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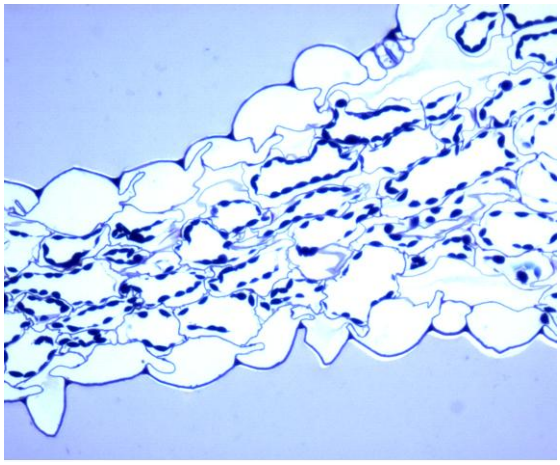
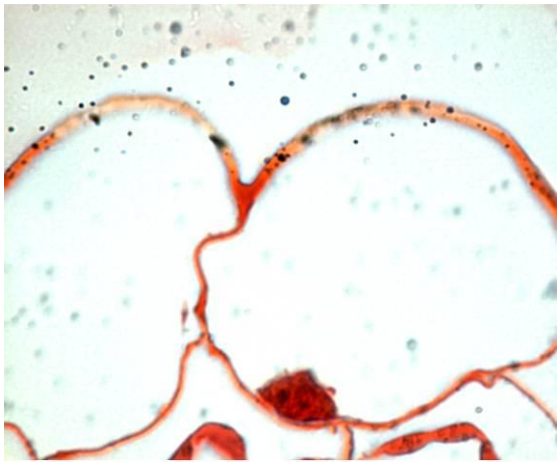
Leaf structure and localization of a transgene protein in barley

Bladstruktur och lokalisering av transgenprotein i korn





by

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Preface and Acknowledgements

This bachelor project was initiated by Dr Salla Marttila at the Department of Plant Protection Biology at SLU, Alnarp. The project is a continuation of earlier studies in the field.

We would like to thank our supervisor Salla Marttila for her never-ending support and engagement. We would also like to thank research engineer Kerstin Brismar for providing us with plant material. Material previously collected by Kristina Santén and Tina Tandrup Poulsen was used, which is greatly acknowledged. Last, but not least, we thank our examinator Professor Tomas Bryngelsson.

Contents

Abstract

Barley is one of the most important cereals cultivated in the Nordic countries. Climate change brings warmer and moisture climate which favors fungal diseases. In the cropland barley can be seriously infested with hard fungus attack. Since it is important that the yield bears a high quality it is of great importance to find varieties more resistant to attacks.

Pathogenesis-related (PR) proteins are stress proteins induced in the plant in response to infection and abiotic stress (van Loon, 1997). PRs are shown to have antimicrobial activity differing between bacteria, fungi and oomycetes (Tandrup Poulsen, 2001). Among the 17 PR-families (van Loon et al., 1994, 2006) PR-5 is one of the most abundant ones in barley (Tandrup Poulsen, 2001, van Loon et al., 2006).

Studies have shown that PR-5 exists naturally in the ground tissue of leaves, but not in the epidermis (Gregersen et al., 1997). In the epidermal cell walls an epidermis-specific promoter for PR-5 has been placed in front of the gene encoding PR-5, this to achieve a more resistant cultivar of barley. Enhanced resistance has been demonstrated, but it is not confirmed that this resistance is due to expression of PR-5 in epidermis (Santén et al., unpublished, Tandrup Poulsen, 2001). In the modified lines, preliminary studies have shown irregular structure of epidermis (Santén et al., unpublished).

The aim of the study was to establish whether there were structural differences between regular and modified barley, and to localize PR-5 in epidermis. Counterstaining was used to be able to study the leaf structure in microscope, and immunocytochemistry was used to localize PR-5.

Results showed irregular cell structure in epidermis, and in a few cases even in the ground tissue, of modified barley. Due to failure in method, unspecific binding of the antibody visualizer occurred and no confident result could be established regarding the localization of PR-5. Nothing indicates an existence of PR-5 in epidermal cell walls of modified barley. This could be a result of a non-working method, undetectable levels of PR-5 or that the gene encoding PR-5 is expressed as mRNA but not translated to protein.

Occurrence of PR-5 has been demonstrated in epidermal cells of infected material. This detection could be due to the fact that the gene needs an infection to be

expressed as the protein. More studies are necessary to establish whether the showed enhanced resistance is due to expression of PR-5 in epidermis.

Sammanfattning

Korn är en av de viktigaste cerealierna som odlas i Norden. Klimatförändringar medför varmare och fuktigare klimat, vilket gynnar svampsjukdomar. I fält kan korn drabbas hårt av svampangrepp. Eftersom det är av stor betydelse att skörden håller en hög kvalitet är det viktigt att hitta sorter som är mer resistenta mot angrepp (Santén et al., opubliserat).

Patogenrelaterade (PR) proteiner är stressproteiner som induceras i växten i respons till infektion eller annan stress (van Loon, 1997). PR-proteiner har uppvisat antimikrobiell aktivitet som skiljer sig mellan bakterier, svampar och oomyceter (Tandrup Poulsen, 2001). Bland de 17 olika PR-familjerna (van Loon et al., 1994, 2006) är PR-5 en av de vanligast förekommande familjerna i korn (Tandrup Poulsen, 2001, van Loon et al., 2006).

