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Investigating the Variation between Lignin Content and the Fracture Characteristics in *Capsicum annuum* Mutant Stems

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Abstract: The cultivation of horticultural plants in controlled greenhouse environments is a pivotal practice in modern agriculture, offering the potential to enhance crop productivity and mitigate climate change effects. This study investigates the biomechanical properties and lignin content of various *Capsicum annuum* mutant lines—'fragile-plant' (*frx*), 'tortuous internodi' (*tti*), and 'puffy-structured stem' (*pfi*)—in comparison to a commercially established variety, 'Garai Fehér'. We employed the acetyl bromide method to quantify lignin content and conducted three-point bending tests to assess rigidity in three distinct regions of the stem. Gene expression analysis of key lignin biosynthetic pathway genes (PAL, C4H, 4CL, CCoAOMT, CAD) was performed using qRT-PCR. The results revealed significant differences in lignin content and breaking force among the genotypes and stem regions. The *tti* mutants exhibited similar lignin content to the control but lower breaking strength, likely due to elongated internodes. The *frx* mutants showed uniformly reduced lignin content, correlating with their fragile stems. The *pfi* mutants displayed abnormally high lignin content in the top region yet demonstrated the lowest stem rigidity in every region. Overexpression of CAD and CCoAOMT was detected in the mutants in specific regions of the stem, suggesting alterations in lignin biosynthesis; however, we could not confirm the correlation between them. Our findings indicate that while lignin content generally correlates with stem rigidity, this trait is complex and influenced by more factors.

Keywords: *Capsicum annuum*; stem rigidity; biomechanical properties; lignin content; gene expression

Citation: Pápai, B.; Kovács, Z.; Tóth-Lencsés, K.A.; Bedő, J.; Chan, K.N.; Kovács-Weber, M.; Pap, T.I.; Csilléry, G.; Szőke, A.; Veres, A. Investigating the Variation between Lignin Content and the Fracture Characteristics in *Capsicum annuum* Mutant Stems. *Agriculture* **2024**, *14*, 1771. https://doi.org/10.3390/agriculture14101771

Academic Editor: Yuan Huang

Received: 13 August 2024
Revised: 16 September 2024
Accepted: 6 October 2024
Published: 8 October 2024

1. Introduction

Horticulture, alongside crop and livestock production, is one of the most crucial sectors in agriculture. The cultivation of horticultural plants in controlled greenhouse environments is a reliable and intensive agricultural practice. These controlled settings allow for the precise regulation of various environmental factors, such as temperature and light [1]. Consequently, the development of novel cultivation methodologies and technological interventions is essential to effectively mitigate the effects of changing climatic conditions.

Modern plant breeding utilizes mutant traits to enhance cultivars, a practice that could lead to innovative cultivation methodologies [2]. While these mutations might be valuable in specialized fields like ornamental breeding or agriculture, in natural ecosystems, mutations causing abnormal stem growth could compromise a plant's fitness and survival [3]. In the context of greenhouse farming, atypical growth mutants might be

beneficial, although their productivity largely depends on factors such as stem elongation and rigidity [4]. The structural role of the stem is pivotal, influencing plant height and biochemical processes, with optimal posture facilitating multifaceted organ interactions [5]. Abnormal stem growth often results from genetic mutations affecting hormone signaling pathways, leading to diverse phenotypes depending on intricate contextual cues.

The mechanical properties of plant stems, which are crucial for normal development, are significantly influenced by the organization of fibers within them. Collenchyma provides flexibility, whereas sclerenchyma imparts rigidity and strength [6]. The noticeable distinctions between fragile and non-fragile stems primarily occur within these mechanical tissues. The absence of interfascicular fibers correlates with a substantial reduction in stem strength [7]. The water content of plant tissues also affects their mechanical rigidity. Turgor pressure, resulting from water uptake in plant cells, helps maintain stem stiffness, while lower moisture reduces firmness [8,9]. Previous reports also indicated that stem strength decreases as stem diameter increases [10]. Differences in cell size, shape, and arrangement may also contribute to changes in rigidity [11].

