % of plants presumably contained both copies of *AOX1a*, as in wild-type plants. Thus, the hybridization corresponded well with the predicted segregation.

Following self-pollination, the F_1 plants produced fruits containing F_2 seeds. These seeds varied considerably in size (Fig. 4) and were classified into three significantly different groups. Notably, the largest seed group did not differ in size from the F_1 seeds, suggesting genotypes of either AAVv or AaVv, as in the F_1 generation. The smallest seeds exhibited degenerative morphological abnormalities; dissection revealed undeveloped embryos (Fig. 6). Most small seeds failed to germinate or died at the seedling stage.

Genotyping of large seeds from the F_2 generation revealed the presence of wild-type copies of the *AOX1a* and *VTC2* genes in their genomes (Fig. 7). In contrast, bands corresponding to wild-type genes were absent in the electrophoregram of DNA extracted from small seeds. This finding confirms that the genotype of small seeds corresponds to the double knockout mutant *aox1a/vtc2*.

Table 3. Punnett square for the F_1 generation from crossing lines SALK_146824C (*vtc2*) and SALK_084897 (*aox1a*)

Line genotype	SALK_084897 (*aox1a*) AaVVEe				
SALK_146824C (*vtc2*) AAvvEE		AVE	aVE	AVe	aVe
	AvE	AAVvEE	AaVvEE	AAVvEe	AaVvEe
	AvE	AAVvEE	AaVvEE	AAVvEe	AaVvEe
	AvE	AAVvEE	AaVvEE	AAVvEe	AaVvEe
	AvE	AAVvEE	AaVvEE	AAVvEe	AaVvEe
		Generation deficient in *VTC2*	Generation deficient in *VTC2* and *AOX1a*	Generation deficient in *VTC2* and *EFS*	Generation deficient in *VTC2*, *AOX1a*, *EFS*

Note. A – wild-type (as in Col-0) *AOX1a* gene; a – insertion in *AOX1a*; V – wild-type (as in Col-0) *VTC2* gene; v – insertion in *VTC2*; E – wild-type (as in Col-0) *EFS* gene; e – insertion in *EFS*.

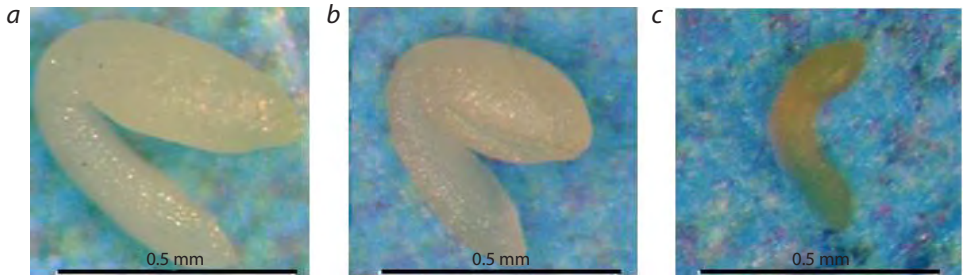

Fig. 6. Embryos dissected from large (*a*), medium (*b*) and small (*c*) seeds of *A. thaliana* crossbreed lines *aox1a/vtc2* of the F_2 generation.

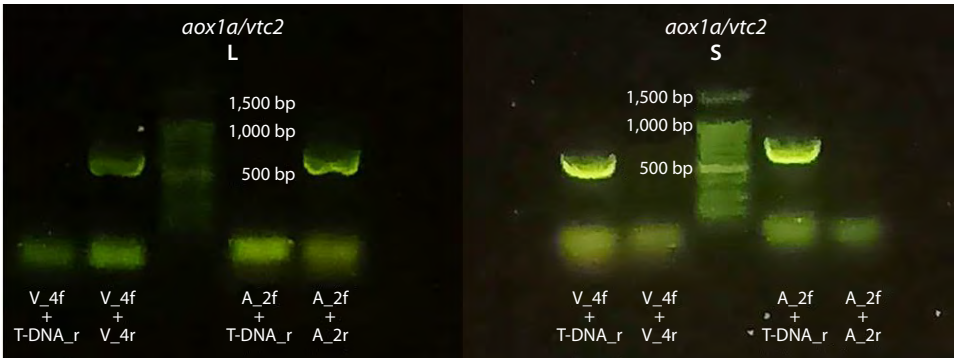

Fig. 7. Electrophoregram of DNA amplicons from large (L) and small (S) seeds of the F_2 generation from the *A. thaliana* crossbreed *aox1a/vtc2* lines.

