An update on polygalacturonase-inhibiting protein (PGIP), a leucine-rich repeat protein that protects crop plants against pathogens

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Polygalacturonase inhibiting proteins (PGIPs) are cell wall proteins that inhibit the pectin-depolymerizing activity of polygalacturonases secreted by microbial pathogens and insects. These ubiquitous inhibitors have a leucine-rich repeat structure that is strongly conserved in monocot and dicot plants. Previous reviews have summarized the importance of PGIP in plant defense and the structural basis of PG-PGIP interaction; here we update the current knowledge about PGIPs with the recent findings on the composition and evolution of pgip gene families, with a special emphasis on legume and cereal crops. We also update the information about the inhibition properties of single pgip gene products against microbial PGs and the results, including field tests, showing the capacity of PGIP to protect crop plants against fungal, oomycetes and bacterial pathogens.

Keywords: polygalacturonase inhibiting proteins (PGIPs), gene family, transgenic plants, plant protection, fungal pathogens, bacterial pathogens

Introduction

Successful colonization of plant tissues by microbial pathogens requires the overcoming of the cell wall. To this end, pathogens produce a wide array of plant cell wall degrading enzymes (CWDEs), among which endo-polygalacturonases (PGs; EC 3.2.1.15) are secreted at very early stages of the infection process (ten Have et al., 1998). PGs cleave the α-(1–4) linkages between the D-galacturonic acid residues of homogalacturonan, the main component of pectin, causing cell separation and maceration of the host tissue. To counteract the activity of PGs, plants deploy the cell wall polygalacturonase inhibiting proteins (PGIPs) that inhibit the pectin-depolymerizing activity of PGs. No plant species or mutants totally lacking PGIP activity have been characterized so far. The structure of PGIPs is typically formed by 10 imperfect leucine-rich repeats (LRRs) of 24 residues each, which are organized to form two β-sheets, one of which (sheet B1) occupies the concave inner side of the molecule and contains residues crucial for the interaction with PGs (Di Matteo et al., 2003). In addition to PG inhibition, the interaction between PGs and PGIPs promotes the formation of oligogalacturonides (OGs), which are elicitors of a variety of defense responses (Cervone et al., 1989; Ridley et al., 2001; Ferrari et al., 2013). Since many aspects of the PGIP biology have
been already summarized in previous reviews (De Lorenzo et al., 2001; De Lorenzo and Ferrari, 2002; D’Ovidio et al., 2004a; Gomathi and Gnanamanickam, 2004; Shanmugam, 2005; Di Matteo et al., 2006; Federici et al., 2006; Cantu et al., 2008; Misas-Villamil and van der Hoorn, 2008; Protsenko et al., 2008; Reignault et al., 2008; Lagart et al., 2009), here we present an overview of the recent findings on genome composition and evolution of pgip gene families and on the efficacy of PGIP to limit the development of diseases caused by microbial pathogens in crop plants.

**PGIP Genes and their Genomic Organization**

Early characterization of a polygalacturonase-inhibiting activity was reported in 1970s (Albersheim and Anderson, 1971) and the first pgip gene was isolated 20 years later in French bean (Toubart et al., 1992). Since then, several PGIPs and a large number of pgip genes have been characterized. Up to now more than 170 complete or partial pgip genes from dicot and monocot plants have been deposited in nucleotide databases (e.g., http://www.ncbi.nlm.nih.gov/). Most of these genes have been identified as pgip genes on the basis of sequence identity but only a few of them have been shown to encode proteins with PG-inhibitory activity.

Genome analysis has shown that pgip genes did not undergo a large expansion and may exist as single genes, as in diploid wheat species (Di Giovanni et al., 2008), or organized into gene families, the members of which are organized in tandem and can vary from two, as in Arabidopsis thaliana (Ferrari et al., 2003), to sixteen, as in Brassica napus (Hegedus et al., 2008). The majority of pgip genes are intronless, however, some of them can contain a short intron as in Atpgp1 and Atpgp2 (Ferrari et al., 2003). Moreover, pgip genes can be inactivated by transposon elements as in cultivated and wild wheat where the occurrence of Copia-retrotransposon and Vacuna transposons has been reported (Di Giovanni et al., 2008). Characterized pgip loci are shown in Figure 1. Like other families of defense-related genes, pgip genes show variation in the expression pattern of the different members, some of which are constitutive, others are tissue-specific and, in most cases, up-regulated following stress stimuli (see reviews indicated above; Table 1). At the protein level, members of a pgip family show both functional redundancy and sub-functionalization (De Lorenzo et al., 2001; Federici et al., 2006). As suggested previously, these features likely have an adaptive significance for combating more efficiently a broad array of pathogens (Ferrari et al., 2003) or responding more rapidly to diverse environmental stimuli (D’Ovidio et al., 2004b). In support of this view, a recent analysis of the genomic organization and composition of the legume pgip families suggested that the forces driving the evolution of the pgip genes follow the birth-and-death model (Kalunke et al., 2014), similarly to what proposed for the evolution of NBS-LRR-type R genes (Michelmore and Meyers, 1998). This possibility is based on genomic features that include inferred recent duplications, diversification as well as pseudogeneization of pgip copies, as found in soybean, bean, barrel clover and chickpea (Kalunke et al., 2014). The organization of the pgip families therefore supports the view that tandem duplications are frequent in stress-related genes and are beneficial for survival in challenging environments (Oh et al., 2012).

### Inhibition Activity of PGIPs

A number of papers deals with the inhibition activity of PGIPs purified from several plant tissues. This aspect has been reviewed several years ago (De Lorenzo et al., 2001); here, we present an update of this information (Table 2). Because purified PGIPs may contain a mix of highly similar PGIP isoforms, the activity detected in a tissue may result from the contribution of the activities of different PGIPs expressed in that tissue. An appropriate approach to study the inhibition activity of individual PGIP isoforms is their expression in a heterologous system. However, only a few of the more than 170 pgip genes isolated so far from different plant species have been investigated. As reported in Table 3, individual heterologous expression and analysis of all members of a pgip family has been performed only for Arabidopsis (Ferrari et al., 2003), common bean (D’Ovidio et al., 2004b), soybean (D’Ovidio et al., 2006; Kalunke et al., 2014) and wheat (Janni et al., 2013). PGIPs have been expressed in prokaryotic systems, as a fusion with the maltose-binding protein (MBP) (Jang et al., 2003; Table 3) or using lower temperature for bacterial growth (Chen et al., 2011), in Pichia pastoris and in plants by stable transformation or, transiently, by virus-mediated expression (Table 3). In some cases, the proteins were successfully expressed, but did not show any inhibitory activity in vitro, as, for example, in the case of some GmPGIPs (D’Ovidio et al., 2006). GmPGIP3, but not GmPGIP1, GmPGIP2, and GmPGIP7 showed inhibitory activity, whereas no expression of GmPGIP5 was obtained (D’Ovidio et al., 2006; Kalunke et al., 2014). Similarly, TaPGIP1 and TaPGIP2, encoded by the two members of the wheat pgip family, were successfully expressed but showed no inhibition activity (Janni et al., 2013).

The absence of inhibition activity in vitro may also reflect the possibility that some PGIPs are active only in the in planta environment, as suggested by Joubert et al. (2006) in the case of the Botrytis cinerea BcPG2 and VvPGIP1 from grapevine (Vitis vinifera L.). These proteins do not interact in vitro, although VvPGIP1 reduces symptoms caused by BcPG2 upon co-infiltration in leaves. The number and sources of PGs tested is also limited; only a few studies have been carried out against PGs of bacteria and insects (Doostdar et al., 1997; D’Ovidio et al., 2004b; Frati et al., 2006; Hwang et al., 2010; Schacht et al., 2011; Kirsch et al., 2012). The limitations of data prevents to draw conclusions about correlations between PGIPs of specific plant families and specific pathogens. Notably, PG produced by a highly detrimental pathogen, Fusarium verticillioides, is not inhibited by any known PGIP (see Table 2). This PG has been a target of an unsuccessful attempt to render PvPGIP2 an efficient inhibitor against this PG (see below, Benedetti et al., 2011a).

The utilization of pgip genes for crop protection relies on the identification of inhibitors with broad specificities against the many PGs produced by phytopathogens and/or the construction of novel PGIPs with stronger and broader inhibitor activity. Many more PGIPs than those reported in Tables 2, 3 exist in...
FIGURE 1 | Schematic representation of the genomic organization of pgip families in rice, wheat, bean, soybean, chickpea, barrel clover, and thale cress. Each block-arrow with compound-type lines represents a predicted pgip gene and a block-arrow with dash type lines represents a predicted pseudo-gene or remnant gene. Vertical line within block-arrow indicates introns (Capgip2, Atpgip1, and Atpgip2) or a Copia retrotransposon (Tapgip3). The direction of the arrow indicates ATG to stop codon. The location of pgip genes of legume species are based on Kalunke et al. (2014), those of rice and wheat on Janni et al. (2006) and Di Giovanni et al. (2008), and those of thale cress on Ferrari et al. (2003). Chr, chromosome.
nature and are likely to have different specificities against microbial PGs, considering that single amino acid changes are able to change specificity of the inhibitors (Leckie et al., 1999). Searching for PGIPs with novel specificities may allow to count on a much larger reservoir of possible genes for crop protection. A direct and simple strategy to isolate PGIPs with recognition capability against a given PG may be based on affinity chromatography methods, similar to that originally used to purify PGIP from P. vulgaris (Cervone et al., 1987), and mass spectrometry. Attempts to drive in vitro evolution of PGIPs to generate proteins with improved inhibition properties have not been successful yet (Benedetti et al., 2011a).

The occurrence of PG-inhibiting activity in crude leaf protein extracts of tetraploid wild wheat (T. dicoccoides) possessing non functional pgip genes (Di Giovanni et al., 2008) suggested the existence of pgip genes with a sequence divergent from the classical one. This possibility, which deserves further investigation, is also supported by the finding that the wheat tissue contains PG-inhibiting proteins with N-terminal sequences (Lin and Li, 2002; Kemp et al., 2003) different from TaPGIP1 and TaPGIP2 (Janni et al., 2013) and from the pgip sequences reported so far (http://www.ncbi.nlm.nih.gov/nucleotide/). Recently, a wheat gene with some sequence similarity to pgip genes has been reported and was shown to be involved in the defense response against Fusarium graminearum (Hou et al., 2014).

