

DARWIN REVIEW

Brassinosteroid action in flowering plants: a Darwinian perspective

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Abstract

The year 2012 marks the 150th anniversary of the publication of Charles Darwin's first botanical book, on the fertilization of orchids (1862), wherein he described pollen grains and outlined his evolutionary principles with respect to plant research. Five decades later, the growth-promoting effect of extracts of Orchid pollen on coleoptile elongation was documented. These studies led to the discovery of a new class of phytohormones, the brassinosteroids (BRs) that were isolated from rapeseed (*Brassica napus*) pollen. These growth-promoting steroids, which regulate height, fertility, and seed-filling in crop plants such as rice (*Oryza sativa*), also induce stress- and disease resistance in green algae and angiosperms. The origin and current status of BR-research is described here, with reference to BR-action and -signal transduction, and it is shown that modern high-yield rice varieties with erect leaves are deficient in endogenous BRs. Since brassinosteroids induce pathogen resistance in rice plants and hence can suppress rice blast- and bacterial blight-diseases, genetic manipulation of BR-biosynthesis or -perception may be a means to increase crop production. Basic research on BR activity in plants, such as *Arabidopsis* and rice, has the potential to increase crop yields further as part of a 21st century 'green biotech-revolution' that can be traced back to Darwin's classical breeding experiments. It is concluded that 'Nothing in brassinosteroid research makes sense except in the light of Darwinian evolution' and the value of basic science is highlighted, with reference to the genetic engineering of better food crops that may become resistant to a variety of plant diseases.

Key words: Biotechnology, brassinosteroids, Charles Darwin, hormone action, signal transduction, steroidal hormones.

Introduction

Three years after Charles Darwin (1809–1882) had published his most famous book *On the origin of species* (Darwin, 1859), a little-known monograph authored by the British naturalist appeared in print wherein plant–insect interactions were described in unprecedented detail. In the 'Introduction' of this first book on a botanical topic, Darwin outlined his motivation as follows: 'The object ... is to show that the contrivances by which Orchids are fertilized, are as varied and almost as perfect as any of the most beautiful adaptations in the animal kingdom; and ... that these contrivances have for their main object the fertilisation of the flowers with pollen brought by insects from a distinct plant' (Darwin, 1862). In more general terms, Darwin (1862) argued that the beauty of orchids is

not the work of a supernatural 'Creator', who wanted to please humans, but the result of natural selection over thousands of subsequent generations to attract insect cross-pollinators. With reference to the earlier work of Christian K. Sprengel (1750–1816), Darwin argued that 'cross-fertilisation is beneficial to most Orchids' and concluded his first botanical book with the statement, 'It is hardly an exaggeration to say that Nature tells us, in the most emphatic manner, that she abhors perpetual self-fertilisation' (Darwin, 1862).

Throughout 'this little treatise', that was published 150 years ago, Darwin (1862) refers to and describes the pollinium of the flowers of orchids, a male sexual organ that consists of a number of wedge-formed packets of

pollen grains (Fig. 1). In a subsequent botanical work on the same topic, he concluded that, ‘With the majority of species, flowers fertilised with their own pollen yield fewer ... seeds than those fertilised with pollen from another individual or variety’ (Darwin, 1876). Hence, in both books on self- versus cross-fertilization in angiosperms, Darwin (1862, 1876) discussed at length the morphology and function of pollen grains.

Seventy-five years ago, a ‘Pollen Research Project’ was established at the US Horticultural Station in Beltsville, Maryland, USA, with the aim of identifying growth-promoting substances from masses of isolated pollen grains (microspores) obtained from different flowering plants. The logic behind this new research agenda was as follows. When attached to the female part of the flower (stigma) of another plant belonging to the same species, pollen grains germinate, and the male gametophyte (pollen tube) rapidly grows towards the ovary. It was suggested that pollen should contain ‘growth-promoting substances’ that may yield, when isolated and purified, novel regulators of plant development, which may be of practical significance.

In this Review Article, which marks the 150th anniversary of Darwin’s seminal book on orchids and insect fertilization, and the 75th ‘birthday’ of a research agenda that led to the discovery of growth-promoting hormones isolated from pollen grains of maize plants (Mitchell and Whitehead, 1941), the origin and current status of brassinosteroid (BR) research is described. BRs are a class of plant-associated polyhydroxy-steroids that are structurally related to steroid hormones in animals (Ashraf *et al.*, 2010; Hayat and Ahmad, 2011). Our discussion of BR action is restricted to green algae and land plants (embryophytes). This monophyletic lineage of photosynthetic eukaryotes

evolved from Characean-like aquatic ancestors more than 400 million years ago (Niklas and Kutschera, 2009; 2010). Our discussion is focused on the model organism Thale cress (*Arabidopsis thaliana*), as well as crops such as rice, maize, wheat, and potato. Finally, new strategies of crop improvements based on biotechnology are outlined, a research agenda that can be traced back to the classical breeding experiments described by Darwin (1859, 1868, 1872).

Origin and evolution of auxin and brassinosteroid research

Although Sachs (1865, 1882) had summarized the basic facts and rules of ‘experimental botany’, the field of plant developmental physiology was still in its infancy when Charles Darwin, with the help of his third son Francis, wrote his famous book on *The Power of movement in plants* (Darwin, 1881). In this volume, the father-and-son team introduced the grass coleoptile as an experimental system and proposed that the organ tip may send off a substance that causes differential growth when the shoot is illuminated (Kutschera and Briggs, 2009; Kutschera and Niklas, 2009).

The subsequent discovery of auxin (indole-3-acetic acid, IAA) by Fritz W Went (1903–1990) in 1928 and the development of a quantitative ‘Darwinian bioassay’ for phytohormone action, the so-called ‘oat (*Avena*) coleoptile test’, marked the beginning of a research agenda that continues to the present (Went and Thimann, 1937; Kutschera and Niklas, 2007; Moulija and Fournier, 2009; Kutschera *et al.*, 2010a; Deng *et al.*, 2012).