The presence of bands corresponding to primer pairs V_4f-V_4r and A_2f-A_2r, characteristic of the wild-type variants of the *VTC2* and *AOX1a* genes, respectively, is evident in large seeds and absent in small seeds. Primer descriptions are provided in Table 1.

Discussion

Studies of vitamin C-deficient lines, primarily *vtc2*, have demonstrated the important role of the L-galactose pathway of vitamin C biosynthesis, and in particularly that of the key enzyme GGP, in replenishing the main pool of this metabolite in plants. However, it is believed that the reduced growth and viability of mutants are associated more with metabolic disturbances and an imbalance in the respiratory ETC than with decreased Asc levels alone (Lim et al., 2016; Matos et al., 2022).

It has been shown that these abnormalities may be linked to a cryptic mutation in the mutant line (Lim et al., 2016). However, the same authors demonstrated that the growth defects of *vtc2* mutants segregate independently of the mutation in the *VTC2* gene, indicating that the potential cryptic mutation is located on a different chromosome. This allows the analysis of the relationship between the phenotype of the offspring and the status of the *VTC2* gene.

The causes of metabolic and growth disturbances of the *vtc2* line have been identified in several studies using mutant

combinations with this line. The growth-reduced phenotype of *vtc2* has been described in combination with mutations causing chronic photooxidative stress (Müller-Moulé et al., 2004), knockout of chloroplast ascorbate peroxidase (Giacomelli et al., 2007), and suppression of the autoimmune response (Zhu et al., 2013). A double *vtc2-1/abi4* mutant, generated by crossing the *A. thaliana vtc2-1* line (a loss-of-function mutant with significantly decreased *VTC2* transcription) with an abscisic acid-insensitive mutant line (*abi4*), exhibited growth and morphology similar to those of wild-type plants (Kerchev et al., 2011). This suggests that abscisic acid contributes to the stunted phenotype of *vtc2-1*. However, all described double knockout mutants retained the ability to grow. The only lethal phenotype was observed in the double knockout of both GGP genes (*VTC2* and *VTC5*) (Dowdle et al., 2007).

Previously, vitamin C-deficient mutants, *vtc1* (Conklin et al., 1996) and *vtc2* (Jander et al., 2002), were initially selected for their sensitivity to ozone. Ozone sensitivity likely results from imbalances in respiratory pathways and increased ROS generation due to low Asc synthesis. Inhibition of the cytochrome pathway under elevated ozone levels occurred simultaneously with a sharp increase in AOX activity (Ederli et al., 2006; Pasqualini et al., 2007). These findings support the hypothesis that enhanced AOX activity in the *vtc2* line protects against of oxidative stress and possible plant death.

This hypothesis was confirmed in our study, where the F_2 generation was nonviable when both *AOX1a* and *VTC2* were knocked out simultaneously. It is possible that the lethal mutation resulted from the interaction of mutations in non-allelic genes, including a cryptic mutation. However, the role of the interaction between the *AOX1a* and *VTC2* mutations – rather than between *AOX1a* and the cryptic mutation – is supported by the observation that all F_2 seeds that failed to germinate carried a mutation in *VTC2*, which appears to segregate independently from the cryptic mutation (Lim et al., 2016). To clarify this issue, more detailed molecular genetic studies are needed, including genomic DNA sequencing and RNA analysis to identify mutations affecting pre-mRNA splicing.

Conclusion

As a result of crossbreeding of the SALK_084897 and SALK_146824C lines, seeds of varying sizes were obtained in the F_2 generation. The smallest seed group was characterized as aberrant, displaying degenerative morphological deviations and an inability to germinate. Genotyping of these seeds revealed the absence of both *AOX1a* and *VTC2*, indicating a lethal mutation arising from the simultaneous double knockout of these genes. This finding highlights the essential joint functioning of two systems: the alternative pathway through AOX and the L-galactose pathway of Asc biosynthesis linked to the mitochondrial ETC. Further investigation into the interrelationship and coordination between the AP and the L-galactose Asc synthesis pathway could focus on producing double mutants with defects in genes involved in ascorbate synthesis, other respiratory electron transport components, and participants in hormonal and redox signaling. Such research may aid in the development of genetic engineering strategies to enhance ascorbate synthesis, thereby improving stress tolerance in significant food-value crops.

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