**Structural Studies on the PG-PGIP Interaction**

Thus, the possibility of new forms of PGIPs depends on the detailed structural knowledge of the PG-PGIP interaction. Several structural studies have been performed (Mattei et al., 2001; King et al., 2002; Benedetti et al., 2011b, 2013; Gutierrez-Sanchez et al., 2012), but a high resolution 3D-structure of the PG-PGIP complex is still missing. The enzyme-inhibitor combinations that have been more extensively investigated, are those that PGIP2 from Phascolos vulgaris (PvPGIP2) forms with PG from A. niger (AnPGII), F. phyllophorum (FpPG) and C. lupini (ClPG). Site-directed mutagenesis has shown that the residues involved in the interaction are located in the concave surface of the inhibitor (Leckie et al., 1999; Federici et al., 2001; Spinelli et al., 2009; Benedetti et al., 2011b, 2013). Computational methods such as the Codon Substitution Model in combination with the Desolvation Energy Calculation and the Repeat Conservation Mapping (RCM; Helft et al., 2011) have pinpointed several residues of PvPGIP2 responsible for the PG-inhibiting activity (Casasoli et al., 2009).

On the other hand, residues of PG that are critical for the interaction with PGIP have been also studied. FvPG is 92.5% identical to FpPG, but is inhibited by neither PvPGIP2 nor other known PGIPs. By both loss- and gain-of-function site-directed mutations, a single amino acid at position 274 of both FvPG and FpPG was demonstrated to act as a switch for recognition by PvPGIP2 (Raiola et al., 2008; Benedetti et al., 2013). Unfortunately, the lack of high-resolution structural information on the PG-PGIP complex does not allow to precisely identify the contacting residue in PGIP. Moreover, both PGs and PGIPs are glycosylated proteins (Caprari et al., 1993; Lim et al., 2009); however, whether glycosylation plays a role in the PGIP-PG interaction requires further investigation. For example, glycosylation in pearl millet PGIP was found to affect pH and temperature stability of the protein but not its capability of inhibiting AnPGII (Prabhu et al., 2015).

A single PGIP may display different mechanisms of PG inhibition (competitive, non competitive and mixed) suggesting that the protein is highly versatile in recognizing different epitopes of various PGs (Federici et al., 2001; King et al., 2002; Sicilia et al., 2005; Bonivento et al., 2008). Consequently, many 3D-models based on docking predictions have been proposed so far (Sicilia et al., 2005; Mauilik et al., 2009; Prabhu et al., 2014). Techniques such as the mass amide exchange mass spectrometry in the case of AnPGII and FpPG and the Small Angle X-ray Scattering (SAXS) in the case of FpPG and CIPG have produced models that, in some cases, are discordant. For example, while the mass amide exchange mass spectrometry predicts that the area of FpPG in contact with PvPGIP2 is located at the N-terminus and predominantly on the underside of the enzyme beta-barrel structures (Gutierrez-Sanchez et al., 2012), the SAXS analysis indicates

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**TABLE 1 | Treatments or stress stimuli affecting pgip expression in some plant species with a well characterized pgip family.**

| Pgip family | Treatments or stress stimuli | References |
|-------------|-------------------------------|------------|
| Rice        | Abscisic acid (ABA), brassinosteroid, gibberellic acid (GA), 3-indole acetic acid (IAA), jasmonic acid (JA), kinetin, naphthalene acetic acid (NAA), salicylic acid (SA); Rhizoctonia solani (necrotrophic fungus) | Janni et al., 2006; Lu et al., 2012 |
| Wheat       | Bipolaris sorokiniana (necrotrophic fungus) and mechanical wounding | Janni et al., 2013 |
| Bean        | Oligogalacturonoidis (OGs); mechanical wounding; Botrytis cinerea, Sclerotinia sclerotiorum (necrotrophic fungus); Colletotrichum lindemuthianum (hemibiotrophic fungus) | Bergmann et al., 1994; Nuss et al., 1996; Devoto et al., 1997; D’Ovidio et al., 2004b; Oliveira et al., 2010; Kalunke et al., 2011 |
| Soybean     | Mechanical wounding; S. sclerotiorum (necrotrophic fungus) | D’Ovidio et al., 2006; Kalunke et al., 2014 |
| M. truncatula | JA, SA, ABA; Colletotrichum trifolii (hemibiotrophic fungus) | Song and Nam, 2005 |
| Rapeseed    | JA, SA, mechanical wounding. S. sclerotiorum | Hegedus et al., 2008 |
| Pepper      | SA, Methyl jasmonate (Me-JA), ABA, wounding, cold treatment | Wang et al., 2013 |
| Arabidopsis | OGs; JA; B. cinerea; Stemphyllum solani (necrotrophic fungus); aluminum, low-pH, cold; geminivirus | Ferrari et al., 2003; Ascencio-Ibanez et al., 2008; Sawaki et al., 2009; Di et al., 2012; Kobayashi et al., 2014 |

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| Plant | Tissue | PGIP preparation | Polygalacturonases | References |
|-------|--------|-----------------|-------------------|------------|
| Tomato (Solanum lycopersici L.) | Stem | Crude extract | Ralstonia solanacearum | Schacht et al., 2011 |
| Tobacco (Nicotiana tabacum L.) | Nectar | | Botrytis cinerea | Thornburg et al., 2003 |
| Potato (Solanum tuberosum L.) | | Gel chromatography | Aspergillus niger Fusarium moniliforme\(\) Fusarium solani isolate 1402 | Machinandiarena et al., 2001 |
| Common Bean (Phaseolus vulgaris L.) | Leaves | PG-Sepharose chromatography | Fusarium anthophilum Fusarium circinatum Fusarium subglutinans Fusarium proliferatum isolate 1162 Fusarium proliferatum PVS-Fu 64 Fusarium sacchari Fusarium fujikuroi F. thapsinum Fusarium moniliforme\(\) FC-10 Fusarium moniliforme\(\) PD | Raiola et al., 2008 |
| Leek (Allium ampeloprasum L.) | Basal leaves | Mono-S chromatography | Fusarium anthophilum Fusarium circinatum Fusarium subglutinans Fusarium proliferatum Fusarium sacchari Fusarium fujikuroi Fusarium verticilloides Fusarium proliferatum ISPAVEmc 1189 Fusarium nygamai | Raiola et al., 2008 |
| Asparagus (Asparagus officinalis L.) | White spear | Mono-S chromatography | Fusarium anthophilum Fusarium circinatum Fusarium subglutinans Fusarium proliferatum Fusarium sacchari Fusarium fujikuroi Fusarium verticilloides Fusarium proliferatum ISPAVEmc 1189 Fusarium nygamai | Raiola et al., 2008 |
| Pepper (Capsicum annuum L.) | Fruit | Ion-exchange chromatography | Colletotrichum gloeosporioides, Colletotrichum capsici, Colletotrichum lindemuthianum Sclerotium rolfsi Fusarium moniliforme\(\) | Shivashankar et al., 2010 |
| Plant                  | Tissue                  | PGIP preparation          | Polygalacturonases                          | References          |
|-----------------------|-------------------------|----------------------------|---------------------------------------------|---------------------|
| Guava (Psidium guajava L.) | Fruit                  | Purified using a Sephadex G-100 | Aspergillus niger                           | Deo and Shastri, 2003 |
| “Oroblanco” grapefruit hybrid (Citrus grandis × C. paradisi Macf.) | Fruit                  | Anion exchange chromatography | Botrytis cinerea                             | D’halluin et al., 2004 |
| Apple (Malus domestica L.) | Fruit                  | Partial purified          | Colletotrichum acutatum Glomerella cingulata | Gregori et al., 2008 |
|                        | Fruit skin              | Partial purified          | Penicillium italicum                        | Lee et al., 2006    |
|                        | Parenchymal tissues     | Partial purified          | Botryosphaeria dothidea                     | Buza et al., 2004   |
| Cantaloupe (Cucumis melo L.) | Fruit                  | Cation exchange chromatography | Phomopsis cucubitae                         | Fish and Davis, 2004 |
|                        |                        |                            | Didymella bryoniae                          |                     |
|                        |                        |                            | Rhizopus PG                                 |                     |
|                        |                        |                            | Fusarium verticilloides                     |                     |
| Cotton (Gossypium hirsutum L.) | Stem                  | PG-affinity chromatography | Aspergillus niger                           | James and Dubery, 2001 |
| Pear (Pyrus communis L.) | Fruit                  | Partial purified          | Verticillium dahliae Botrytis cinerea Venturia rashicola | Ladu et al., 2012; Faize et al., 2003 |
| Pearl millets (Pennisetum glaucum (L) R. Br.) | Seedlings              | Crude extract             | Aspergillus niger                           | Prabhu et al., 2012 |
| Grass pea (Lathyrus sativus L.) | Seeds                 | Gel-filtration chromatography | Aspergillus niger Rhizopus spp              | Tamburino et al., 2012 |
| Orange (Citrus reticulate L.) | Fruit                  | Partial purified          | Diaprepes abbreviatus                       | Doostdar et al., 1997 |
| Blue mustard (Chorispora bungeana) | Leaves, stem, root     | Partial purified          | Aspergillus niger Stemphytium solani        | Di et al., 2009     |
| Ginseng (Panax ginseng L.) | Crude extract           |                            | Colletotrichum gloeosporioides Phytium ultimum Fusarium oxysporum Rhizoctonia solani | Sathiyaraj et al., 2010 |
| Bread wheat (Triticum aestivum L.) | Leaves                | Cation exchange chromatography | Cochliobolus sativus                        | Kemp et al., 2003    |

(Continued)
that the protein region in contact with PvPGIP2 is located at the C-terminus of the enzyme and includes the loops surrounding the active site cleft. A site-directed mutagenesis analysis has been used to validate this second view (Benedetti et al., 2013). In general, low resolution techniques such as SAXS analysis or mass amide exchange mass spectrometry require validation by site-directed mutagenesis to locate the contacting residues in a protein complex.

