Phytochemistry 69 (2008) 2655–2667

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Review

Primary or secondary? Versatile nitrilases in plant metabolism

Markus Piotrowski *

Department of Plant Physiology, Ruhr-Universität Bochum, Universitätsstrasse 150, 44801 Bochum, Germany

ARTICLE INFO

Article history:
Received 25 July 2008
Accepted 26 August 2008
Available online 6 October 2008

Keywords:
Nitrilase
Cyanide detoxification
Cyanogenic glycosides
Glucosinolates
Auxin biosynthesis
Arabidopsis thaliana
Sorghum bicolor
Zea mays

ABSTRACT

The potential of plant nitrilases to convert indole-3-acetonitrile into the plant growth hormone indole-3-acetic acid has earned them the interim title of "key enzyme in auxin biosynthesis". Although not widely recognized, this view has changed considerably in the last few years. Recent work on plant nitrilases has shown them to be involved in the process of cyanide detoxification, in the catabolism of cyanogenic glycosides and presumably in the catabolism of glucosinolates. All plants possess at least one nitrilase that is homologous to the nitrilase 4 isoform of *Arabidopsis thaliana*. The general function of these nitrilases lies in the process of cyanide detoxification, in which they convert the intermediate detoxification product β-cyanoalanine into asparagine, aspartic acid and ammonia. Cyanide is a metabolic by-product in biosynthesis of the plant hormone ethylene, but it may also be released from cyanogenic glycosides, which are present in a large number of plants. In *Sorghum bicolor*, an additional nitrilase isoform has been identified, which can directly use a catabolic intermediate of the cyanogenic glycoside dhurrin, thus enabling the plant to metabolize its cyanogenic glycoside without releasing cyanide. In the Brassicaceae, a family of nitrilases has evolved, the members of which are able to hydrolyze catabolic products of glucosinolates, the predominant secondary metabolites of these plants. Thus, the general theme of nitrilase function in plants is detoxification and nitrogen recycling, since the valuable nitrogen of the nitrile group is recovered in the useful metabolites asparagine or ammonia. Taken together, a picture emerges in which plant nitrilases have versatile functions in plant metabolism, whereas their importance for auxin biosynthesis seems to be minor.

Contents

1. Introduction

Nitrilases (nitrile aminohydrolases, E.C. 3.5.1.x) are enzymes that catalyze the hydrolytic cleavage of nitriles (organic cyanides) into their corresponding carboxylic acids and ammonia (Fig. 1A).

* Tel.: +49 (0) 234 3224290; fax: +49 (0) 234 3214187.
E-mail address: Markus.Piotrowski@ruhr-uni-bochum.de.

0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.08.020

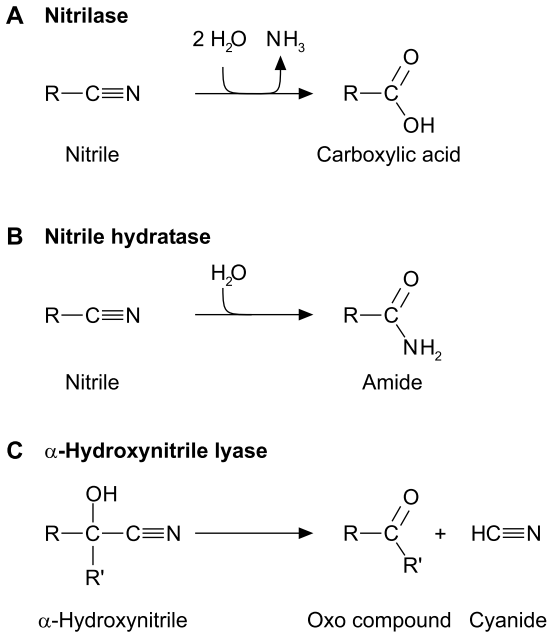

Fig. 1. Nitrile-converting enzymes.

