Molecular and morphological aspects of lodging resistance in spring wheat (*Triticum aestivum* L.)

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I Summary

Lodging is a major problem in the production of cereals. It can reduce grain yield directly by interfering with dry matter accumulation or indirectly by hindering the harvest of a crop. Lodging may also affect grain quality. The severity of lodging, and the losses resulting from it depend on many environmental factors as well as on numerous plant characters.

Glycine-rich proteins (GRPs) are an important class of cell wall proteins. The first GRP-encoding gene was discovered and isolated from petunia in 1986. Since then genes for GRPs have been isolated from many other plant species. The biological function of these proteins is still unknown. It is suggested that GRPs have a structural function, providing the cell wall with tensility, strength, and elasticity.

The aim of the field experiments in this study was to find morphological plant traits related to lodging-resistance. At the molecular level, genes encoding glycine-rich proteins should be isolated from the wheat genome and, based on data from the field, should be used to test the relationship of glycine-rich proteins to mechanical stability (lodging resistance) of wheat.

A two year field study was conducted to determine the relationship of morphological traits to lodging resistance in 15 spring wheat (Triticum aestivum L.) genotypes. In the first year a total of 29 plant traits was measured at two growth stages and correlated with the lodging score; in the second year only six traits were measured and correlated with the lodging score. Significant correlations between the lodging score and single morphological traits were found for stem diameter and stem weight (fresh weight) per cm. Greater stem diameters and heavier stems per cm (g per cm) indicated better lodging resistance. Stem weight per cm explained 49.7% of the phenotypic variation in lodging resistance. Multiple linear regression equations, which predict lodging resistance, indicated that 77.2% of lodging resistance is probably explained by the stem weight per cm and by the weight of the ear.
At the molecular level, genomic clones encoding cell wall glycine-rich proteins were isolated from a wheat genomic library. One of the genes (wGRP1) was sequenced. The derived amino acid sequence contains 391 amino acids 65% of which are glycine (Gly). The glycine-rich part of the sequence shows the repetitive, (Gly-x)$_n$ structural arrangement in pairs which is typical of plant GRPs. wGRP1 has an amino terminal hydrophobic signal peptide of 27 amino acids which suggests that the protein is transported to the cell wall compartment. The in vitro translation of wGRP1 produced a protein of 28 kD molecular weight which is in close agreement with the expected molecular weight of 28.73 kD. This protein was specifically precipitated by a French bean (Phaseolus vulgaris L.) anti-GRP1.8 antibody. Southern blot analysis showed three copies of the gene in the wheat genome. Comparative RFLP analysis with seven enzymes did not detect any polymorphisms between lodging-resistant and lodging-susceptible spring wheat genotypes. Northern analysis of six spring wheat genotypes revealed two tissue-specific mRNAs at 0.9 kb and at 1.5 kb (as expected for wGRP1). The signal at 0.9 kb was significantly stronger in shoots of genotypes previously assessed as being lodging-resistant. This result supports the hypothesis that GRPs influence the mechanical properties of the cell wall and, thereby, the lodging resistance of the plant.
Zusammenfassung


Die glycin-reichen Proteine (GRPs) bilden eine wichtige Klasse von Zellwandproteinen. Erstmals wurden GRPs 1986 aus Petunie isoliert. Seither wurden GRPs in vielen anderen Pflanzenarten nachgewiesen und charakterisiert. Die biologische Funktion der GRPs ist im wesentlichen immer noch unbekannt. In der Literatur wird jedoch vorgeschlagen, dass GRPs strukturelle Funktionen haben und dass sie die Zugfestigkeit und die Elastizität der Zellwand verbessern.


In einem zweijährigen Versuch an je zwei Standorten wurde ein Sortiment von 15 Sommerweizen-Genotypen (Triticum aestivum L.) geprüft. Im ersten Jahr wurden 29 Pflanzenparameter an zwei Erntezeitpunkten gemessen und mit der Standfestigkeitsbonitur korreliert. Im zweiten Jahr wurden nur noch sechs Parameter erfasst. Es wurden signifikante Korrelationen zwischen Stengeldurchmesser, Stengelgewicht pro cm (Frischgewicht) und der Standfestigkeitsbonitur gefunden. Das Stengelgewicht pro cm erklärte 49,7% der phänotypischen Standfestigkeits-Variation. Eine multiple lineare Regression mit den Parametern Stengelgewicht pro cm und Aehrenengewicht erklärte 77,2% der Variation bezüglich Standfestigkeit.
II General Introduction

A major problem in crop production throughout the world is yield loss which occurs before and at harvest as a result of lodging. Lodging may influence yield in a number of crop species including cereals such as rice (Hoshikawa & Wang, 1990), barley, and wheat (Pinthus, 1973) as well as non-cereals such as tobacco (Menchey et al., 1993). Morphological and physiological traits as well as environmental constraints affect the lodging resistance of the plants and may limit yield and quality. Lodging is triggered by strong wind and/or rain and can be intensified by an attack of insects or fungal pathogens. The rotation of the whole plant in the soil is referred to as root lodging, and the buckling of the stem (normally at a basal internode) is referred to as stem lodging. The developmental stage at which lodging of the plant occurs is highly significant for the extent of economic losses due to lodging. Lodging around anthesis, for example, causes much greater reductions in yield than does lodging which occurs later.

The use of dwarf genotypes and chemicals to control the height of wheat seemed to reduce the incidence of lodging. However, increased public interest in more ecological systems of agricultural production and ecological necessities demand a reduced input of agrochemicals into the environment. The Swiss Federal Office of Agriculture launched a system of wheat production (Extenso-wheat) in 1992 which subsidizes the production of bread wheat without using insecticides, fungicides, or agents to limit the height of the plants. The goal to breed lodging-resistant cereal varieties has, therefore, taken on renewed importance.

Glycine-rich proteins (GRPs) represent a relatively recent discovery of plant cell wall proteins. Other important classes of cell wall proteins are the hydroxyproline-rich cell wall proteins (HRGPs) or extensins and the proline-rich proteins (PRPs). The first GRP was isolated by Condit and Meagher (1986) from petunia. Other research groups have also isolated and characterized GRP cDNAs or genomic DNAs from bean (Keller et al., 1988), Arabidopsis (de Oliveira et al., 1990), barley (Rohde et al., 1990), tomato (Showalter et al., 1991), rice (Fang et al., 1991; Lei et al., 1991), and
tobacco (Brady et al., 1993). These proteins are characterized by their repetitive primary structure which contains up to 70% glycine. Most of these GRP clones also have one common protein feature: the presence of an amino terminal signal peptide (Showalter, 1993). The assumption that these GRPs are localized in the cell wall has been verified by immunolocalization studies with antibodies against GRP1 from petunia (Condit et al., 1990) and GRP1.8 from French bean (Keller et al., 1988, 1989).

The biological function of cell wall GRPs is largely a matter of speculation. GRPs are probably structural proteins which play roles in plant vascular systems and wound healing (Showalter, 1993). The predicted β-pleated sheet secondary structure of cell wall GRPs (Condit & Meagher, 1986) may provide elasticity and tensile strength during vascular development. It has also been suggested that GRPs either serve as nucleation sites for lignification (Keller et al., 1989) or form links with other wall proteins and polymers.

Previous research in the area of lodging resistance has mainly concentrated on the relationships between morphological, biochemical, and physical parameters of the plant. The goals of this work were (i) to conduct a field study to find easily measurable morphological traits related to lodging resistance, (ii) to isolate and characterize genes encoding wheat GRPs, and, with regard to the hypothetical functions of GRPs mentioned above, (iii) to determine whether a functional relation between GRPs and the lodging resistance of wheat exists. These studies may aid in breeding cereal varieties with improved lodging resistance. This approach represents a synthesis of classical agronomic and modern molecular methods in applied plant science.
III Morphological traits associated with lodging resistance of spring wheat (*Triticum aestivum* L.)

1. **Abstract**

Lodging in cereals causes high annual losses in yield worldwide. A two year field study was conducted to determine the relationship of morphological traits to lodging resistance in spring wheat (*Triticum aestivum* L.) genotypes and to find easily measurable traits which are related to lodging resistance. A set of 15 genotypes, representing a preselected range of plant height and lodging resistance, was evaluated.

In the first year a total of 29 morphological parameters was measured at two growth stages and correlated with the lodging score. The harvest at anthesis (DC 65) showed higher correlations than the harvest at maturity (DC 92), and the fresh weight traits were generally better correlated to lodging resistance than were the corresponding dry matter parameters.

In the second year, only six selected traits were measured at anthesis and correlated with the lodging score. Significant correlations between the lodging score and single morphological traits were found for stem diameter and stem weight per cm. Thicker stems and heavier stems per cm (g per cm) indicated better lodging resistance. Stem weight per cm explained 49.7% of the phenotypic variation in lodging resistance. Multiple linear regression equations indicated that 77.2% of the variation in lodging resistance was based on stem weight per cm and on the weight of the ear even though root lodging only had occurred.
2. Introduction

Lodging is a serious problem in cereal production and may reduce grain yield by up to 30% (Pinthus, 1973). In addition, it can hinder harvesting and may cause a deterioration in grain quality. Lodging is caused mainly by strong wind and/or rain and can be intensified by damage to the plants caused by insects or foot rot (pathological lodging). In this study pathological lodging was not considered; the experimental plants were treated with fungicides and insecticides. Lodging can be caused by buckling of the stem at a basal internode (stem lodging) or by the rotation of the whole plant in the soil (root lodging).

Numerous articles on lodging in small grain cereals were published before 1980 (Atkins, 1938; Baier, 1965; Brady, 1934; Clark & Wilson, 1933; Heyland, 1956 and 1960; Neenan & Spencer-Smith, 1975; Pinthus, 1973; Vaidya & Malkani, 1963; v Wettstein, 1952). Most of these publications described the correlations between morphological traits and lodging resistance as observed on plants with natural or artificially induced lodging. More recent studies have been concerned with establishing mechanical models for lodging resistance based on a physical formula (Crook & Ennos, 1993; Ennos, 1991) or have focussed on the components of the plant and their histological distribution: Kokubo (1989) reported high correlations between the cellulose content in barley cell walls and the maximum bending stress. Gartner (1984) found that silica deposits in the epidermis of wheat culms were more abundant in a lodging resistant variety than in a variety more sensitive to lodging.

Some of the results of these investigations are incompatible. Many high correlations between plant parameters and lodging resistance were found in the individual publications, but no single trait or group of traits has proven to be reliable as a general index for lodging resistance.

Research findings about the roles played by stem lodging and root lodging are contradictory. Neenan (1975) postulated that structural failure in small grain cereals occurred as a result of buckling rather than being due to loss of anchorage, whereas
Crook & Ennos (1993) reported that, in modern varieties, stem lodging was relatively uncommon as a result of selecting for shorter, more rigid stems over the last 40 years. Thus, the divergence of old and modern breeding material seems to be partly responsible for the conflicting results. Modern varieties show a lower root : shoot ratio as compared to older varieties (Siddique et al., 1990). This may be one reason for the higher frequency of root lodging in modern varieties.

In the present study we evaluated a range of modern spring wheat genotypes from Switzerland in order to find easily measurable morphological traits associated with lodging resistance. The results suggest that the parameters stem weight per cm and ear weight may be of value in breeding for lodging-resistant varieties.
3. **Materials and methods**

3.1 **Plant material**
A set of 15 spring wheat genotypes of the Swiss Federal Research Station for Agronomy Zurich-Reckenholz (FAP) was used in these experiments. See Appendix 1 for the pedigree of this breeding material.