Lignins play a crucial role in plant development by contributing to the structural integrity and support of plant tissues. These complex polymers, derived from phenolic compounds, provide strength to cell walls, aiding in resistance against environmental stressors and mechanical forces. Lignins also participate in water transport, defense mechanisms, and overall plant resilience, making them integral to the growth, protection, and adaptability of plants throughout their life cycle. Lignin deficiency can cause abnormal stem development, underscoring the complex interplay between genetics and biochemical processes in shaping plant architecture [12,13]. Lignin content varies depending on factors such as plant species, genotype, and tissue type [14]. However, when plants are cultivated under standardized conditions, the variability in lignin content within the same tissue diminishes significantly. Although several factors contribute to the development, structure, and rigidity of the stem, comprehensive lignin deficiency is rare and typically results in severe growth and developmental abnormalities [15]. Measuring lignin content is a common research focus because it is crucial for determining plant rigidity, reinforcing cell wall strength, and contributing to the overall mechanical robustness of the plant.

Given that lignins have a profound impact on various agricultural fields, there is a growing emphasis on breeding strategies aimed at modifying the expression level of the genes' intervening lignin content and composition [16]. The expression levels of several genes within the lignin biosynthetic pathway have been analyzed in previous studies to assess their impact on lignin content in plants. These studies suggest that the overexpression of genes encoding key enzymes in this pathway, specifically Phenylalanine Ammonia-Lyase (PAL) [17], Cinnamate 4-Hydroxylase (C4H) [18], 4-Coumarate: CoA Ligase (4CL) [19], Caffeoyl-CoA O-Methyltransferase (CCoAOMT) [20], and Cinnamyl Alcohol Dehydrogenase (CAD) [21], results in increased lignin content. Consequently, the downregulation of these genes is hypothesized to reduce lignin content, potentially resulting in more friable stems.

Recent investigations into the mechanical properties of plant stems have predominantly explored their utility as biological resources for energy and industrial applications. Studies have examined the mechanical attributes, including compressive strength and bending characteristics, of stalks from sorghum [22], corn [23], wheat [24], cotton [25], alfalfa [26], rice [27], and sugarcane [28]. These results demonstrated that increasing secondary cell wall constituents, such as lignin, contributed to enhanced stem strength. In addition, the augmentation of lignin content improved the stems' resistance to bending and breaking. Unfortunately, experimental insights concerning *Solanaceous* plants are scarce, with available data primarily relating to tomato stems [29]. Given the lack of data on bell peppers, this study aims to investigate the biomechanical properties and lignin content of various *Capsicum* mutant plants. This exploration seeks to uncover their potential applicability to different cultivation methods and their capacity for optimal fruit-bearing performance.

2. Materials and Methods

2.1. Mutant Lines Involved in the Experiment

This study involved a comparative analysis of three distinct *Capsicum annuum* breeding lines, characterized by aberrant stem development and juxtaposed against a commercially established processing variety serving as the control. The seeds of the mutant pepper breeding lines were sourced from Gábor Csilléry, a prominent Hungarian pepper breeder associated with PepGen Kft. in Budapest, Hungary. The three mutant breeding lines—namely, 'fragile-plant' (*frx*), 'tortuous internodi' (*tti*), and 'puffy-structured stem' (*pfi*)—were procured and maintained through self-pollination. Subsequent experimentation was conducted using self-fertilized lineages derived from these mutants. For the purpose of comparative evaluation, a Hungarian cultivar was chosen as a control, 'Garai Fehér', featuring indeterminate growth. The cultivar is produced by Royal Sluis Magrovet Kft. in Kecskemét, Hungary. The plants were grown under greenhouse conditions and the samples were gathered 12 weeks after germination.

The *frx* mutants always exhibit a very fragile stem and at the breaking point; there is always a clear-cut fracture (Figure 1A) [30]. The breeding line exhibiting the puffy-structured stem (Figure 1B) originated from the collection of Bergh and Lippert [31]. The *tti* phenotype is characterized by elongated internodes and the development of extended spiral-like slender stems (Figure 1C) [32].

