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The cinnamyl alcohol dehydrogenase gene family is involved in the response to *Fusarium oxysporum* in resistant and susceptible flax genotypes

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Flax (*Linum usitatissimum* L.) is used for the production of textile, oils, pharmaceuticals, and composite materials. Fusarium wilt, caused by the fungus *Fusarium oxysporum* f. sp. *lini*, is a very harmful disease that reduces flax production. Flax cultivars that are resistant to Fusarium wilt have been developed, and the genes that are involved in the host response to *F. oxysporum* have been identified. However, the mechanisms underlying resistance to this pathogen remain unclear. In the present study, we used transcriptome sequencing data obtained from susceptible and resistant flax genotypes grown under control conditions or *F. oxysporum* infection. Approximately 250 million reads, generated with an Illumina NextSeq instrument, were analyzed. After filtering to exclude the *F. oxysporum* transcriptome, the remaining reads were mapped to the *L. usitatissimum* genome and quantified. Then, the expression levels of cinnamyl alcohol dehydrogenase (*CAD*) family genes, which are known to be involved in the response to *F. oxysporum*, were evaluated in resistant and susceptible flax genotypes. Expression alterations in response to the pathogen were detected for all 13 examined *CAD* genes. The most significant differences in expression between control and infected plants were observed for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B*, with strong upregulation of *CAD1B*, *CAD5A*, and *CAD5B* and strong downregulation of *CAD4A*. When plants were grown under the same conditions, the expression levels were similar in all studied flax genotypes for most *CAD* genes, and statistically significant differences in expression between resistant and susceptible genotypes were only observed for *CAD1A*. Our study indicates the strong involvement of *CAD* genes in flax response to *F. oxysporum* but brings no evidence of their role as resistance gene candidates. These findings contribute to the understanding of the mechanisms underlying the response of flax to *F. oxysporum* infection and the role of *CAD* genes in stress resistance.
Key words: flax; *Linum usitatissimum*; resistant cultivars; *Fusarium oxysporum*; RNA-Seq; transcriptome; *CAD*.

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Семейство генов дегидрогеназ коричного спирта вовлечено в ответ устойчивых и восприимчивых генотипов льна на заражение *Fusarium oxysporum*

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Лен (*Linum usitatissimum* L.) используется для производства текстиля, масел, фармацевтических препаратов и композитных материалов. Крайне вредоносным заболеванием, снижающим урожайность льна, является фузариозное увядание, вызываемое грибом *Fusarium oxysporum* f. sp. *lini*. Созданы устойчивые к фузариозному увяданию сорта льна и определены гены, вовлеченные в ответ на *F. oxysporum*, однако механизмы устойчивости *L. usitatissimum* к этому патогену до сих пор неясны. В настоящем исследовании мы использо-

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вали данные секвенирования транскриптомов восприимчивых и устойчивых генотипов льна, выращенных в контрольных условиях или зараженных *F. oxysporum*. Проанализировано около 250 миллионов прочтений, полученных на секвенаторе NextSeq Illumina. После фильтрации прочтений для исключения транскриптома *F. oxysporum* оставшиеся прочтения картировали на геном *L. usitatissimum* и провели их количественный анализ. Оценили экспрессию генов семейства *CAD*, которые, как известно, участвуют в ответе на заражение *F. oxysporum*, у устойчивых и восприимчивых к фузариозному увяданию генотипов. Изменение экспрессии в ответ на возбудителя выявили для всех 13 исследованных генов *CAD*. Наиболее значительные различия в экспрессии между контрольными и инфицированными растениями наблюдались для генов *CAD1B*, *CAD4A*, *CAD5A* и *CAD5B*: сильное повышение экспрессии выявлено для *CAD1B*, *CAD5A* и *CAD5B*, а сильное снижение – для *CAD4A*. Для большинства генов *CAD* уровни экспрессии были близкими при одинаковых условиях выращивания для всех изученных генотипов льна. Статистически значимое различие в изменении экспрессии между группами устойчивых и восприимчивых генотипов выявлено только для гена *CAD1A*. Наше исследование указывает на активное участие генов *CAD* в ответе растений льна на *F. oxysporum*, но не приводит свидетельств их роли в качестве кандидатов в гены устойчивости. Полученные результаты вносят вклад в понимание механизмов ответа льна на заражение *F. oxysporum* и роли генов *CAD* в устойчивости к стрессовым воздействиям.

Ключевые слова: лен; *Linum usitatissimum*; устойчивые сорта; *Fusarium oxysporum*; RNA-Seq; транскриптом; *CAD*.