In a seminal study, Laibach and Kornmann (1933) used the classical ‘oat coleoptile bending test’ to resolve another problem—the question whether or not extracts from different plant organs can cause growth. They observed that extracts from orchid pollen grains, which were investigated five decades earlier by Darwin (1862) (Fig. 1), promote cell elongation. Moreover, these researchers documented that the stems of seedlings of dicotyledonous plants bend following unilateral application of pollen extracts. These and other related studies formed the basis of a ‘pollen research project’ at the US Horticultural Station in Beltsville, Maryland, USA, which was established during the second half of the 1930s. Seven decades ago, Mitchell and Whitehead (1941), using pinto bean (*Phaseolus vulgaris*) seedlings as a bioassay-system, showed that extracts from pollen of corn (*Zea mays*) plants, mixed with lanolin, causes a large promotion of internode elongation. This growth stimulation of the first internode of pinto bean plants is caused by a corresponding enhancement in cell enlargement (Fig. 2A, B). Pollen extract-induced promotion of cell (and organ) elongation only occurred in light-grown seedlings with sturdy first internodes. When applied to etiolated stems, no such effect was detected (Mitchell and Whitehead, 1941).

Three decades later, a research team lead by JW Mitchell working at the (re-named) US Department of Agriculture

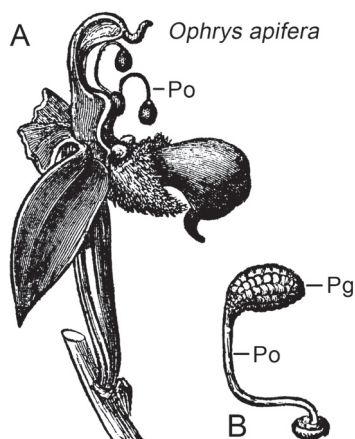


Fig. 1. Side view of the flower of the Bee Orchid (*Ophrys apifera*), with the upper sepal and the two upper petals removed (A). This herbaceous perennial angiosperm belongs to the family Orchidaceae and inhabits semi-dry grasslands throughout Europe. A separate pollinium (Po), i.e. a coherent mass of pollen grains (Pg) produced by one anther, is shown at higher magnification (B) (adapted from Darwin, 1862. *On the various contrivances by which British and foreign orchids are fertilized by insects*. London: John Murray).

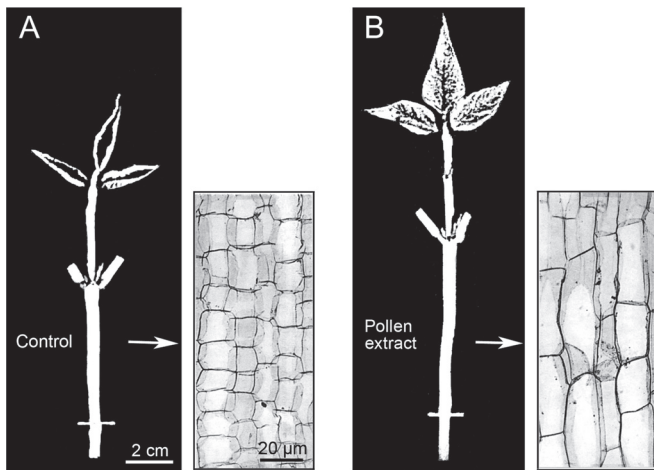


Fig. 2. The discovery of growth-promoting substances from pollen extracts of maize (*Zea mays*) plants using the bean (*Phaseolus vulgaris*) second internode bioassay. Control experiment (A) and effect of pollen extract on stem- and cell elongation in light-grown pinto beans (B) (adapted from Mitchell and Whitehead, 1941). Response of vegetative parts of plants following application of extracts of pollen from *Zea mays*. *Botanical Gazette* **102**, 770–791, with permission from the University of Chicago Press).

(USDA) in Beltsville, Maryland, reported the discovery of ‘new hormones, termed brassins’, extracted from rapeseed (*Brassica napus*) pollen (Fig. 3A, B). In a ‘bean second internode test’, the oily product named ‘brassins’ (defined as a crude lipid extract) induced an enhancement in the rate of organ elongation similar to that observed in the classical experiment depicted in Fig. 2A, B (Mitchell *et al.*, 1970). Nine years later, the structure of the plant growth-promoting substance, extracted from 500 pounds of bee-collected rapeseed pollen, resulting in 10 mg of active crystalline material, was determined and named brassinolide (Fig. 3C). The authors of this study (Grove *et al.*, 1979) documented that externally applied crystalline brassinolide causes a strong growth response in the bean second internode bioassay. Moreover, the chemical structure of brassinolide (BL), and that of the second steroidal plant hormone, castasterone (CS) discovered in 1982, was found to be similar to that of ecdysone, the insect moulting steroid hormone (Yokota, 1997; Thummel and Chory, 2002; Haubrick and Assmann, 2006; Ashraf *et al.*, 2010).

Fifteen years ago, the key question of whether or not brassinolide and related compounds (brassinosteroids) are, in fact, naturally occurring endogenous growth regulators was definitively answered. Four independent studies published in 1996 documented the isolation of brassinolide (BR)-insensitive or -deficient mutants of the model plant *Arabidopsis thaliana*. All BR-mutants exhibited a conspicuous dwarf phenotype, which could be rescued to that of the wild type by the external application of BR solution (Altmann, 1999). Since two of the BR-deficient mutants were caused by lesions in genes that encode steroid biosynthetic enzymes, it was generally accepted that brassinolide and related compounds (brassinosteroids, BRs) are

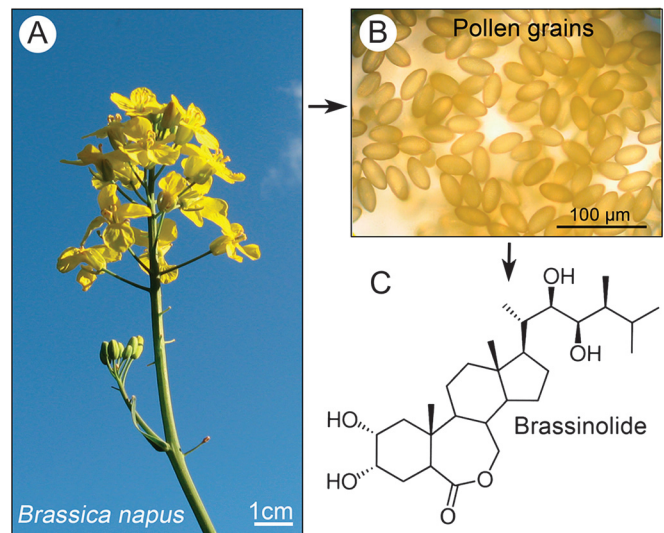


Fig. 3. Flowering stalk of a rapeseed (*Brassica napus*) plant (A), pollen grains isolated from the mature stamens (B) and the structure of the steroidal phytohormone brassinolide (C). In 1979, brassinolide was isolated from bee-collected rape pollen and its chemical structure determined by X-ray analysis.