3.2 **Experimental design and growing conditions**
The 15 genotypes were grown for two years at two locations in randomized complete block designs with four replications. The trials were located at Zurich-Reckenholz (Swiss Federal Research Station for Agronomy (FAP)) in 1992 and 1993, at Ellighausen (Experimental Station of the FAP) in 1992, and at Eschikon (Institute of Plant Sciences, Swiss Federal Institute of Technology (ETH), Zurich) in 1993. All three locations are situated in the eastern part of Switzerland between 400 and 600 meters above sea level. In 1992 the soil at the FAP site was plot-specifically a gleyic cambisol. All other trials were grown on a eutric cambisol (FAO classification). The plot size was 7.2 m² with eight rows at intervals of 16 cm. The seed rate was 400 seeds m⁻². Seeding dates were 5 March (Reckenholz) and 10 March (Ellighausen) in 1992 and 15 March (Reckenholz) and 16 March (Eschikon) in 1993. Nitrogen (N) was applied as NH₄NO₃ at a rate of 120 kg N ha⁻¹ at seeding (including mineral N in the soil to a depth of 100 cm). A second application of 30 kg ha⁻¹ N was made at the first node stage (DC 30, Zadoks et al., 1974). Chemicals were applied to avoid pathological lodging, and herbicides containing no growth regulators were used. Precipitation was recorded during the vegetation period (March - August). The amounts of precipitation were 521 mm (1992) and 614 mm (1993) at the FAP, 430 mm (1992) at Ellighausen, and 640 mm (1993) at Eschikon.

In our experiments lodging was naturally induced by strong winds and rainfall. In both years two or three climatic occurrences were registered which caused significant changes in the lodging score. The earliest lodging was observed after complete anthesis (DC 69), and only root lodging was observed.
3.3 Lodging score
Lodging at maturity was scored on a scale from 1 (totally upright) to 9 (totally lodged), depending on the deviation of the plants from the vertical and the frequency of lodged plants within a plot.

3.4 Plant sampling
In 1992 plants at Reckenholz were harvested at anthesis (DC 65) and at maturity (DC 92). At Ellighausen in 1992 and at both locations in 1993, plants were harvested at DC 65 only. Seven strong tillers from each plot were selected randomly and cut at ground level. The internodes were numbered from top to bottom. Stem diameter was measured at the center of the fifth internode. Ear length and length of the internodes were determined, and plant height was calculated as the sum of these measurements. Ears, stems, and leaves were weighed and then dried at 105°C for one day. Internodes and leaves from the upper part of the plant (internodes 1 and 2 and leaves from internodes 1 and 2) were pooled and weighed separately from the pooled samples of the lower part of the plant (internodes 3 to 6 and corresponding leaves). The number of vascular bundles from the trial at FAP (anthesis) was determined at the fourth internode (18 replications per breeding line) by histochemical staining of stem sections with phloroglucine-HCl.

Of the 29 parameters measured in 1992 (Tab. 1) the three traits correlated best with lodging resistance at both locations; stem weight cm⁻¹ (calculated from stem weight and stem length), stem diameter, and plant height were tested again in 1993 (Tab. 1 (bold print)). Stem weight per cm of internodes 1 and 2 (r = -0.74 at FAP and r = -0.59 at Ellighausen) was omitted due to close intercorrelation with total stem weight per cm (r = -0.72 at FAP and r = -0.55 at Ellighausen) which was easier to measure. Ear weight was measured again in 1993 since this parameter is thought to contribute to the plant's leverage on the root system (Heyland, 1960).
Table 1: Phenotypic correlations\(^1\) between 29 traits of the above-ground parts of the plant and the lodging score for 15 spring wheat genotypes at two locations in 1992

<table>
<thead>
<tr>
<th>Trait</th>
<th>FAP anthesis (DC 65)</th>
<th>FAP maturity (DC 92)</th>
<th>Ellighausen anthesis (DC 65)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fresh weights:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear(^2)</td>
<td>-0.26</td>
<td>-0.05</td>
<td>-0.17</td>
</tr>
<tr>
<td>Leaf 1+2</td>
<td>-0.30</td>
<td>-0.17</td>
<td>-0.27</td>
</tr>
<tr>
<td>Leaf 3-6</td>
<td>-0.43</td>
<td>-0.30</td>
<td>-0.33</td>
</tr>
<tr>
<td>Internode 1+2</td>
<td>-0.21</td>
<td>-0.33</td>
<td>-0.32</td>
</tr>
<tr>
<td>Internode 3-6</td>
<td>-0.21</td>
<td>-0.24</td>
<td>-0.06</td>
</tr>
<tr>
<td><strong>Stem</strong></td>
<td>-0.26</td>
<td>-0.30</td>
<td>-0.21</td>
</tr>
<tr>
<td><strong>Dry weights:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ear</td>
<td>-0.15</td>
<td>-0.01</td>
<td>-0.13</td>
</tr>
<tr>
<td>Leaf 1+2</td>
<td>-0.26</td>
<td>-0.15</td>
<td>-0.25</td>
</tr>
<tr>
<td>Leaf 3-6</td>
<td>-0.30</td>
<td>-0.26</td>
<td>-0.27</td>
</tr>
<tr>
<td>Internode 1+2</td>
<td>0.09</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>Internode 3-6</td>
<td>-0.07</td>
<td>-0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Stem</td>
<td>0.04</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Lengths:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant</td>
<td>0.57</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td>Ear</td>
<td>-0.07</td>
<td>-0.02</td>
<td>-0.13</td>
</tr>
<tr>
<td>Internode 1</td>
<td>0.49</td>
<td>0.48</td>
<td>0.45</td>
</tr>
<tr>
<td>Internode 2</td>
<td>0.31</td>
<td>0.13</td>
<td>0.35</td>
</tr>
<tr>
<td>Internode 3</td>
<td>0.50</td>
<td>0.41</td>
<td>0.36</td>
</tr>
<tr>
<td>Internode 4</td>
<td>0.36</td>
<td>0.29</td>
<td>0.47</td>
</tr>
<tr>
<td>Internode 5</td>
<td>0.17</td>
<td>-0.01</td>
<td>-0.07</td>
</tr>
<tr>
<td>Internode 6</td>
<td>0.12</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td>Stem</td>
<td>0.59</td>
<td>0.43</td>
<td>0.44</td>
</tr>
<tr>
<td><strong>Stem weights / cm:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total stem (FW)(^3) (SWCM)</td>
<td>-0.72</td>
<td>-0.46</td>
<td>-0.55</td>
</tr>
<tr>
<td>Total stem (DM)</td>
<td>-0.52</td>
<td>-0.36</td>
<td>-0.33</td>
</tr>
<tr>
<td>Internode 1+2 (FW)</td>
<td>-0.74</td>
<td>-0.42</td>
<td>-0.59</td>
</tr>
<tr>
<td>Internode 1+2 (DM)</td>
<td>-0.56</td>
<td>-0.19</td>
<td>-0.32</td>
</tr>
<tr>
<td>Internode 3-6 (FW)</td>
<td>-0.46</td>
<td>-0.33</td>
<td>-0.24</td>
</tr>
<tr>
<td>Internode 3-6 (DM)</td>
<td>-0.37</td>
<td>-0.22</td>
<td>-0.16</td>
</tr>
<tr>
<td><strong>Others:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem diameter</td>
<td>-0.61</td>
<td>-0.59</td>
<td>-0.45</td>
</tr>
<tr>
<td>Number of vascular bundles</td>
<td>-0.25</td>
<td>ND(^4)</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^1\) Correlation values above 0.27 and 0.35 are significant at the 0.05 and 0.01 probability level respectively

\(^2\) Parameters in bold print were measured during the second year (1993)

\(^3\) FW = fresh weight; DM = dry matter

\(^4\) ND not determined
3.5 Statistical analyses
Data were analyzed each year at each location. Mean values were calculated across all environments. The results of correlation and multiple regression analyses showed the relationship between morphological traits and lodging resistance. Genotypic correlations were determined as described by Falconer (1984) using the computer programs Plabstat (Utz, 1989) and Plabcov (Utz, 1991). Heritabilities in a broad sense were estimated as described by Hallauer and Miranda (1981).
4. Results and discussion

The breeding material from the Swiss Federal Research Station Zurich-Reckenholz was divided into short, medium, and tall plants (Tab. 2). The heights of the 15 genotypes varied from 66 to 113 cm (mean: 92 cm) and the lodging scores from 1.0 to 7.6 (mean: 4.5). The plants were divided into three groups according to height in order to integrate a wide range of combinations of plant height and lodging resistance and so as to have lodging-resistant and lodging-susceptible genotypes in each group.

The comparison of the data from the two harvests at FAP in 1992 showed higher correlation coefficients for most traits for the harvest at anthesis (DC 65) than for the harvest at maturity (DC 92) (Tab. 1). The diminished water content of the vegetative parts at maturity as compared to anthesis results in a large reduction in the weight and area of the leaves. This, in turn, may directly affect lodging caused by wind or may have an indirect effect on the accuracy of evaluating the effective morphology at earlier periods of lodging. As a result, the factors measured at maturity may show reduced correlation values. This is supported by the generally smaller correlation values for parameters based on dry matter as compared to the corresponding traits based on fresh weight in 1992. The differences between the correlation values based on fresh weight and the corresponding values based on dry matter were somewhat smaller at anthesis than at maturity. Only the measurements based on fresh weight at anthesis were included in later investigations.

Correlation values at Ellighausen in 1992 were lower than at Reckenholz for most of the traits. This result may be due to the generally lower level of lodging at Ellighausen than at Reckenholz. The average lodging score at Ellighausen in 1992 was 4.1 (SE 2.51; range 1.0 - 6.8), whereas the corresponding value at Reckenholz was 5.7 (SE 3.23; range 1.0 - 9.0).
Table 2: Traits of above-ground parts of three groups (short, medium, tall) of 15 spring wheat genotypes and heritabilities (h²) in a broad sense. Average of two locations and two years.

<table>
<thead>
<tr>
<th>Height</th>
<th>Genotype</th>
<th>Plant height (cm)</th>
<th>Stem length (cm)</th>
<th>Stem diameter (mm)</th>
<th>Ear weight (g)¹</th>
<th>Stem weight (g)¹</th>
<th>Stem lodging weight (mg)¹</th>
<th>Lodging score²</th>
</tr>
</thead>
<tbody>
<tr>
<td>short</td>
<td>1</td>
<td>66.3</td>
<td>59.0</td>
<td>3.6</td>
<td>1.65</td>
<td>4.0</td>
<td>68</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>79.2</td>
<td>71.1</td>
<td>3.4</td>
<td>1.93</td>
<td>4.6</td>
<td>64</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>82.0</td>
<td>72.7</td>
<td>4.1</td>
<td>2.35</td>
<td>6.5</td>
<td>90</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>86.4</td>
<td>76.5</td>
<td>3.8</td>
<td>2.07</td>
<td>6.3</td>
<td>83</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>86.5</td>
<td>76.8</td>
<td>3.7</td>
<td>1.98</td>
<td>5.3</td>
<td>68</td>
<td>6.1</td>
</tr>
<tr>
<td>medium</td>
<td>6</td>
<td>91.2</td>
<td>83.1</td>
<td>3.4</td>
<td>1.30</td>
<td>4.5</td>
<td>54</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>91.4</td>
<td>83.3</td>
<td>3.7</td>
<td>1.25</td>
<td>5.2</td>
<td>63</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>92.1</td>
<td>81.9</td>
<td>3.9</td>
<td>1.88</td>
<td>5.8</td>
<td>72</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>92.9</td>
<td>83.5</td>
<td>4.0</td>
<td>1.58</td>
<td>5.6</td>
<td>67</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>96.3</td>
<td>87.6</td>
<td>3.7</td>
<td>2.15</td>
<td>5.5</td>
<td>62</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>96.5</td>
<td>88.3</td>
<td>3.4</td>
<td>1.32</td>
<td>4.8</td>
<td>54</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>99.0</td>
<td>91.4</td>
<td>3.4</td>
<td>1.23</td>
<td>4.2</td>
<td>46</td>
<td>7.6</td>
</tr>
<tr>
<td>tall</td>
<td>13</td>
<td>104.9</td>
<td>97.1</td>
<td>3.8</td>
<td>1.53</td>
<td>6.0</td>
<td>61</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>105.4</td>
<td>96.9</td>
<td>3.3</td>
<td>1.46</td>
<td>5.2</td>
<td>54</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>112.7</td>
<td>103.5</td>
<td>3.4</td>
<td>1.79</td>
<td>5.8</td>
<td>56</td>
<td>6.6</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>92.11</td>
<td>83.52</td>
<td>3.64</td>
<td>1.690</td>
<td>5.29</td>
<td>64.2</td>
<td>4.49</td>
</tr>
<tr>
<td>LSD (0.05)</td>
<td></td>
<td>3.14</td>
<td>3.11</td>
<td>0.08</td>
<td>0.113</td>
<td>0.25</td>
<td>3.4</td>
<td>0.70</td>
</tr>
<tr>
<td>h²</td>
<td></td>
<td>0.98</td>
<td>0.98</td>
<td>0.93</td>
<td>0.97</td>
<td>0.98</td>
<td>0.98</td>
<td>0.96</td>
</tr>
</tbody>
</table>

¹) Fresh weight at anthesis
²) Scale of 1 to 9 (1 = no lodging and 9 = total lodging)
We found only few significant differences for the number of vascular bundles in the different genotypes (average number of vascular bundles: 30.1; SE 1.52; range 26.1 - 33.0); the correlation with the lodging score was low ($r = -0.25$). This finding is in agreement with a publication by Dunn et al. (1988) who did not observe a relationship between the number of vascular bundles and lodging resistance in five barley cultivars.