Introduction

Flax (*Linum usitatissimum* L.) is an agricultural crop with numerous uses. High-quality fiber can be obtained from flax stems and is used for the production of textile and fiber-based materials for the healthcare, military, aerospace, and electronics industries (Costa et al., 2018). Flaxseed is also used in the production of pharmaceuticals, functional foods, and other products for human consumption, while linseed is used in paints, varnishes, and animal feed (Singh et al., 2011; Goyal et al., 2014). *Fusarium oxysporum* f. sp. *lini* is a harmful pathogen that reduces flax production and quality (Rashid, 2003). Flax genotypes showing resistance to Fusarium wilt have been identified, and cultivars with improved resistance have been bred (Diederichsen et al., 2008; Rozhmina et al., 2017). However, the molecular mechanisms underlying resistance to Fusarium wilt remain unclear, and the search for genes involved in the response to *F. oxysporum* is an area of active research. The involvement of pathogenesis-related proteins in the response to *F. oxysporum* infection was demonstrated (Wrobel-Kwiatkowska et al., 2004; Wojtasik et al., 2014; Galindo-Gonzalez, Deyholos, 2016), and the roles of antioxidants, polyamines, and phenolic compounds in response to the pathogen were shown (Lorenc-Kukula et al., 2007, 2009; Boba et al., 2011, 2016; Zeitoun et al., 2014; Wojtasik et al., 2015). Moreover, *F. oxysporum*-infected flax plants show cell wall rearrangements (Wojtasik et al., 2015, 2016; Boba et al., 2016).

Cinnamyl-alcohol dehydrogenases (CADs) are involved in the biosynthesis of lignin, which can function as a barrier against pathogens, and the role of *CAD* genes in the response to *F. oxysporum* was previously shown (Wrobel-Kwiatkowska et al., 2007; Preisner et al., 2014, 2018). Plants with downregulated *CAD* showed reduced (Wrobel-Kwiatkowska et al., 2007) or slightly decreased (Preisner et al., 2014) resistance to *F. oxysporum*. Sixteen *CAD* genes were identified in *L. usitatissimum* and their roles in plant growth and stress responses were examined in the Nike cultivar, which is relatively resistant to *Fusarium* infection (Preisner et al., 2018). In the present study, we evaluated the expression of *CAD* genes in resistant and susceptible flax genotypes under control conditions and *F. oxysporum* infection to determine the general trends in response to the pathogen and genotype-specific alterations in expression.

Materials and methods

Two *F. oxysporum*-susceptible flax cultivars (TOST and AP5), two resistant cultivars (3896 and Dakota), and two resistant BC_2F_5 populations (3896 × AP5, recurrent parent AP5, and Dakota × AP5, recurrent parent AP5) were used in the present study. Seeds were obtained from the Institute for Flax (Torzhok, Russia) and sterilized, first in 70 % ethanol for 1 min and then in 1 % sodium hypochlorite for 20 min. The plants were grown in 15 ml glass tubes on Murashige–Skoog medium in a growth chamber at 22 °C under a 16/8 h day/night cycle for seven days. Then, half of the plants were inoculated with *F. oxysporum* (pathogenic isolate #39 from the phytopathogen collection of the Institute for Flax); the remaining uninoculated plants were used as controls. Forty-eight hours later, the root tips were collected and frozen in liquid nitrogen. In total, the material was obtained from 240 plants.

Total RNA was extracted from the pooled plants (10–12 plants each) using the RNeasy Plant Mini Kit (Qiagen, USA). We obtained 24 RNA samples from the TOST, AP5, 3896, Dakota, 3896 × AP5, and Dakota × AP5 plants under control and infection in duplicate. The quality and concentration of the isolated RNA were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and Qubit 2.0 fluorometer (Life Technologies, USA). The TruSeq Stranded Total RNA Sample Prep Kit (Illumina, USA) was used to prepare the cDNA library. The libraries were sequenced on a NextSeq500 sequencer (Illumina) with 80-nucleotide paired-end reads (SRP119227, Sequence Read Archive).