plant hormones essential for normal growth and development (Bishop, 2003; Ashraf *et al.*, 2010). The subsequent analysis of BR mutants of pea (*Pisum sativum*), tomato (*Lycopersicon esculentum*), and rice (*Oryza sativa*) revealed that BRs, a class of more than 60 structurally different polyhydroxylated sterol derivatives, are a new group of growth-promoting steroidal hormones (Yokota, 1997; Clouse and Sasse, 1998; Khrupach *et al.*, 1999, 2000; Kim and Wang, 2010; Tang *et al.*, 2008, 2010; Clouse, 2011; Hayat and Ahmad, 2011).

Occurrence of brassinosteroids and its evolutionary implications

Over the past four decades, brassinosteroids, a group of polyhydroxy lactones with a common 5 α -cholestane skeleton, have been isolated from a variety of plant species. These growth-promoting steroidal hormones were extracted from pollen grains, anthers, seeds, stems, leaves, roots, flowers, and other organs. In addition, BRs were isolated from insect and crown galls of plants such as the Japanese chestnut (*Castanea crenata*). The highest BR-concentrations were measured in pollen and immature seeds (1–100 $\mu\text{g kg}^{-1}$ fresh mass) (Bajguz and Tretyn, 2003).

Since the discovery of brassinolide (BL) in 1979 (Fig. 3), 69 chemically different brassinosteroids have been isolated from 61 species of embryophytes: 53 angiosperms (12 mono- and 41 dicotyledonous plants), 6 gymnosperms, one pteridophyte (*Equisetum arvense*), and one bryophyte (*Marchantia polymorpha*). In addition, BRs have also been discovered in two species of single-celled green freshwater algae (Chlorophyta) (*Chlorella vulgaris* and *Hydrodictyon reticulatum*), and in the marine brown alga *Cystoseira*

myrica (Hayat and Ahmad, 2011). Since it is well established that green algae are the closest living relatives of land plants (Scherp *et al.*, 2001; Niklas and Kutschera, 2009, 2010), these results indicate that the last common unicellular ancestor of the chlorophytes/embryophytes may have already been capable of synthesizing BRs (Fig. 4A, B).

The relative abundance of the 69 BRs among different groups of embryophytes is variable. Castasterone (CS) is the most widely distributed BR (53 species), followed by brassinolide (BL) (37 species). In the hypocotyl of sunflower (*Helianthus annuus*) seedlings, an organ that has been used as a model system for the elucidation of auxin action and the biophysical basis of stem elongation (Kutschera and Niklas, 2007), CS is the only BR (concentration: *c.* 10 ng g⁻¹ dry mass), whereas the level of BL was close to zero (Kurepin *et al.*, 2012). However, in pollen grains of adult *H. annuus* plants, four BRs (BL, CS, dolichosterone, norcastasterone) have been identified, and a conversion of CS to BL in metabolically active plant cells is documented (Kurepin *et al.*, 2012). It should be noted that the two polyhydroxysteroids CS and BL have been found in *Chlorella vulgaris*, an ancient, aquatic C₃-type photosynthesizer, as well as in the terrestrial, drought-adapted C₄ plant *Zea mays* (source: pollen grains and primary roots of mature sporophytes) (Bajguz, 2011; Hayat and Ahmad, 2011). Interestingly, in cultured *Chlorella* cells (Fig. 4A), the concentrations of BL, auxin (IAA), zeatin, and abscisic acid were found to be similar (*c.* 0.1–0.2 fg per cell). This finding indicates that, in these green freshwater algae, growth and reproduction may be regulated via the same phytohormones as in land plants, such as maize (Fig. 4B) (Bajguz, 2011). Populations of *C. vulgaris* inhabit freshwater ecosystems, the soil, or the moist bark of trees. In these fluctuating habitats, unicellular photosynthetic eukaryotes and other organisms are regularly exposed to toxic substances.

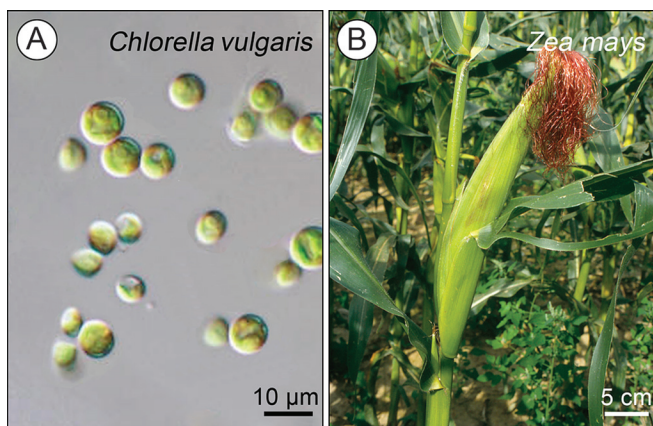


Fig. 4. Brassinosteroids occur in the aquatic unicellular freshwater alga *Chlorella vulgaris* (A) and in the maize plant *Zea mays* (B). This complex multicellular terrestrial organism is characterized by the efficient C₄-mode of photosynthesis, whereas *Chlorella* is a C₃-photosynthesizer. It is likely that BRs were already present in the last common ancestor of these representative members of the evolutionary green lineage.

What role do plant hormones play during stress adaptation in green algae? Laboratory experiments with *Chlorella* cultures yielded the following results. In the presence of heavy metal stress (cadmium, lead, and copper at concentrations of 0.1 mM), the level of BL was not changed, but that of IAA showed a large enhancement, and that of the other hormones increased slightly. Based on these and other data, Bajguz (2011) concluded that BL enhances, in a heavy metal-dependent way, the level of other phytohormones, and thus contributes to the survival of *Chlorella* cells under stressful environmental conditions.