Plant height, stem length, and stem diameter were not significantly different in the two experimental years, whereas ear weight, stem weight, stem weight per cm, and lodging score showed significant differences (Tab. 3). Genotype x environment interactions, however, were small (data not shown) as compared to the differences between genotypes and between environments. For this reason the data of all four experiments were pooled for further analysis.

Highly significant differences were found among genotypes for all traits when combined over the four environments (Tab. 2). The heritabilities in a broad sense were equal or above 0.97 for all traits except stem diameter ($h^2 = 0.93$) and lodging score ($h^2 = 0.96$).

Correlation analyses of the pooled data showed positive but insignificant phenotypic correlations between plant height ($r = 0.49$) or stem length ($r = 0.51$) and lodging score (Tab. 4a). The corresponding genotypic correlation coefficients of both these traits (Tab. 4b) were quite similar to the phenotypic coefficients. The generally better lodging resistance of short varieties has been reported for small grain cereals (Murthy & Rao, 1980 (barley): $r = 0.56$; Oehme, 1989 (rye): $r = 0.77$ at DC 67/69 ). However, Atkins (1938) found inconsistent correlations between plant height or stem length and lodging for wheat. Baier (1965) reported significant correlations between the length of the lower three internodes of barley and lodging resistance ($r = 0.495$ -0.730, depending on the internode). As in our experiments, the correlation with plant height was insignificant.
Table 3: Traits of above-ground plant parts and lodging score for 15 spring wheat genotypes. Averages of two locations in 1992 and 1993.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Year</th>
<th>Difference between 1992 and 1993</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1992</td>
<td>1993</td>
</tr>
<tr>
<td>Ear weight, g (^1)</td>
<td>1.61</td>
<td>1.77</td>
</tr>
<tr>
<td>Stem weight, g (^1)</td>
<td>5.42</td>
<td>5.16</td>
</tr>
<tr>
<td>Plant height, cm</td>
<td>90.9</td>
<td>93.3</td>
</tr>
<tr>
<td>Stem length, cm</td>
<td>82.3</td>
<td>84.7</td>
</tr>
<tr>
<td>Stem diameter, mm</td>
<td>3.67</td>
<td>3.62</td>
</tr>
<tr>
<td>Stem weight per cm, mg (^1)</td>
<td>66.8</td>
<td>61.7</td>
</tr>
<tr>
<td>Lodging score, 1-9 (^2)</td>
<td>4.9</td>
<td>4.1</td>
</tr>
</tbody>
</table>

\(^*\), ** Significant at the 0.05 and 0.01 probability level respectively; NS = non-significant

\(^1\) Fresh weight at anthesis

\(^2\) Scale of 1 to 9 (1 = no lodging; 9 = total lodging)
Table 4a: Phenotypic correlations between six traits of the above-ground plant parts and the lodging score for 15 spring wheat genotypes (based on mean values across two locations in 1992 and 1993)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Ear weight</th>
<th>Stem weight</th>
<th>Plant height</th>
<th>Stem length</th>
<th>Stem diameter</th>
<th>Stem weight cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem weight</td>
<td>0.62*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant height</td>
<td>-0.30</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem length</td>
<td>-0.34</td>
<td>0.24</td>
<td>1.00**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem diameter</td>
<td>0.56*</td>
<td>0.64**</td>
<td>-0.28</td>
<td>-0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem weight cm(^{-1})#</td>
<td>0.78**</td>
<td>0.65**</td>
<td>-0.54*</td>
<td>-0.58*</td>
<td>0.77**</td>
<td></td>
</tr>
<tr>
<td>Lodging score</td>
<td>-0.23</td>
<td>-0.37</td>
<td>0.49</td>
<td>0.51</td>
<td>-0.70**</td>
<td>-0.71**</td>
</tr>
</tbody>
</table>

* ** Significant at the 0.05 and 0.01 probability level respectively.
* fresh weight

Table 4b: Genotypic correlations between six traits of the above-ground plant parts and the lodging score for 15 spring wheat genotypes (based on two locations in 1992 and 1993)

<table>
<thead>
<tr>
<th>Trait</th>
<th>Ear weight</th>
<th>Stem weight</th>
<th>Plant height</th>
<th>Stem length</th>
<th>Stem diameter</th>
<th>Stem weight cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem weight</td>
<td>0.64</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant height</td>
<td>-0.29</td>
<td>0.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem length</td>
<td>-0.38</td>
<td>0.24</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem diameter</td>
<td>0.59</td>
<td>0.66</td>
<td>-0.28</td>
<td>-0.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem weight cm(^{-1})#</td>
<td>0.80</td>
<td>0.65</td>
<td>-0.53</td>
<td>-0.58</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Lodging score</td>
<td>-0.24</td>
<td>-0.37</td>
<td>0.51</td>
<td>0.53</td>
<td>-0.73</td>
<td>-0.72</td>
</tr>
</tbody>
</table>

* fresh weight
The negative phenotypic correlations between stem diameter, stem weight per cm, and lodging score were highly significant in this study. Heyland (1960) calculated a close correlation \( r = -0.65 \) between the lodging resistance of wheat and the carrying capacity of the culm (at maturity) which was calculated from the weight per unit length of the culm basis (g per 10 cm), the plant height, and the weight of the ear. Jezowski (1987) found that the lower internodes of lodging-resistant F₁ hybrids derived from crosses between lodging-resistant and lodging-susceptible barley cultivars were shorter and thicker at maturity than those of their lodging-susceptible siblings. The upper internodes were not measured separately in that study. Vaidya & Malkani (1963) suggested that the greater diameter of the lower internodes and the heavier stem basis per unit of *Durum* wheat were responsible for the better lodging resistance.

The apparent but unexpected negative correlation \( r = -0.23 \) between ear weight and lodging score, even though this relationship is insignificant (Tab. 4a), is actually a correlation between ear weight and the characteristics of the stem. Ear weight showed a significant phenotypic correlation with stem weight per cm \( r = 0.78 \) and stem diameter \( r = 0.56 \) (Tab. 4a). Heavier ears are obviously not direct indications of better lodging resistance, despite the negative correlation between ear weight and the lodging score; the better lodging resistance is the result of thicker and heavier stems. We observed the same apparent negative correlation between ear weight, the weight of leaves, and the lodging score in 1992 (Tab. 1).

According to simple regression analysis we found that 49.7% of the variation in the lodging score could be explained by stem weight per cm (SWCM) (Fig. 1). The correlation value between lodging score and SWCM was lower \( R^2 = 0.35 \) when the five short genotypes (66 - 87 cm) (Tab. 2) were analyzed separately. The genotypes of medium height (91 - 99 cm) showed an \( R^2 \) value of 0.47, and the three tall genotypes (104 - 113 cm) showed an \( R^2 \) value of 0.91. This implies that SWCM is of less importance to lodging resistance for plants shorter than 90 cm.
Figure 1: Linear regression between stem weight per cm (SWCM) and lodging score (LS) of 15 spring wheat genotypes (Nr. 1-15). Genotypes belonging to the same group (height) (s. Table 1) are indicated by the same symbol (= short, ° = medium, □ = tall). Fresh ear weight is indicated in brackets. Average of two locations and two years.

\[ R^2 = 0.4970 \]
\[ LS = 14.5 - 156.1 \times SWCM \]
A multiple linear regression, including stem weight per cm (SWCM) and ear weight (EW), of all 15 genotypes explained 77.2% of the variation in lodging score (lodging score = 13.7 - 0.3 x SWCM + 6.0 x EW) (Tab. 5). Arranged according to height (Tab. 2), the corresponding values were 89.4% for the short genotypes, 85.3% for the medium genotypes, and 100.0% for the tall genotypes. All genotypes with ear weights above 1.8 g (FW) were situated above the regression line of SWCM (lodging score), whereas the genotypes with lighter ears were below the regression line (Fig.1). Genotypes with heavier ears probably have to attain higher SWCM values if they are to reach the same lodging resistance as genotypes with lighter ears.

Stepwise inclusion of additional variables in a multiple regression model resulted in slight increases in the correlation values (Tab. 5). SWCM includes length and weight of the stem. Because of the close phenotypic correlation between stem length and plant height (r = 1.00) (Tab. 4a) and between stem weight per cm and stem diameter (r = 0.77), plant height and stem diameter were regarded as being integrated in SWCM. Another multiple regression model (Tab. 5) including stem diameter (the trait which correlated second best with the lodging score), plant height, and ear weight revealed a lower $R^2$ value (0.64) than the first model.

Apart from the root system, the above-ground characteristics of a genotype also seem to play important roles in the occurrence of root lodging. It has been shown (Crook & Ennos, 1993; Ennos, 1991) that breeding for longer, more rigid coronal roots or for larger root spreading angles (Pithus, 1967) could increase resistance to root lodging. The traits stem weight per cm and ear weight, however, seem to be accurate indicators of the leverage of the above-ground plant on the root system.