The X-ray crystallography, successfully used to solve several high-resolution structures of PGs (van Santen et al., 1999; Federici et al., 2001; Bonivento et al., 2008) and that of PvPGIP2 (Di Matteo et al., 2003), was so far unsuccessful in the case of the PG-PGIP complex. This is probably due to the intrinsic instability of the PG-PGIP interaction, which only occurs, under apoplastic conditions of pH and ionic strength, through the contact of only a few, sometimes only one, residues (Leckie et al., 1999). The use of a cross-linker for stabilizing the PG-PGIP complex coupled to techniques that allow the protein analysis directly in solution, such as SAXS and NMR spectroscopy (Wand and Englander, 1996; Nietlispach et al., 2004), may be a valid alternative in order to obtain a detailed map of the contacting residues but this requires a subsequent validation by site-directed mutagenesis.

**PGIPs Engineered in Dicot Crops**

The important role of PGIP in plant defense has been demonstrated by overexpressing *pgip* genes in several plant species. In these experiments, the source of the used genes was either the same plant species utilized for transformation or a different one (Table 4). The transformation of the model plant *A. thaliana* has been particularly useful to highlight the potentiality of several *pgip* genes, namely the endogenous *Atpgip1* and *Atpgip2*, the bean *Pvpgip2* and the rapeseed (*Brassica napus*) *Bnpgip1* or *Bnpgip2*. Arabidopsis plants overexpressing *Atpgip1* or *Atpgip2* showed a significant reduction of disease symptoms caused by *B. cinerea* (Ferrari et al., 2003) and were less susceptible against the hemibiotrophic fungal pathogen *F. graminearum* (Ferrari et al., 2012), the major causal agent of Fusarium head blight (FHB). Conversely, silencing of their expression using an antisense *Atpgip* led to enhanced susceptibility (Ferrari et al., 2006). Arabidopsis plants expressing *Pvpgip2*, encoding an efficient inhibitor of the *B. cinerea* PG (ten Have et al., 1998), showed reduction of disease symptoms caused by *B. cinerea* and those expressing the rapeseed genes *Bnpgip1* and *Bnpgip2* delayed the symptoms caused by *S. sclerotiorum* (Bashi et al., 2013).

The protective potential of *pgip* genes has also been demonstrated in transgenic crops. The first transgenic crop plant obtained by using a *pgip* gene and tested against pathogenic microorganisms were tomatoes expressing PvPGIP1 from *P. vulgaris*. These plants, however, did not show any increased resistance against *Fusarium oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *Alternaria solani*. The negative result was due to the inability of PvPGIP1 to inhibit the PGs secreted by these fungi, as shown by in vitro inhibition assays and led to discovery of other forms of PGIPs and eventually to the existence of a complex PGIP family in French bean (Desiderio et al., 1997). A few years later, transgenic tomato plants expressing a pear (*Pyrus communis L.*) PGIP (PcPGIP) capable of inhibiting the PGs secreted by *B. cinerea*, showed a reduction of disease lesions caused by this fungus both on ripening fruit (15% reduction) and leaves (about 25% reduction). The initial establishment of infection was not affected in the transgenic plants but the later colonization of the host tissue was significantly reduced (Powell et al., 2000).

Tobacco has been the most used crop plant for testing the effect of PGIP expression on resistance to pathogens. Constitutive and high-level expression of *Pvpgip2* (from *P. vulgaris*), *Vvpgip1* (from *V. vinifera*), *Cagpgip1* (from pepper (*Capsicum annuum*)) and *Brpgip2* (from *B. rapa*) have been obtained in transgenic tobacco. Plants expressing *PvPGIP2* showed about 35% reduction of symptoms caused by *B. cinerea* (Manfredini et al., 2005) and, more recently, were shown to display reduced disease symptoms against *R. solani* and two oomycete pathogens, *Phytophthora parasitica* var. *nicotianae* and the blue mold-causing agent *Peronospora hyoscyami* f. sp. *tabacina* (Borras-Hidalgo et al., 2012). Notably, the experiments against *P. hyoscyami* f. sp. *tabacina* were performed in the field during seasonal conditions that favor the pathogen spreading. In agreement with what observed under controlled conditions, resistance of transgenic plants was comparable to that exhibited by *Nicotiana* species (*N. rustica*, *N. debneyi* and *N. megalosiphon*) that are highly resistant to blue mold disease. These transgenic plants expressing *PvPGIP2* represented the first example of PGIP-expressing plants subjected to field trials. Recently, transgenic rice expressing OsPGIP1 showed also improved resistance against *R. solani* in field experiments (Wang et al., 2014b).

Transgenic tobacco plants expressing the grapevine *pgip* gene *Vvpgip1* (Joubert et al., 2006) also showed a reduced (from 47 to 69%) disease susceptibility to *B. cinerea* infection. As for plants expressing *PvPGIP2*, the resistance phenotype correlated with the accumulation of VvPGIP1 as well as with its capability of inhibiting the activity of PG secreted by *B. cinerea*, namely

| Plant                          | Tissue     | PGIP preparation | Polygalacturonases Inhibited | Polygalacturonases Not inhibited | References                  |
|-------------------------------|------------|------------------|-----------------------------|---------------------------------|-----------------------------|
| Durum wheat *(Triticum turgidum ssp. dicoccoides)* | Leaves     | Crude extract    | Fusarium graminearum         | Fusarium phyllophyllum          | Janni et al., 2013          |

*Reclassified as Fusarium phyllophyllum (Mariotti et al., 2008).*
| Species                 | Gene   | Heterologous systems            | Origin of purified PG                          | References                  |
|------------------------|--------|---------------------------------|------------------------------------------------|-----------------------------|
| Common bean (Phaseolus vulgaris L.) | PvPGIP1 | Transgenic tomato               | Fusarium oxysporum                             | Desiderio et al., 1997      |
|                        |        |                                 | Botrytis cinerea                               |                             |
|                        |        |                                 | Alternaria solani                              |                             |
|                        |        |                                 | Aspergillus niger                              | Berger et al., 2000         |
|                        |        |                                 | Stenocarpella maydis                           |                             |
|                        |        |                                 | Aspergillus niger                              | D’Ovidio et al., 2006; Frati et al., 2006 |
|                        |        | PVX/Nicotiana benthamiana       | Aspergillus niger                              |                             |
|                        |        |                                 | Fusarium moniliforme                          |                             |
|                        |        |                                 | Stenocarpella maydis                           |                             |
|                        |        |                                 | Colletotrichum acutatum                        |                             |
|                        |        |                                 | Botrytis cinerea                               |                             |
|                        |        |                                 | Lygus rugulipennis                             |                             |
|                        |        |                                 | Adelphocoris lineolatus                        |                             |
|                        |        |                                 | Orthops kalmi                                  |                             |
|                        |        |                                 | Closterotomus norwegicus                       |                             |
|                        |        |                                 | Fusarium phyllophilum FC-10                   | Janni et al., 2008; Volpi et al., 2013 |
|                        |        |                                 | Fusarium phyllophilum 25305                   |                             |
|                        |        |                                 | Fusarium verticillioides 62264                 |                             |
|                        |        |                                 | Fusarium verticillioides PD                    |                             |
|                        |        | PVX/Nicotiana benthamiana       | Fusarium phyllophilum FC-10                   | Mariotti et al., 2008       |
|                        |        |                                 | Fusarium phyllophilum 25219                   |                             |
|                        |        |                                 | Fusarium phyllophilum 25218                   |                             |
|                        |        |                                 | Fusarium phyllophilum FC10                    |                             |
|                        |        |                                 | Fusarium phyllophilum 25219                   |                             |
|                        |        |                                 | Fusarium phyllophilum 25218                   |                             |
|                        |        | PVX/Nicotiana benthamiana       | Fusarium moniliforme                          | Farina et al., 2009         |
|                        |        |                                 | Aspergillus niger                              |                             |
|                        |        |                                 | Colletotrichum lupini                         |                             |
|                        |        |                                 | Botrytis cinerea                               |                             |
|                        |        | PWPGIP2                         | PVX/Nicotiana benthamiana                      |                                        |
|                        |        |                                 | Fusarium moniliforme                          |                                        |
|                        |        |                                 | Aspergillus niger                              |                                        |
|                        |        |                                 | Colletotrichum lupini                         |                                        |
|                        |        |                                 | Botrytis cinerea                               |                                        |
|                        |        | Tepary bean (Phaseolus acutifolius L.) | PVX/Nicotiana benthamiana                      | Positive                      |
|                        |        |                                 | Fusarium moniliforme                          | Farina et al., 2009         |
|                        |        |                                 | Aspergillus niger                              |                             |
|                        |        |                                 | Colletotrichum lupini                         |                             |
|                        |        |                                 | Botrytis cinerea                               |                             |
|                        |        | Lima bean (Phaseolus lunatus L.) | PVX/Nicotiana benthamiana                      | Positive                      |
|                        |        |                                 | Fusarium moniliforme                          | Farina et al., 2009         |
|                        |        |                                 | Aspergillus niger                              |                             |
|                        |        |                                 | Colletotrichum lupini                         |                             |
|                        |        |                                 | Botrytis cinerea                               |                             |
|                        |        | Soybean (Glycine max L.)         | PVX/Nicotiana benthamiana                      | Positive                      |
|                        |        |                                 | Sclerotinia sclerotiorum PGb                  | D’Ovidio et al., 2006; Frati et al., 2006 |
|                        |        |                                 | Sclerotinia sclerotiorum PGa                  |                             |
|                        |        |                                 | Fusarium moniliforme                          |                             |
|                        |        |                                 | Botrytis aclada                                |                             |
|                        |        |                                 | Aspergillus niger                              |                             |
|                        |        |                                 | Botrytis cinerea                               |                             |
|                        |        |                                 | Colletotrichum acutatum                        |                             |
|                        |        |                                 | Fusarium graminearum                          |                             |
|                        |        |                                 | Lygus rugulipennis                             |                             |
|                        |        |                                 | Adelphocoris lineolatus                        |                             |
|                        |        |                                 | Orthops kalmi                                  |                             |
|                        |        |                                 | Closterotomus norwegicus                       |                             |