Figure 1. (**A**) *frx*—fragile-plant (on the left) compared to a control (right), (**B**) *tti*—tortuous internodi plants grown in greenhouse, (**C**) *pfi* exhibiting the puffy-structured stem.

2.2. Determining the Lignin Content of the Stems

The lignin content in the stems of various *Capsicum annuum* lines was extracted and quantified employing the acetyl bromide method, as outlined by Moreira-Vilar et al., 2014 [33]. For the test, five biological replicates together with three technical replicates were applied. The concentration of lignin was determined using a calibration curve constructed with a dilution series of alkali lignin (Sigma-Aldrich, St. Louis, MO, USA). For the calibration curve 0, 1, 2, 3, 4, 5, 6, 7, and 8 mg lignin were used in generating the equation of $y = 62.714x + 0.0345$, $R^2 = 0.8989$. Based on this, as the path lenght is 1 cm, the $\varepsilon = 62.71\ M^{-1}\ cm^{-1}$. Currently, no extinction coefficient is available for lignin in pepper stems, so the extinction coefficient for lignin in *N. Benthamiana* stems is generally used instead. The outcomes are expressed as %.

2.3. Determining the Rigidity of the Stems Using 3-Point Bending Fracture

We measured the breaking force of the three mutant breeding lines and the control cultivar. Due to visually observable differences in various regions of the stems, we have

divided them into three distinct regions as follows: the top region, located immediately below the shoot apex; the middle region, found at the geometric center of the plant stem; and the bottom region, located right at the base of the plant (Figure 2). For all four different genotypes, ten samples were measured from each region of the stem. For our measurement, we chose stems without visible disease symptoms or any damage caused by pests.

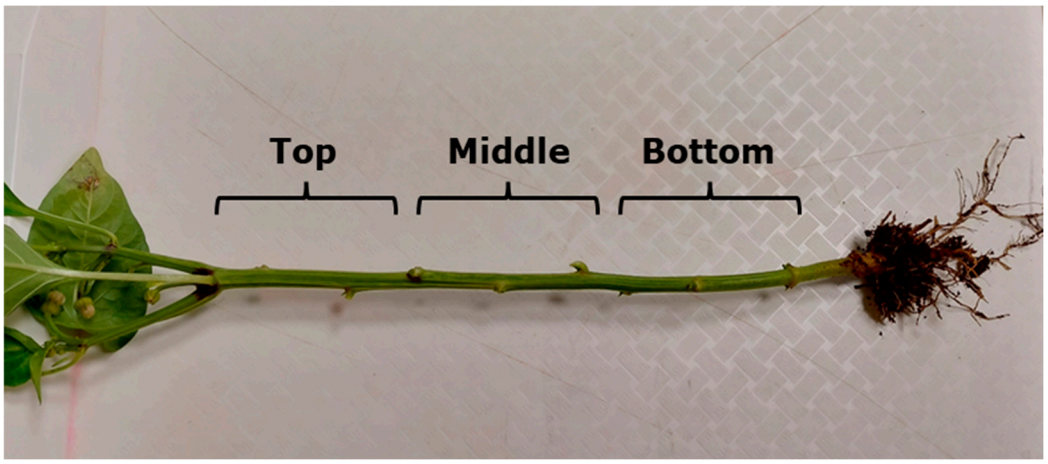

Figure 2. Sampling regions of the stem for lignin content and mechanical property evaluation.

Three-point bending tests were conducted utilizing the TA.XTplusC Texture Analyzer (Stable Micro Systems) to assess the mechanical properties of the samples. Each sample was cut to a resembling length (approx. 8 cm) and positioned on a pedestal. The fracture was always made in the middle of the internodes [34]. Subsequently, a consistent pressure was applied to the samples until fracture or the attainment of maximum flexure. The machine facilitated controlled loading on the samples, allowing for precise evaluation of their flexural characteristics. The outcomes of the three-point bending tests were systematically recorded and subsequently analyzed using the Exponent Connect computer program. During our experiments, breaking force (N) was recorded, which measures the maximum force that the material can bear before it experiences mechanical stress failure.