Bauer (1963) reported close correlations between weight ($r = -0.95$) and diameter ($r = -0.96$) of the basal stem (second internode from bottom to top) and the mechanical elasticity of the culm. The weight of the stem as a measure of the elasticity of the culm is included in SWCM along with the length of the stem. Thus, SWCM is an easily measurable trait which combines plant height and the mechanical characteristics of the culm.
Table 5: Influence of selected morphological traits on correlation values; the lodging score is the dependent variable (two locations in 1992 and 1993)

<table>
<thead>
<tr>
<th>No. of independent variables</th>
<th>$R^2$</th>
<th>Traits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>Ear weight (EW)</td>
</tr>
<tr>
<td>1</td>
<td>0.13</td>
<td>Stem weight (SW)</td>
</tr>
<tr>
<td>1</td>
<td>0.24</td>
<td>Plant height (HT)</td>
</tr>
<tr>
<td>1</td>
<td>0.26</td>
<td>Stem length (SL)</td>
</tr>
<tr>
<td>1</td>
<td>0.48</td>
<td>Stem diameter (SD)</td>
</tr>
<tr>
<td>1</td>
<td>0.50</td>
<td>Stem weight per cm (SWCM) (= SW / SL x 1000)</td>
</tr>
<tr>
<td>2</td>
<td>0.77</td>
<td>SWCM, EW</td>
</tr>
<tr>
<td>3</td>
<td>0.80</td>
<td>SWCM, EW, SD</td>
</tr>
<tr>
<td>4</td>
<td>0.81</td>
<td>SWCM, EW, SD, HT</td>
</tr>
<tr>
<td>5</td>
<td>0.84</td>
<td>SWCM, EW, SD, HT, SL</td>
</tr>
<tr>
<td>6</td>
<td>0.84</td>
<td>SWCM, EW, SD, HT, SL, SW</td>
</tr>
<tr>
<td>2</td>
<td>0.58</td>
<td>SD, HT</td>
</tr>
<tr>
<td>3</td>
<td>0.64</td>
<td>SD, HT, EW</td>
</tr>
</tbody>
</table>
In conclusion, it is suggested that increasing the SWCM may be of value in breeding for better lodging resistance in years with no differentiation in lodging. At the very least, SWCM should prove to be an adequate selection criterion for eliminating genotypes which are highly susceptible to lodging.
IV A novel glycine-rich protein in wheat (*Triticum aestivum* L.): characterization and functional relation to the mechanical stability (lodging resistance) of wheat

1. Abstract

The objective of the present study was to investigate the functional relations between structural cell wall proteins and the mechanical stability (lodging resistance) of wheat. Genomic clones encoding cell wall glycine-rich proteins were isolated from a wheat genomic library. One of the genes (wGRP1) was sequenced. The derived amino acid sequence contains 391 amino acids, 65% of which are glycine (Gly). The sequence shows a highly repetitive, (Gly-x)ₜ structural arrangement, in pairs, in the Gly-rich part of the protein (where x is frequently also Gly). wGRP1 shows a hydrophobic signal peptide of 27 amino acids which suggests that the protein is transported to the cell wall compartment. We transcribed and translated the wGRP1 gene *in vitro*. A protein with a molecular weight of 28 kD (the expected molecular weight was 28.73 kD) was produced after translation. This protein was specifically precipitated by a French bean (*Phaseolus vulgaris* L.) anti-GRP1.8 antibody. Southern blot analysis showed three copies (one per homoeologous group) of the gene in the wheat genome. Comparative RFLP analysis with seven enzymes did not detect polymorphisms between spelt wheat (*Triticum spelta* L. var. Oberkulmer) and bread wheat (*Triticum aestivum* L. var. Arina) or between lodging-resistant and lodging-susceptible spring wheat breeding lines. Northern analysis of six spring wheat breeding lines (shoots and roots of 10 day old seedlings) revealed two tissue-specific bands at 0.9 kb and at 1.5 kb (as expected for wGRP1). The signal at 0.9 kb was significantly stronger in shoots of genotypes previously assessed as lodging-resistant.
2. Introduction

The major structural part of the plant cell is the cell wall. As well as many other functions, it determines the shape and size of the plant cell and helps the cell to withstand mechanical pressures and tensions which affect the plant. The major components of the cell wall are carbohydrates (cellulose, hemicelluloses, pectins) and lignin. The cellulose fibers are embedded in a matrix of hemicelluloses, pectins, lignin, and proteins (for a review see Carpita & Gibeaut, 1993). Cell walls of higher plants normally contain relatively small amounts of proteins, most of which are thought to be structural, although their biological function is still largely a matter of speculation. Grasses, for example, contain about 0.5% (dry weight of un lignified primary cell wall) extensins (Fry, 1988). The content of glycine-rich proteins may be significantly lower.

Three types of structural cell wall proteins have been characterized to date: hydroxyproline-rich proteins (HRGPs) or extensins, proline-rich proteins (PRPs), and glycine-rich proteins (GRPs) (Keller, 1993; Showalter, 1993). The amino acids proline (Pro) (or hydroxyproline (Hyp)) and glycine (Gly) are major constituents of these proteins. High contents of glycine and proline (hydroxyproline) are also found in other biological structures: the structural proteins of skin (epidermal proteins), hair, silk cocoons, and spider webs consist mainly of these amino acids. Glycine and proline (hydroxyproline), appear, therefore, to be suitable building elements for structural proteins.

GRPs contain 50 - 70% glycine. Although researchers have found many proteins with such high contents of glycine, only two GRPs are known to be part of the cell wall: GRP1 from petunia (Condit et al., 1990) and GRP1.8 from Phaseolus vulgaris L. (Keller et al., 1988; Ryser and Keller, 1992). The GRPs from Arabidopsis, rice, barley, and tomato are likely to be cell wall proteins. Their primary structures are arranged similarly which suggests that GRPs have a β-pleated sheet secondary structure (Condit and Meagher, 1986).
GRP1.8 from *Phaseolus vulgaris* L. has been specifically localized in the vascular tissue (Keller et al., 1988). It has been detected mainly in the nonlignified primary cell walls of the oldest protoxylem elements of French bean hypocotyls by means of tissue printing as well as in phloem. Ryser and Keller (1992) suggested that GRP1.8 is involved in a repair or 'filling-in' mechanism in the primary walls, making up for dead protoxylem cells that are subjected to intensive passive stretching. It is likely that GRPs (and other wall proteins) contribute to some of the functional properties of the cell wall in which carbohydrates, pectins, and lignins are not involved. GRPs may be responsible for elasticity and strength similar to the GRPs that are found in the strong and flexible spider web (Lewis, 1992).

Our results suggest that structural cell wall proteins influence the mechanical stability of the cell wall and the plant to a certain degree. The objectives of the present study were (i) to isolate and characterize genes encoding structural cell wall proteins (especially GRPs) in wheat and (ii) to investigate the functional relations between these proteins and the mechanical stability (lodging resistance) of wheat.
3. Material and methods

3.1 Screening a wheat genomic library

3.1.1 Library plating and filter replicas

To isolate the wGRP1 gene we used a wheat genomic library (Triticum aestivum L. var. Cheyenne) in λEMBL3 (Clontech Laboratories Inc., Palo Alto USA). The average insert size of the library was 15 kb, and the number of independent clones was indicated by 4.1x10⁶.

For plating the library a culture of the E.coli strain NM 538 grew overnight at 37°C in LB (10 g/l bacto tryptone, 10 g/l NaCl, 5 g/l bacto yeast extract, pH 7.5) containing 0.2% maltose. NM 538 (permissive host of λEMBL3) produces larger and more defined plaques than the restrictive strain NM 539.

For each culture plate a sample of the phage library which contained about 30,000 pfu (diluted in 0.1ml SM buffer: 0.1 M NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-HCl pH 7.5, 0.1% (w/v) gelatin) was prepared.

A total of 30 plates (9x10⁵ phages excluding replicas) was screened to cover the wheat genome.

The phage sample was mixed with 0.3 ml SM buffer and 0.3 ml host cell culture and then incubated at 37°C for 20 minutes.

The cell suspension was mixed with 10 ml of molten top agarose (47°C) and poured onto a warm 150 mm LB plate (37°C). The plate was swirled quickly during pouring so that the agarose was evenly distributed on the plate.

The plates were inverted and incubated at 37°C for about eight hours and then chilled at 4°C for an hour.

Nitrocellulose filters (Schleicher & Schuell BA 85 0.45μm; 132 mm diameter) were numbered and, using forceps, were placed on the top agarose. The filters were marked at three asymmetric locations by piercing the filter with an 18-gauge needle attached to a syringe containing waterproof black ink which came into contact with the agar.
After 60 seconds the filters were carefully peeled off and floated (plaque side up) on DNA denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 seconds. The filters were then immersed for another 60 seconds in the solution.

The filters were removed and immersed in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for five minutes.

The filters were rinsed briefly in 3XSSC and placed on Whatman 3MM paper to dry. While the filters were still damp, the DNA on the filters was fixed using 120 mJ of UV light (Stratagene UV Stratalinker 1800).

A second filter (duplicate) was placed on the same plate and marked with ink at the same locations. The duplicate was peeled off after three minutes and treated as described above.

3.1.2 Hybridization with the fbGRP1.8 (Keller et al., 1988) probe

10 filters were incubated in 50 ml of (pre)hybridization solution (50% formamide, 5x SSPE, 5X Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA) in a glass dish at 42°C for four hours.

The 1.6 kb fbGRP1.8 DNA probe was cut (EcoRI / BamHI) from the plasmid vector pSP64 and labelled with ³²P-dCTP using a random priming kit (Amersham International).

The DNA probe was denatured by heating at 100°C for five minutes and then chilled on ice.

The (pre)hybridization solution was replaced by 50 ml of fresh solution, and the denatured probe was added.

The filters were incubated at 42°C overnight.

After hybridization the (pre)hybridization solution was carefully discarded, and the filters were washed in a large volume of 2X SSC plus 0.05% SDS for one hour.

The filters were washed twice for an hour in a solution of 1X SSC plus 0.1% SDS at 68°C.
The filters were dried on Whatman 3MM paper at room temperature. The duplicate filters were paired and taped onto a piece of filter paper cut to the size of the X-ray film used for autoradiography.

- The filter paper was marked at three asymmetric spots with radioactive ink.
- The filters were covered with Saran wrap and autoradiographed overnight at -70°C using an intensifying screen to enhance the signal.
- After developing, the film was aligned with the filter to transfer the three marked locations.
- The film was aligned with the plate containing the plaques (transilluminator), and an agar plug containing several plaques around the positive signal was placed into 1 ml sterile SM buffer (plus one drop of chloroform).
- The phage lysate was replated to obtain about 400 plaques on a 150 mm plate. These plaques were then rescreened as described above. After three screening cycles, single, well-isolated positive plaques were selected.

3.1.3 Isolation of $\lambda$ DNA

- A culture of 2 ml *E.coli* (NM 538) was grown overnight. The culture was centrifuged at 4000 rpm (SS 34 rotor in Sorvall super speed centrifuge) for five minutes.
- The pellet was resuspended in sterile 10 mM MgSO$_4$ and diluted to an OD$_{600}$ of 0.5.
- $10^5$ pfu of the desired positive plaque were prepared in 0.1 ml SM buffer and mixed with 1 ml of the *E.coli* / MgSO$_4$ suspension.
- The *E.coli* / phage suspension was incubated at 37°C for 20 minutes.
- In a sterile 250 ml Erlenmeyer, 30 ml LB plus 5 mM CaCl$_2$ were prewarmed to 39°C.
- The *E.coli* / phage suspension was added to the LB / CaCl$_2$ mixture and incubated in a shaker for two hours at 39°C.
- After lysis 100 µl of chloroform were added, and the mixture was incubated at 39°C for ten minutes. (If lysis did not occur then 30 ml of prewarmed LB / CaCl$_2$ were added, and the mixture was incubated for another three to four hours before chloroform was added.)
- The mixture was centrifuged at 12,000 g (10,000 rpm, SS34 rotor, Sorvall refrigerated superspeed centrifuge) for 10 minutes, and the supernatant was transferred to a fresh SS34 tube.

- DNase and RNase were added to give concentrations of 1 µg/ml and 10 µg/ml respectively, and the lysate was incubated for 20 minutes at 37°C.

- Solid NaCl was added to give a concentration of 1 M. The samples were incubated on ice for one hour and then centrifuged at 10,000 g for 10 minutes.

- The supernatant was transferred to a fresh SS34 tube.

- Solid PEG (MW 6,000) was added to give a concentration of 10%. The PEG was dissolved on a shaker at room temperature or at 37°C.

- The mixture was incubated on ice overnight and centrifuged at 15,000 g for 10 minutes. The supernatant was discarded.

- The remaining pellet was dissolved in 0.5 ml TE.

- After being transferred to an Eppendorf tube, 20 µl of EDTA (0.5 M), 12 µl of SDS (20%) and 2.5 µl of proteinase K (20 mg/ml) were added.

- The mixture was incubated for one hour at 37°C and extracted with 1:1 phenol:chloroform.

- The upper phase was transferred to a fresh Eppendorf; 10 µl of 5 M NaCl and 500 µl of ice-cold isopropanol were added.

- The samples were incubated on ice for 30 minutes and centrifuged at 15,000 rpm for 15 minutes.

- The isopropanol was removed, and the pellet was washed with EtOH (70%) and dissolved in 100 µl TE.