(Continued)
TABLE 3 | Continued

| Species          | Gene    | Heterologous systems       | Origin of purified PG                                      | References                  |
|------------------|---------|----------------------------|------------------------------------------------------------|-----------------------------|
|                   |         |                            | Sclerotinia sclerotiorum PGb                              | D’Ovidio et al., 2006; Frati et al., 2006 |
|                   |         |                            | Sclerotinia sclerotiorum PGa                              |                             |
|                   |         |                            | Fusarium moniliforme§                                      |                             |
|                   |         |                            | Botrytis aclada                                            |                             |
|                   |         |                            | Aspergillus niger                                          |                             |
|                   |         |                            | Botrytis cinerea                                           |                             |
|                   |         |                            | Colletotrichum acutatum                                    |                             |
|                   |         |                            | Fusarium gramineanum                                      |                             |
| GmPGIP3           | PVX/Nicotiana benthamiana |                           | Sclerotinia sclerotiorum PGb                              | D’Ovidio et al., 2006; Frati et al., 2006 |
|                   |         |                            | Sclerotinia sclerotiorum PGa                              |                             |
|                   |         |                            | Fusarium moniliforme§                                      |                             |
|                   |         |                            | Botrytis aclada                                            |                             |
|                   |         |                            | Aspergillus niger                                          |                             |
|                   |         |                            | Botrytis cinerea                                           |                             |
|                   |         |                            | Colletotrichum acutatum                                    |                             |
|                   |         |                            | Fusarium gramineanum                                      |                             |
| GmPGIP4           | PVX/Nicotiana benthamiana |                           | Sclerotinia sclerotiorum PGb                              | D’Ovidio et al., 2006; Frati et al., 2006 |
|                   |         |                            | Sclerotinia sclerotiorum PGa                              |                             |
|                   |         |                            | Fusarium moniliforme§                                      |                             |
|                   |         |                            | Botrytis aclada                                            |                             |
|                   |         |                            | Aspergillus niger                                          |                             |
|                   |         |                            | Botrytis cinerea                                           |                             |
|                   |         |                            | Colletotrichum acutatum                                    |                             |
|                   |         |                            | Fusarium gramineanum                                      |                             |
| GmPGIP7           | PVX/Nicotiana benthamiana |                           | Sclerotinia sclerotiorum                                   | Kalunke et al., 2014        |
|                   |         |                            | Fusarium gramineanum                                      |                             |
|                   |         |                            | Colletotrichum acutatum                                    |                             |
|                   |         |                            | Aspergillus niger                                          |                             |
| Pepper (Capsicum annuum L.) | CaPGIP1, CaPGIP2 | Escherichia coli           | Alternaria alternata                                       | Wang et al., 2013           |
|                   |         |                            | Colletotrichum nicotianae                                  |                             |
| Rapeseed (Brassica napus L.) | BrPGIP1 | Pichia pastoris            | Sclerotinia sclerotiorum PG6                              | Bashi et al., 2013          |
| Chinese cabbage (Brassica rapa L.) | BrPGIP2 | Transgenic Brassica rapa | Pectobacterium carotovorum                                 | Hwang et al., 2010          |
| Chinese cabbage (Brassica rapa L.) | BrPGIP2 | Escherichia coli           | Sclerotinia sclerotiorum                                   | HuangFu et al., 2014        |
| Grapevine (Vitis vinifera L.) | VvPGIP1 | Transgenic tobacco         | Botrytis cinerea PG1                                       | Joubert et al., 2006        |
|                   |         |                            | Botrytis cinerea PG4                                       |                             |
|                   |         |                            | Botrytis cinerea PG6                                       |                             |
|                   |         |                            | Aspergillus niger PGB                                       |                             |
|                   |         |                            | Aspergillus niger PGB                                       |                             |
| Apple (Malus domestica Borkh.) | MdPGIP1 | Transgenic tobacco         | Colletotrichum lupini                                      | Oelofse et al., 2006        |
|                   |         |                            | Botrytis cinerea                                           |                             |
|                   |         |                            | Botrytis cinerea                                           |                             |
|                   |         |                            | Diaporthe ambigua                                          |                             |
|                   |         |                            | Verticillium dahliae                                        | Gazendam et al., 2004       |
| Pear (Pyrus communis L.) | PpPGIP | Transgenic grape           | Botrytis cinerea                                           | Agüero et al., 2005         |
|                   |         |                            | Botrytis cinerea                                           | Powell et al., 2000         |
|                   |         |                            | Transgenic tomato                                          |                             |
|                   |         |                            | Botrytis cinerea                                           | Tamura et al., 2004         |
|                   |         |                            | Transgenic persimmon                                       |                             |
| (Continued)       |         |                            |                                                             |                             |
TABLE 3 | Continued

| Species                | Gene          | Heterologous systems | Origin of purified PG | References                  |
|------------------------|---------------|----------------------|-----------------------|------------------------------|
| Raspberry (Rubus idaeus L.) | RiPGIP        | Transgenic pea       | Stenocarpella maydis  | Richter et al., 2006         |
|                        |               |                      | Colletotrichum lupini |                              |
| Wheat (Triticum aestivum L.) | TaPGIP1, TaPGIP2 | PVX/Nicotiana benthamiana | Fusarium phyllophylu  | Janni et al., 2013           |
|                        |               |                      | Stenocarpella maydis  |                              |
|                        |               |                      | Bipolaris sorokiniana |                              |
|                        |               |                      | Fusarium graminearum  |                              |
| Rice (Oryza sativa L.) | OsPGIP1       | PVX/Nicotiana benthamiana | Sclerotinia sclerotiorum | Janni et al., 2006           |
|                        |               |                      | Fusarium moniliforme |                              |
|                        |               |                      | Fusarium graminearum  |                              |
|                        |               |                      | Aspergillus niger     |                              |
|                        |               |                      | Botrytis cinerea      |                              |
| Pearl millet [Pennisetum glaucum (L.) R. Br.] | OsFOR1, PglPGIP1 | Escherichia coli BL21, Escherichia coli SHuffle® T7 Express | Aspergillus niger PG | Jang et al., 2003 |
| Arabidopsis thaliana  | AtPGIP1, AtPGIP2 | Transgenic Arabidopsis | Colletotrichum gloeosporioides | Frati et al., 2006; Ferrari et al., 2012, 2003 |
|                        |               |                      | Stenocarpella maydis  |                              |
|                        |               |                      | Botrytis cinerea      |                              |
|                        |               |                      | Fusarium graminearum  |                              |
| §Reclassified as Fusarium phyllophilum FC10 (Marriott et al., 2008). |

BcPG1, BcPG3, and BcPG6. Several observations, however, suggest that PGIP may improve resistance by mechanisms other than classical PGIP-PG inhibition. For example, non-infected transgenic tobacco plants expressing Vvpgip1 show modified expression patterns of genes involved in various metabolic pathways (Alexandersson et al., 2011) and an altered cell wall structure (Ngueuma-Ona et al., 2013). In these plants, lignin accumulation and arabinoxylolucan-cellulose re-organization leads to a general strengthening/reinforcing of the cell wall that may contribute to an improved resistance against B. cinerea.

A reduction of disease symptoms (about 50%) caused by Alternaria alternata and Colletotrichum nicotianae was also observed in transgenic tobacco lines expressing the pepper CaPGIP1 and, once again, resistance correlated with the inhibition capacity of purified CaPGIP1 against PG activity of both fungal pathogens (Wang et al., 2013).

Within the Solanaceae family, transgenic potato (Solanum tuberosum) plants expressing the gene StPGIP1 from S. torvum showed a 50% reduction of wilt disease symptoms caused by Verticillium dahliae and a normal plant growth (Guo et al., 2014). Transgenic potato plants overexpressing the apple pgip1 gene showed protection against the same fungal pathogen but displayed an extended juvenile phase (Gazendam et al., 2004).

Transgenic grapevine (V. vinifera) plants constitutively expressing the pear PcPGIP gene represent an interesting example of the potential of PGIP for protection against pathogens other than fungi and oomycetes. These plants show a delayed development of the Pierce’s disease (PD) caused by bacterial pathogen Xylella fastidiosa (Agüero et al., 2005). Not only leaf scorching and Xylella titre were reduced but also plants showed a better re-growth after pruning compared to infected untransformed controls. Moreover, an inverse dose-effect relationship was shown between development of PD and levels of PcPGIP activity in the tissues. The improved resistance of the grapevine plants expressing PcPGIP against a bacterial pathogen was unexpected, because until then the PGIP inhibition activity was thought to be limited to fungal and insect PGs (Cervone et al., 1990; Johnston et al., 1993; D’Ovidio et al., 2004b). It was later shown that pear PcPGIP inhibits the PG encoded by X. fastidiosa and that PG activity is a virulence factor of this pathogen (Roper et al., 2007; Pérez-Donoso et al., 2010). The observation that PcPGIP is present in xylem exudates of non-transgenic scions grafted on transgenic rootstocks suggests that grafting of non transgenic varieties on transgenic rootstocks represents, in this case, a useful agronomical practice for plant protection (Agüero et al., 2005).