2.4. Gene Expression

The total RNA was isolated from the three different regions of the stems using the Omega E.Z.N.A.® Plant RNA Kit (Norcross, GA, USA). The quantity and the quality of the total RNA were checked by agarose gel electrophoresis and the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively. From the RNA, cDNA was synthesized with the RevertAid H Minus First Strand cDNA Synthesis Kit by Thermo Fisher Scientific (Waltham, MA, USA) with oligo-dT and random primers applied, according to the manufacturer's instructions. The qRT-PCR was carried out in a Stratagene MX3000p instrument using actin as a reference gene. For PCRs, we used the Power Up™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions, with 10 µL as the final volume: 5 µL 2× PowerUp™ SYBR Green Master Mix, 1-1 µL 500 nmol primer, 1 µL diluted cDNA (1/50). The machine is set for standard cycling mode: UDG activation 50 °C 2 min, activation (Dual-Lock™ DNA polymerase) 95 °C 2 min, denature 95 °C 15 s, anneal/extend 60 °C 1 min; the denature and anneal/extend are repeated in 40 cycles. The primers used in this study were designed by Primer3 using Zunla's genome as a reference (Table 1). The qPCR data were evaluated by the ddCT method, where 'Garai Fehér' was used as a reference.

Table 1. Primers used for qRT-PCR.

Primer	Sequence	TM (°C)	Expected Fragment Size
CaPAL Fw	gcagagtcattgaaaggtagcc	55.9 °C	168 bp
CaPAL R	tgcatcctcagataactccact	55.4 °C	
CaC4H Fw	attatcctagcgctgccaattc	56 °C	219 bp
CaC4H R	tatcagatttctccagagcccc	55.3 °C	
Ca4CL Fw	acctgatgtgaaaatccagcct	56.7 °C	178 bp
Ca4CL R	gcaacacatcaacacgtcttca	56.1 °C	
CaCCoAMOT Fw	gttggtggactgattggctatg	55.9 °C	160 bp
CaCCoAMOT R	gaagctggcagatttcgattct	55.4 °C	
CaCAD Fw	cgatgttaagcgcttcaaagtt	54.1 °C	157 bp
CaCAD R	agtaactgtaccatccgtgtct	55.2 °C	

2.5. Statistical Analysis

The recorded results from the lignin content measurements underwent thorough analysis. The graphical representation of the data was visualized using the computer program IBM® SPSS® Statistics version 23. To assess the statistical significance of the acquired breaking force (N) and the lignin content (% DW) data, a multivariate analysis of variance (MANOVA) was employed. This statistical method was chosen to systematically evaluate potential differences among the experimental groups.

3. Results

3.1. Comparative Analysis of Lignin Content and Breaking Value

The total lignin content of the stem cell walls was assessed during our experiments. The data results are given in % of the cell wall in dry weight (DW). The breaking force of the stems from different genotypes is given in (N) force (Table 2).

Table 2. Mean values of lignin content and breaking value of the different genotypes.

Sample	Place	Lignin [% DW]	Breaking Force [N]
'Garai Fehér'	Bottom	8.06 ± 0.40^{a}	24.85 ± 2.42^{a}
	Middle	8.08 ± 0.23^{a}	20.38 ± 1.96^{a}
	Top	10.31 ± 0.52^{b}	11.29 ± 1.20^{b}
frx	Bottom	3.17 ± 0.29^{a}	7.63 ± 0.53^{a}
	Middle	2.53 ± 0.25^{a}	11.51 ± 1.17^{b}
	Top	3.05 ± 0.30^{a}	5.88 ± 0.37^{a}
tti	Bottom	7.98 ± 0.27^{a}	11.65 ± 1.12^{a}
	Middle	8.49 ± 0.33^{a}	8.76 ± 0.80^{a}
	Top	8.36 ± 0.46^{a}	3.89 ± 0.35^{b}
pfi	Bottom	4.33 ± 0.37^{a}	4.50 ± 0.36^{a}
	Middle	4.63 ± 0.37^{a}	2.32 ± 0.14^{b}
	Top	7.39 ± 0.26^{b}	1.15 ± 0.10^{c}

Note: Values in the same row and subtable not sharing the same superscript are significantly different at $p < 0.05$ in the two-sided test of equality for column means. Tests assume equal variances.