Studier har visat att PR-5 finns naturligt i grundvävnaden i blad, men inte i epidermis (Gregersen et al., 1997). I cellväggen hos epidermis har en epidermis-specifik promotor för PR-5 placerats framför genen som kodar för PR-5, detta för att hitta en mer resistent kornsort. Ökad resistens har påvisats, men det är inte fastställt om den ökade resistensen beror på uttryck av PR-5 i epidermis (Santén et al., opubliserat, Tandrup Poulsen, 2001). I de transgena linjerna har preliminära studier påvisat oregelbunden struktur i epidermis (Santén et al., opubliserat).

Syftet med studien var att fastställa om det fanns någon strukturell skillnad mellan vanligt och modifierat korn, och att lokalisera PR-5 i epidermis. Kontrastering användes för att kunna studera bladstrukturen i mikroskop, och immunolokalisering användes för att lokalisera PR-5.

Resultaten visade på en oregelbunden cellstruktur i epidermis, och i ett fåtal fall även i grundvävnaden, hos modifierat korn. På grund av en felande metod förekom ospecifik inbindning av antikropparnas visualiseringsämne, vilket medförde att inget säkert resultat kunde fastställas gällande lokalisering av PR-5. Ingenting pekar på en förekomst av PR-5 i epidermiscellväggarna hos modifierat korn. Detta kan vara resultatet

av en icke-fungerande metod, ej detekterbar nivå av PR-5 eller att genen som kodar för PR-5 uttrycks som mRNA istället för som protein.

Förekomst av PR-5 har påvisats i epidermis hos infekterat material. Denna förekomst kan bero på att genen kräver en infektion för att uttryckas som proteinet. Ytterligare studier är nödvändiga för att fastställa om den ökade resistensen beror på uttryck av PR-5 i epidermis.

Introduction

Barley

Barley (*Hordeum vulgare L.*) is a member of the *Poaceae* family and is one of the oldest cultivated plants in the world. The cultivation areas range from temperate to subtropical and tropical countries (Wikipedia, 2009). In 2007 the world production of barley was estimated to 136209179 tonnes, of which 1439000 tonnes were produced in Sweden (FAOSTAT, 2009). In Scandinavia barley is cultivated mainly for animal feed and malt production (Wikipedia, 2009).

Several diseases infest barley. Among these are rusts, leaf spots, gall midges, aphids and powdery mildew (SJV a). One of the most crucial diseases of barley is powdery mildew. Without use of fungicides a yield reduction of approximately 10 % is estimated in cooler climates (Tandrup Poulsen, 2001).

Powdery mildew

According to Agrios (2005), different cereals, for example wheat and barley, are most severely affected by powdery mildew. The severity is due to the fact that chemical control of plant diseases is impractical, difficult or not cost effective. The species of fungi that affect barley is called *Blumeria graminis*.

The symptoms of powdery mildew are white/grayish spots or patches with a powdery appearance. The powder covers especially the upper side of leafs of young plant tissue. Other organs affected are the underside of leafs, young shoots, young stems, buds, flowers and young fruits. Powdery mildew almost never kills the plant. Instead the fungus uses the plants nutrients, reduce their photosynthesis, increase their respiration and transpiration, impair the plants growth and reduce yields. The reduction of the yields can be as high as 20 – 40 % (Agrios, 2005).

In barley, the fungus only infests the cells in the epidermis, the outermost cell layer of leaf (Thordal-Christensen et al., 2000). By sending out haustoria, a feeding

organ, the fungus is able to penetrate the epidermal cells of the plant and obtain nutrients (Agrios, 2005).

Powdery mildew is most severe in warm and dry climate but does also affect plants in cool or warm humid areas. As long as the relative humidity in the air is relatively high, no water film on the plant surface is needed for the fungi to be able to release spores, germinate and cause infection (Agrios, 2005).

Due to an increased amount of resistant cultivars of spring barley the need to control powdery mildew has decreased in the south of Sweden. However, during the recent years hard attacks have become more common in the east of Kalmar County than in the rest of the country. In the midst of Sweden the need for control is usually small. The same tendency counts for cultivars of fall barley, which is grown foremost in the south of Sweden (SJV b). Due to current climate changes, a warmer and moisture climate is to expect. These conditions favor fungal growth, which may lead to increased fungal attacks. To be prepared to this situation plant breeding resulting in more resistant cultivars is needed.

Plant defense

Plants have different defense strategies to protect themselves against threats from pathogens and pests (Taiz & Zeiger, 2006). Layers of cutin and suberin function as mechanical holdbacks, preventing pathogens and pests to penetrate the surface (Kolattukudy, 1985). Bryngelsson and Collinge (1992) pointed out that lignified secondary walls seem more efficient in protecting the plant from penetration than non-lignified primary ones do, this due to the properties of lignin, making it resistant both chemically and physiologically.

Secondary metabolites, such as phenolics, nitrogen-containing compounds and terpenes, are decay products without involvement in growth and development in the plant. These substances can, however, defend the plant by their toxical properties (Taiz & Zeiger, 2006).

In addition to the constant defense the plant is able to induce defense as a response to infection. Hypersensitive response (HR) results in programmed cell death in

the infected tissue. Further spread of the pathogen is prevented by nutrient deficiency in the dead cells. When a plant is infested by a pathogen and survives, it triggers an enhanced defense throughout the plant body. This phenomenon is known as systemic acquired resistance (SAR) and in time of subsequent infection the plant body is more resistant (Taiz & Zeiger, 2006, van Loon, 1997).