The results obtained with X. fastidiosa prompted further investigations on the capability of PGIP of controlling bacterial diseases (summarized in Table 4). Transgenic tobacco plants expressing B. rapa BrPGIP2 were resistant against Pectobacterium carotovorum, the causal agent of the soft rot disease, with a strong reduction (66–88%) of the symptoms as compared
**TABLE 4 | List of transgenic crops produced using the gene coding for PGIP and their response to fungal, oomycetes or bacterial phytopathogens.**

| Transgenic crops | PGIP gene\(c\) | Tested against fungal, oomycetes or bacterial phytopathogens | References |
|------------------|-----------------|---------------------------------------------------------------|------------|
| Tomato\(a\) (Solanum lycopersicum L.) | PcPGIP, PvPGIP1 | Botrytis cinerea\(^*\), Fusarium oxysporum f.sp. lycopersici\(†\), Botrytis cinerea\(†\), Alternaria solani\(†\) | Powell et al., 2000, 1994, Desiderio et al., 1997 |
| Tobacco\(a\) (Nicotiana tabacum L.) | PpPGIP1 | Botrytis cinerea\(^*\), Rhizoctonia solani\(^*\), Phytophthora parasitica\(^*\), Peronospora hyoscyami\(^*\), Alternaria alternata\(^*\), Colletotrichum nicotianae\(^*\) | Manfredini et al., 2005, Borras-Hidalgo et al., 2012 |
| Peanut\(a\) (Arachis hypogaea L.) | BrPGIP1, BrPGIP2 | Botrytis cinerea\(‡\), Alternaria solani\(‡\), Alternaria alternata\(‡\), Colletotrichum nicotianae\(‡\) | Joubert et al., 2006, Hwang et al., 2010 |
| Potato\(a\) (Solanum tuberosum L.) | MdPGIP1, StPGIP | Verticillium dahliae\(‡\), Verticillium dahliae\(^*\) | Gazendam et al., 2004, Guo et al., 2014 |
| Brassica rapa\(a\) | BrPGIP2 | Pectobacterium carotovorum\(^*\) | Hwang et al., 2010 |
| Rapeseed\(a\) (Brassica napus L.) | BrPGIP2 | Sclerotinia sclerotiorum\(^*\) | HuangFu et al., 2014 |
| Pea\(a\) (Pisum sativum L.) | RpPGIP | Glomus intraradices\(^\text{w}\) | Hassan et al., 2012 |
| Grapevine\(a\) (Vitis vinifera L.) | PcPGIP, OvPGP1 | Botrytis cinerea\(^*\), Xylella fastidiosa\(^*\), Rhizoctonia solani | Agüero et al., 2005, Wang et al., 2014b |
| Rice\(a\) (Oryza sativa L.) | BrPGIP1 | Botrytis cinerea\(^*\) | Janni et al., 2008, Ferrari et al., 2012, Volpi et al., 2013, Wang et al., 2014a |
| Wheat\(b\) (Triticum aestivum L., Triticum durum Desf.) | PpPGIP2, GmPGIP3, BrPGIP1, BrPGIP2 | Bipolaris sorokiniana\(^*\), Fusarium graminearum\(^*\), Claviceps purpurea\(†\), Bipolaris sorokiniana\(†\), Gaeumannomyces graminis var. tritici\(†\) | Janni et al., 2008, Ferrari et al., 2012, Volpi et al., 2013, Wang et al., 2014a |
| Arabidopsis thaliana L.\(a\) | PpPGIP2, AtPGIP1, AtPGP2, BrPGIP1, BrPGIP2 | Botrytis cinerea\(^*\), Fusarium graminearum\(^*\), Sclerotinia sclerotiorum\(^*\) | Manfredini et al., 2005, Ferrari et al., 2012, Basri et al., 2013 |

\(a\)The transgenic gene was under control of CaMV 35S promoter.
\(b\)The transgenic gene was under control of Ubiquitin promoter.
\(c\)Pc, Pyrus communis; Pv, Phaseolus vulgaris; Ca, Capsicum annuum; Vv, Vitis vinifera; Br, Brassica rapa; Md, Malus domestica; St, Solanum torvum; Ri, Rubus idaeus; Ac, Actinidia delicosa; At, Arabidopsis thaliana; Br, Brassica rapa.
\(\text{w}\)No evidence of enhanced resistance.
\(\text{‡}\)No effect on mycorrhization.

To wild-type plants (Hwang et al., 2010). The resistance correlated with the inhibitory activity against *P. carotovorum* PG activity found in the total protein extracts of the transgenic plants (Hwang et al., 2010). No phenotypic abnormalities were, instead, found in transgenic tobacco plants expressing BrPGIP2 (Hwang et al., 2010), nor in rapeseed plants overexpressing the *B. napus BnpGp2* The latter plants displayed a significant reduction of rot caused by the necrotrophic fungal pathogen *S. sclerotiorum* (HuangFu et al., 2014).

No phenotypic abnormalities were, instead, found in transgenic tobacco plants expressing BrPGIP2 (Hwang et al., 2010), nor in rapeseed plants overexpressing the *B. napus BnpGp2*. The latter plants displayed a significant reduction of rot caused by the necrotrophic fungal pathogen *S. sclerotiorum* (HuangFu et al., 2014).

Additional PGIP-transgenic crops include pea (*Pisum sativum* L.), transformed with Ripgip from raspberry (*Rubus idaeus* L.) (Richter et al., 2006), persimmon (*Diospyros kaki* L.) and apple (*Malus domestica* Borkh.) transformed with pear PcPGIP (Szankowski et al., 2003; Tamura et al., 2004), sugarbeet (*Beta vulgaris* L.) transformed with bean Pvpgip2
chickpea transformed with either Ripgip or a ppgp gene from kiwi fruit (Senthil et al., 2004), tobacco transformed with PpPGIP gene from Pyrus pyrifolia Nakai (Liu et al., 2013) and maize (Zea mays L.) transformed with bean Pppgip1 (O’Kennedy et al., 2001). The response of these plants to pathogens has not been reported yet. Transgenic pea plants expressing RiPGIP were instead evaluated for their response to beneficial microorganisms. Glomus intraradices, an arbuscular mycorrhizal fungus, colonized roots of transgenic plants at an extend comparable to that observed in control non transgenic plants, indicating that the expression of RiPGIP does not affect mycorrhization (Hassan et al., 2012).

PGIPs Engineered in Monocot Crops

Although the low lectin content of cereal species like wheat and rice indicates that this cell wall component may have a marginal role during infection, results show that the expression of PGIP in transgenic plants limits some diseases caused by fungal pathogens (Janni et al., 2008; Ferrari et al., 2012; Wang et al., 2014a,b). In our labs, the bean Pvp pgip2 gene was used under the constitutive promoter of the maize ubiquitin gene (Ubi-1) to transform both durum and bread wheat by particle bombardment. PvpPGIP2 was correctly targeted to the apoplast and the transgenic plants did not show any major morphological and growth defects. Transgenic wheat showed a significant reduction (46–50%) of foliar spot blotch symptoms caused by the hemibiotrophic fungal pathogen Bipolaris sorokiniana and improved resistance (25–30%) against the hemibiotrophic fungal pathogen F. graminearum (Ferrari et al., 2012), the major causal agent of FHB in wheat. A reduced degradability of the transgenic tissue by PG treatments correlated with the capacity of PvpPGIP2 to inhibit PG activity of B. sorokiniana and less strongly PG of F. graminearum (Janni et al., 2008; Ferrari et al., 2012). An interesting aspect of the wheat plants expressing PvpPGIP2 is that, under moderate infection with F. graminearum, the reduced FHB symptoms are concomitant with a greater amount of total starch in the grains as compared to control plants (D’Ovidio et al., 2012). On the other hand, wheat plants expressing PvpPGIP2 were susceptible to the biotrophic fungal pathogen Claviceps purpurea, the causal agent of ergot disease probably because PvpPGIP2 is not able to inhibit the activity of C. purpurea CpPG1 and CpPG2 (Volpi et al., 2013). Recently, transgenic wheat expressing the soybean GmPGIP3 was shown to be resistant to both take-all and common root rot diseases caused by the fungal pathogen Gaeumannomyces graminis var. tritici and B. sorokiniana, respectively; symptoms were reduced of about 47–83% and 42–60%, respectively (Wang et al., 2014a). Similarly, the expression of OsPGIP1 in transgenic rice enhanced resistance against Rhizoctonia solani in field tests and resistance was related with the expression levels of OsPGIP1 (Wang et al., 2014b).

Concluding Remarks and Future Challenges

The results reported in this review clearly indicate that PGIP is useful to improve resistance in different crop species. High-level expression of PGIP does not prevent infection but limits significantly the colonization of the host tissue with a consequent positive impact on crop yield and product quality. The efficacy of PGIP to control diseases has been demonstrated against fungi, oomycetes and bacteria and is equally efficient against necrotrophic and hemibiotrophic pathogens. The experiments performed with biotrophs do not allow to draw any clear conclusion since the only fungal biotrophic pathogen analyzed, C. purpurea, produced PG activity that was not inhibited by the PGIP expressed in the transgenic plants (Volpi et al., 2013). The identification and development of PGIPs with stronger and broader inhibitory capacities may be useful to utilize these proteins in crop protection. Germplasm analysis to identify novel PGIPs is still limited (Farina et al., 2009) and the initial attempts to drive in vitro evolution of PGIP to generate proteins with improved inhibition properties have not been particularly successful (Benedetti et al., 2011a). Structural studies should be implemented in order to obtain a detailed map of the contacts between various PGs and PGIPs. This is necessary not only for constructing novel inhibitors with stronger activities but also for future programs of genome editing in which the existing genes of a plant species may be ameliorated to better adapt to new virulent strains of microorganisms evolving in nature.

The available results support the notion that inhibition of the microbial PG by PGIP is a prerequisite of the inhibitors to confer resistance to transgenic plants against microbes. The delay of symptoms is often related to the capacity of PGIP to inhibit the PG activity secreted by the pathogens and, consequently, to reduce both tissue maceration and favor the release of OGs, as summarized in Figure 2. However, this aspect of the PGIP’s biology needs further investigation. In some cases PGIP has been reported to confer resistance without any evidence of PG-inhibition in vitro (Joubert et al., 2006). Moreover, some evidence suggests that the capability of reducing tissue maceration is associated with the property of PGIP to bind pectin, likely shielding this component of the cell wall from PG activity (Spadoni et al., 2006). In this regard the observation that transgenic plants expressing PGIPs exhibit an altered gene expression and cell wall composition is also intriguing. It is not yet clear the mechanism that links the ectopic expression of PGIP to alteration of gene expression and whether this contributes to disease resistance (Alexandersson et al., 2011; Nguema-Ona et al., 2013).