In the 'Garai Fehér' variety, a significant difference was observed in lignin content, particularly in the top region of the stem, where the measured lignin content was 10.31 ± 0.52% DW. This was much higher compared to other parts of the stem, where the measured values were 8.08 ± 0.23% DW in the middle region and 8.06 ± 0.40% DW in the bottom region. This increase in lignin content negatively correlates with the results from the breaking force measurements, which showed the lowest breaking force of 11.29 ± 1.20 N in the top

region. For the *frx* samples, the lignin content was found to be consistent across all regions, with 3.17 ± 0.29% DW in the bottom region, 2.53 ± 0.25% DW in the middle region, and 3.05 ± 0.30% DW in the top region. Surprisingly, a significantly higher breaking force was measured in the middle region of the stem compared to the other two regions, presenting a breaking force of 11.51 ± 1.17 N. Similarly, the *tti* samples exhibited uniform lignin content throughout the stem, with 7.98 ± 0.27% DW in the bottom region, 8.49 ± 0.33% DW in the middle region, and 8.36 ± 0.46% DW in the top region. However, the top region showed a markedly lower breaking force of only 3.89 ± 0.35 N. The *pfi* group displayed a much higher lignin content in the top region, with 7.39 ± 0.26% DW, while the bottom region had 4.33 ± 0.37% DW, and the middle region had 4.63 ± 0.37% DW, which is similar to the control group. Nonetheless, significant differences were detected between all regions of the stems when evaluating their breaking force, presenting 4.50 ± 0.36 N in the bottom region, 2.32 ± 0.14 N in the middle region, and 1.15 ± 0.10 N in the top region, which were the lowest among all genotypes.

The correlation between lignin content and breaking strength across different genotypes is illustrated in Figure 3 using linear regression.