The λ DNA obtained was digested with SaII and separated on an agarose gel. The gel was blotted on a nitrocellulose membrane (Southern transfer; Ausubel et al., 1987) and hybridized with the fbGRP1.8 probe. Positive bands were cut out from a new gel and ligated into the plasmid vector pBluescript. Opened with SaII.
3.2 Deletions of the plBI25 constructs

The plBI25 constructs described above were deleted using Exo III and S1 nuclease (Nested deletion kit; Pharmacia P-L Biochemicals). The procedures are described in the manufacturer's instructions. The deletions were again blotted on nitrocellulose membranes and hybridized with the fbGRP1.8 probe. Deletions still hybridizing were sequenced.

3.3 DNA Sequencing

DNA was sequenced using a T7 sequencing kit (Pharmacia P-L Biochemicals) based on the dideoxy method according to Sanger et al. (1977).

3.4 In vitro transcription and translation of wGRP1

The plasmid containing the complete wGRP1 gene was linearized with EcoRI and transcribed in vitro with T7 RNA polymerase. The transcription was performed using an mRNA capping kit (Stratagene, La Jolla USA). The in vitro translation was completed in a cell-free rabbit reticulocyte lysate incorporating ^35S labelled methionine (In vitro Express Translation Kit, Stratagene). The in vitro translation products were analyzed on a 12.5% SDS-PAGE gel (Laemmli, 1970).

3.5 Immunoprecipitation of the wGRP1 gene product

The ^35S labelled wGRP1 gene product from the in vitro translation was specifically immunoprecipitated (Ausubel et al., 1987) with an anti-fbGRP1.8-serum (Keller et al., 1988):

- 147.5 μl of dilution buffer (0.1% Triton X-100 (Fluka) and 0.1% bovine hemoglobin, prepared in TSA (0.01 M Tris-HCl pH 8.0, 0.14 M NaCl)), were added to 2.5 μl of the labelled translation product.
- 1 μl of diluted rabbit serum (1:3 with H2O) and 40 μl of anti-lg-serum were added (rabbit serum Sigma S-2632, anti-lg-serum Dako Z421), and the
reaction was incubated at 4°C for 12 hours (preclearing).

- The reaction was centrifuged at 1000 g for 10 minutes, and the supernatant was transferred to a fresh Eppendorf tube.
- Fresh Eppendorf tubes were filled with lysis buffer (1% Triton X-100, 1% bovine hemoglobin (Fluka), 1 mM iodoacetamide (Fluka), 1 mM PMSF (Fluka), and Aprotinine (Fluka) to give a final concentration of 0.2 trypsin inhibitor units per ml (buffer prepared in TSA) and was stored at room temperature for 10 minutes (precoating minimizes antigen absorption to the Eppendorf).
- 25 µl of the precleared translation product (10^5 - 10^6 cpm) were transferred to a precoated Eppendorf, and the volume was brought up to 200 µl with dilution buffer.
- 1 µl of anti-fbGRP1.8-serum was added (control reaction: 1 µl rabbit serum Sigma S-2632), and the reaction was incubated at 4°C for two hours.
- 40 µl of anti-lg-serum were added, and the reaction was incubated at 4°C for 12 hours.
- The reactions were washed four times as follows:
  - One ml of washing agent was added.
  - The reaction was centrifuged at 1000 rpm in an Eppendorf tube for seven minutes.
  - The supernatant was discarded and the pellet rewashed.

Washing agents:
First and second wash: dilution buffer  
Third wash: TSA  
Fourth wash: 0.05 M Tris-HCl pH 6.8
- 25 µl of SDS PAGE sample buffer were added to the pellet after the fourth wash (no vortex), and the reactions were incubated for one hour at 56°C and for five minutes at 100°C.
- The reactions were vortexed and centrifuged at 13,000 rpm for five seconds.
- The supernatants were analyzed on a 12.5% SDS PAGE and fluorographed (Amersham Amplify) for 20 days before autoradiography.
3.6 Southern blots

Genomic DNA was isolated (Graner et al., 1990) from bread wheat (*Triticum aestivum* L. var. Arina), spelt wheat (*Triticum spelta* L. var. Oberkulmer), and two spring wheat breeding lines which were previously assessed in field experiments as lodging-resistant and lodging-susceptible. The isolated DNA was digested with seven restriction enzymes, separated on agarose gels, and blotted on nylon membranes. These Southern blots were hybridized with a 2.2kb *wGRP1* DNA probe (deletion 3/3 (Fig. 3) in pBl25 cut HindIII / SalI) as described in chapter IV, 3.1. Southern blotting was performed according to Graner et al. (1990). The hybridized blot was autoradiographed for six days.

3.7 Northern blots

Total RNA of 10 day old seedlings was isolated as described by Jones et al. (1985). 15 μg of total RNA was separated on a formaldehyde-MOPS agarose gel and blotted on a nylon membrane (Pall Biodyne B transfer membrane 0.45 μm) (northern transfer) (Ausubel et al., 1987). The northern blots were then stained with methylene blue (0.3 M NaAc pH 5.5, 0.02% methylene blue) for three minutes and partially destained with 20% EtOH (three - five minutes) to control the steady-state level of RNA on the blots. Before hybridization, the membranes were completely destained in 0.2 x SSPE, 1% SDS (15 minutes). The hybridization was performed as described in chapter IV, 3.1 with the same *wGRP1* probe used for the Southern blots (chapter IV, 3.6). After hybridization, equal loading of the different RNA samples on the northern blots was tested again using a 1.4 kb *EcoRI* fragment (cloned into the *EcoRI* site of pSP65) of the constitutively expressed nuclear gene encoding the radish 18 S rRNA subunit (fragment G of the λRA2 clone as described by Delcasso et al., 1988). The hybridized blot was autoradiographed for four days (*wGRP1* probe) and for three hours (constitutive probe) respectively.
3.8 Western blots

- Leaves of 10 day old seedlings were vacuum-infiltrated with 20 ml extraction buffer (10 mM CaCl₂, 20 mM MES-NaOH pH 6.0) and centrifuged at 1100 rpm for 10 minutes (Falcon tubes 60ml).
- The supernatant (crude extract) was precipitated with acetone (1:4 crude extract:acetone) for 30 minutes at -20°C and then centrifuged at 8,000 g for 10 minutes (8,000 rpm, SS34 rotor, Sorvall refrigerated superspeed centrifuge).
- The pellet was dried under vacuum and resuspended in 200 µl of resuspension buffer (20 mM Tris-HCl, 5 mM DTT pH 7.5).
- The protein content of the sample was measured using a Bio-Rad protein assay.
- Equal amounts of protein (about 40 µl of sample plus 20 µl of SDS PAGE buffer) were separated on a 12.5% SDS PAGE.
- The gel was electroblotted (western blot) at 150 V for 75 minutes using a Bio Rad Trans Blot Cell (transfer buffer: 28.8 g glycine, 6 g tris, 400 ml MeOH, 2ml SDS 10%, water to 2 l) (Hybond ECL membrane Amersham).
- Immunostaining with anti-fbGRP1.8-serum was performed according to the instructions in the Enhanced Chemiluminescence Kit (Amersham).
- Dilutions: primary antibody 1:3,000; secondary antibody 1:5,000.

3.9 Preparing samples for protein sequencing

- The protein samples were separated on a 12.5% SDS-PAGE gel (Laemmli et al., 1970).
- The gel was blotted (150 V, 50 minutes) on a PVDF membrane (Millipore Immobilon P Membrane 0.45µm) using CAPS transfer buffer (10 mM CAPS (Fluka), 10% MeOH, pH 11.0).
- After blotting the membrane was soaked in staining solution (50% MeOH, 0.1% Coomassie blue R250 (Bio-Rad) for five minutes and destained for another five minutes (50% MeOH, 10% acetic acid).
The two bands of interest (i.e., the bands which had reacted specifically with the fbGRP1.8 antibody) were cut out and stored at \(-20^\circ\text{C}\) until they were sequenced.

3.10 Trypsin digest of protein samples

- The protein samples were separated on a 12.5\% SDS PAGE.
- The gel was soaked in ice-cold 0.1 M KCl for 10 minutes.
- The two bands which had specifically reacted with the fbGRP1.8 antibody were cut out and cut into small pieces.
- The gel pieces were eluated in ammoniumbicarbonate buffer (0.1 M, pH 8.6) for 12 hours at 4\(^\circ\text{C}\).
- The supernatant was dialyzed against 0.1 M ammoniumbicarbonate buffer (pH 8.6) overnight and lyophilized.
- The lyophilized sample was resuspended in 25 \(\mu\text{l}\) of 0.1 M ammoniumbicarbonate buffer (pH 8.6).
- Trypsin was added to a relation protein:enzyme of 50:1 (w/w) (trypsine was prepared in 1 mM HCl, 2 mM CaCl\(_2\)).
- The reaction was incubated for 24 hours at 37\(^\circ\text{C}\).
- The enzyme was inactivated by adding 100 \(\mu\text{M}\) 3,4-dichloroisocoumarine (Fluka) (prepared in DMSO).
- The digestion products were analyzed on a 12.5\% SDS PAGE.

3.11 Expression of the \(wGRP1\) promoter in tobacco

3.11.1 Production of GUS-reporter gene constructs and transformation of tobacco plants

- The \(wGRP1\) deletion 3/102 (Fig. 3) was cut with \(Pvu\text{II} / X\text{baI}\) to obtain a 600 bp fragment containing the \(wGRP1\) promoter and part of the leader sequence (cutting site after amino acid 20 or bp 394 (relative to Fig. 4)).
- The plasmid pBl 101.3 (containing the GUS gene) was cut \(SmaI / X\text{baI}\).
The DNA fragments were ligated and transformed into *E.coli* HB 101 (kanamycine selection).

After sequencing the ligation site (*SmaI / PvuII*) the constructs were transferred from *E.coli* HB 101 to *Agrobacterium tumefaciens* LBA 4404 as described by Bevan (1984). Pieces of tobacco leaves (*Nicotiana tabaccum cv Xanthi*) were transformed according to Horsch et al. (1984). Transformed plants were selected on MS-medium (Murashige and Skoog, 1962) with 150 μg/ml of kanamycine and 500 μg/ml of carbenicilline (500 μg/ml of cefotaxime in the first two weeks). Analyses were performed when the transformants had reached a height of about 8 cm.

### 3.11.2 GUS activity measurements

The preparation of tissues, and the determination of enzyme activity were carried out according to Jefferson et al. (1987). The concentration of 4-methylumbelliferone per mg protein and minute which was produced by the enzyme was used for the calculation of GUS activity (transformation of 4-methylumbelliferyl-glucuronide (Sigma) to 4-methylumbelliferone). The reaction took place in 200 μl at 37°C for 60 minutes and was stopped by adding 800 μl ice-cold Na₂CO₃ (0.2 M). The concentration of the reaction product was measured fluorometrically.

### 3.11.3 Histochemical staining

Histochemical staining was carried out as described by Jefferson et al. (1987). Tissues were fixed in formaldehyde (0.3%) and incubated in bromo-4-chloro-3-indolyl-β-D-glucuronic-acid (1 mM) (Biosynth, CH-9422 Staad) for 15 hours at room temperature. The reaction was stopped by adding EtOH (70%) which also removed chlorophyll.
3.12 Production of a glutathione S-transferase (GST) - wGRP1 fusion protein in *E.coli*

3.12.1 Production of GST - wGRP1 constructs

The first step in producing a GST-wGRP1 fusion was to cut a 600 bp fragment coding for 187 amino acids of the glycine-rich part of wGRP1 out of wGRP1 (wGRP1 deletion 3/6 (Fig. 3) cut *PvuII / HindIII*). This fragment was treated with Klenow polymerase to produce blunt ends and then ligated to the plasmid pGEX-4T-2 (Pharmacia P-L Biochemicals) which contained GST and which had been digested with *SmaI* and treated with alkaline phosphatase. The ligation site of the transformants was sequenced to control the reading frame.