An important but very little explored aspect of the PGIP biology is its possible role in processes of growth and development. Although plants overexpressing PGIPs do not show obvious morphological alterations, indeed several reports point to PGIP as a player in development. PGIP are induced, not only by phosphate deficiency, but also by auxin treatment and in mutants defective in SIZ1, a SUMO (small ubiquitin-related modifier) E3 ligase that is involved in several stress responses, including Pi starvation, and flowering (Sato and Miura, 2011). Suppression of PGIPs under the control ABA insensitive 5 (ABI5) transcription factor accompanies promotion of seed germination by the peroxisomal ABC transporter PED3 (Kanai et al., 2010). Upregulation of PGIP2 correlates with the acquisition of competence to form green callus in an auxin-rich callus induction medium (Che et al., 2007) and occurs in Arabidopsis tissue culture lines
in which the expression of the peroxidases PRX33 and PRX34 is knocked down by antisense expression (O’Brien et al., 2012), whereas PGIP1 was identified in a proteomic study performed on Arabidopsis etiolated hypocotyls used as a model of cells undergoing elongation followed by growth arrest within a short time (Irshad et al., 2008). Finally, both PGIP1 and PGIP2 are associated with cell wall stabilization at low pH under the control of the zinc-finger protein STOP1 (Sensitive to Proton Rhizotoxicity 1) and STOP2 (Kobayashi et al., 2014). A role of PGIP not only in defense but also in growth and development implies that the inhibitor may affect one or more of the many endogenous PGs expressed by plants. This is also an unexplored aspect of the PGIP biology and, at the moment, only one very old evidence is available showing that PGIP may have a plant-derived PG partner (Cervone et al., 1990).

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Superhydrophobicity in perfection: the outstanding properties of the lotus leaf

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Abstract

Lotus leaves have become an icon for superhydrophobicity and self-cleaning surfaces, and have led to the concept of the ‘Lotus effect’. Although many other plants have superhydrophobic surfaces with almost similar contact angles, the lotus shows better stability and perfection of its water repellency. Here, we compare the relevant properties such as the micro- and nano-structure, the chemical composition of the waxes and the mechanical properties of lotus with its competitors. It soon becomes obvious that the upper epidermis of the lotus leaf has developed some unrivaled optimizations. The extraordinary shape and the density of the papillae are the basis for the extremely reduced contact area between surface and water drops. The exceptional dense layer of very small epicuticular wax tubules is a result of their unique chemical composition. The mechanical robustness of the papillae and the wax tubules reduce damage and are the basis for the perfection and durability of the water repellency. A reason for the optimization, particularly of the upper side of the lotus leaf, can be deduced from the fact that the stomata are located in the upper epidermis. Here, the impact of rain and contamination is higher than on the lower epidermis. The lotus plant has successfully developed an excellent protection for this delicate epistomatic surface of its leaves.

Introduction

Since the introduction of the ‘Lotus concept’ in 1992 [1,2], the lotus leaf became the archetype for superhydrophobicity and self-cleaning properties of plant surfaces and a model for technical analogues [3,4]. Lotus (Nelumbo nucifera) is a semi-aquatic plant and develops peltate leaves up to 30 cm in diameter with remarkable water repellency. As an adaptation to the aquatic environment – some of the leaves float occasionally on the water surface – the stomata are located in the upper
epidermis. The lower epidermis consists of convex cells covered with wax tubules and contains only few stomata. The upper epidermis features the distinctive hierarchical structure consisting of papillae with a dense coating of agglomerated wax tubules, which is the basis for the famous superhydrophobicity (Figure 1).

Figure 1: (a) Lotus leaves, which exhibit extraordinary water repellency on their upper side. (b) Scanning electron microscopy (SEM) image of the upper leaf side prepared by ‘glycerol substitution’ shows the hierarchical surface structure consisting of papillae, wax clusters and wax tubules. (c) Wax tubules on the upper leaf side. (d) Upper leaf side after critical-point (CP) drying. The wax tubules are dissolved, thus the stomata are more visible. Tilt angle 15°. (e) Leaf underside (CP dried) shows convex cells without stomata.

However, a hierarchical surface structure which induces strong water repellency and contact angles above 150° is not a special feature of lotus leaves. It has been known for a long time that plant surfaces covered with epicuticular wax crystals are water repellent, and that this feature is enhanced when the epidermis has additional structures such as papillae or hairs [5,6]. Neinhuis and Barthlott (1997) [7] presented an overview of more than 200 species with contact angles >150° and their surface morphologies. Many studies, in which the properties of lotus leaves were compared with those of other superhydrophobic plants, have shown the superiority of the upper side of the lotus leaf. A standard tool for the determination of wettability or water repellency is the measurement of the static contact angle by the ‘sessile drop’ method. Neinhuis and Barthlott (1997) [7] for example, measured contact angles on the lotus leaf of 162°, which are among the highest of the compared species, but many other (43%) of the tested superhydrophobic plants also showed contact angles between 160 and 163°. Even some species with flat epidermis cells but with a dense layer of epicuticular wax crystals, such as *Brassica oleracea* or some *Eucalyptus* species, can exhibit contact angles >160°. Thus, the contact angle alone is not suitable for a differentiated comparison of superhydrophobic samples. Other values such as contact angle hysteresis or roll-off (tilting) angle show more clearly the differences between the species. Mockenhaupt et al. (2008) [8] compared the tilting angles and the stability of the superhydrophobicity of various plants under moisture condensation conditions. Only the lotus leaves showed no significant loss of water repellency when water vapour condensed on the surface of the cooled samples at 5 °C. Wagner et al. (2003) [9] examined the morphology of the epidermal structures and the wettability with liquids of varying surface tension such as methanol–water mixtures. They reported the lowest wettability by these liquids for the lotus leaves in comparison to other species. They also described the unique shape of the papillae and a very high papillae density (number per area). Chemical analyses [10] and crystal structure analysis by X-ray diffraction [11] showed unique properties of the epicuticular wax of the lotus. The high content of nonacosanediols leads to a high melting point as well as a strongly disturbed crystal structure which is the basis for the formation of tubules. The visualization of the contact zone between leaves and droplets with cryo-scanning electron microscopy demonstrated the extremely reduced contact area for lotus [12]. Zhang et al. (2008) [13] made detailed measurements of the water repellency of the papillose lotus leaf surface in comparison with the non-papillose leaf margin. The importance of the nanoscopic wax crystals for the water repellency was demonstrated by Cheng et al. (2006) [14]. They reported a strong decrease of the contact angle after melting of the waxes. A limited air retaining capability of submerged lotus leaves was reported by Zhang et al. (2009) [15] after the leaves were held at a depth of 50 cm for 2 h. Bhushan et al. (2010) [4] used the surface structures of the lotus leaf as model for the development of artificial biomimetic superhydrophobic structures.

It became obvious that the outstanding and stable superhydrophobicity of the lotus leaf relies on the combination of optimized features such as the surface topography, robustness and the unique properties of the epicuticular wax. The aim of this article is to integrate the relevant features of the lotus leaf, and to compare them with superhydrophobic leaves of other plant species in order to illustrate their significance.
Results and Discussion
The properties of the lotus leaves
The lotus leaf shows an outstanding water repellency particularly on its upper (adaxial) side, which is more robust and less sensitive to mechanical damage than the under (abaxial) side. The reasons for these superior properties can be ascribed to the combination of micro- and nano-structures with optimized geometry and the unique chemical composition of the epicuticular waxes. These properties are illustrated in the following sections and compared with those of other superhydrophobic leaves. (The species are listed in the Experimental section).

Minimization of the water-to-leaf contact area: The epidermis cells of the upper leaf side form papillae of varying height and with a unique shape. The diameter of the papillae is much smaller than that of the epidermis cells and each papilla apex is not spherical but forms an ogive (Figure 2).

The whole surface is covered with short wax tubules which often accumulate in clusters. In comparison with other papillose plant surfaces, lotus has the highest density of papillae, but the lotus papillae have much smaller diameters which reduces the contact area with water drops; strictly speaking, the area of heterogeneous contact between surface and water. The contact area depends on the hydrophobicity of the surface and on the pressure of the water or on the kinetic energy or velocity of the striking water drops. At low pressures, caused by resting or rolling water droplets, the contact area is determined by the local contact angle of the surface structures. For the surface of a papilla coated with wax tubules, a superhydrophobic behavior with a local contact angle of >140° can be assumed. So, the diameter of the contact areas can be estimated from the SEM images and the cross sections of the selected samples (Figure 2).

The minimized contact area is the basic cause for the very low adhesion of water and, thus, the small roll-off (tilting) angles. Compared with lotus, the papillae on the leaves of the other plants (E. myrsinites, C. esculenta, A. macrorrhiza) (Figure 3, see also Figure 7) have much larger diameters and tip radii, and are covered with different wax types, wax platelets or wax film, respectively, which have a lower water repellency than wax tubules.
The varying height of the papillae further reduces the adhesion between water drops and the surface (Figure 4). Small resting or sliding water drops touch only the highest papillae [12]. At higher pressures, e.g., at the impact of raindrops, the water intrudes deeper between the papillae (Figure 4a) and forms a meniscus at the still superhydrophobic wax tubules coating. The deformation of the non-wetting droplet surface due to surface tension causes a repellent force (‘re’, Figure 4). When the water retracts, either at the receding side of a moving drop or if the drop is lifted off the surface, the contact areas decrease and the papillae release their contact to the water one by one (Figure 4b, Figure 4c), so that only few of the papillae are simultaneously in the adhesive state (‘ad’). Finally, before the drop loses contact with the leaf, only few of the papillae are still in contact and cause a small adhesive force. In contrast, artificial superhydrophobic samples with pillars of equal height lead to stronger adhesion during drop retraction when all the pillars are simultaneously in the adhesive state before the contact breaks (Figure 4d). The measurement of the adhesive and repellent forces between a superhydrophobic papilla-model (with ten times larger tip radius than a lotus papilla) and a water drop is shown in Figure 5.