Many defense-related substances, such as proteins, defend the plant by the formation of enzymes. By attacking the cell walls, the enzymes have a direct effect on the pathogen (Taiz & Zeiger, 2006).

Pathogenesis-related (PR) proteins

The definition of pathogenesis-related (PR) proteins is "*proteins encoded by the host plant but induced specifically in pathological or related situation*" (van Loon, 1999). The PR proteins were first identified 30 years ago (for review, see Tandrup Poulsen, 2001). Being the most studied group of defense response proteins, they are found to exist throughout the Plant Kingdom (van Loon, 1999) and are stress proteins induced in response to infections caused by pathogens such as bacteria, viruses and fungi. Among the studied PRs the ones present in tobacco and tomatoes are the most characterized ones (van Loon, 1997).

PRs are divided into 17 families (Table 1) and separated due to differences in amino acid sequences, serological relationship and enzymatic or biological activity (van Loon et al., 1994, 2006). Each PR family may occur in several different plant families but all plant families do not comprise representatives from every PR family (van Loon, 1997). Each PR family is divided further into different classes, differing in isoforms with high and low pI-values (van Loon et al., 2006).

Salicylic acid, jasmonic acid and ethylene are signaling compounds which induce PRs (van Loon et al., 2006) both near the infection site and systemically throughout the entire plant body (Liljeroth et al., 2005). PRs are shown to have antimicrobial activity differing between bacteria, fungi and oomycetes (Tandrup Poulsen, 2001). Chitinase and glucanase could affect fungi due to their ability to limit pathogen activity, growth and spread. Nematodes and herbivorous insects are suppressed by the chitinase-containing PR

families as well as the proteinase-inhibiting family; PR-6. The chitinase in the PR-8 family as well as the defensins (PR-12), thionins (PR-13) and lipid-transfer proteins (PR-14) have antibacterial properties. A further property of PR-12, -13 and -14 is their antifungal effect. PR-1 and PR-5 are families associated with an inhibiting effect on oomycetes (van Loon et al., 2006).

Trials with transgenic plants over-expressing PR proteins have tended to show an enhanced resistance to diseases (Tandrup Poulsen, 2001). Datta et al. (2001) transformed rice with genes encoding PR-3 and PR-5. Both trials resulted in enhanced resistance against fungal attack. The PRs are believed to defend the plant by activating hydrolysis of fungal cellular components or by inhibiting fungal growth by its toxic effect (Muthukrishnan et al., 2001).

Table 1 displays the properties of the different PR families found so far. All except PR-7 and PR-9-12 have been found and studied in barley as reviewed by Santén (2007).

Table 1. Recognized families of pathogenesis-related proteins (modified from van Loon et al., 2006).

PR family	Properties	Example
1	Unknown	Tobacco PR-1a
2	β-1,3-glucanase	Tobacco PR-2
3	Chitinase class I, II, IV-VII	Tobacco P, Q
4	Chitinase class I, II	Tobacco R
5	Thaumatin-like	Tobacco S
6	Proteinase-inhibitor	Tomato inhibitor I
7	Endoproteinase	Tomato P_{69}
8	Chitinase class III	Cucumber chitinase
9	Peroxidase	Tobacco lignin-forming peroxidase
10	Ribonuclease-like	Parsley "PR-1"
11	Chitinase, type I	Tobacco "class V" chitinase
12	Defensin	Radish Rs-AFP3
13	Thionin	Arabidopsis THI2.1
14	Lipid-transfer protein	Barley LTP4
15	Oxalate oxidase	Barley OxOa (germin)
16	Oxalate-oxidase-like	Barley OxOLP
17	Unknown	Tobacco PRp27

PR-5

One of the most important PR-families in barley is PR-5. Due to similarities in sequence to the protein thaumatin another name for PR-5 is thaumatin-like protein (TLP) (Tandrup Poulsen, 2001, van Loon et al., 2006). *In vitro,* several PR-5 proteins have shown antifungal activity by inhibiting hyphal growth, spore germination or development of germ tubes (Abad et al., 1996; Vigers et al., 1992). Tandrup Poulsen (2001) ascertains in her Ph.D-thesis; "*The antifungal activity of PR-5s depends on the combination of isoforms and fungal species or even the strains of a species, suggesting a target specificity between PR-5 proteins and the fungi*". The mode of action seems to be the same within the family but the regions of recognition seem to differ between the individual proteins. The balance between the amount of PR-5 binding to the fungal cell wall and the amount of barriers blocking the plasma membrane access determines the result of the interaction. (Tandrup Poulsen, 2001)

Osmotic stress protection (Kononowicz et al., 1992) and freezing tolerance are other functions of PR-5 (Hon et al., 1995).

Kuboyama (1998) found PR-5 to be expressed constitutively in the stigma and style of tobacco. Wang et al. (2006) observed that the spatial expression of *pr-5* genes in potato leaves accumulated strongest and earliest in the tissue closest to the local infection site. The same tendency counts for barley; regardless of which plant part is infected trials showed that PR-5 accumulated earliest and strongest closest to the infection site (Santén, 2007).