These novel *Chlorella* data document that BRs (as well as IAA, cytokinin, and abscisic acid) represent ubiquitous, phylogenetically ancient phytohormones. These growth regulators may have evolved in the Pre-Cambrian, at a time during the evolution of life on Earth, when the split between uni- and multicellular green algae (which later gave rise to the embryophytes) had not yet occurred (Fig. 4A, B) (Kutschera and Niklas, 2004, 2005; Ross and Reid, 2010).

Brassinosteroid action and signal transduction

As described in the last section, brassinolide, the first and most active BR, was isolated from rape pollen (Fig. 3C). Using the model plant *Arabidopsis thaliana*, a relative of rape (family Cruciferae), and adopting a genetic approach, a number of mutants were identified that are impaired in BR biosynthesis, metabolism, signalling, and response. The most conspicuous feature of BR biosynthesis and signalling in *Arabidopsis* mutants is that they are dwarfs and display a dark-green colour. Some of these BR-minus-mutants exhibit a de-etiolation phenotype when raised in darkness. In other words, these mutated *Arabidopsis* plants perform a kind of ‘photomorphogenic’ development when they grow in the absence of light (Bishop, 2003).

Based on these mutants and sophisticated proteomic analyses, the signal transduction pathway of BRs, from receptor kinases to transcription factors, has been elucidated and described in detail (Deng *et al.*, 2007; Tang *et al.*, 2008, 2010; Kim *et al.*, 2009, 2012; Kim and Wang, 2010; Sun *et al.*, 2010; She *et al.*, 2011; Clouse, 2011; Ye *et al.*, 2011).

In competent cells, BRs are perceived at the outer surface of the plasma membrane by BRI 1, a member of a large group of leucine-rich repeat receptor-like kinases. Thereafter, the incoming BR-signal is transduced from perception by the receptor kinase into the nucleus of the cell, where a small family of transcription factors is activated. These proteins, that bind to specific DNA sequences, regulate, via the recruitment of RNA polymerases, the expression of hundreds of nucleus-encoded genes in a BR-dependent pattern, and hence modulate growth and development. The elucidation of the BR-signal transduction pathway has helped to answer the question of how one hormone can affect a wide spectrum of different developmental processes, inclusive of the regulation of stomatal development (Kim *et al.*, 2012). In addition, these insights have revealed the

basic modes of how BRs interact with other plant hormones, such as auxins and ethylene (Gendron *et al.*, 2008; Kim and Wang, 2010; Clouse, 2011).

In 2010, BR-deficient and -insensitive mutants were not only reported for *Arabidopsis thaliana*, but also for the following crop species: pea, tomato, rice, and barley (*Hordeum vulgare*) (Wang *et al.*, 2010). It should be noted that no 'BR-minus-mutants' have yet been described for maize (*Zea mays*), a major crop species that displays the evolutionary advanced C₄ mode of photosynthesis (Kutschera *et al.*, 2010b; Zhu *et al.*, 2010). However, gibberellin-deficient *Zea mays* mutants have been discovered, and coleoptiles of this species are very sensitive to applied auxin (Kutschera *et al.*, 2010a). Currently, the question as to the role of BRs in maize development is still a matter of debate (Arora *et al.*, 2008; Hartwig *et al.*, 2011).

Recent studies have yielded insights into the possible mode of BR action regarding the regulation of cell elongation. A detailed analysis by Wang *et al.* (2010) revealed that BR-insensitive and -deficient rice mutants (*d61-4* and *brd1-3*, respectively), which are characterized by very short stems and roots, display a number of drastic proteomic changes compared with the wild type. Among the numerous up- or down-regulated proteins, the large decrease in a vacuolar H⁺-ATPase (V-ATPase) was of special significance, because these electrogenic proton pumps on the tonoplast and the membranes of the Golgi-dependent secretory pathway may be important for cell wall biosynthesis. In an accompanying proteomic study, Kutschera *et al.* (2010a) concluded that the cessation of cell elongation in rye coleoptiles is due to the degradation of the V-ATPases. As a result, the sensitivity of the organ towards added auxin is lost. Taken together, these proteomic studies indicate that in the rice BR-minus mutants *d61-4* and *brd1-3*, as well as in mature rye coleoptiles, the intracellular secretory pathway is down-regulated. Thus, wall biosynthesis, a requirement for continued cell elongation to occur, may no longer be possible in these organs (Wang *et al.*, 2010; Kutschera *et al.*, 2010a).

In summary, the analysis of BR-mutants in rice have shown that steroidal phytohormones regulate key parameters such as plant height, fertility, seed filling, and leaf angle (Sakamoto *et al.*, 2006). This last feature, which is of considerable economic importance, has been studied in detail and is therefore the subject of the next section.

Brassinosteroids and the morphology of the rice plant

In his *Origin of species*, and the accompanying monograph on *The Variation of animals and plants under domestication*, Darwin (1859, 1868, 1872) described numerous breeding experiments on crop plants from Europe. However, today, with respect to the number of humans that depend on it, the Asian cereal species rice (*Oryza sativa*) is the most important crop of the world (Khush, 1997, 1999; Kutschera and Kende, 1988). In China, average rice grain yields of

6.4 t ha⁻¹ were obtained between 1987 and 1997, but no further enhancements have been achieved over the past decade (Zhu *et al.*, 2010). In addition to the modulation of the mechanism of photosynthetic carbon dioxide assimilation (C₃, as in rice, versus C₄, as in maize, see Kutschera *et al.*, 2010b; Zhu *et al.*, 2010), the morphology of the adult, photosynthetically active rice plant is a key factor for yield improvements. Rice cultivars with narrow, erect leaves, which increase light absorbency for photosynthesis and nitrogen storage for grain filling, have higher grain yields than varieties with a larger leaf angle (wild types) (Sinclair and Sheehy, 1999). Two independent lines of evidence document that brassinosteroids are involved in these developmental processes that are responsible for erect leaves in rice plants.