They form a group of homologous enzymes that are widely distributed in (but not restricted to) bacteria, fungi and plants. Nitrilases form one branch of a much larger superfamily known as C–N hydrolases (Bork and Koonin, 1994) or the nitrilase superfamily (Pace and Brenner, 2001). In the older literature, the term "nitrilase" is sometimes also applied to other nitrile-converting enzymes, i.e., nitrile hydratases (E.C. 4.2.1.84) and hydroxynitrile lyases (E.C. 4.1.2.x). Nitrile hydratases are bacterial metallo enzymes consisting of α/β subunits that catalyze the conversion of nitriles into their corresponding amides (Fig. 1B) (Banerjee et al., 2002; Kobayashi and Shimizu, 2000). The resulting amides may then be further hydrolyzed to carboxylic acids by bacterial amidases. Nitrile hydratases are of interest for the industrial biotransformation of nitriles into amides and have been successfully applied to the production of acrylamide and nicotinamide (Yamada et al., 2001). Hydroxynitrile lyases, on the other hand, are involved in the wound-dependent release of cyanide from cyanogenic glycosides in plants, where they catalyze the cleavage of α-hydroxynitriles to cyanide and an oxo compound (aldehyde or ketone, Fig. 1C) (Wajant and Effenberger, 1996). They are of different phylogenetic origins and thus seem to have evolved several times independently (Purkarthofer et al., 2007). Their common name "oxynitrilase" represents one of the earliest usages of the term "nitrilase" in the scientific literature (Rosenthaler, 1908).

Nitrilases, nitrile hydratases and hydroxynitrile lyases are not phylogenetically related to each other. However, nitrilases are closely related to fungal cyanide hydratases and bacterial cyanide dihydratases, which convert cyanide to formamide or formic acid and ammonia, respectively (for review see O'Reilly and Turner (2003)).

2. History of nitrilase research

2.1. The pre-cloning years

The history of nitrilase research is tightly connected to the plant growth hormone (auxin) indole-3-acetic acid (IAA). Its beginning may be dated to 1952, when Jones and colleagues purified 900 mg of a "new" auxin from 500 kg of cabbage and elucidated its structure as indole-3-acetonitrile (IAN). It had already been described before that nitrile derivatives of other auxins (e.g., 1-naphthaleneacetonitrile and phenylacetonitrile) also had auxin activity, which was explained by a possible conversion of these nitriles into their active carboxylic acids within the cell (Steiner, 1948; Zimmerman and Wilcoxon, 1935). Most interestingly, IAN proved to be 5–10 times more active than the original auxin IAA in promoting elongation growth of oat (*Avena sativa*) coleoptiles (Bentley and Housley, 1952; Thimann, 1953), suggesting that the effect of IAN was not due to its conversion to IAA. However, IAN was inactive or only slightly active in a number of different bioassays with other plants, in which IAA consistently gave positive results (Bentley and Bickle, 1952; Jones et al., 1952; Thimann, 1953). Because of these data, Thimann suggested that conversion of IAN to IAA is nevertheless the reason for the auxin activity of IAN; thus, only species that carry out this reaction effectively would be sensitive to IAN. Indeed, he could show that IAN-sensitive oat coleoptiles converted IAN into a compound that had auxin activity on IAN-insensitive pea stems, and he identified this compound as IAA (Stowe and Thimann, 1954; Thimann, 1953). The higher activity of IAN on oat coleoptiles in comparison to IAA could be explained by a 2 to 10 times greater uptake of the more lipophilic IAN into the coleoptile tissue (Poole and Thimann, 1964). Thimann continued his experiments using barley (*Hordeum vulgare*), which, like oat, had high IAN-hydrolyzing activity. The activity in the barley extract was soluble, did not require oxygen, could be precipitated with ammonium sulfate or acetone and was destroyed after boiling the extract (Thimann and Mahadevan, 1958). Thus, the activity was assigned to a soluble enzyme that catalyzes the hydrolysis of IAN and was therefore entitled "indolacetonitrilase". This was changed to "nitrilase" when it was shown that the barley enzyme accepted many other nitriles as substrates and quite a few of them were hydrolyzed with higher velocities than IAN (Mahadevan and Thimann, 1964). In a survey with 29 plant species covering 21 families, IAN-hydrolyzing nitrilase activity was observed in only 10 plants from the families Brassicaceae, Poaceae and Musaceae (Thimann and Mahadevan, 1964). The first attempts to purify the barley nitrilase resulted in a moderate, 29-fold enrichment, but the final preparation was not homogeneous with respect to protein composition.