Aim

To enhance resistance against powdery mildew in barley trials have been done where barley was transformed by its own defense protein; PR-5 (Tandrup Poulsen, 2001). Previously, it has been shown that the protein exists in the inner leaf tissue, but not in the epidermis (Gregersen et al., 1997). By placing an epidermis-specific promoter for PR-5 in epidermis the hypothesis is that it would be expressed even in the epidermis. Enhanced

resistance has been demonstrated, but it is not confirmed that this resistance is due to expression of PR-5 in epidermis (Tandrup Poulsen, 2001).

Structural differences between common and modified barley have been observed in epidermis in a preliminary study (Santén et al, unpublished). If there are differences maybe they affect the plant development and growth in general. The aim of this bachelor thesis was to establish whether there are structural differences in leaves of regular and modified barley, by microscopy studies of leaf tissues. Furthermore, the aim was also to determine if the enhanced resistance that has been observed could be due to expression of PR-5 in epidermal cells of modified barley.

Material and Method

Plant material

Barley (*Hordeum vulgare, L.*) was cultivated in a green house during the summer of 2002 at KVL, Copenhagen. Cv. Golden Promise (GP) was a negative, untransformed control. Line 8.3K, another control, was a transformed line without transgene. Lines 5.1, 5.2 and 8.3 were transformed lines with a transgene encoding PR-5.

A part of the material was ready to use, already being fixated and embedded in London Resin White (LRW). The rest of the material was fixated and stored in a buffer.

London Resin White (LRW) embedding

London Resin White (LRW) is an embedding media based on methacrylate used for immunochemistry. Due to its low viscosity LRW efficiently penetrates tissues. The medium is non-toxic, but work should take place in the hood. Because of its irritating features gloves are recommended.

Leaf material had been fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in phosphate buffer pH 7.2 for 2 h at RT year 2002. Vacuum treatment had been done in the beginning. Part of material had been embedded in LRW directly, and part of material was kept in buffer at 4°C, and embedded the spring 2009.

Fixed leaf samples were pre-embedded with agarose (Marttila and Santén, 2007). The embedding ensures that the samples are localized in the middle of the block tip. This procedure is not necessary but useful when the samples are flat, as with leaves.

The agarose was dispersed to a mold; a leaf sample was added and covered by additional agarose. Redundant agarose was excised before dehydration with ethanol. After dehydration the samples were infiltrated with LRW.

The samples were embedded in a mold, covered by LRW, and allowed to polymerize 24 h at 58^{o}C. The samples were cut into sections of 1 µm and placed on coated object slides (Superfrost Plus).

Counterstaining

Counterstaining of leaf tissue enable microscopy studies by visualizing cell structures and giving sufficient contrast for microscopy.

Prepared sections of leaf tissue were counterstained with 0.1 % Toluidine Blue O in 1 % borax (TB). A drop of TB was filtered directly on sections and kept on a hot plate for 30 seconds. The cuttings were rinsed with water from redundant TB and then allowed to dry before mounting.

Immunocytolocalization

"…the definition of immunocytochemistry is the use of labeled antibodies as specific reagents for localization of tissue constituents (antigens) in situ." (Polak & Van Noorden, 1997). To identify the localization of an antigen, immunocytochemistry is the only method possible (Polak & Van Noorden, 1997).

The immunocytolocalization was started by blocking to reduce the risk that the antibodies bind unspecifically. 5 % goat normal serum and 1 % BSA in PBS buffer made sure the unspecific binding sites were blocked. The blocking was followed by incubation at 4^{o}C with primary antibody raised against PR-5 protein (Bryngelsson et al., 1994). The unbound antibodies were washed off with buffer before incubation at 37^{o}C with secondary antibody. The secondary antibody consisted of goat anti-rabbit IgG-gold conjugate (NanoProbe), and was added due to the fact that the primary antibody (and also the PR-5) needs a secondary antibody to be visible. Once again, the unbound antibodies were washed off, and silver enhancer was added to visualize the proteins. To visualize the leaf structures (and consequently the location of the proteins) the cuttings were counterstained with Safranine O as in Santén et al. (2005).

Results

Anatomy of leaves

Figure 1 shows anatomy of barley leaf and the naming of tissues used in the thesis. Ground tissue constitutes the majority of the leaf tissue and is the site of photosynthesis. Vascular tissue is embedded in the ground tissue and contains the phloem and xylem, which transport water and nutrients through the plant body. Epidermis is the outermost layer of leaves and constitutes the plants mechanical protection (Raven et al., 2005).