Five decades ago, Maeda (1960) reported that explants cut from etiolated rice seedlings display a phytohormone-mediated bending response after incubation in the corresponding test solutions. The excised organ sections consisted of the lamina, the lamina-sheath-joint, and 2 cm of the sheath of the second leaf. In this bioassay, auxins and gibberellin caused a weak response. Based on Maeda's (1960) work, the 'lamina joint inclination assay' for BR action was developed. Thirty years ago, Wada *et al.* (1981) reported that brassinolide and other BRs caused a much stronger lamina inclination than auxins or gibberellins when applied at very low concentrations. A modified version of this specific BR-bioassay is shown in Fig. 5. Rice seedlings were grown for 10 d in closed plastic boxes

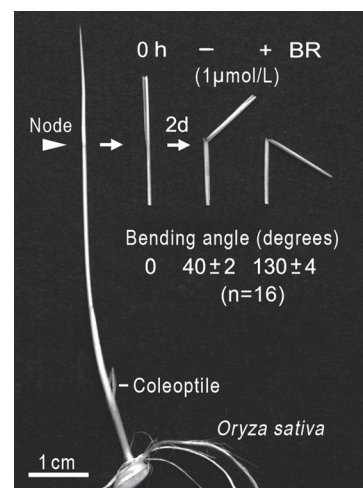


Fig. 5. Brassinolide (BL)-induced promotion of leaf bending in etiolated seedlings of rice (*Oryza sativa*). Thirty-two explants, c. 2.5 cm in length, were cut in the region of the node and collected on distilled water in green safelight. Thereafter, half of the explants (16) were incubated on a shaker either in the absence (–) or presence (+) of BR (1 μmol l⁻¹). After 2 d of incubation (25 °C, darkness), the explants were photographed and the leaf bending angles determined. The results show that in water (control) a significant leaf bending response occurred, which was promoted by a low concentration of BL (data represent means ± sem, n=16).

on moist filter paper (darkness, 25 °C). Shoots of average length (10.5 ± 0.3 cm, mean \pm sem, $n=40$) were cut. Thereafter, explants were excised from the shoots and either incubated in water or BL-solution ($1 \mu\text{mol l}^{-1}$). After 2 d of incubation (25 °C, darkness), the explants were photographed and the leaf bending angles determined. The results show that in water (control) a significant response occurred, which was promoted by low concentrations of BL.

This BR-bioassay documents that leaf angle in rice seedlings is dependent on the intracellular level of brassinosteroids. These phytohormones promote the rate of cell elongation on the upper (adaxial) side of the lamina joint, which causes leaf bending (Wada *et al.*, 1981). Subsequent studies indicated that leaf angle and hence the morphology of the adult, green rice plant is regulated via endogenous brassinosteroids. Three lines of evidence support this conclusion. First, mutants that are deficient in brassinosteroids (*osdwarf 4-1*) have erect leaves (Sakamoto *et al.*, 2006). Second, a rice mutant with an enhanced lamina inclination (*ili1-D*) (Fig. 6) may be hypersensitive to endogenous BR (Zhang *et al.*, 2009). Finally, transgenic rice plants with a reduced level of endogenous BR display the 'erect leaf-phenotype' of brassinosteroid-deficient mutants, such as *osdwarf 4-1* (Wang *et al.*, 2009). Taken together, these studies, combined with the BR-bioassay (Fig. 5), document that rice plants with erect leaves are deficient in endogenous brassinosteroids (Morinaka *et al.*,

2006; Sakamoto *et al.*, 2006; Zhang *et al.*, 2009; Wang *et al.*, 2009, 2010) (Fig. 6).

It follows that two separate research agendas may contribute to the improvement of rice yields: the development of 'evolutionary advanced' C_4 -like photosynthesizers (Kutschera *et al.*, 2010b; Zhu *et al.*, 2010), and the breeding of BR-deficient *Oryza sativa* lines with erect leaves (Morinaka *et al.*, 2006; Sakamoto *et al.*, 2006). A third approach, related to plant disease prevention, is outlined in the next two sections.

Are brassinosteroids activators of endogenous fungicides?

In nature, plants are steadily exposed to the spores of pathogenic (disease-causing) fungi. Under suitable conditions, these propagules germinate and, via wounded regions or the stomata, send their hyphae into the tissues of their host organism. Plant organs infected by pathogenic fungi develop dead spots or die, so that crop yields may become severely reduced. Can BRs protect the plant from these omnipresent, co-evolved fungal pathogens?

In a research paper published by Russian scientists, it is documented that, although higher doses of applied BRs protected crop plants against pathogens such as the oomycete *Phytophthora infestans*, very low concentrations of two brassinosteroids sprayed onto the plants caused a negative effect (Vasyukova *et al.*, 1994). Although these results were not conclusive, they originated a series of related studies that are summarized here. Six years later, more detailed results on this topic were published. Roth *et al.* (2000) extracted a mixture of brassinosteroids from seeds of the flowering plant 'sticky catchfly' (*Lychnis viscaria*), a species from which horticulturists had previously extracted 'plant strengthening substances'. The application of these *L. viscaria*-derived aqueous BR-solutions caused, at low concentrations, an enhanced resistance of crop plants (tomato, cucumber, tobacco) to viral and fungal pathogens of up to +36 %, compared with the controls. Based on biochemical analyses, Roth *et al.* (2000) concluded that BRs mediate (or elicit) the activation of defence mechanisms in the treated crop plants. In addition, Roth *et al.* (2000) analysed whether or not these extracted BRs cause a direct anti-fungal effect. However, in a mycelium growth-assay, no inhibitory action of BRs on the spread of *P. infestans* mycelia, which consist of hyphae, was reported.