Between 1964 and 1992, little work was published on plant nitrilases. Nitrilase activity was enriched from Chinese cabbage (28-fold) and swede (Rausch et al., 1981; Rausch and Hilgenberg, 1980). Like the barley nitrilase, both enzyme preparations showed higher maximum activities with 3-cyanopyridine than with IAN, while the K_M values for IAN were about 0.5 mM, approx. 10 times higher than that measured for the barley enzyme. Finally, in 1993, a 3-phenylpropionitrile-hydrolysing nitrilase was purified to apparent homogeneity from seedlings of *Brassica napus* (Bestwick et al., 1993). The three-step purification procedure required an enrichment of only 15-fold to obtain a homogenous preparation, indicating a high concentration of the enzyme in rape seedlings. However, at that time, nitrilases from bacteria and plants had already been cloned and expressed as recombinant proteins in *Escherichia coli* (Bartling et al., 1992; Kobayashi et al., 1992a, 1992b; Stalker and McBride, 1987), which may be the reason that this enzyme was not studied in more detail afterwards.

2.2. Cloning the first nitrilases

Although little progress was made in research on plant nitrilases in the 1970s and 1980s, research on microbial nitrilases progressed well in that time. Among others, the bacterium *Klebsiella ozaenae* was isolated and shown to be able to metabolize the herbicide bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) due to the presence of a specific nitrilase (McBride et al., 1986). The gene for this nitrilase was located on a plasmid, which could be transferred

to *E. coli* resulting in heterologous expression of the enzyme (Stalker and McBride, 1987). The gene locus could be narrowed down to a DNA fragment of 1.2 kbp, which allowed determination of its complete coding sequence by DNA sequencing (Stalker et al., 1988a). Transferring the bromoxynil nitrilase to tobacco conferred resistance against the herbicide to these plants (Stalker et al., 1988b) and transgenic, bromoxynil resistant cotton and canola that express this nitrilase have been produced and used commercially.

The first plant nitrilase was cloned by Bartling and colleagues in 1992 in an attempt to identify plasma membrane localized proteins from plants. They screened a cDNA expression library of *Arabidopsis thaliana* with an antibody raised against purified plasma membranes of this plant. Among a number of clones, one that showed homology to the published nitrilase sequence from *Klebsiella ozaenae* was identified, and the encoded protein (nitrilase 1, NIT1) was shown to have IAN-hydrolyzing nitrilase activity (Bartling et al., 1992). Re-screening of the cDNA library with a *NIT1* probe led to the identification of a second nitrilase (NIT2) (Bartling et al., 1994). NIT2 and two additional nitrilases of *A. thaliana* (NIT3 and NIT4) were at the same time independently identified by Bartel and Fink by screening cDNA and genomic libraries with a *NIT1* probe (Bartel and Fink, 1994). Thus, all four nitrilases encoded by the Arabidopsis genome had been identified. The *NIT1*, *NIT2* and *NIT3* genes were found to be tandemly arranged in the order *NIT2*/*NIT1*/*NIT3* on chromosome 3, while *NIT4* was found to be localized on chromosome 5 (Bartel and Fink, 1994; Hillebrand et al., 1996, 1998). Beyond Arabidopsis, full or partial nitrilase sequences were identified shortly afterwards from Chinese cabbage, *Brassica oleracea*, tobacco and rice (Bischoff et al., 1995; Chiba et al., 1999; Grsic et al., 1999; Tsunoda and Yamaguchi, 1995).