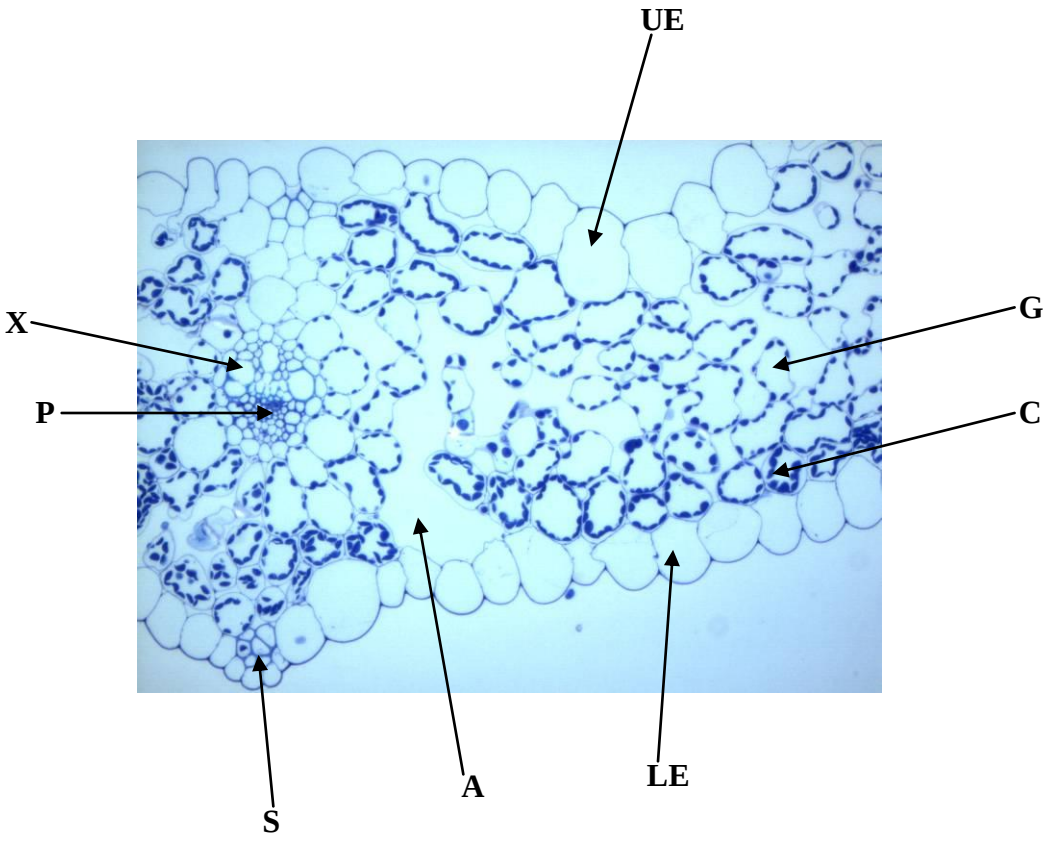

Figure 1. Leaf anatomy of barley. A = Air space, C = Chloroplast, G = Ground tissue, LE = Lower epidermis P = Phloem, S = Sclerenchyma, UE = Upper epidermis, X = Xylem.

Three blocks of each line except for 8.3K were studied. Only two blocks of the line 8.3K could be studied, due to damaged and unusable material in block three. During microscopy studies both upper and lower epidermal cells were especially observed. Both sides of the leaf showed an explicit structural difference in epidermal cells between regular and modified barley.

A majority of the epidermal cells of regular barley (Fig. 2) displayed a round, even structure with turgor. As visible in figure 2, the upper epidermis of GP showed occasional irregularity. No divergent structure was demonstrated in the line 8-3K (Fig. 2).

The cells of the ground tissue appeared in the same manner, and air spaces could be observed. The vascular tissue appeared normal, containing both phloem and xylem.