In a related experimental analysis, Korableva *et al.* (2002) treated intact potato tubers with the highly active 24-epibrassinolide (EB). This application resulted in a prolonged dormancy period, an enhancement in ethylene production, and a higher content of abscisic acid in the buds. The cytological basis of these EB-effects in the tubers was also elucidated by the authors. They observed a decrease in cell volume and the number of vacuoles per cell. These and other studies revealed that potato plants, as well as other crops, respond, after they were sprayed

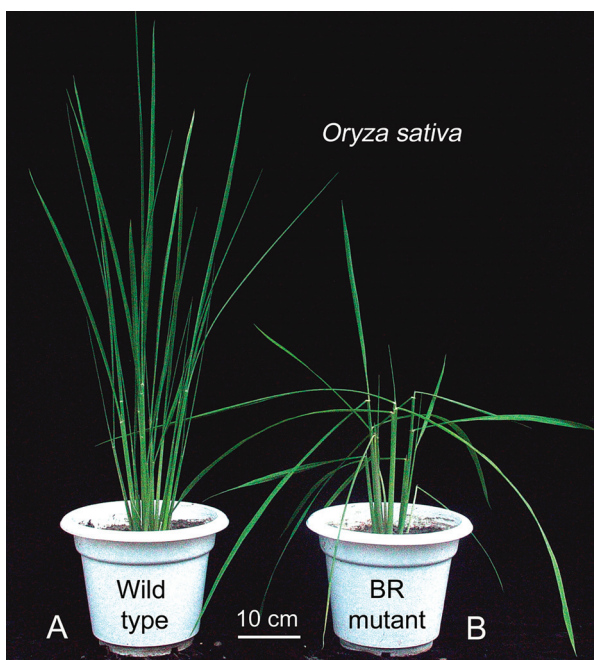


Fig. 6. Photographs of a tillering-stage wild-type rice (*Oryza sativa*) plant (A) and the mutant *ili1-D* (B), grown in soil. Note that, in the rice mutant, the increased lamina inclination phenotype is similar to that caused by treatment of explants from wild-type seedlings with brassinolide (BL) (see Fig. 5) (adapted from a photograph provided by Liying Zhang, Institute of Botany, Chinese Academy of Sciences, Beijing, China, with kind permission from Liying Zhang).

with BR-solutions, with an enhanced resistance towards *P. infestans* infections.

This documented brassinosteroid-mediated plant resistance against fungal pathogens was attributed to enhanced levels of abscisic acid, ethylene, and the accumulation of phenolics and terpenoids (Khripach *et al.*, 1999, 2000; Krishna, 2003; Bajguz and Hayat, 2009; Hayat and Ahmad, 2011). However, more work is required to elucidate the effects of BRs in their postulated role as activators of unknown 'endogenous fungicides' in potato, tomato, and other dicotyledonous plants of economic value. Much more is known about the corresponding effects of BRs in monocots such as rice and wheat. These studies are summarized in the next section.

Brassinosteroids and disease resistance in cereals

In his *Origin of species*, Darwin (1859, 1872) mentioned that mixtures of wheat cultivars are more productive than single varieties grown in monoculture. However, the British naturalist was unable to explain this observation. Decades later it became obvious that mixtures of plants restrict the spread of pathogens, such as fungi and bacteria, and, as a result, of diseases. The most significant disease in rice is caused by a plant-pathogenic fungus (*Magnaporthe grisea*, syn. *M. oryzae*) (Fig. 7A), which can also infect other agriculturally important cereals, such as wheat or barley (*Hordeum vulgare*), and cause severe crop losses (Ou, 1985; Schaffrath and Delventhal, 2011). The devastating symptoms caused by this 'cereal killer' (Talbot, 2003), i.e., dramatic lesions on the leaves, are shown in Fig. 7B.

A decade ago, Zhu *et al.* (2000) documented that 'Darwin's mixture principle', when applied in field trials,

yields positive results. Six rice strains (varieties), planted together across thousands of farms in China, were found to be more resistant to *M. grisea* than single varieties grown in monocultures. Concomitantly with the publication of this remarkable report (Zhu *et al.*, 2000), field trials using brassinosteroids as 'agro-chemicals' led to the conclusion that these phytohormones can protect crop plants, such as rice and wheat, from diseases (Khripach *et al.*, 2000). However, the mechanism of this BR-mediated disease resistance remained unknown.

In a seminal study, Nakashita *et al.* (2003) have shown that, in *Oryza sativa* (Fig. 6), brassinolide (Fig. 3C) induces resistance to rice blast (*M. grisea*) (Fig. 7). In addition, BRs prevent bacterial blight disease caused by epiphytic microbes, such as *Xanthomonas oryzae*. In contrast to most plant-associated bacteria that are commensals causing no harm to their green host organism (Schauer and Kutschera, 2008, 2011), *X. oryzae* is pathogenic and can lead, under certain environmental conditions, to high yield losses (Ou, 1985).

In addition to their studies on rice, Nakashita *et al.* (2003) documented that wild-type tobacco (*Nicotiana tabacum*) plants treated with brassinolide exhibited enhanced resistance to the viral pathogen tobacco mosaic virus, the bacterial pathogen *Pseudomonas syringae*, and the fungus *Oidium* sp. Based on these results, the authors suggested that brassinolide (as well as other BRs) function as part of the innate immune system of land plants (Nakashita *et al.*, 2003). This general concept, which is largely based on work on monocots (rice, wheat), is currently under investigation (Jones and Dangl, 2006; Friebe, 2006; Xia *et al.*, 2008).

How can BRs modulate plant immunity? Innate immune responses in organs, such as stems or leaves, are triggered via the recognition of conserved microbe-associated molecular patterns (flagellin, chitin, etc) at the outer surface of host cells. Recent experiments led to the hypothesis that brassinosteroids regulate plant immunity at multiple levels through signalling steps downstream of the cell surface receptors (Wang, 2012). However, more work is required to corroborate further this speculative molecular model of BR-modulated plant immune responses.

Brassinosteroids and plant tolerance to abiotic stresses

Throughout their ontogeny, green freshwater algae and their evolutionary descendants, the embryophytes (Fig. 4A, B), are constantly exposed to a variety of stresses exerted by other organisms, and by changing environmental conditions. The most important biotic stresses (pathogen infections) have been discussed in the preceding sections. The role of BRs is summarized here with respect to plant tolerance against abiotic stresses (drought, flooding, extreme temperatures, salinity), and toxic substances, such as heavy metals, UV-radiation, and ozone, with reference to Darwin's seminal insights on this topic.

