Thesis of Doctoral Dissertation

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In vitro and biomechanical investigation of pepper mutants with abnormal stem growth, and their molecular genetic analysis.

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1. Background and objectives

Nowadays, vegetable production is a dynamically developing and continuously evolving sector in which innovation, as well as the adoption of new cultivation methods and plant materials, is essential to meet market demands. The need for plant breeding has existed for centuries, as it plays a fundamental role in increasing both yield quantity and quality. Spontaneously occurring mutant plants found in nature are of particular importance in breeding programs, as their favorable agronomic traits can be effectively exploited.

Hungarian-bred sweet and spice pepper (*Capsicum annuum* L.) is an important element of traditional cuisine and represents a significant staple food crop in Hungary and Central Europe. Pepper is cultivated both in open-field conditions and under protected cultivation; however, its production is currently challenged by high production costs, necessitating the development of novel cultivation strategies.

Stem growth, development, and structural properties of plants have a substantial impact on yield. Pepper typically develops a strong, upright stem; however, mutations may give rise to alternative growth habits. Prostrate, drooping, or creeping phenotypes are primarily utilized in ornamental plant breeding, yet in pepper they may offer new opportunities for greenhouse production systems, such as vertical or high-wire support systems that have already proven successful in other vegetable crops.

These mutant traits are presumed to result from disturbances in lignification, alterations in phytohormone levels, or impaired light or gravity perception. Laboratory-based investigations and the elucidation of their genetic background may enable the tracking of these traits in breeding programs and their targeted modification through biotechnological approaches.

The aim of this study is to elucidate the physiological, biomechanical, and molecular background of stem growth mutants in pepper. The main objectives of the research are as follows:

- To determine the light- and gravitropic responses of the mutants, as well as the dynamics of their gravitropic adaptation.
- To investigate the role of starch content in gravitropic perception.
- To perform quantitative and qualitative analyses of lignin content and to examine the gene expression background of lignin biosynthesis.
- To analyze the expression of gibberellin oxidase genes in relation to altered growth phenotypes.
- To conduct a comparative evaluation of the mechanical properties of the stem.
- To analyze physiological responses to auxin treatment.

2. Material and method

2.1. Plant material

Four mutant breeding lines exhibiting abnormal stem growth were included in the experiments. These lines originate from the collection of Gábor Csilléry (PepGen Ltd., Budapest, Hungary).

The *Pcx* (procumbent plant) mutant phenotype can be recognized as early as the cotyledonary stage. Plants exhibit a prostrate, creeping growth habit, with high phenotypic variability among progeny.

In case of he *tti* (tortuous internodi) mutant the internodes are significantly elongated (10–15 cm), while the number of nodes remains unchanged, leading to reduced mechanical stability of the stem.

The *frx* (fragile plant) mutant is characterized by a short root system and stems that break easily with a snapping fracture, resulting in a smooth fracture surface.

The *pfi* (puffy-structured stem) mutant is difficult to identify at early developmental stages. Stem thickening is restricted exclusively to the internodes and does not affect the nodes, suggesting a mutation associated with increased tissue volume.

2.2. *2.2. In vitro experiments for the investigation of photo- and gravitropism*

All *in vitro* experiments were conducted on Murashige and Skoog (1962) MS medium. After surface sterilization, pepper seeds were grown in vitro for two and three weeks, after which the inclination angle of the hypocotyls was documented. Three-week-old seedlings were rotated by 90 degrees, and their responses to the altered gravitational stimulus were recorded after 24 hours.

The experiment was complemented by an additional setup in which a subset of plants was grown in a closed system under controlled conditions in a phytotron, exposed exclusively to unilateral illumination, while another group was grown in complete darkness, shielded from all light stimuli.

2.3. Auxin hormone treatment

Pcx mutant plants, which do not exhibit gravitropic responses, were germinated and cultivated on MS medium supplemented with different concentrations of auxin (indole-3-acetic acid; 0, 10 $\mu M\ L^{-1}$, and 20 $\mu M\ L^{-1}$). The aim was to determine whether exogenously applied auxin exerts its physiological effect on the plants and whether it is capable of disrupting the prostrate phenotype.

2.4. Qualitative and quantitative determination of starch content

Qualitative detection of starch content in the root tips of three-week-old seedlings was performed using Lugol's solution (iodine–potassium iodide). Quantitative analytical determination of starch content in the stems of fully developed plants (12 weeks after germination) was carried out using the Sigma-Aldrich® Starch (HK) Assay Kit, following the manufacturer's instructions.