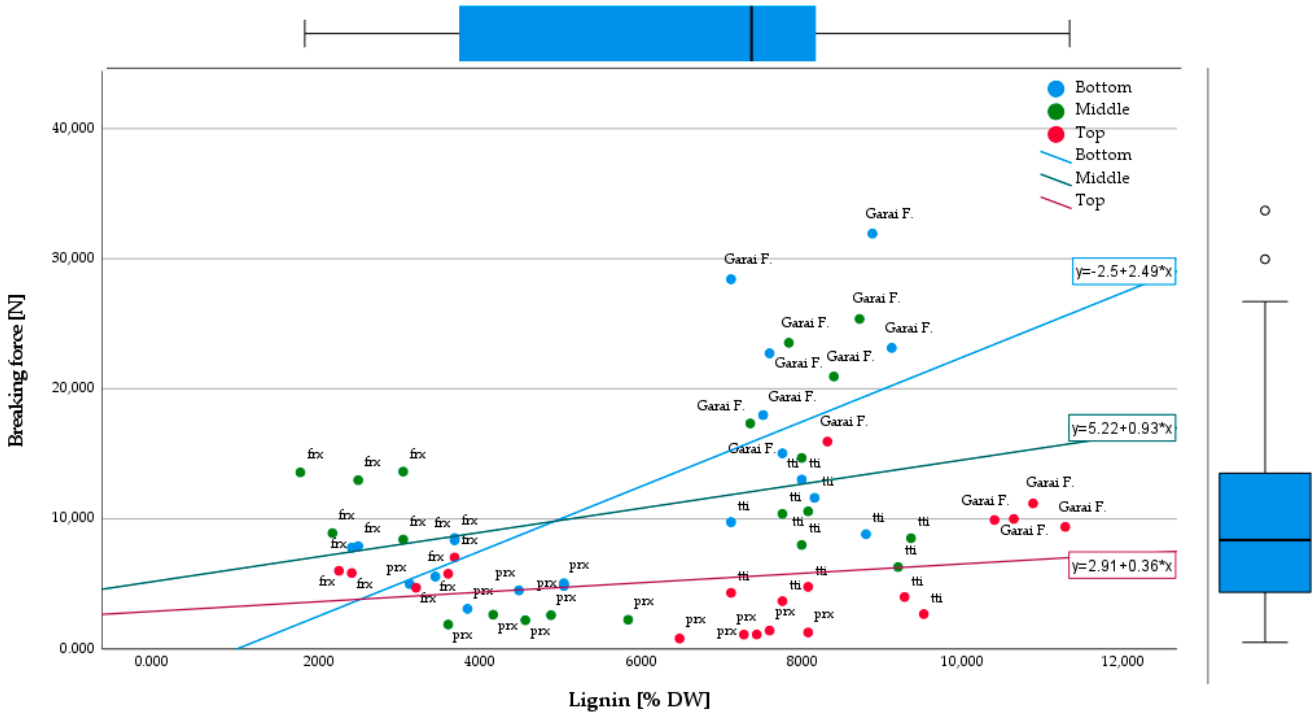

Figure 3. Regression plot summarizing the correlation between lignin content and breaking value among the genotypes.

As shown in Figure 3, changes in lignin content at the lower part of the plants exert a greater effect on breaking force resistance compared to changes in the upper parts. Samples can be grouped by genotype, but clear separation based on stem region is not evident. Samples with a lower lignin content such as the *frx* mutant show lower breaking force resistance as well.

A MANOVA was conducted to determine whether there was a statistically significant interaction between the genotypes and stem regions of the dependent variables studied. The F values are displayed in Table 3, with the variables exerting the greatest role indicated in bold. The combined effect proved to be significant, $F(12, 94) = 6.248$, $p = 0.00$, Wilks' $\lambda = 0.309$. The genotype having a significant effect on the lignin content and on the breaking force was also proved, $F(3, 48) = 186.112$, $p = 0.00$, and $F(3, 48) = 109.031$, $p = 0.00$, respectively. The stem region analyzed also had a significant effect on lignin content, $F(2, 48) = 20.851$, $p = 0.00$, and on the breaking force as well, $F(2, 48) = 38.400$, $p = 0.00$.

Table 3. F values of lignin content and breaking value.

	Lignin [% DW]	Breaking Force [N]
Genotype (G)	**186.112**	**109.031**
Stem region (Sr)	20.851	38.400
G × Sr	6.081	6.855

Note: genotype (G); stem region (Sr).

3.2. Difference in Expression Levels of the Genes Playing a Role in Lignin Biosynthesis

Using qPCR analysis, we examined the expression of five different genes involved in lignin biosynthesis across three regions of various genotypes, namely the Phenylalanine Ammonia-Lyase (PAL), Cinnamate 4-Hydroxylase (C4H), 4-Coumarate: CoA Ligase (4CL), Caffeoyl-CoA O-Methyltransferase (CCoAOMT), and Cinnamyl Alcohol Dehydrogenase (CAD) (Figure 4). According to previous reports, it is anticipated that an upregulation in the expression levels of the specified genes will correlate with an increase in lignin content in the stems.