Figure 4: The contact between water and superhydrophobic papillae at different pressures. At moderate pressures the water intrudes into the space between the papillae, but an air layer remains between water and epidermis cells (a). The superhydrophobic surface of the papillae causes a repellent force (‘re’). When the water recedes, then the papillae lose contact one after the other (b, c). At a certain water level, the meniscus is flat and the force is neutral (‘n’). Just prior to the separation an adhesive force (‘ad’) arises at the almost horizontal area of the papilla tip, which is small on tips with intact wax crystals and larger when the wax is damaged or eroded. On artificial superhydrophobic structures with equal height (d) the adhesive forces during water receding occur simultaneously at all contacts.

Figure 5: Measured forces between a superhydrophobic papilla-model and a water drop during advancing and receding. The images corresponding to the marks (arrows) in the diagram show the repellent (a) and adhesive (b) meniscus. (c) Papilla-model tip shown with SEM.

Contact angle measurements are the standard tool for the determination of hydrophobicity. But the measurement of very high contact angles is often inaccurate due to difficulties in the determination of the exact drop shape [16], particularly on uneven leaf surfaces. For many superhydrophobic plant surfaces, the contact angles are very close together [7] such that the inaccuracies are larger than the differences between the samples. This may prevent a meaningful comparison. A more differentiated comparison of water repellency has been achieved by the measurement of the adhesion between surface and water during retraction of a drop [13], similar to the measurement shown in Figure 5. Table 1 shows, in addition to other relevant properties, the maximal adhesion forces of water drops on fresh lotus leaves and leaves of other species with intact wax. The adhesion forces are strongly dependent on...
Table 1: Comparison of water repellency relevant properties of lotus and other selected species.

|                        | Nelumbo nucifera (Lotus) (upper side) | Colocasia esculenta (upper side) | Euphorbia myrsinites (upper side) | Alocasia macrorrhiza (lower side) | Brassica oleracea (upper side) |
|------------------------|---------------------------------------|----------------------------------|----------------------------------|----------------------------------|-------------------------------|
| papillae density (per mm²) | 3431                                  | 2662                             | 1265                             | 2002                             | 0                             |
| contact angle (static) | 163°                                  | 165°                             | 162°                             | 157°                             | 161°                          |
| drop adhesion force (µN) | 8–18                                   | 28–55                            | 30–58                            | 90–127                           | 7–48                          |
| wax type               | tubules                               | platelets                        | platelets                        | film on cuticular folds          | rodlets and tubules           |
| wax melting point (°C) | 90–95                                 | 75–78                            | 75–76                            | n.a. b                           | 65–67                         |
| main components [11]   | C₂₉-diols                             | C₂₆-1-ol                         | C₂₆-1-ol                         | n.a. b                           | C₂₉-ketones, C₂₉-alkanes       |

*provided by D. Mohr, Nees Institute, Bonn; *the wax film of A. macrorrhiza has not been isolated and analyzed; no data available.

surface defects which cause pinning of the drops. In contrast, advancing contact angles depend weakly on such irregularities. Thus, the adhesion data correlate better with receding contact angles and hysteresis and indicate the perfection and defects of superhydrophobic surfaces.

**Mechanical protection of the wax crystals by papillae:** The highest water repellency occurs when the water drops touch the tips of the epicuticular wax crystals only. Thus, the best properties are found on leaves with an intact coating of wax crystals on the epidermal cells (Figure 6). The waxes are, however, relatively soft materials so that older leaves often show patches of eroded or damaged wax (Figure 7), which cause an increased adhesion of water. Neinhuis and Barthlott (1997) [7] have reported that papillae protect the wax crystals between them. On papillose epidermis cells only the wax on the papillae tips appears damaged while the wax between the papillae remains intact (Figure 7a, Figure 7b). Thus, lotus leaves retain their water repellency up to the end of their lifetime. In contrast, the non-papillose surfaces of Brassica oleracea and Yucca filamentososa (Figure 7c, Figure 7d) often show larger damaged areas which cause a stronger pinning of water. The efficiency of the protective properties can easily be tested by wiping across the leaf with the finger, which destroys only the wax on the papillae tips (Figure 8a, Figure 8b), but the leaves remained superhydrophobic. In the case of the non-papillose surface of a B. oleracea leaf (Figure 8c), the waxes are completely destroyed and superhydrophobicity is lost; the contact angle decreased from 160° to ca. 130°. On a Y. filamentososa leaf (Figure 8d) with convex epidermis cells, most of the wax crystals were destroyed and the contact angle dropped from 150° to ca. 110°.

![Figure 6: Papillose and non-papillose leaf surfaces with an intact coating of wax crystals: (a) Nelumbo nucifera (Lotus); (b) Euphorbia myrsinites; (c) Brassica oleracea; (d) Yucca filamentososa. Even the non-papillose leaves are superhydrophobic. The contact angle of B. oleracea can exceed 160°.](image)

![Figure 7: Traces of natural erosion of the waxes on the same leaves as in Figure 6: (a) Nelumbo nucifera (Lotus); (b) Euphorbia myrsinites; (c) Brassica oleracea; (d) Yucca filamentososa. On the papillose leaves (a,b) the eroded areas are limited to the tips of the papillae. On non-papillose cells, the damaged areas can be much larger (c,d), causing stronger pinning of water droplets.](image)
Figure 8: Test for the stability of the waxes against damaging by wiping on the same leaves: (a) Nelumbo nucifera (Lotus); (b) Euphorbia myrsinites; (c) Brassica oleracea; (d) Yucca filamentosa. On the papillose surfaces only the waxes on the tips of the papillae are destroyed. The waxes between the papillae are protected and remain intact. On the non-papillose surfaces, most of the waxes are destroyed, adhesion of water drops (pinning) is strongly increased, and the superhydrophobicity is lost.

Figure 9: SEM and LM images of cross sections through the papillae. Lotus (a,b) and Euphorbia myrsinites (c,d) have almost massive papillae, those of Alocasia macrorrhiza (e,f) have a relatively thick outer wall; the epidermal cells of Colocasia esculenta have thin walls (g,h). The arrow in (b) marks a stoma.

The basis for the ability to protect the leaf surface in lotus is the robustness of its leaf papillae in combination with their high density. Cross sections (Figure 9) show that they are almost massive at least in the apical part, in contrast to the fragile papillose cells found on many flower petals. However, papillae of other superhydrophobic leaves show various architectures: Euphorbia myrsinites has completely massive papillae; those of the lower epidermis of Alocasia macrorrhiza have quite thick outer walls, whereas the epidermal cells of Colocasia esculenta have very thin walls with slight thickening at the protrusions.

Properties of the lotus wax

Both the upper side and the lower side of the lotus leaf are covered with wax tubules. But, as can be seen on the SEM images (Figure 10a, Figure 10b), the waxes of both sides look quite different. The wax tubules of the lower side are longer (1 to 2 µm) and thicker (ca. 150 nm) and are typical ‘nonacosanol tubules’ which commonly occur on many plant species [7]. In contrast, the wax tubules of the upper leaf side are very short (0.3–1 µm) and thin (80–120 nm) but the density is very high. Figure 10 shows on a clearly arranged area, approximately 200 tubules per 10 µm² on the upper side, but only about 63 tubules per 10 µm² on the lower side of the same leaf. The spacing between the tubules on the upper side of the lotus leaf is much smaller than that of other wax crystals such as platelets (Figure 10c, Figure 10d) and other tubular waxes (Figure 10b, Figure 10e, Figure 10f). These distances between the hydrophobic wax crystals determine the pressure (capillary pressure) which is necessary for an intrusion of a water droplet between them.

The chemical analyses of the waxes give an explanation for the different properties. It is known that the epicuticular wax of lotus contains a high percentage of nonacosanediols [10], but the older analyses were made from the entire wax of the leaves, which was obtained as a chloroform extract and also contained intracuticular lipids. The new analyses of the separately isolated waxes from both sides (Figure 11) show that the wax of the upper side contains ca. 65% of various nonacosanediols and only 22% of nonacosan-10-ol, whereas the wax of the underside contains predominantly nonacosan-10-ol (53%) and only 15% of diols, together with 18% of alkanes. The remaining 13% and 14% could not be identified.

This high content of nonacosanediols provides extraordinary properties to the upper side wax. The melting point of 90 to 95 °C is very high for normal (aliphatic) waxes and indicates the influence of hydrogen bonding in the crystal lattice which increases the stability. A comparison of different aliphatic wax components with similar chain length shows that the melting points increase with the occurrence of polar OH-groups. Strong
The upper side of the lotus leaf has the highest crystal density (number per area) of wax crystals and the smallest spacings between them.

Lotus upper side (a) ca. 200 tubules per 10 µm²; (b) Lotus underside ca. 63 tubules per 10 µm²; (c) Euphorbia myrsinites ca. 50 platelets per 10 µm²; (d) Yucca filamentosa ca. 17 platelets with over 80 jags per 10 µm²; (e) Brassica oleracea ca. 22 rodlets and tubules; and (f) Eucalyptus macrocarpa ca. 50 tubules per 10 µm². The larger spacing between the wax crystals of the other surfaces compared to the lotus upper side is obvious.

Hydrogen bonding effects have been measured recently by Coward (2010) [17] in nonacosanol wax using FTIR spectroscopy. The effects on the crystal structure should be even stronger for the nonacosanediols. Although the secondary alcohols (nonacosan-10-ol and nonacosanediols) contain polar OH-groups in their molecules, the resulting wax tubules are known to feature strong and relatively stable water repellency, particularly the diols of the lotus leaf. This seems paradoxical, but X-ray diffraction analyses (Figure 12) are in accordance with a layer structure model in which the OH-groups are buried deep in the layer, while the layer surface consists only of nonpolar methyl groups [11, 18]. In contrast, primary alcohols such as the widespread octacosan-1-ol, which occurs in many platelet-shaped epicuticular waxes, can present the OH-group on the surface, e.g., if they are in contact with a polar environment (water). Holloway (1969) [19] studied the hydrophobicity and water contact angles of various plant waxes and pure wax components. He found the highest contact angles for aliphatic waxes which present only methyl groups on the surface.