2.5. Qualitative and quantitative determination of lignin content

Qualitative detection of lignin was performed using phloroglucinol staining, which produces a purplish coloration when applied to sections of three-week-old seedlings. Quantitative determination of lignin content in the stems of fully developed plants (12 weeks after germination) was based on the acetyl bromide method described by Moreira-Vilar *et al.* (2014).

2.6. Mechanical analysis of stem rigidity

Stem rigidity of fully developed mutant breeding lines and control pepper plants (12 weeks after germination) was determined using a three-point bending test with a TA.XTplusC (Stable Micro Systems) texture analyzer.

2.7. Gene expression analyses

2.7.1. RNA isolation

RNA was isolated from stem segments of pepper plants 12 weeks after germination. RNA extraction was performed using the OMEGA E.Z.N.A.® Plant RNA Kit according to the manufacturer's instructions. RNA quality and concentration were assessed using a NanoDrop® ND-1000 UV–Vis spectrophotometer, and samples were stored at −70 °C until further use.

2.7.2. Reverse transcription and cDNA synthesis

The cDNA synthesis was carried out using the Thermo Scientific™ Maxima H Minus First Strand cDNA Synthesis Kit, following the manufacturer's protocol.

2.7.3. Primer design

Target genes were selected based on previously published scientific studies describing similar mutations. Pepper gene sequences were identified in the NCBI (National Center for Biotechnology Information, USA) database.

Primers required for PCR reactions were designed using the Primer3 software based on the corresponding GenBank accession sequences.

2.7.4. Genes analyzed

Lignin biosynthesis proceeds through multiple consecutive enzymatic steps. Previous studies have demonstrated that differences in the expression of genes involved in this pathway can significantly affect total lignin content in plants. Based on these findings, five key enzymes of the pathway were selected: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate–CoA ligase (4CL), cinnamyl alcohol dehydrogenase (CAD), and caffeoyl-CoA O-methyltransferase (CcoAOMT).

Gibberellin biosynthesis is strongly influenced by GA20-oxidase (GA20ox) and GA3-oxidase (GA3ox), enzymes that act in the cytoplasm and convert biologically inactive gibberellins into active forms. According to the literature, overexpression of genes encoding these enzymes has resulted in elongated stem structures in several plant species. In parallel, plants can directly reduce levels of biologically active gibberellins through the activity of GA2-oxidase (GA2ox), an enzyme responsible for gibberellin inactivation.

Based on these considerations, the expression levels of genes encoding the above-mentioned enzymes were determined.

3. Results and discussion

3.1. Results of *In Vitro* Experiments

Using *in vitro* methods, we demonstrated that all mutants are able to perceive light and bend toward it. Our experiments showed that in the case of the *Pcx 1* procumbent mutant, the prostrate trait results from a lack of gravity perception. When examining the time required for reorientation, we found that *Pcx 2* mutants with normal stem growth responded to the altered gravitational stimulus and reoriented at the same speed as the control plants, whereas the hypocotyls of *Pcx 1* did not bend even after 420 minutes. All other mutant lines responded to gravity; in addition, the tti and pfi mutants exhibited a slightly drooping phenotype, which is due to weaker stem strength. The *Pcx 1* procumbent mutants were treated in vitro with varying amounts of auxin (IAA), but this did not eliminate the prostrate trait.

3.2. Starch Content Analysis

Staining with Lugol's solution demonstrated that the root tips of the studied plants have an average starch content, indicating that abnormal stem growth in the mutants is not caused by the absence of statoliths. Although large variations in hypocotyl growth were observed among the *Pcx* plants during *in vitro* experiments, only minor differences were seen in starch content between genotypes. In all cases, the mutants showed results similar to the control, suggesting that starch content does not influence the expression of the mutant phenotype.

3.3. Lignin Content and Stem Strength Analysis

We determined the lignin content and stem strength in three different regions of the stems of *tti*, *frx*, and *pfi* mutants. In the *tti* mutant, although the lignin content was similar to that of the control genotype, the stems required much lower force to break in all regions. For this mutant, it is advisable to also consider plant height and internode length in future studies, as the distribution of lignin within the cell wall may vary, potentially causing changes in mechanical properties even if the total lignin content is the same. Elongated internodes may have altered cell structures, which can affect overall tissue strength.

The *pfi* and *frx* mutants had much lower lignin content compared to the control. An unexpected result was observed in *pfi* mutants, where the upper stem region showed exceptionally high lignin content. This result aligns with breeding observations, indicating that the stem structure characteristic of the *pfi* mutant is more dominant in the upper regions. The *pfi* stems have higher lignin content than *frx* stems, even though this genotype has the lowest measured stem-breaking forces. In the *frx* mutant, based on its brittle phenotype, low lignin content was expected, which was confirmed by the measurements.