A second construct with a shorter wGRP1-part was made as follows: deletion 3/50 (Fig. 3) was cut *HindIII / SacI* to obtain a 300 bp fragment coding for 96 amino acids of the glycine-rich part of wGRP1. This fragment was ligated into pSP72 (Promega Corp.) (cut *HindIII / SacI*). The ligation pSP72 / 3/50 was cut *EcoRI / XhoI*, and the 300 bp fragment obtained was ligated into the GST-containing plasmid pGEX-4T-3 (Pharmacia). The ligation site was sequenced again.

For the third construct, two complementary 64 bp oligonucleotides (Fig. 1), representing a 20 amino acid sequence of wGRP1 (amino acid 256 - 275 relative to Fig. 4) were synthesized (Microsynth, CH-5200 Windisch). The codon usage was adapted to the preferred pattern in *E.coli* (Sharp et al., 1988). The oligonucleotides were purified on a 9% denaturing PAGE (Ausubel et al., 1987) and phosphorylated with T4 polynucleotide kinase. Then the two corresponding oligonucleotides were combined and incubated for two minutes at 85°C, for 15 minutes at 65°C, for 15 minutes at 37°C, and for 15 minutes at 25°C; they were placed on ice for 15 minutes. The hybridized oligonucleotides were then ligated with pGEX-4T-2 (cut *EcoRI / XhoI*), and the construct was transformed into *E.coli DH5α*. Plasmids from transformants were sequenced. The fourth and last construct was similarly produced with two complementary 113 bp oligonucleotides (Fig. 2) encoding 34 amino acids (aa 256 - 289 relative to Fig. 4).
Fig. 1: Sequences of the two complementary 64 bp oligonucleotides (indicated in standard letters) used for the third GST - wGRP1 construct mentioned in the text. The codon usage of these oligonucleotides, which encode 20 amino acids of wGRP1 (aa 256 - 275 relative to Fig. 4, indicated in bold letters), was adapted to the preferred pattern in E. coli (Sharp et al., 1988).

Fig. 2: Sequences of the two complementary 113 bp oligonucleotides (indicated in standard letters) used for the fourth GST - wGRP1 construct mentioned in the text. The codon usage of these oligonucleotides which encode 34 amino acids of wGRP1 (aa 256 - 289 relative to Fig. 4; indicated in bold letters) was adapted to the preferred pattern in E. coli (Sharp et al., 1988). Arrows indicate two important restriction sites.
3.12.2 Expression of the GST-wGRP1 constructs in *E.coli* DH5α

Protein expression from pGEX plasmids is under the control of the tac promoter which is induced by the lactose analog isopropyl β-D-thiogalactoside (IPTG). The expression of the recombinant pGEX plasmids was performed according to the instructions in the GST purification module (Pharmacia P-L Biochemicals).
4. Results

4.1 Isolation and structure of the \textit{wGRP1} clone

To isolate genes which encode GRPs from wheat, a genomic library was screened with a GRP-DNA probe from \textit{Phaseolus vulgaris} L. (\textit{fbGRP1.8}; Keller et al., 1988). From a total of 900,000 plaques, three strongly and two weakly hybridizing \text{\textlambda}EMBL3 clones were isolated, and the crosshybridizing \textit{SalI} fragments were subcloned into the plasmid vector \textit{pIBI25}. The insert size of the first strongly hybridizing clone which contained the \textit{wGRP1} gene was about 4 kb. Deletions of this fragment were made using \textit{S1} and \textit{Exo III} nuclease. Deletions still hybridizing with the \textit{fbGRP1.8} probe in a range of 1 kb to 2.8 kb were sequenced (Fig. 3). A second strongly hybridizing \text{\textlambda}EMBL3 clone contained a gene identical to \textit{wGRP1}.

The nucleotide sequence of the \textit{wGRP1} gene from wheat and its deduced amino acid sequence is shown in Figure 4. The derived sequence (expected MW 28.73 kD) contains 391 amino acids of which 65\% are glycine. It shows the pairwise (Gly-x)$_n$ arrangement typical of plant GRPs. \textit{wGRP1} has a very hydrophobic signal sequence of 27 amino acids which suggests that the protein is transported to the cell wall compartment. The most abundant amino acids in \textit{wGRP1} after glycine are alanine (11.8\%), leucine (6.7\%), phenylalanine (4.6\%), valine (2.3\%), and histidine (2.3\%). The high contents of alanine, leucine, phenylalanine, and valine in the signal peptide of \textit{wGRP1} causes its high hydrophobicity. Fig. 5 shows the hydrophatic index of \textit{wGRP1} (compare Kyte and Doolittle, 1982). The signal peptide is easily recognized as a hydrophobic region at the amino terminus of the protein. Kyte and Doolittle developed a hydrophathy scale, whereby the hydrophilic and hydrophobic properties of each of the 20 amino acid side-chains are taken into consideration. The scale is based on experimental observations derived from literature. In the case of membrane-bound proteins, the portions of their sequences that are located within the lipid bilayer are clearly delineated by uninterrupted areas on the hydrophobic side of the line passing through the midpoint.
Fig. 3: Sequencing strategy of **wGRP1**: a) location of the gene in the 4 kb pIBI25 insert. The 4 kb SalI fragment was subcloned from a lambda EMBL 3 clone isolated from a wheat genomic library. b) sequenced part of the 4 kb fragment with names and sequencing starts (numbers in brackets relative to Figure 4) of the most important deletions of the primary and the complementary strand used for sequencing.
Fig. 4: Nucleotide sequence of the wGRP1 clone and its deduced amino acid sequence. Numbers indicate nucleotides, and bold numbers indicate amino acids. The TATA box and the polyadenylation site are underlined.
Fig. 5: Hydropathic index (Kyte and Doolittle, 1982) of the predicted amino acid sequence of wGRP1, computed using an interval of five amino acids. The hydropathicity is represented as a function of the amino acid number. 0 on the hydropathic index specifies the threshold of hydrophobicity and hydrophilicity.
The third \textit{\texttt{EML3}} clone (\textit{wGRP2}) contained an insert of about 9 kb. This insert was also cloned into \textit{pBl225}. The plasmid was then digested with a set of 10 restriction enzymes, and a single, very strongly hybridizing \textit{SphI} fragment of about 2 kb was isolated and subcloned into \textit{pBl225}. This fragment contained the putative \textit{wGRP2} gene which was only partially sequenced (Fig. 6). In agreement with \textit{wGRP1}, the deduced amino acid sequence of \textit{wGRP2} shows the pairwise (Gly - x) arrangement. However, in contrast to \textit{wGRP1}, the partial \textit{wGRP2} sequence contains four methionine residues (\textit{wGRP1}: two methionine residues in the whole gene), one lysine residue (\textit{wGRP1}: 0), and three arginine residues (\textit{wGRP1}: three arginine residues in the Gly-rich part of the whole gene). Thus, the amino acid composition of \textit{wGRP2} is quite different from that of \textit{wGRP1}.

\subsection*{4.2 In vitro transcription and translation of \textit{wGRP1}}

To confirm the open reading frame of the \textit{wGRP1} gene and to determine the apparent molecular weight of \textit{wGRP1} on a SDS-PAGE gel, the gene was transcribed \textit{in vitro} using T7 RNA polymerase. With regard to the putative presence of pseudogenes in the wheat genome (Mauch et al., 1991) we wanted to confirm that \textit{wGRP1} is a functional gene. The \textit{wGRP1} mRNA which was not degraded (tested on a denaturing PAGE gel) was translated in a rabbit reticulocyte lysate incorporating $^{35}$S labelled methionine. \textit{wGRP1} contains two methionine residues (aa 1 and 38 relative to Fig. 4) which can be labelled by $^{35}$S. The \textit{in vitro} translation of \textit{wGRP1} (Fig. 7) produced a protein of about 28 kD molecular weight which is in close agreement with the expected molecular weight of 28.73 kD. The control reaction (\textit{wGRP1} mRNA was not added to the reticulocyte lysate) did not show a signal.

\subsection*{4.3 Immunoprecipitation}

To analyze the cross-reactivity of the anti-GRP1.8 serum from \textit{Phaseolus vulgaris} L., the $^{35}$S-labelled \textit{in vitro} translated \textit{wGRP1} gene product was immunoprecipitated. Immunoprecipitation of the gene product with the anti-GRP1.8 serum resulted in a strong band at 28 kD (Fig. 8). A serum of a non-immunized rabbit resulted in a very
Fig. 6: Partial nucleotide sequence from the glycine-rich part of the putative wGRP2 gene and its deduced amino acid sequence. Numbers indicate nucleotides; bold numbers indicate amino acids. The sequence shows distinct differences in its amino acid composition as compared to wGRP1.
Fig. 7: Translation of the *wGRP1* mRNA in a rabbit reticulocyte lysate incorporating $^{35}$S labelled methionine. Lane 1: *wGRP1* translation product (expected MW 28.73 kD). Lane 2: control without addition of mRNA. Lane 3: Translation of BMV (brome mosaic virus) mRNA as a control of the reticulocyte system. Proteins were separated on a 12.5% SDS polyacrylamide gel.
Fig. 8: Immunoprecipitation of the $^{35}$S labelled in vitro wGRP1 translation product with the anti-fbGRP1.8 serum. Lane 1: Immunoprecipitation with the bean antibody. Lane 2: control reaction with non-immunized rabbit serum. Proteins were separated on a 12.5% SDS polyacrylamide gel.
weak signal. This suggests that the fbGRP1.8 antibody was able to react specifically with wGRP1 and that wGRP1 and fbGRP1.8 are immunologically related.

4.4 GRP sequences in the wheat genome

To determine the copy number of wGRP1 in the wheat genome and to assess the degree of polymorphism at this locus in different wheat genotypes, Southern analyses were made. The analysis (Fig. 9) of two spring wheat genotypes (previously assessed in field experiments as lodging-resistant and lodging-susceptible) using wGRP1 as a probe showed three strongly hybridizing bands, suggesting that the hexaploid wheat genome contains three copies of the wGRP1 gene (one derived from each of the three wheat progenitor genomes A, B, and D). Comparative RFLP analyses of the two spring wheat genotypes (Fig. 9), of bread wheat (Triticum aestivum L. var. Arina), and of spelt wheat (Triticum spelta L. var. Oberkulmer) (not shown) revealed no polymorphisms with seven enzymes.

4.5 Expression of the wGRP1 gene in wheat seedlings

RNA analyses were performed to measure the expression of the wGRP1 gene in genotypes previously assessed as being lodging-resistant or lodging-susceptible and to determine whether or not a functional relation between GRPs and the mechanical stability of the plant exists.

The spring wheat genotypes used were 1 (1.0), 3 (1.3), and 4 (2.1) (lodging-resistant) and 6 (7.2), 14 (6.5), and 15 (6.6) (lodging-susceptible). Values in brackets indicate lodging scores on a scale of 1 to 9 (1 = no lodging; 9 = total lodging). The genotypes and the field experiments for the evaluation of lodging-resistance are described in chapter III.

Total RNA was extracted from roots and shoots of 10 day old wheat seedlings and blotted on nylon membranes. These membranes were then hybridized with the wGRP1 clone. The analysis of the six spring wheat genotypes (Fig. 10a) revealed one
Fig. 9: Southern analysis of two spring wheat genotypes (3 (lodging-resistant)) and 14 (lodging-susceptible)) with the *wGRP1* probe and seven restriction enzymes (indicated at the bottom). The genotypes and the assessment of lodging-resistance are described in chapter III.
Fig. 10a: Northern blot of 10 day old seedlings from six spring wheat genotypes. Contents of total RNA in the lanes (roots and shoots): 15μg. The genotypes, and the assessment of their lodging resistance are described in chapter III.