Reads were trimmed using Trimmomatic (Bolger et al., 2014) and filtered against the *F. oxysporum* reference genome as described in our previous study (Dmitriev et al., 2017). Then, the remaining reads were mapped to the *L. usitatissimum* genome (GenBank assembly: GCA_000224295.2) using STAR (Dobin et al., 2013) and quantified using BEDTools (Quinlan, Hall, 2010). The genome sites where the *CAD* family genes (*CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, *CAD7*, and *CAD8*) are localized were identified in the latest *L. usitatissimum* genome assembly (GenBank assembly: GCA_000224295.2) using the data of Preisner et al. (2018). Namely, the sequences of the scaffold regions of the old *L. usitatissimum* genome assembly (GenBank: AFSQ00000000.1) that encode *CAD* transcripts were mapped to the latest *L. usitatissimum* ge-

nome assembly (GCA_000224295.2) and new coordinates were identified for the *CAD* transcripts. The counts per million (CPM) values were determined for 13 *CAD* genes in each cultivar and population under both control conditions and *Fusarium* infection, and then the log(*CPM Fusarium*/*CPM control*) values were calculated for each cultivar and BC_2F_5 population. This was performed using the equipment of the Genome Center of Engelhardt Institute of Molecular Biology (http://www.eimb.ru/rus/ckp/ccu_genome_c.php).

Results

In our previous study on the response of *L. usitatissimum* to *F. oxysporum*, we used RNA-Seq data for *de novo* transcriptome assembly and annotation and then quantified the expression levels of the identified transcripts (Dmitriev et al., 2017). Unfortunately, we had failed to identify a significant number of *CAD* family genes in our *de novo* transcriptome assembly. In the present study, we used an improved assembly of the *L. usitatissimum* genome (GCA_000224295.2), in which the scaffolds were mapped to specific chromosomes, as a reference for RNA-Seq read mapping, and *CAD* gene sequence data (Preisner et al., 2018) were used to identify the genome sites in which the *CAD* genes are located. This approach enabled us to evaluate the expression levels of all presently identified *CAD* genes in flax. Unique chromosome locations were determined for *CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, and *CAD7*, while *CAD8A*, *CAD8B*, *CAD8C*, and *CAD8D* were mapped to the same region of chromosome Lu7. Therefore, we performed an expression analysis of *CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, *CAD7*, and *CAD8* genes.