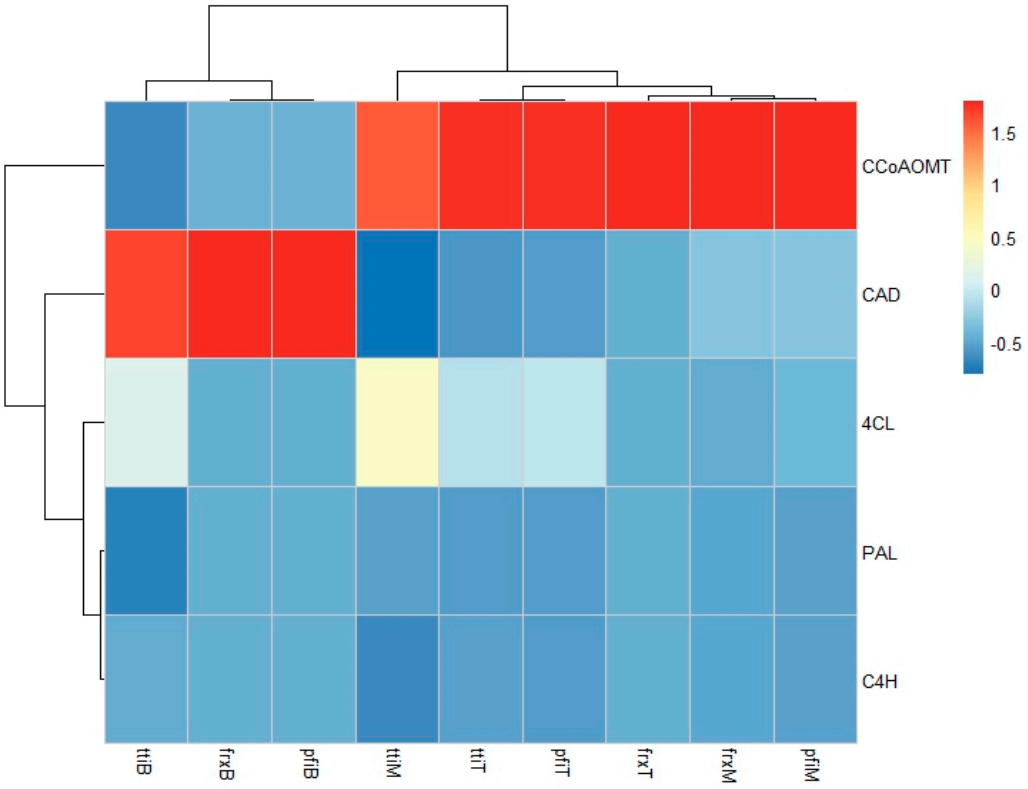

Figure 4. The heat map illustrates the differential relative gene expression levels across various regions of different mutant lines compared to the control genotype 'Garai Fehér'.

According to our results, differences were observed among the genotypes. Compared to the control genotype, the *frx*, *tti*, and *pfi* genotypes presented abnormal stems, exhibiting overexpression of the CCoAOMT gene in both the top and middle regions. Additionally, these mutant lines showed overexpression of the CAD gene specifically in the bottom region, which should all result in a higher lignin content according to previous reports; however, we cannot confirm this in the case of our mutant lines. Hierarchical clustering analysis of the genes effectively illustrates their relationships and grouping. Lower branches indicate higher similarity among gene expression profiles, while higher branches signify greater dissimilarity. Notably, PAL and C4H enzymes exhibit correlation, representing the initial steps of the phenylalanine pathway, which subsequently involves the 4CL enzyme.

CCoAOMT is integrated later in the metabolic process, while CAD serves as the final step in forming monolignol units [35].

4. Discussion

In the case of the *tti* mutant, despite the similarity in lignin content compared to the control, the stems exhibit less rigidity in each region. It is prudent to consider incorporating assessments of plant height and internode length in subsequent analyses. The distribution of lignin within the cell wall might differ, leading to changes in the mechanical properties even if the total lignin content is similar [36]. Elongated internodes may also have altered cellular structures that affect overall tissue firmness. In the cases of the *pfi* and *frx* genotypes, markedly lower lignin content was measured. An unexpected result was observed in the *pfi* mutant, displaying a remarkably higher lignin content in the top region compared to the others. However, this aligns with breeding observations indicating that the stem's puffy nature is more prominent in the upper region. The *pfi* mutant stem contains more lignin; however, its breaking value is the lowest among the genotypes. In the examination of the breaking force of the *frx* mutant, we expected a lower lignin content, which would align with the observed characteristic of the mutant's notably facile stem breakage. It is acknowledged that stem hardness is not solely governed by lignin but also by other constituents within the secondary cell wall [37]. Furthermore, in fragile stems, mutations affecting cellulose deposition led to decreased stem strength due to deficiencies in cellulose deposition in the cell wall [38]. As we have already noted, the mutant stem does not break in a fibrous manner. Significant distinctions were identified among these mutant genotypes, acknowledging that variations in lignin content may arise not just by the genotype but as a response to gravitropic stimulation or mechanical stress [39].