1) + = lodging-resistant genotype; - = lodging-susceptible genotype

Fig. 10b: Hybridization of the northern blot shown in Fig. 10a with the constitutively expressed gene encoding the radish 18S rRNA subunit (described in chapter IV 3.7). This hybridization was performed to control equal loading of RNA in the 12 lanes of the blot.
signal at 0.9 kb and one signal at 1.5 kb (the expected length of the \textit{wGRP1} mRNA as deduced from the genomic clone is about 1.4 to 1.5 kb). The 1.5 kb signal was detected in roots of all lines but not in shoots, whereas the 0.9 kb signal was present in both roots and shoots. The strength of the 1.5 kb signal was similar in all six genotypes. At 0.9 kb, the genotypes 1, 3, and 4, which had proven to be lodging-resistant in the previous field experiments, showed a significantly stronger expression signal in roots and especially in shoots than did the lodging-susceptible genotypes 6, 14, and 15 (Fig. 10a). The methylene-blue staining of the northern membrane (not shown) and the hybridization of the membrane with the constitutive rRNA probe (chapter IV, 3.7) (Fig. 10b) after the hybridization with the \textit{wGRP1} probe demonstrated that equal amounts of total RNA were present on the northern blot.

4.6 Expression of the \textit{wGRP1} promoter in transgenic tobacco

To investigate a possible tissue or cell-type specificity of the \textit{wGRP1} promoter, expression patterns of translational fusion gene constructs with the \(\beta\)-glucuronidase (GUS) reporter gene were analyzed in transgenic tobacco. The results of such an experiment have to be interpreted very carefully because of the expression of a monocot promoter in a dicot plant. Previous publications, however, have reported that monocot promoters may be active in transgenic tobacco. Zhu et al. (1993) showed that a rice chitinase promoter (inducible by wounding and by a fungal elicitor) supported high level expression of the GUS reporter gene in tobacco. The same chitinase promoter / GUS fusion was also active in transfected rice protoplasts. The authors concluded that signal pathways for stress induction are conserved between monocot and dicot plants. We assumed that the \textit{wGRP1} promoter was also active in dicot plants.

Histochemical staining of transgenic tobacco plants containing the \textit{wGRP1}-promoter / GUS fusion did not show visible staining of stems, leaves, or roots. Determination of GUS-activity, however, revealed a weak expression of the reporter gene in roots. The enzyme activity was significantly higher in roots than in stems or leaves (Tab. 1). The high standard error of the transgenic roots may be due to the position effect.
<table>
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<tr>
<th>Tissue</th>
<th>GUS-activity$^1$</th>
<th>GUS-activity$^1$</th>
<th>GUS-activity$^1$</th>
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<td></td>
<td>roots</td>
<td>stems</td>
<td>leaves</td>
</tr>
<tr>
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<td>6.6 +/- 2.1</td>
<td>3.1 +/- 1.8</td>
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<td>(N = 15)</td>
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<td>3.4 +/- 1.7</td>
</tr>
<tr>
<td>(N = 8)</td>
<td>(N = 8)</td>
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</tbody>
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$^1$ indicated as pmol produced 4-methylumbelliferone per mg protein and minute

Tab. 1 GUS-activity in tobacco plants transformed with a translational wGRP1-promoter / GUS-reporter gene fusion (LSD 99% = 7.86)

### 4.7 Glycine-rich proteins in the intercellular fluid of wheat leaves

After having confirmed the cross-reactivity of the anti-fbGRP1.8 serum with wGRP1 in the immunoprecipitation, we investigated the occurrence of GRPs in extracts of the intercellular fluid from vacuum infiltrated wheat leaves of 10 day old seedlings. The anti-fbGRP1.8 serum (Keller et al., 1988) was raised against the glycine-rich region of French bean GRP1.8 (consisting of amino acids 15 - 331 of the immature French bean protein which represents approximately 68% of the mature protein). The vacuum extraction of wheat leaves represents a simple method for isolating soluble proteins from the cell wall compartment of the plant. Figure 11 shows a Coomassie stained SDS-PAGE gel of these leaf extracts.

In our western blots of 15 spring wheat genotypes the antibody recognized one strongly reacting band at about 25 kD and one weakly reacting band at about 23 kD in all the tested genotypes (not shown). We speculated that the 25 kD protein is the processed form of wGRP1 or at least a member of the GRP family. To confirm this assumption, the 25 kD protein was digested with trypsin; we expected a fragment of 19 kD for wGRP1 due to the specificity of trypsin (-P,-P,'; P\(_i\) = Lys, Arg; P\(_i\)' = non-specific) as indicated by the manufacturer (Boehringer Manneim). The trypsin digest
Fig. 11: Coomassie stained SDS - PAGE gel of extracts of the intercellular fluid from 10 day old wheat seedlings. The leaves from the 15 spring wheat genotypes, described in the first part of this thesis, were vacuum infiltrated as described in chapter IV 3.8. The two protein bands at 25 and 23 kD, which reacted specifically with the fbGRP1.8 antibody, are clearly recognizable. There were no significant differences between the genotypes.
of the 25 kD protein did not leave a fragment with an MW above 10 kD as we
expected of a GRP (no bands were visible on a SDS-PAGE gel). The 25 kD protein
was partially sequenced using a 470 A protein sequencer (Applied Biosystems,
Fosters City, CA USA). The first 39 amino acids of the protein are shown in Fig. 12.
Although there are a few glycine residues present in the sequenced protein, it is
clearly not a GRP. By means of a databank search, we found a rice leaf protein (Fig.
12) with 78% homology to the wheat 25 kD protein (Tsugita et al., 1994). The function
of the rice protein is unknown.

4.8 Production of a glutathione S-transferase (GST) - wGRP1 fusion protein
in E. coli DH5α

Since we were unsuccessful in detecting a wheat GRP with the anti-fbGRP1.8
antibody, we decided to produce a GST - wGRP1 fusion protein to raise a new
antibody with a higher specificity against wheat GRPs.

The expression products of the first and second GST - wGRP1 constructs expressed
in E.coli (containing wGRP1 sequences encoding 187 amino acids and 96 amino
acids) were not visible on Coomassie stained gels. Blotting of the gels and
immunostaining with anti-GST serum resulted in faint bands at the expected molecular
weight as well as in smears below it, indicating degradation of the fusion proteins in
both cases (not shown).

The third construct containing a synthetic oligonucleotide encoding 20 amino acids of
wGRP1 (aa 256 - 275 relative to Fig. 4; codon usage adapted to the preferred pattern
of E.coli) showed a strong overexpression of the fusion protein at the expected
molecular weight (Fig. 13). Therefore, we synthesized an elongated oligonucleotide
encoding 34 amino acids of wGRP1 (aa 256 - 289 relative to Fig. 4). This construct
also showed a strong overexpression in E.coli (Fig. 13). The fusion proteins of the
third and fourth GST - wGRP1 constructs are suitable for producing antibodies
against wGRP1. Improved antibodies against wGRP1 may contribute to the detection
of wheat GRPs on a protein level (s. chapter IV, 4.7).
Fig. 12: Alignment of the 25 kD protein recognized by the fbGRP1.8 antibody in extracts of wheat leaves (first 39 amino acids in bold letters) and the homologous (78 %) rice leaf protein (25 kD; 20 amino acids in italics) reported by Tsugita et al. (1994).
Fig. 13: Expression of glutathione S-transferase (GST) - wGRP1 fusion proteins in E. coli. Lane 1: fusion protein encoding 34 amino acids of wGRP1 (fourth construct mentioned in chapter IV 3.12.1); lane 3: fusion protein encoding 20 amino acids of wGRP1 (third construct mentioned in chapter IV 3.12.1); lane 5: GST vector without fusion to wGRP1 (control). Lanes 2, 4, and 6 show control samples of the corresponding lanes 1, 3, and 5 without induction of the fusion protein expression by IPTG (s. chapter IV 3.12.2).
The construct containing the 113 bp oligonucleotides mentioned above may, at a later date, be elongated with two new synthetic oligonucleotides. After ligation of the construct (opened at the \textit{BbrP1} site (blunt end) indicated in Fig. 2) to the new oligonucleotides, a new construct encoding about 60 amino acids of wGRP1 may be obtained. This fusion protein may be of value in examining the secondary structure of wGRP1.
5. Discussion

5.1 Structure of wGRP1 and its deduced amino acid sequence

The wheat protein under investigation has structural features in common with previously published GRP sequences from other plant species (e.g. petunia, French bean, barley, rice): it has a high glycine content (65 %) and a repetitive structure and is preceded by a hydrophobic putative leader sequence (Condit & Meagher, 1986; Keller et al., 1988; Rohde et al., 1990; Fang et al., 1991). The putative amino-terminal signal sequence of wGRP1 (Fig. 4) is about 27 amino acids long and has three physico-chemically distinct regions which are typical for signal sequences: a positively charged amino terminal region (amino acids 6 and 7), a central hydrophobic region (aa 9 - 17), and a more polar carboxy-terminal region (aa 19 - 23). The putative cleavage site of the signal peptide is located between amino acid 32 and amino acid 33 (numbered amino acids correspond to Fig. 4) (v Heijne, 1988). The putative hydrophobic signal peptide is easily recognizable in the plot of the hydropathic index of wGRP1 (Fig. 5).

Beyond the putative signal peptide the deduced amino acid sequence of wGRP1 can, for the most part, be described by the formula (Gly - x)n where x is alanine, glutamine, histidine, isoleucine, leucine, phenylalanine, valine, or often glycine. This repetitive, paired arrangement of the amino acids is broken three times (at amino acids 138, 346 and 384). However, following those pairs which lack glycine in the first position, the (Gly - x)n sequence is resumed. Analogous to silk fibroin, the primary amino acid structure of wGRP1 suggests a secondary structure of a β-pleated sheet (Condit & Meagher, 1986).

wGRP1 has a potential TATA box about 90 bp upstream of the ATG start codon and a putative polyadenylation signal (AATAA) 61 bp downstream of the stop codon (TGA). There is no intron-like sequence in the coding region.
5.2 *In vitro* translation of *wGRP1* and immunoprecipitation of the gene product

The successful *in vitro* translation of *wGRP1* confirms the presence of an open reading frame as deduced from the sequence analysis. The apparent molecular weight (MW) of the *in vitro* translation product of *wGRP1* on SDS gels is about 28 kD. The close agreement with the MW predicted from the gene sequence (28.73 kD) contrasts with other GRPs. GRP1.8 from *Phaseolus vulgaris* L. (Keller et al., 1988), for example, shows an apparent MW of 53 kD which is 16 kD larger than predicted. The predicted MW of *wGRP1* matches its apparent MW on SDS gels in spite of the unusual sequence of the amino acids. The predicted MW for the cell wall extracted protein is 25.50 kD due to the removal of the signal peptide after secretion of the protein (assuming that there are no other post-translational modifications).

The fbGRP1.8 antibody was able to detect *wGRP1* in the immunoprecipitation experiment. From that we concluded that *wGRP1* is immunologically related to fbGRP1.8 and that the fbGRP1.8 antibody would also react with wheat GRPs on a western blot. However, the 25 kD protein detected by this antibody in western blots of vacuum infiltrated wheat leaves was not a GRP (Fig. 12). Thus, we did not find *wGRP1*. We concluded, therefore, that *wGRP1* is not expressed in wheat leaves, is masked by the strong signal of the 25 kD protein, or is very rapidly insolubilized in the cell. The insolubilization of fbGRP1.8 was reported by Keller et al. (1989). The insolubilization of structural proteins upon secretion into the wall has been discussed in a recent review by Iyama et al. (1994): it is assumed that this insolubilization is the result of covalent cross-linkages within and between structural wall proteins. The relatively weak signal in the sensitive immunoprecipitation assay suggests a low affinity of the antibody to the wheat GRP. The results from the northern analysis (chapter IV 4.5) and from the expression of the *wGRP1* promoter in transgenic tobacco (chapter IV 4.6) support the hypothesis that *wGRP1* is either not expressed or only very weakly expressed in leaves.
5.3 GST - wGRP1 fusion proteins as antigens for the production of a specific anti-wGRP1 antibody

For the future production of a more specific antibody against wGRP1, we expressed GST - wGRP1 fusions in E.coli. The expression of the first and second GST - GRP constructs encoding 187 and 96 amino acids respectively showed no visible fusion protein bands on SDS PAGE gels. Immunoblots of the expression products, probed with a GST antibody, indicated a partial degradation of the fusion protein. Additional reasons for the very weak expression may be the insolubilization of the fusion protein or the codon usage for glycine in wGRP1 which is very unusual for E.coli. Sharp et al. (1988) reported that the codon usage for glycine, as found in highly expressed genes of E.coli, was about 1% GGG and 0% GGA (57% GGU, 42% GGC). In contrast, the codon usage for glycine found in wGRP1 is 28% GGA and 10% GGG. This species-dependent codon usage might interfere with a strong expression of wGRP1 in E.coli. Our third and fourth GST - wGRP1 constructs, containing synthetic oligonucleotides which encoded 20 and 34 amino acids of wGRP1 respectively, were adapted to the preferred codon usage of E.coli and were strongly overexpressed. We do not know whether this overexpression was a result of the shorter GRP sequences in the constructs (compared to the first and second constructs) or if the adapted codon usage also played an important role.