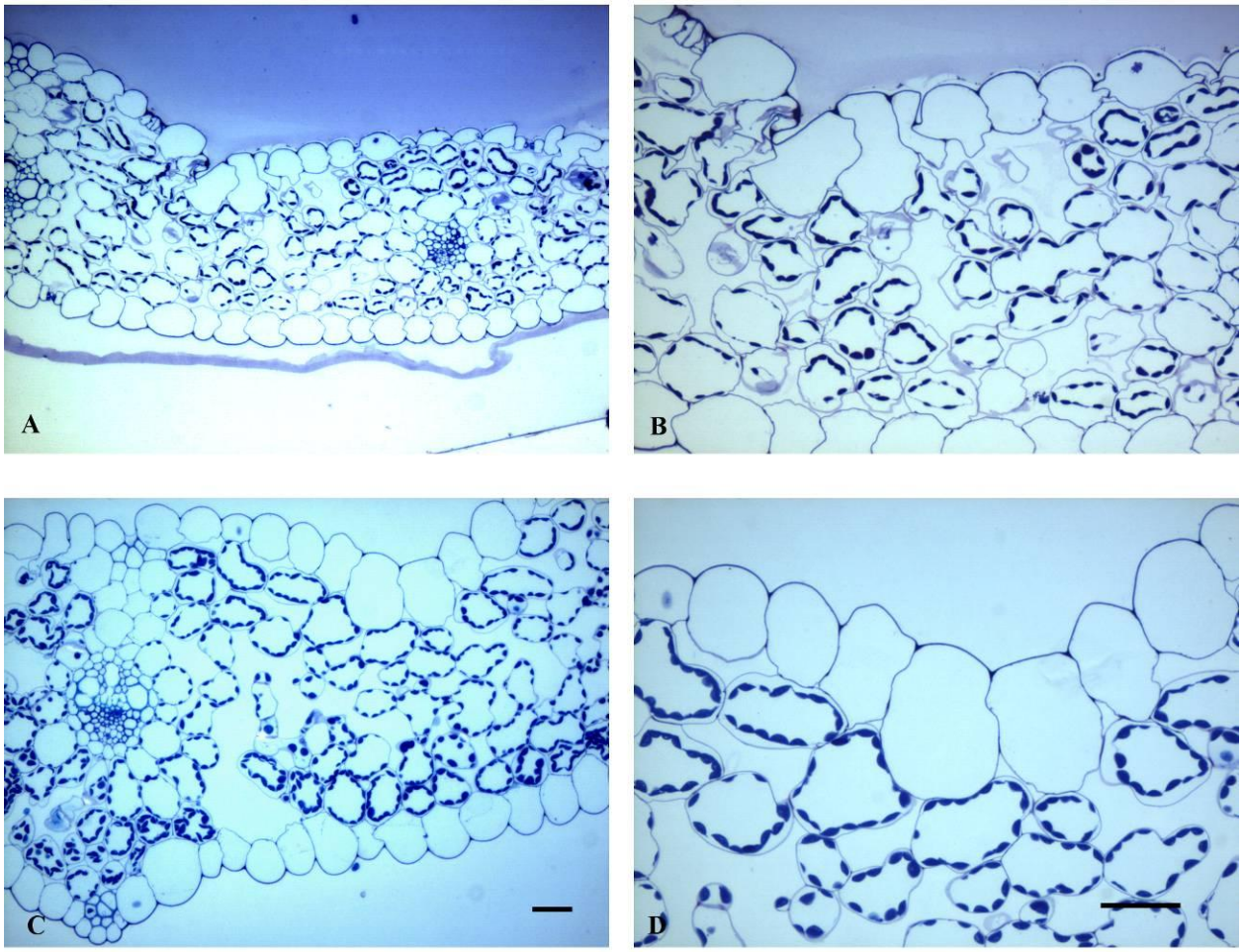

Figure 2. The structure of regular barley leaves photographed with 10x (left column) and 20x objective (right column). Bars 50 µm. A = Golden Promise (GP), B = Golden Promise (GP), C = transformed line, without transgene (8.3K), D = transformed line, without transgene (8.3K).

The epidermal cells of modified barley (Fig. 3) showed a divergent, uneven structure. Instead of being expanded and circular they were suppressed and irregular. The total of the leaf structure demonstrated a suppressed impression showing only few and small air spaces in the ground tissue. The suppressed impression counted especially for the lines 5.2 and 8.3. The line 5.1 displayed a ground tissue more similar to the ground tissue of the lines GP and 8.3K. However, the epidermis of the line 5.1 diverged from normal epidermal structure, displayed a divergent and uneven structure. Both sides of epidermis in the lines 5.2 and 8.3 demonstrated the same tendency in the epidermal structure as in the case of line 5.1. However, the upper epidermis of the line 5.2 demonstrated a structure extremely different from the normal one.

In comparison, no divergences were observed in the vascular tissues or stomata between modified and regular barley.