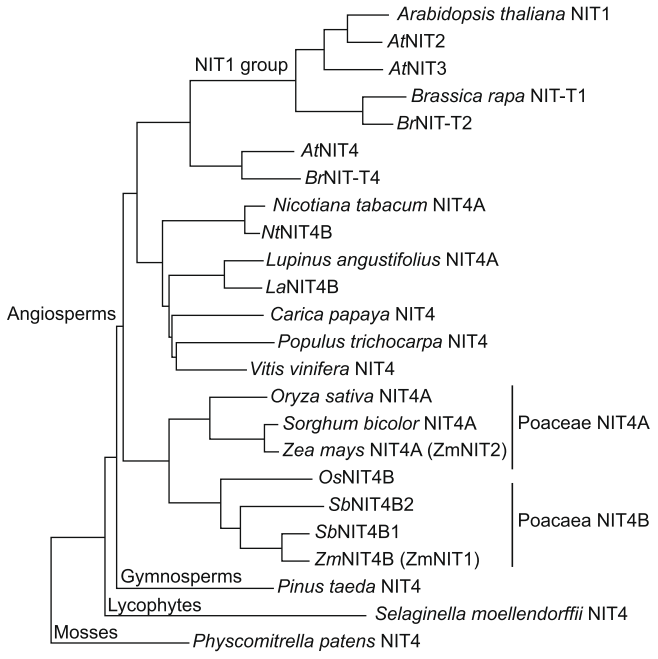

Fig. 2. Distribution of plant nitrilases within the plant kingdom. A neighbor-joining phylogenetic analysis was performed with selected sequences of plant nitrilases covering important taxonomic ranks, nitrilases from plants with published full-genome sequences and nitrilases that have been functionally characterized.

3. Phylogeny of plant nitrilases

Thimann and Mahadevan stated in 1964 that "[nitrilase] is not common in the plant kingdom" because they could detect nitrilase activity in only 10 of 29 plants tested. However, since they only tested for enzyme activity in green leaves using the substrate IAN, they would have missed nitrilases that are expressed in different tissues or at different developmental stages and nitrilases that prefer other substrates over IAN. Rausch and Hilgenberg (1980), for example, found no nitrilase activity in crude extracts of Chinese cabbage with IAN as a substrate, but they were successful when using 3-cyanopyridine. Indeed, IAN is a bad substrate for most plant nitrilases (see below). Today, with the complete and nearly complete genome sequences of several plant species at hand and access to millions of plant EST and cDNA sequences covering a wide range of additional plant species, it is clear that nitrilases are ubiquitous in the plant kingdom (Fig. 2). Nitrilase sequences are known not only from both monocots and dicots of the angiosperms and from gymnosperms but also from the moss *Physcomitrella patens*. Thus, possession of nitrilase genes is the ancient state in higher plants, and they may exist in all land plants. In contrast, nitrilases seem to be absent from algae. It should be noted that BLAST searches in several databases reveal hits to sequences from algae that are annotated as, for example, "nitrilase-like protein" or "nitrilase-superfamily member"; these are, however, orthologs of other members of the C–N-hydrolase superfamily, but not nitrilases.

All plant nitrilase genes known so far consist of five exons with strictly conserved splice positions and intron phases (Table 1). The internal exons 2, 3 and 4 are highly conserved in length, while the flanking exons 1 and 5, encoding the N- and C-termini of the