In Chapter III of his *Origin of species*, Darwin (1859, 1872) explained the key term 'struggle for life' in the

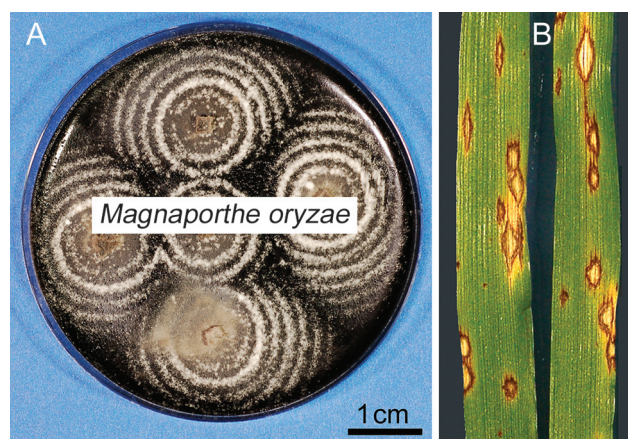


Fig. 7. The phytopathogenic fungus *Magnaporthe oryzae*, cultivated in a Petri dish on nutrient medium (A), and infected leaves of a barley (*Hordeum vulgare*) plant (B). Healthy, green leaves were inoculated with the fungus *M. oryzae*, cultivated, and photographed when the symptoms (red circles) emerged (adapted from Schaffrath and Delventhal, 2011. *Wie wird aus Wirt Nichtwirt? Labor and more* 7, issue 2, 24–27, with permission from succidia AG).

following words: ‘... I use this term in a large and metaphorical sense, including dependence of one being on another, and including (which is more important) not only the life of the individual, but success in leaving progeny. Two canine animals in a time of dearth, may be truly said to struggle with each other which shall get food and live. But a plant on the edge of a desert is said to struggle for life against the drought, though more properly it should be said to be dependent on the moisture.’ Hence, Darwin (1859, 1872) clearly recognized that land plants struggle with and adapt to adverse environmental conditions, such as a lack of water etc. However, only decades later, plant biologists elucidated the biochemical basis of these survival strategies under stressful environmental conditions, notably after the discovery of the phytohormones abscisic acid and the brassinosteroids.

Twenty years ago, a series of preliminary studies indicated that the treatment of crop plants with BRs may enhance the tolerance of these green, sessile organisms against drought and high salt concentrations (Krishna, 2003). However, no details on the mode of BR action were known at that time.

More recently, it was shown that treatment of *Arabidopsis thaliana*, and its relative, rapeseed (*Brassica napus*) (Fig. 3A), with BRs enhances seedling tolerance to drought and cold stress. These effects of exogenously applied BRs were attributable to phytohormone-mediated modulations of the expression of drought- and cold-stress marker genes (Kagale *et al.*, 2007). However, the exact biochemical link between the BR-signal cascade and stress tolerance remained a mystery.

It has been known for many years that stressed land plants can generate a surplus of reactive oxygen species (ROS), which, at high levels, are toxic by causing damage to lipids, proteins, and DNA. These oxygen derivatives comprise both free radical and molecular forms (for instance, hydroxyl radicals or hydrogen peroxide, respectively). A series of detailed studies revealed that low levels of ROS may have regulatory functions in plant stress responses (Gill and Tuteja, 2010), but, until recently, no ROS–BR relationship was known.

Three years ago, it was documented that one ROS-species, hydrogen peroxide (H_2O_2), mediates the transcriptional induction of defence- or antioxidant genes caused by BR. Experiments with cucumber (*Cucumis sativus*) plants revealed that intracellular BR levels were positively correlated with the tolerance of the entire organism to abiotic stresses. Moreover, BR-treatment enhanced NADPH oxidase activity and increased the H_2O_2 -level in the apoplast. Based on these and other findings, it was proposed that perception of BR by receptors leads to the activation of plasma membrane-bound NADPH oxidase, which results in elevated levels of H_2O_2 , a signal that functions to activate stress response-pathways in the plant (Xia *et al.*, 2008). However, it should be noted that this molecular model for the induction of BR-mediated tolerance to abiotic stresses is only a crude scheme of brassinosteroid action in plants that grow under sub-optimal (or unfavourable) environmental condition (Yuan *et al.*, 2010).

The physiological and biochemical processes that occur in different plant species under salt stress (i.e. in the presence of enhanced levels of sodium chloride at concentrations of *c.* 150 mM NaCl), with reference to BR action, have recently been summarized by Ashraf *et al.* (2010). The reader is referred to this excellent presentation of this area of plant research, since this topic is beyond the scope of the present article.

In summary, the results discussed above indicate that in their ongoing ‘struggle for life’ under unfavourable environmental conditions (Darwin, 1859, 1872), land plants are capable of survival via BR-mediated anti-stress-responses. However, other phytohormones, such as abscisic acid (ABA), salicylic acid (SA), ethylene etc are also important signalling molecules during the plant’s struggle to withstand these abiotic stresses (Ashraf *et al.*, 2010; Hayat and Ahmad, 2011).

Brassinosteroids and the second green biotech revolution

During the 1960s, the so-called ‘green revolution’ technology was developed. At that time, conventional plant breeding and the use of fertilizers helped to increase crop yields according to the demand of the growing world population. Using the Darwinian principles of the creation of variability by hybridization, followed by artificial selection of desirable recombinants with modified phenotypes, crop cultivars with increased yields were produced over numerous generations. These domesticated cultivars had evolved under the direction of plant breeders and led, for instance, to rice varieties with a drastically modified architecture (Fig. 8A, B). Compared with their progenitors (Khush, 1997, 1999), these high-yielding, fertilizer-responding varieties of *Oryza sativa* have erect leaves, more tillers, and a higher harvest index. However, the full yield potential of these crops has not yet been realized, because of the grain losses caused by diseases elicited by fungi, bacteria, and insect pests (Ou, 1985; Khush, 1997, 1999; Heinrichs and Miller, 1991). It is estimated that, in cereal crops such as wheat, rice, and barley, diseases and insects (i.e. biotic stress factors) cause yield losses of up to 25% per year (Huckelhoven and Schweizer, 2011).

Moreover, crop yields are reduced as a consequence of additional abiotic stresses, such as drought or excess water, abnormal temperatures, mineral deficiencies and toxicities etc (Khush, 1997, 1999). As Sakamoto and Matsuoka (2004) have pointed out, the principles of plant biotechnology, notably the modulation of BR-dependent traits, may lead to a second ‘green biotech-revolution’ via the genetic engineering of stress-resistant crops.