According to the layer structure model, the tubules are strongly curved helically growing layers. While straight long-chained alkanes form flat layers and regular platelet crystals, secondary alcohols and ketones carry lateral oxygen atoms which inhibit a tight package of the molecules. Thus the resulting layers have a strong curvature and form tubules with a circular cross-section (Figure 13). Today, the progress in molec-
For a stable superhydrophobicity – that means the retention of the Cassie state with only partial contact between surface and water – an intrusion of water between the surface structures must be avoided. When the air layer is displaced by water, the water repellency is lost and the surface becomes wet (Wenzel state). The pressure which is necessary to press water into the space between hydrophobic structures depends on the local contact angle and the size of the spacing. This pressure (capillary pressure) is reciprocal to the size of the spacing and can be deduced from the Young–Laplace equation. Due to the irregular spacing, it can be estimated roughly. Water droplets with a radius <100 nm may be able to intrude between the wax tubules; this curvature corresponds to a Laplace pressure of >1.4 MPa (14 bar). Varanasi et al. (2009) [22] calculated the capillary pressures of hydrophobic test samples with structure dimensions roughly similar to those of the lotus leaf: The capillary pressure for spacing of 5 µm between hydrophobic pillars is 12 kPa (120 mbar); a nanoporous structure with 90 nm pore diameter has a capillary pressure of 1.6 MPa (16 bar). Thus the capillary pressure of the lotus papillae with spacing of ca. 10 µm is sufficient to carry the load of resting or rolling water drops. But impacting raindrops generate higher pressure pulses and can intrude into the space between the papillae. The maximal pressure for a drop impact on a rigid material can be calculated from the ‘water hammer’ equation: 

\[ p_{WH} = 0.2 \rho c v \]

where \( \rho \) is the density of the liquid, \( c \) is the speed of sound in the liquid, and \( v \) is the velocity of the droplet. Varanasi et al. (2009) [22] calculated the ‘water hammer pressure’ of raindrops with a velocity of 3 m/s as 0.9 MPa (9 bar). However, drop impacts on flexible surfaces generate considerably lower pressures [23]. Due to the small spacing between the wax tubules of the lotus leaf and their strong hydrophobicity, their capillary pressure is obviously higher than the impact pressure of raindrops and sufficient to prevent water intrusion. However, it is unproven and hypothetical whether the larger spacing in other waxes causes an intrusion of raindrops. Mechanical damage to the waxes by the impacting drops is a more likely cause for degradation.

Biological models serve as an inspiration for the development of technical superhydrophobic materials [4]. So the question arises whether the lotus leaf presents an optimal architecture for superhydrophobicity. In biological surfaces, several different strategies can be found. The lotus leaf with the largely reduced contact area seems optimal for low adhesion of contaminants and water, observable as small roll-off angles. A disadvantage is the relatively soft wax material, which is too fragile for most technical applications. A different architecture is found on some species with hairy leaf surfaces. The water fern (some species of the genus Salvinia) and Pistia stratiotes leaves retain a relatively thick air layer between hydrophobic hairs when sub-

Figure 13: Model of a wax tubule composed of layers of nonacosan-10-ol and nonacosanediol molecules. The OH-groups (red) occupy additional space so that the dense package is disturbed and the layer is forced into a curvature which leads to the formation of a tubule. The polar OH-groups are hidden in the layer, only the CH₂-groups appear at the surface of the layers and tubules.

**Resistance against environmental stress**

The excellent superhydrophobic properties of the upper side of the lotus leaf are a result of several unique optimizations. The question then arises whether this development has a certain reason or whether it is a ‘freak of nature’. On most plants, the undersides of the leaves show the highest water repellency, or more precisely, those sides which are equipped with stomata. It is obvious that the water repellency serves as a protection to keep the stomata dry [7]. On some species only the cells around the stomata are covered with wax crystals. This is in accordance with the fact that the lotus leaf is epistomatic; it bears the stomata on the upper side, which possesses the higher water repellency. The upper side of a leaf is strongly exposed to environmental impacts such as rainfall and deposition of contaminants. Obviously it is a greater challenge to keep the upper side of a large leaf dry and clean than the underside or the surfaces of vertically growing leaves (grasses etc.). On most plants, the upper sides of the leaves bear no stomata and are more robust than the undersides [21]. Thus the extremely stable and durable water repellency of the lotus leaf, which persists up to the end of its lifetime in autumn, seems to be a successful evolutionary adaptation to the aquatic environment, which led to the placing of the stomata in the upper epidermis and the development of an effective protection through specialized epidermal structures.

For a stable superhydrophobicity – that means the retention of the Cassie state with only partial contact between surface and water – an intrusion of water between the surface structures must be avoided. When the air layer is displaced by water, the water repellency is lost and the surface becomes wet (Wenzel state). The pressure which is necessary to press water into the space between hydrophobic structures depends on the local contact angle and the size of the spacing. This pressure (capillary pressure) is reciprocal to the size of the spacing and can be deduced from the Young–Laplace equation. Due to the irregular spacing, it can be estimated roughly. Water droplets with a radius <100 nm may be able to intrude between the wax tubules; this curvature corresponds to a Laplace pressure of >1.4 MPa (14 bar). Varanasi et al. (2009) [22] calculated the capillary pressures of hydrophobic test samples with structure dimensions roughly similar to those of the lotus leaf: The capillary pressure for spacing of 5 µm between hydrophobic pillars is 12 kPa (120 mbar); a nanoporous structure with 90 nm pore diameter has a capillary pressure of 1.6 MPa (16 bar). Thus the capillary pressure of the lotus papillae with spacing of ca. 10 µm is sufficient to carry the load of resting or rolling water drops. But impacting raindrops generate higher pressure pulses and can intrude into the space between the papillae. The maximal pressure for a drop impact on a rigid material can be calculated from the ‘water hammer’ equation: 

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mersed in water [24]. This provides sufficient buoyancy to avoid long-term submerging. Although superhydrophobic leaves retain an air layer when they are submerged, they are not designed for continuously living under water. All permanently submerged plant surfaces are hydrophilic without hydrophobic waxes [25]. Superhydrophobic surfaces which feature permanent air retention under water are found on animals (some birds, spiders and insects). An outstanding air-retention capability is found, for example, for the aquatic insect *Notonecta glauca* (‘backswimmer’) [26,27]. Here the water repellency is created by a two-level structure consisting of coarse hairs which can hold a relatively thick air layer, and extremely fine hairs which ensure a high capillary pressure. The biopolymers used in these structures have the advantage of a much higher strength than waxes. On the other hand, the plant surfaces have the capability to regenerate damaged or lost waxes.

**Conclusion**

It is true that lotus exhibits outstanding water repellency on the upper side of its leaves. The basis of this behaviour is the hierarchical surface structure. In comparison to other species with a hierarchical structure composed of papillae and wax crystals, the lotus leaf shows special optimization of some of its features. The morphology of the papillae, particularly the small tip radius, minimizes the contact area to water drops but also the area where erosion and damaging of the waxes occurs. The robustness of the papillae ensures protection of the wax crystals between them. The chemical composition of the epicuticular wax with the high content of nonacosanediols leads to the growth of a dense layer of very small wax tubules with a permanently hydrophobic surface. The unique combination of these properties provides the lotus leaves with unrivaled superhydrophobic and self-cleaning properties as an effective protection of the delicate epistomatic surface.

**Experimental**

In addition to the data from the literature, some new examinations provided material for this publication. Plant leaves were taken from the Botanical Gardens, University of Bonn: *Alocasia macrorrhiza* (Elephant ear), *Brassica oleracea* var. gongylodes (Kohlrabi), *Colocasia esculenta* (Taro), *Euphorbia myrsinites*, *Nelumbo nucifera* (Lotus), *Yucca filamentosa*.

For scanning electron microscopy, a Cambridge Stereoscan S200 SEM was used. Depending on the sample properties, different preparation methods were applied: Slowly drying leaves were examined as fresh-hydrated samples (*Euphorbia myrsinites, Alocasia macrorrhiza, Brassica oleracea, Yucca filamentosa*). The other species were critical-point dried or freeze dried (Lotus). Air-dried samples were used for high-magnification imaging of epicuticular waxes. These preparation methods are described in detail elsewhere [28]. The samples for thin sections were prepared following a standard protocol for transmission electron microscopy preparation [29]: fixation in glutaraldehyde, dehydration with acetone, embedding in epoxy resin (Agar Low Viscosity Kit, Plano GmbH, Wetzlar, Germany). Sections of ca. 0.5 µm thickness were stained with ‘Rapid dye’ (Azur II and Methylene blue) for light microscopy.

Wax samples for chemical analyses were isolated mechanically using a ‘cryo-adhesion’-method using triethylene glycol as preparation liquid [30]. The wax was analysed by gas chromatography (HP 5890 series II, Avondale, USA) after ‘derivatization’ by the reaction with N,O-bis(trimethylsilyl)trifluoroacetamide [31]. X-ray powder diffraction diagrams were recorded with a diffractometer PW 1049/10 (Philips, Eindhoven, The Netherlands) [6].

Contact angles of water drops on the sample surfaces were measured with a contact angle measurement system (OCA 30-2, Dataphysics Instruments GmbH, Filderstadt, Germany) using drops of 10 µL. The adhesion of water drops on the samples was measured with a self-developed device by recording force–distance curves while the drop was attached to and detached from the surface with constant velocity. Drops of 10 µL with a diameter of 2.5 mm were attached until the contact area was 0.7 mm in diameter. Then the maximal adhesion forces during retraction were measured and compared. Low adhesion forces correlate with strong water repellency. The robustness of the leaf surface structures was tested by wiping the leaves with a finger, with a vertical force of 1 N and a contact area of 2.5 cm².

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