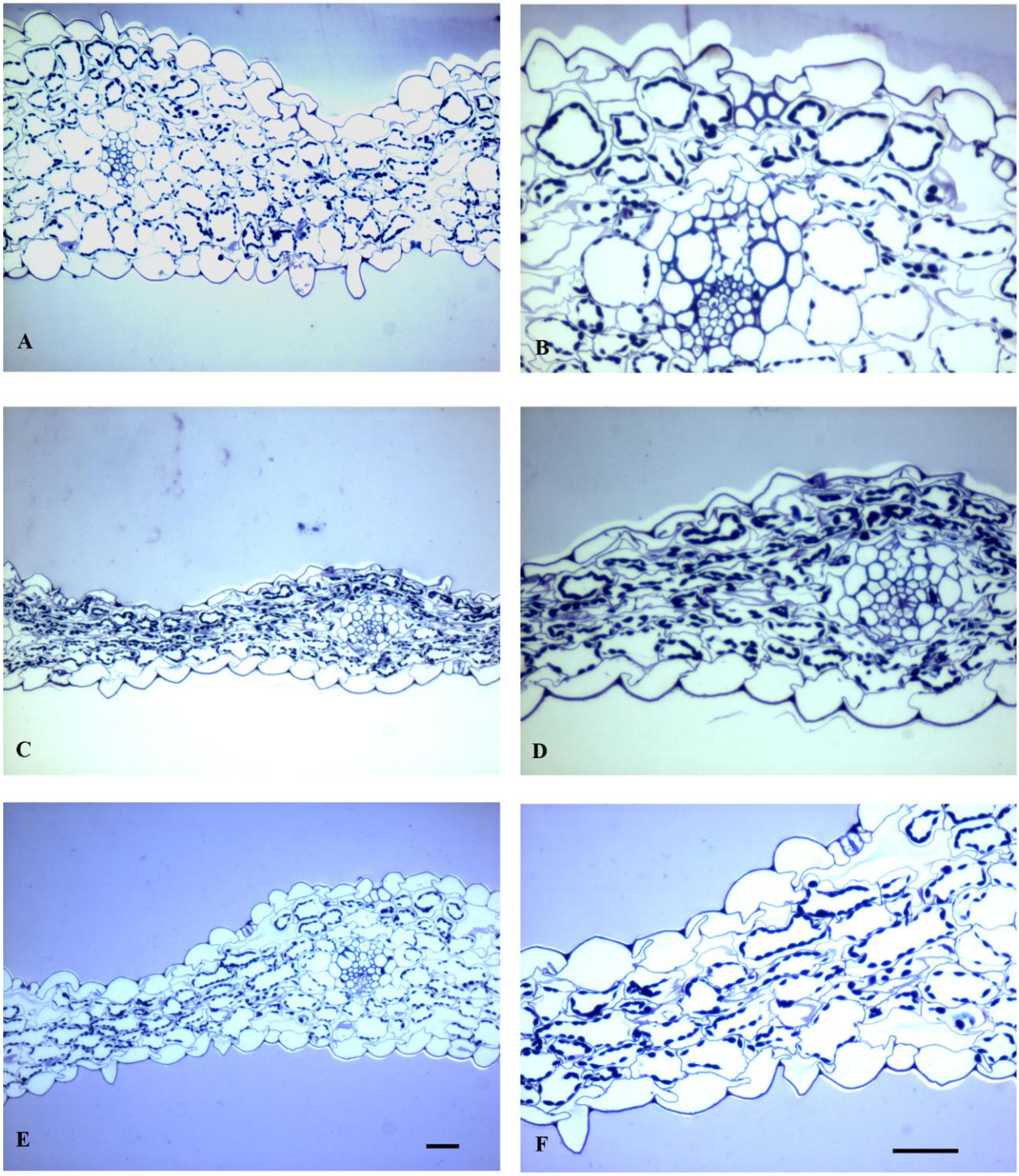

Figure 3. The structure of modified barley leaves photographed with 10x (left column) and 20x (right column) objective. Bars 50 μm. A = transformed line (5.1) with a transgene encoding PR-5, B = transformed line (5.1) with a transgene encoding PR-5, C = transformed line (5.2) with a transgene encoding PR-5, D = transformed line (5.2) with a transgene encoding PR-5, E = transformed line (8.3) with a transgene encoding PR-5, F = transformed line (8.3) with a transgene encoding PR-5.

Localization of PR-5 in leaves

Several trials were performed to find an optimal primary antibody concentration and time of silver enhancement. All trials performed gave rise to unspecific binding of the silver, resulting in a silver staining evenly spread on the sections and the surrounding LRW.

Immunolocalization of PR-5 showed little or no occurrence of PR-5 in epidermal cell walls of regular and modified barley. In regular barley no visible difference was demonstrated in comparison between control and antibody-treated sections (Fig. 4, GP and 8.3K). In a few occasions a comparison between the modified line 5.1 and the regular GP and 8.3K showed an increased amount of silver staining in the cell wall of the epidermal cells (Fig. 4). The sample of the modified line 5.1 in figure 4 represent the most significant accumulation of silver. The remaining modified samples (5.1, 5.2 and 8.3) showed less or no silver accumulation in epidermal cells.