Overall, the results suggest that increased lignin content generally correlates with higher force required to break the stem, as described in previous studies in other plant species. In contrast, for mutants exhibiting abnormal stem growth, this correlation cannot be simply applied when considering results from different stem regions. It is likely that not only the quantity but also the structure and quality of lignin (ratios of H, G, S units) are important; therefore, total lignin content does not necessarily directly reflect mechanical properties, as supported by previous literature in other plant species.

3.4. Gene Expression Analysis

The aim of these experiments was to determine how genes potentially involved in abnormal stem growth are expressed compared to control plants in different stem regions.

The analysis detected differences between genotypes. Compared to the control, mutants showing abnormal stem growth (*tti*, *frx*, and *pfi*) exhibited overexpression of the *CCoAOMT* gene in the upper and middle stem regions. All mutant genotypes also showed overexpression of the *CAD* gene, but this was only observed in the lower stem regions. Although the function of *CAD* and *CCoAOMT* genes and their relationship with lignin content has been widely demonstrated, this correlation could not be confirmed in the mutant genotypes.

The analysis also detected differences between the tti genotype and the control variety ‘Garai Fehér’. Expression of the *GA3ox1* gene was much higher in the control compared to tti, but this was only detected in the lower stem region. In tti, overexpression was also observed in the lower region for *GA20ox* and *GA20ox1*. These results are consistent with literature reports indicating that overactivity of these genes provides continuous gibberellin signaling, which leads to stem elongation.

4. Conclusions and Recommendations

We successfully adapted the in vitro methodology to study the light and gravity responses of mutant pepper genotypes. Most genotypes responded to these stimuli as expected, except for the *Pcx* mutants, which exhibited random stem growth in the dark, indicating a defect in gravity perception. This is contradicted by the fact that the starch content of their roots and stems was similar to that of the control plants.

We measured the lignin content of the stems and the force required to break them, which revealed significant differences between genotypes and stem segments. In the *tti* mutants, lignin content was similar to the control; however, due to elongated internodes, the stems were mechanically weaker. In the *frx* and *pfi* mutants, lower lignin content was observed, which may be associated with their brittle or weak stem structure, although the contribution of other cell wall components (e.g., cellulose) cannot be ruled out.

Gene expression analyses showed overexpression of *CCoAOMT* in the upper and middle stem regions and *CAD* in the lower regions of the mutant lines, but this was not associated with higher lignin content. Currently, no literature exists on this phenomenon in pepper. In *tti* mutants, overexpression of gibberellin biosynthesis-related genes *GA3ox1*, *GA20ox*, and *GA20ox1* was confirmed, which is consistent with the elongated stem phenotype characteristic of these mutants.

The genetic basis of stem growth in the other mutants could not be determined. Future studies should investigate genes involved in lignin biosynthesis, auxin transport (e.g., *PIN*, *LAX*), and cell wall formation.

Based on these results, *Pcx* and *tti* mutants could potentially be used in vertical or inclined cultivation systems, while the brittle phenotype of the *frx* mutant may facilitate mechanical harvesting. Additionally, due to their unique appearance, *Pcx*, *tti*, and *pfi* mutants may have ornamental value, although greenhouse trials are still needed to confirm their practical applicability.

5. New scientific results

1. We adapted an *in vitro* methodology for peppers to study phototropism and gravitropism.

2. In the case of the *Pcx* mutant, we confirmed that the prostrate phenotype results from a lack of gravity perception.

3. We determined the lignin content and stem strength of the mutants in three stem regions. Based on the results, the average load the pepper stem can support can be estimated, which is also practically useful in pepper cultivation.

4. In all *tti*, *frx*, and *pfi* mutants, we detected overexpression of the *CAD* gene in the lower stem regions and overactivity of the *CCoAOMT* gene in the middle and upper regions; however, this was not associated with increased lignin content. This finding contradicts previous literature, and although lignin contributes to stem strengthening, it is not the sole factor determining stem mechanical strength.

5. We demonstrated that stem elongation and long internodes in *tti* mutants result from overexpression of the *GA3ox1*, *GA20ox*, and *GA20ox1* genes, which we detected exclusively in the lower stem region. This is a novel observation in the literature, but it is consistent with the idea that enhanced expression of these genes can induce internode elongation.

6. The author's publication activity

Publications related to the dissertation in international scientific journals with IF:

Pápai, Bánk ; Kovács, Zsófia ; Tóth-Lencsés, Kitti Andrea ; Bedő, Janka ; Chan, Khin Nyein ; Kovács-Weber, Mária ; Pap, Tibor István ; Csilléry, Gábor ; Szőke, Antal ; Veres, Anikó
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