Since 1996, it has been known that, without BRs, land plants are tiny, infertile dwarfs, with reduced roots and impaired stress tolerance (Yokata, 1997; Altmann, 1999; Bishop, 2003; Clouse and Sasse, 1998; Haubick and Assmann, 2006; Kim and Wang, 2010). More recently, it was concluded that BRs function as ‘master regulators’ that

co-ordinate plant growth, inclusive of stomatal development (Kim *et al.*, 2012). Hence, BRs are key components in aims to improve the productivity and quality of agricultural products, such as seeds of rice and other cereal species.

According to Divi and Krishna (2009), the practical application of BR research in plant biotechnology has not yet been fully explored. Since BRs control many agronomic traits, such as plant architecture, seed yield, and tolerance to biotic as well as abiotic stresses (drought, salinity etc), the genetic manipulation of BR biosynthesis or perception may lead to further increases in crop yields. Using these new techniques, the quality and amount of plant-based food, fibre, and renewable raw materials may be enhanced, without negative effects on the environment (Bajguz and Hayat, 2009; Divi and Krishna, 2009; Kim and Wang, 2010; Hayat and Ahmad, 2011) (Fig. 8C).

Conclusions: A Darwinian view of brassinosteroid research

It is well known that the concept of ‘descent with slow and slight successive modifications (principle of evolution)’ by ‘means of natural selection’ (Darwin, 1859, 1872) was, at

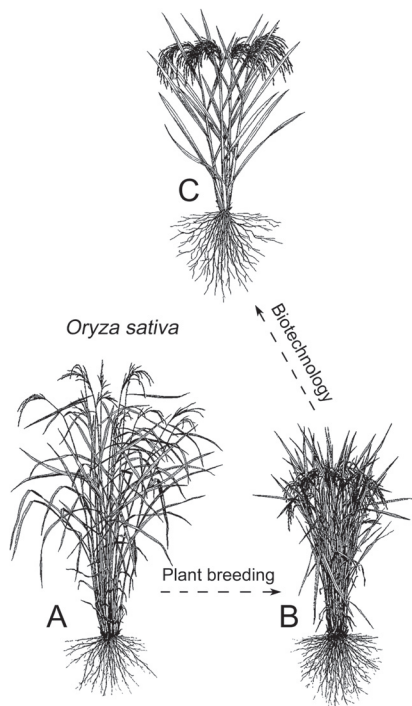


Fig. 8. The evolution of modern rice (*Oryza sativa*) cultivars that originated from wild-type like plants. Conventional hybridization and artificial selection led, over many generations, from a wild-type plant (A) to an improved high-yielding high-tillering rice variety with a different phenotype (B). Using the tools of modern biotechnology, rice breeders aim to produce a low-tillering, disease- and stress-resistant ideotype with a higher harvest index than their progenitors (C) (adapted from G S Khush 1999, Green revolution: preparing for the 21st century. *Genome* **42**, 646-655. © 2008 Canadian Science Publishing or its licensors. Reproduction with permission).

least in part, deduced on the basis of breeding experiments (Fig. 8A, B). In a little-known supplementary two-volume ‘species book’, Darwin (1868) summarized a large number of facts and data concerning animal and plant breeding under domestication. Thomas Henry Huxley (1825–1895), who was also known as ‘Darwin’s Bulldog’, pointed out in his review of the first edition of the *Origin* that ‘all species have been produced by the development of varieties from common stocks ... into new species, by the process of natural selection, which process is essentially identical with that artificial selection by which man has originated the races of domestic animals’ (Huxley, 1860). Hence, evolutionary biology emerged, at least in part, on the basis of breeding experiments carried out with economically important organisms (Hill and Kirkpatrick, 2010). Today, the evolutionary sciences are diverse disciplines of both theoretical and practical significance (Kutschera and Niklas, 2004; Kutschera, 2008, 2009). Hence, basic research and applied science (or technology) are interrelated agendas: New discoveries generated as a result of pure curiosity of the researcher often led to unpredictable practical applications (Fig. 9).

With respect to brassinosteroid research, it should be remembered that the insect/flowering plant interaction that Darwin (1862) analysed in detail was employed decades later, when the BRs were discovered. The new steroidal lactone called brassinolide was isolated from large quantities of rape pollen that had been collected by bees (Grove *et al.*, 1979) (Fig. 3B). In a speculative article, Maugh (1981) suggested that these new steroidal plant hormones, which were of considerable theoretical significance for developmental physiologists, may ‘promise larger crops’. Three decades later, we know that this prediction was correct.

In this article, novel facts from a variety of research areas have been summarized that led us to conclude that ‘Nothing in brassinosteroid research makes sense except in the light of Darwinian evolution’. Data were presented suggesting that plant traits that are regulated via the intracellular level

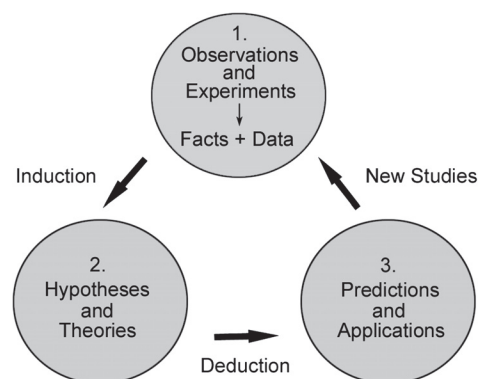


Fig. 9. Scheme depicting the cyclical method of induction–deduction in the natural sciences, with respect to basic research and practical applications. Charles Darwin was an eminent 19th century scientist who applied these principles to a variety of research agendas that continue to the present (for instance, studies on brassinosteroid action in green algae and land plants).

of BRs, such as the erect leaf phenotype in rice, may become a primary target for genetic engineering of novel food crops (Figs 8, 9). Thus, breeding experiments that led to Darwin's great theoretical insights may permit, in the course of the 21st-century 'green biotech-revolution', the generation of larger, disease-resistant high-quality crops. Since the world population is still growing, and 'much more crop production will probably be needed to guarantee future food security' (Foley *et al.*, 2011), these novel plants will be of significant economic importance and may contribute to the well-being of mankind on all continents of planet Earth.

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