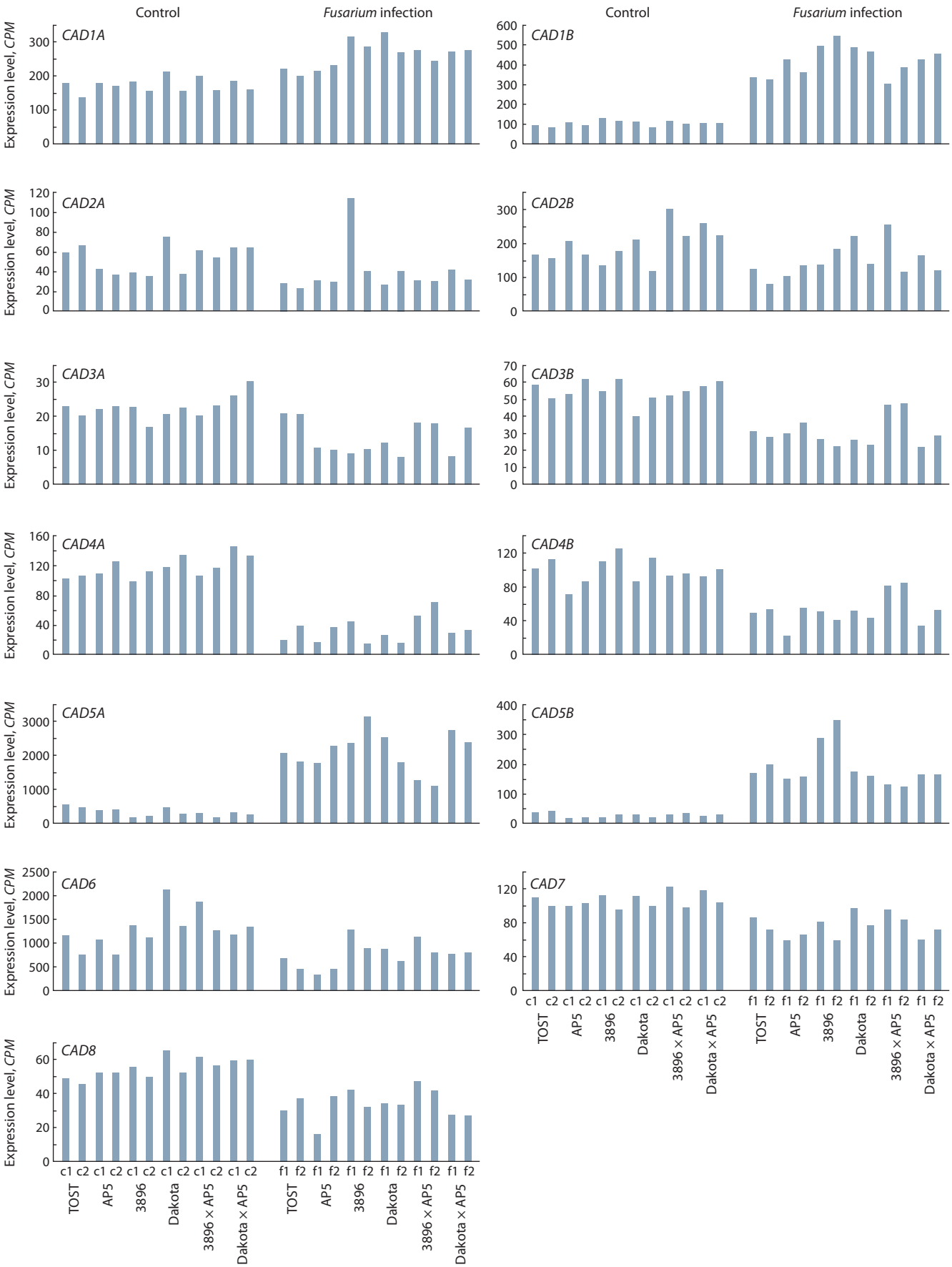
The expression levels of the 13 *CAD* family genes in six flax cultivars and populations grown under control conditions or inoculated with a pathogenic isolate of *F. oxysporum* were evaluated based on RNA-Seq data. The results are shown in the Figure, which presents the CPM values for each studied gene in resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and susceptible (TOST and AP5) genotypes under control conditions and 48 h after inoculation with *F. oxysporum* in biological replicates. Under both conditions, the expression levels of *CAD5A* and *CAD6* were the highest, and the expression levels of *CAD2A*, *CAD3A*, *CAD3B*, and *CAD8* were the lowest. Compared to control conditions, statistically significant alterations in expression under *Fusarium* infection were observed for all 13 genes ($p < 0.01$ for all genes except *CAD2B*, which was $p < 0.05$, Mann–Whitney test). The most significant differences in expression between the control and infected plants were observed for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B*; *CAD1B*, *CAD5A*, and *CAD5B* showed strong upregulation under *F. oxysporum* infection, and *CAD4A* showed strong downregulation. Under the same conditions, the expression levels were similar in all studied flax genotypes for most *CAD* genes; however, some exceptions were observed. For example, the expression levels of *CAD3A*, *CAD3B*, *CAD4A*, and *CAD4B* in the infected plants of the 3896 × AP5 population were higher than those in other infected genotypes.

To identify genotype-specific expression alterations in response to *F. oxysporum* infection, the log(*CPM Fusarium*/*CPM control*) values were calculated (see the Table). In all studied genotypes, the most significant (more than 3-fold in average) increases in expression were observed for *CAD1B*, *CAD5A*, and *CAD5B*, while the most significant decreases in expression were observed for *CAD4A*. For *CAD2A*, *CAD3A*, *CAD3B*, *CAD4B*, and *CAD6*, downregulation was observed in the majority of genotypes after *F. oxysporum* infection. For *CAD2B*, *CAD7*, and *CAD8*, the decrease in expression was only slight, and for some genotypes, no decrease was observed. Statistically significant differences in expression between resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and susceptible (TOST and AP5) genotypes were only observed for the *CAD1A* gene ($p < 0.05$, Mann–Whitney test).

Expression alterations in the *CAD* genes of *F. oxysporum* resistant and susceptible flax cultivars and populations

Gene	log(*CPM Fusarium/CPM control*)					
	Susceptible genotypes		Resistant genotypes			
	TOST	AP5	3896	Dakota	3896 × AP5	Dakota × AP5
CAD1A	0.42	0.36	0.82	0.70	0.54	0.65
CAD1B	1.86	1.91	2.08	2.24	1.67	2.04
CAD2A	–1.28	–0.38	1.04*	–0.73	–0.89	–0.80
CAD2B	–0.65	–0.64	0.04	0.14	–0.49	–0.75
CAD3A	–0.07	–1.10	–1.03	–1.09	–0.26	–1.17
CAD3B	–0.88	–0.80	–1.26	–0.87	–0.18	–1.23
CAD4A	–1.80	–2.08	–1.80	–2.56	–0.86	–2.14
CAD4B	–1.05	–1.03	–1.34	–1.07	–0.19	–1.14
CAD5A	1.91	2.32	3.66	2.51	2.22	3.09
CAD5B	2.14	2.85	3.56	2.66	1.98	2.55
CAD6	–0.73	–1.18	–0.19	–1.21	–0.69	–0.70
CAD7	–0.40	–0.70	–0.57	–0.28	–0.30	–0.75
CAD8	–0.47	–0.93	–0.50	–0.77	–0.37	–1.13

* A 3-fold difference between biological replicates was observed for the *CAD2A* gene in cultivar 3896 under *Fusarium* infection (see the Figure).