While studies have identified lignin deficiency and its consequential impact on plant fitness, particularly in mutant plants [40], it is noteworthy that much of this research has been predominantly confined to grasses and field crop plants. In our results, substantial variations in breaking force were evident among sugarcane genotypes, with a normal distribution spanning from 6.6 N to 32.8 N. This observed range signifies the diverse mechanical properties inherent within different sugarcane varieties, offering insights into the variability of breaking forces across the examined genotypic spectrum [41]. This option may open up new research possibilities. In the context of a comparative experiment involving tomato stems, wherein test groups were subjected to mechanical stress to assess their rigidity, the mean breaking force for the treated plants was recorded as 21.13 N. In comparison, the breaking force for the control plants was lower by 9.8 N. This disparity in breaking force values suggests that the mechanical stress may improve the rigidity of the plant stems [42]. Upon comparing the results obtained from the tomato with those of our mutants, it is evident that our mutants exhibit higher stem hardness than the control tomato. Notably, in the instance of the *pfi* mutant, a significantly lower stem hardness value is observed.

Based on our investigations, it can be generally stated that an increase in lignin content correlates with an increase in the breaking force, as found by others in their studies [43,44]. However, when examining peppers with abnormal stem growth, this relationship is not clearly demonstrated when analyzed regionally. It has been suggested that not all lignin is mechanically relevant, implying that the total amount of lignin may not directly indicate the mechanical properties [45].

During gene expression analysis, we detected differences in the expression of the CAD and CCoAOMT genes out of a total of five genes examined. In both cases, we found overexpression compared to the control genotype. Previous studies suggest that overexpression of these genes should result in higher lignin content; however, in our mutants, we could not confirm this positive correlation, as all mutant plants exhibit lower lignin content overall compared to the control plants.

5. Conclusions

Our research revealed significant differences in lignin content and breaking force both among genotypes and across different regions of the stems. Although the *tti* mutants exhibited lignin content similar to the control, their breaking strength was notably inferior, potentially due to the elongated structure of the internodes. Conversely, all *frx* mutants demonstrated reduced lignin content, which likely contributes to their friable stems. The *pfi* mutants presented the worst results among all tests. Based on this study, their economic value can be defined by their abnormal traits. They can be valuable breeding material; however, it needs to be considered that their maximum weight-bearing capacity is more limited than that of commercially available cultivars. As for the expression levels of the key genes playing a role in lignin biosynthesis, we found that in the abnormally growing mutant stems, CAD presented overexpression exclusively in the bottom region, and CCoAOMT presented higher expression levels in the middle and top regions. According to previous studies, these differences should lead to higher lignin content; however, in the case of our mutants, we could not confirm this. Measuring other secondary cell wall constituents and the expression levels of their encoding genes is highly suggested. Using these mutants could enable new cultivation methods for pepper, such as on-wire cultivation or vertical farming, particularly in cases of indeterminate abnormal growth. Additionally, the fragile mutant may prove beneficial for mechanical harvesting if the brittleness can be concentrated in the areas supporting the fruits.

Author Contributions: Conceptualization, G.C., A.S. and A.V.; methodology, B.P., M.K.-W., T.I.P. and K.N.C.; validation, Z.K., J.B. and K.A.T.-L.; formal analysis, A.S. and A.V.; data curation, Z.K., J.B. and K.A.T.-L.; writing—original draft preparation, B.P.; writing, review, and editing, B.P. and Z.K.; visualization, B.P. and Z.K.; supervision, A.S., A.V., G.C. and M.K.-W. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest: The authors declare no conflicts of interest.

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