5.4 The occurrence of the wGRP1 gene in the wheat genome

The occurrence of three major bands in the Southern blot analyses of bread wheat, spelt wheat, and two spring wheat genotypes suggests the existence of three copies of wGRP1 in the wheat genome. We assume that each copy of the gene originates from one of the three wheat progenitor genomes (homoeologous genes). The absence of polymorphisms in the four tested genotypes in the comparative RFLP analysis with seven enzymes showed that there are probably no different alleles at the wGRP1 locus in the tested wheat genotypes.
5.5 Expression of the \textit{wGRP1} promoter in transgenic tobacco

The expression of the \textit{wGRP1} promoter in tobacco showed very weak activity of the GUS reporter gene in general. Histochemical staining did not reveal tissue or cell specific expression. This is in contrast to the vascular-specific expression of the bean \textit{GRP1.8} gene (Keller et al., 1988). Considering the transformation of a dicot plant with a reporter gene under control of a monocot promoter, these results should not be overemphasized. However, the expression of the GUS gene in roots, measured in GUS activity assays, was significantly higher in transgenic plants than in control plants. This result is in agreement with the specific expression of \textit{wGRP1} in the roots of wheat seedlings as found on northern blots (chapter IV 4.5).

5.6 Secondary structure and possible function of \textit{wGRP1}

As mentioned above, predictions of the secondary structure for cell wall GRPs indicate that these proteins exist as $\beta$-pleated sheets (Condit & Meagher, 1986). Such a structure may, similar to silk fibroin, provide elasticity as well as tensile strength (Showalter, 1993). Considering, for example, the properties of spider silk which has an extraordinary tensile strength combined with flexibility, GRPs may contribute to the extensibility of the cell wall (Keller et al., 1989). Table 2 compares molecular weights and amino acid compositions of two cell wall GRPs (GRP1.8 from bean and GRP1 from petunia) and five putative cell wall GRPs. In contrast to the relatively high content in tyrosine found in the GRPs of rice (6.6%), barley (6.5%), bean (6.9%), and \textit{Arabidopsis} (8.9%), the GRPs of wheat and petunia do not contain tyrosine residues. On the other hand, the GRPs from wheat and petunia contain phenylalanine (4.6% and 4.2% respectively), whereas the other GRPs shown in Table 2 have lower contents of phenylalanine. It has been proposed that the tyrosine residues and their regular arrangement in the molecule (e.g. fbGRP1.8; Keller et al., 1988) either have a catalytic effect on the oxidative polymerization chain reactions of lignin synthesis or are involved in interactions with other wall components (Keller et al., 1989; Fry, 1986). The many phenylalanine residues in the GRPs from wheat and petunia may be involved in hydrophobic interactions with matrix polymers in the cell wall. Fry (1986)
Tab. 2: Comparison of molecular weights (kD) and compositions of amino acids (mol%) of two cell wall GRPs (GRP1.8 from bean and GRP1 from petunia) and five putative cell wall GRPs from three monocot and four dicot plants.

<table>
<thead>
<tr>
<th>Gene</th>
<th>wGRP1</th>
<th>Osgrp-1</th>
<th>GRP</th>
<th>GRP1.8</th>
<th>GRP1</th>
<th>atGRP3</th>
<th>tgGRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>wheat</td>
<td>rice</td>
<td>barley</td>
<td>bean</td>
<td>petunia</td>
<td>arabidopsis</td>
<td>tobacco</td>
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<tr>
<td>Ref.</td>
<td>this thesis</td>
<td>Lei et al., 1991</td>
<td>Rohde et al., 1990</td>
<td>Keller et al., 1988</td>
<td>Condit et al., 1986</td>
<td>Oliveira et al., 1990</td>
<td>Brady et al., 1993</td>
</tr>
<tr>
<td>MW (kD)</td>
<td>28.73</td>
<td>13.50</td>
<td>18.11</td>
<td>36.54</td>
<td>28.77</td>
<td>14.39</td>
<td>21.44</td>
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<th>aa (mol%)</th>
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<tbody>
<tr>
<td>Gly</td>
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<td>Phe</td>
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<td>Lys</td>
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reported that the gelling of pectin is an example of hydrophobic interactions in the cross-linking of matrix polymers.

5.7 Expression of the \textit{wGRP1} gene in wheat seedlings

Northern analyses were conducted on a set of spring wheat breeding lines to determine whether or not functional relationships exist between the expression of wheat GRPs and the mechanical stability of wheat. The analyzed genotypes had been assessed for their lodging resistance in field experiments. The occurrence of two transcripts of different molecular weights (0.9 kb and 1.5 kb) in the northern analysis suggests that one related GRP gene in the wheat genome is significantly shorter than \textit{wGRP1}. Rohde et al. (1990) found a GRP gene in \textit{Hordeum vulgare} which is transcribed into a 1.0 kb mRNA. This barley GRP may be related to the 0.9 kb mRNA found in our northern blots.

Based on the available data, we can not yet exclude that the 0.9 kb transcript represents a proline-rich protein (PRP). Because of the sequence complementarity between stretches of CCX which encodes proline and stretches of GGX which encodes glycine, the double-stranded \textit{wGRP1} probe may also hybridize to a sequence encoding a PRP. In other words, the noncoding strand of a PRP gene shares sequence homology with the coding strand of a GRP gene and vice versa (Keller et al., 1988; Showalter, 1993).

The putative \textit{wGRP1} signal at 1.5 kb was detected in the RNA of wheat roots only. This result corresponds to the \textit{wGRP1} promoter expression in tobacco and to the absence of a \textit{wGRP1} signal in western blots of wheat leaves. The second signal of 0.9 kb was, dependent of the genotype, detected in roots and shoots of the six examined genotypes. The correlation between the abundance of the 0.9 kb mRNA and the lodging score of the genotypes (Fig. 10) supports the hypothesis that GRPs influence the mechanical properties of the cell wall and of the plant. However, the set of six screened genotypes is too small to prove it.
V Conclusions and Prospects

The results of our field trials with 15 spring wheat genotypes show that there is no single morphological trait which is responsible for more than 49.7% of the variation in lodging resistance. It is difficult to predict a complex plant characteristic such as lodging resistance which is affected by many genotypic and environmental factors with only one easily determinable morphological trait. The best correlated trait, stem weight per cm, may, however, help to eliminate very susceptible genotypes from breeding programs, especially in years without natural lodging. The multiple regression which includes stem weight per cm and ear weight explains 77.2% of the phenotypic variation in lodging resistance. To improve this high correlation root traits or quantitative biochemical parameters may have to be taken into consideration.

Many publications have reported that correlations between morphological traits and lodging-resistance are valid for a limited spectrum of genotypes and probably for specific environments. It is uncertain, therefore, as to whether generally reliable parameters for estimating lodging resistance will be found in agronomic-morphological investigations. The value of such investigations is the detailed characterization of a set of modern genotypes with different lodging resistance which may, in the future, be used as a basis for finding molecular markers (QTLs) for lodging resistance.

At the molecular level, the isolation of wGRP1 has proven the existence of GRP genes in wheat. The large genotypical differences in the expression of the 0.9 kb mRNA, which is likely to represent a GRP, and the correlation between the abundance of this mRNA and the lodging score in our field experiments support the hypothesis that GRPs affect the mechanical stability of wheat. To prove this hypothesis, a larger set of genotypes and different growth stages must be examined. It would also be interesting to isolate the 0.9 kb mRNA after screening a cDNA library with the wGRP1 probe used in the northern analyses. Positive clones would have to be cloned and sequenced.
If the relationship between lodging resistance and the expression of the 0.9 kb mRNA were to be confirmed on a larger set of genotypes, heritability studies could be performed with inbred lines of crosses between lodging-resistant and lodging-susceptible genotypes.
VI References


### VII Appendix

Appendix 1: Pedigree of the genotypes used in this work (according to FAP Zurich-Reckenholz)

<table>
<thead>
<tr>
<th>Genotype no. (this thesis)</th>
<th>Line no. FAP</th>
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<th>Pedigree</th>
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<tbody>
<tr>
<td>1</td>
<td>94985</td>
<td>1984</td>
<td>KENT/2xB564//SO/3/CL/MS</td>
</tr>
<tr>
<td>2</td>
<td>94896</td>
<td>1983</td>
<td>GA-CU//KAW/SX/3/MS</td>
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<tr>
<td>3</td>
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<td>95074</td>
<td>1983</td>
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<tr>
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<td>95064</td>
<td>1985</td>
<td>MS</td>
</tr>
<tr>
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<td>94664</td>
<td>1982</td>
<td>MS/3/KENT/2xB564//SAP/4/KENT/2xB564//SAP</td>
</tr>
<tr>
<td>8</td>
<td>94702</td>
<td>1983</td>
<td>ALBIS/GA-CU</td>
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<td>9</td>
<td>94767</td>
<td>1982</td>
<td>HEGE312-75-262/CHAT'S</td>
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<td>11</td>
<td>94853</td>
<td>1984</td>
<td>SCER/CAL//35399/3/TA/4/MS/5/FAM/6/MS</td>
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<tr>
<td>12</td>
<td>94737</td>
<td>1984</td>
<td>MS/BOW'S'</td>
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<td>1982</td>
<td>MS//EXSR400/SEL</td>
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<tr>
<td>15</td>
<td>92572</td>
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<td>BESSO (Swiss variety)</td>
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Abbreviations:

- **B564**: LNL//GARANT
- **BB**: Blue bird
- **BOW'S'**: Bobwhite 'S'
- **CAL**: Calanda
- **CHAT'S**: Chat's
- **CL**: Combination line
- **CNO**: Ciano
- **EXSR400**: Hlx2/2284
- **FAM**: Famos
- **GA-CU**: Gallo-Cuckoox
- **GRN**: Granat
- **HEGE**: Hege breeding line
- **KAL**: Kalyansona
- **KAW**: Kawkas
- **KENT**: Kentana
- **KIME**: Kime 23
- **MS**: Male-sterility programme FAP
- **PCI**: Pichihuila
- **PROB**: Probat
- **RRIV68**: Red River line V68
- **SAP**: Sappe
- **SCER**: Siete-Cerros
- **SEL**: Selpek
- **SO**: Solo
- **SX**: Super X
- **TA**: Tano
- **WA**: Walter
- **WT**: Triticale (wheat types)

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Curriculum vitae

November 24, 1964  Born in Horgen / ZH

1971 - 1977  Primary school in Horgen / ZH

1977 - 1983  High school in Zurich (Matura Typus B)

1984 - 1989  Student at the Faculty of Agriculture of the Swiss Federal Institute of Technology (ETH) in Zurich

1989  Diploma in Agronomy (Major in Plant Sciences)

1989 - 1991  Employee in the chemical industry

1991 - 1994  Scientific collaborator at the Institute of Plant Sciences of the Swiss Federal Institute of Technology (ETH) in Zurich, Switzerland (PhD candidate)