Expression of *CAD* genes in resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and susceptible (TOST and AP5) flax genotypes under control conditions (c1 and c2) and 48 h after inoculation with *F. oxysporum* (f1 and f2) in biological replicates.

Discussion

Recent progress in molecular analysis has provided novel opportunities for plant studies (Kage et al., 2015; Poland, 2015). Using high-throughput sequencing, genome and transcriptome sequences for many plant species can be obtained in a short time period and used for further research (Varshney et al., 2009; He et al., 2014). The draft genome sequence for flax was obtained in 2012 (Wang et al., 2012), and in 2018, the genome assembly was improved and chromosome-scale pseudomolecules were obtained using BioNano genome optical mapping (You et al., 2018). Flax transcriptomes and small RNAs obtained from different tissues, development stages, and under biotic and abiotic stresses have been sequenced. Such data has enabled the identification of genes and miRNAs that are expressed in particular organs and in the definite time and could play key roles in plant development, as well as the discovery of genes and miRNAs with altered expression under unfavorable conditions that are likely involved in the response to stress (Yu et al., 2014, 2016; Melnikova et al., 2015, 2016; Dmitriev et al., 2016, 2017, 2019; Galindo-Gonzalez, Deyholos, 2016; Dash et al., 2017; Gorshkova et al., 2018; Zyablitsin et al., 2018; Gorshkov et al., 2019; Krasnov et al., 2019; Wu et al., 2019).

In the present study, we used RNA-Seq data from *F. oxysporum*-resistant (3896, Dakota, 3896 × AP5, and Dakota × AP5) and -susceptible (TOST and AP5) flax genotypes grown under control conditions or 48 h after inoculation with *F. oxysporum* to evaluate the expression of *CAD1A*, *CAD1B*, *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD5A*, *CAD5B*, *CAD6*, *CAD7*, and *CAD8* and identified the *CAD* genes that were involved in the response to the pathogen. The genes showed different expression changes after *F. oxysporum* infection: in most genotypes, *CAD1A*, *CAD1B*, *CAD5A*, and *CAD5B* were upregulated, while *CAD2A*, *CAD2B*, *CAD3A*, *CAD3B*, *CAD4A*, *CAD4B*, *CAD6*, *CAD7*, and *CAD8* were downregulated. In the study by Preisner et al. (2018), decreased expression was observed for most *CAD* genes at 24 and 96 h after *F. oxysporum* infection in the flax cultivar Nike. In the present study, the greatest expression changes in infected plants were observed for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B*; three of these genes were upregulated, while *CAD4A* was downregulated. Our data for *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B* are consistent with the results of the previous study at 24 h after infection (Preisner et al., 2018). However, the changes observed by us were more significant, with a 3–5-fold change for *CAD1B*, a 2–5-fold change for *CAD4A*, a 4–13-fold change for *CAD5A*, and a 4–12-fold change for *CAD5B*. Based on our results, we suggest that *CAD1B*, *CAD4A*, *CAD5A*, and *CAD5B* are the most involved in the response of flax to *F. oxysporum*.

Searching for genes with diverse expression alterations in resistant and susceptible genotypes under stress conditions is important for the identification of resistance genes. In our study, statistically significant differences in expression between resistant and susceptible genotypes in response to the pathogen were observed only for *CAD1A*. Therefore, this gene could be involved in resistance to *F. oxysporum*. However, the changes were not pronounced. Thus, further investigations are necessary.

Conclusion

We performed expression analysis of *CAD* family genes after *F. oxysporum* inoculation based on RNA-Seq data and identified genes with significant up- and down-regulation after pathogen infection. The results of the present study indicate the involvement of *CAD* genes in response to *Fusarium* infection, but their role as resistance genes in the studied cultivars and populations is questionable. Our data also contribute to the understanding of the role of *CAD* genes in stress response and resistance.

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