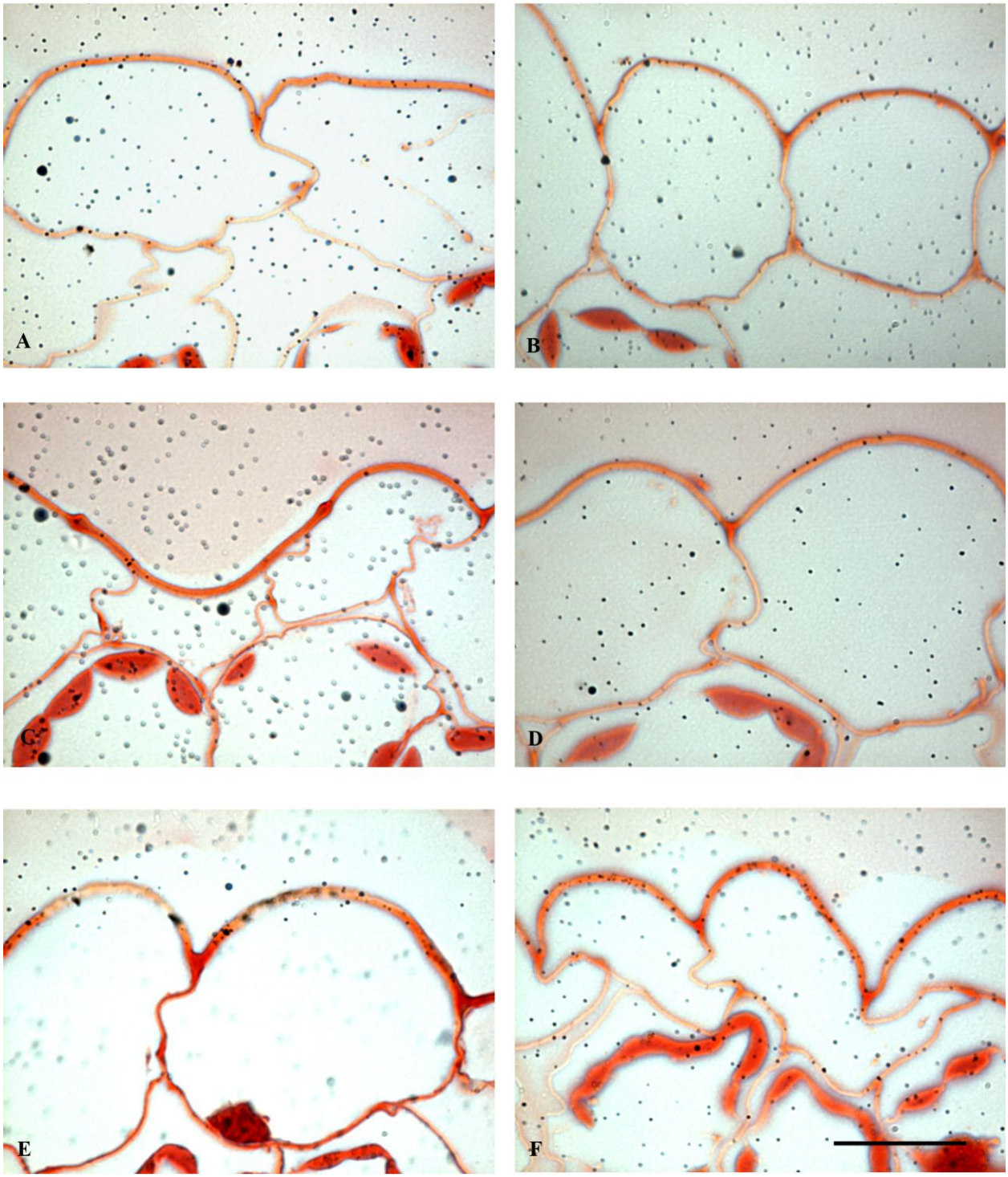

Figure 4. Immunolocalization of PR-5 in epidermis photographed with 100x objective. The pictures in the left column show tissue treated with an antibody and the pictures in the right column show the corresponding control without any antibody. Bars 20 µm. A = Golden Promise (GP), B = Golden Promise (GP, control), C = transformed line without transgene (8.3K), D = transformed line without transgene (8.3K, control), E = transformed line (5.1) with a transgene encoding PR-5, F = transformed line (5.1, control) with a transgene encoding PR-5.

Discussion

Structural changes in leaves

The tendency to irregularity observed in GP in a few cases represents a natural variation leaving no influence on the result.

Due to the fact that the modified line 8.3K demonstrated a normal leaf structure, the irregular structure of the other lines of modified barley is likely to depend on the transgene and not the transformation process. Differences in all three transgenic lines were observed. Not only were the epidermal cells abnormal, even cells in the ground tissue displayed partially an irregular structure. This could have been due to a lack of a supporting function of the abnormal epidermal cells leading to a collapse of the leaf structure. The collapse resulted in a suppressed tissue without the expected air spaces. The differences that were observed could be due to the tissue treatment, such as vacuum pumping during fixation. However, as the samples have been treated the same way and the structure of the controls is normal, this is not likely to be the reason to the irregular structure of the transgene lines.

Despite the irregular structure of modified barley an enhanced resistance against powdery mildew has been observed (Tandrup Poulsen, 2001). Nonetheless, the abnormal structure gives rise to the question; how resistant the crop would be against other pathogens out in the field? Could it perform photosynthesis at a normal rate?

Structural differences between regular and modified crops have, to our knowledge, not yet been studied by others.

Attempt to localize PR-5 in leaves

The regular lines showed little or no silver accumulation in the epidermal cells. The accumulation that was observed in some of the samples indicates failure in the method because no accumulation is expected in the epidermal cells of regular barley.

Little or no accumulation of silver was visible in epidermal cells of the modified lines of barley. The reason to the lack of PR-5 in epidermal cells could be due to an

undetectable level of PR-5. Another possible reason might be that the gene encoding PR-5 is expressed as mRNA but not translated to protein and therefore not visible until after infection.

The increased amount of silver enhancement visible in a few cases might just depend on an accidental accumulation of silver. This leaves us with an untrustworthy result and it is therefore impossible to draw a conclusion that PR-5 is expressed in epidermal cells of modified barley.

Considering the method; the silver staining gave rise to problems. Instead of just binding to PR-5 detected by the secondary antibody conjugate, much silver bound unspecifically and resulted in an even spread of silver on both the leaf sections and the surrounding LRW. To resolve the problem the method was repeated to make sure nothing in the procedure was inaccurately performed. In addition to repeated trials; shorter silver exposure time was tried out and Tween, a detergent, was added to the washing buffer, this to prevent too much unspecific binding of the silver. A possible reason to the unspecific binding of silver might be a failure in the blocking procedure. Due to the fact that the Goat normal serum and the buffer are supposed to block the unspecific binding sites something with the solution or the procedure might have been incorrect. Another possible reason for the unspecific binding could be that the oven thermostat was unreliable. Temperatures above recommended level might lead to denaturation of the secondary antibody, resulting in unspecific binding of the silver enhancer.

PR-5 has been located in epidermal cells in leaf tissue of barley infected with the fungus *Bipolaris sorokiniana* (Santén, 2007). If the gene is expressed as mRNA instead of PR-5 protein an infection might be a requirement for PR-5 to become visible. If sufficient time had been dedicated to the project infected material would have been an interesting benchmark for further studies. Earlier studies have shown varying results; analysis using western blot method could not display any difference in the level of PR-5 in inoculated epidermal cells between regular and modified barley. On the other hand trials using PCR analysis have shown the opposite result; occurrence of PR-5 in epidermal cells of modified barley (Tandrup-Poulsen, 2001). Existing results are ambiguous; some of them pointing at an existence of PR-5 in epidermis, and some of

them do not. This makes it impossible to confirm that the shown enhanced resistance is due to PR-5 expression in epidermis – further studies are necessary.

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Appendix 1

Solutions

Agarose 3 %

Ethanol 30, 50, 70, 90, 95, 100 %

London Resin White (LRW) 30, 60, 100 % in ethanol

0.1 % Toluidine Blue O in 1 % Borax

Sterilized water

Biomount

Xylene

5 % goat normal serum and 1 % Bovine Serum Albumine (BSA) in Phosphate-Buffered Saline (PBS)

PBS and 1 % Tween

Goat anti-rabbit IgG-gold conjugate

Silver enhancer

0.5 % Safranine O