Table 1
Structure of selected nitrilase genes

	Length (nt)								
	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Intron 3	Exon 4	Intron 4	Exon 5
*At*NIT1	130	92	180	629	294	93	282	86	155
*At*NIT2	109	71	180	547	294	92	282	92	155
*At*NIT3	130	95	180	752	294	83	282	104	155
*At*NIT4	163	81	180	1030	294	99	282	86	149
*La*NIT4A	142	282	180	673	294	103	282	702	152
*La*NIT4B	145	267	180	615	294	91	282	765	152
*Os*NIT4A	147	1018	192	908	294	232	282	149	173
*Os*NIT4B	148	541	180	571	294	253	282	222	170
*Pp*NIT4	133	134	180	179	294	543	285	186	152
*Sb*NIT4A	160	1140	192	557	294	168	282	135	167
*Sb*NIT4B1	145	932	180	929	294	114	282	456	146
*Sb*NIT4B2	154	1911	180	112	294	143	282	228	179
*Zm*NIT4A	145	3983	192	549	294	168	282	123	173
*Zm*NIT4B	145	1640	180	884	294	267	282	121	155

At: *Arabidopsis thaliana*, La: *Lupinus angustifolius*, Os: *Oryza sativa*, Pp: *Physcomitrella patens*, Sb: *Sorghum bicolor*, Zm: *Zea mays*.

proteins, respectively, are more diverse. Intron size may vary considerably, resulting in genes of 1800–5900 bp in length.

Most plant nitrilases are homologs of NIT4 from *A. thaliana* (Fig. 2). Only a few are homologs of Arabidopsis NIT1 (like Arabidopsis NIT2 and NIT3), and these have been found only in the family Brassicaceae so far. The *NIT1* ancestor most likely arose from a duplication of the *NIT4* gene early in the phylogeny of the Brassicaceae (our unpublished data). Also, an early *NIT4* gene duplication in the Poaceae resulted in two *NIT4* paralogs, which are conserved in all Poaceae species analyzed so far (Jenrich et al., 2007). Both gene duplications are in all likelihood the result of ancient polyploidization events, which have been described for both families (Paterson et al., 2004; Schranz and Mitchell-Olds, 2006). We also identified a monophyletic branch of NIT3 sequences nested within the NIT1 branch, while Arabidopsis NIT2 seems to be a direct paralog of Arabidopsis NIT1 (our unpublished data).

Since the *NIT1*, *NIT3* and *NIT4* genes are each of monophyletic origin and the NIT1 and NIT4 enzymes use different substrates (see below), it is recommended that all plant nitrilases are designated NIT1, NIT3 or NIT4 depending on their homology rather than using a simple enumeration in each species. Otherwise, confusion is bound to happen, exemplified by the confusion of Arabidopsis NIT2 (which is a NIT1 homolog) with maize NIT2 (which is a NIT4 homolog) (e.g., D'Haese et al., 2006).

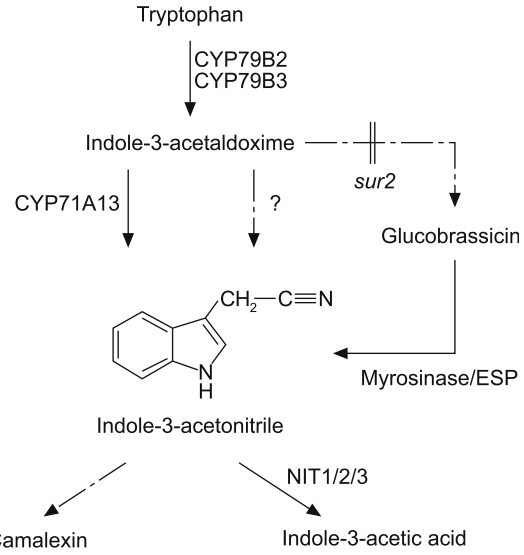

Fig. 3. Possible sources of indole-3-acetonitrile as a precursor for indole-3-acetic acid in *Arabidopsis thaliana*. Interrupted arrows indicate multi-step reactions. ESP: epithiospecifier protein.

4. Physiological functions

4.1. Nitrilases in auxin biosynthesis? Yes and no

As outlined above, nitrilase research was initiated by the observation that IAN, the nitrile derivative of the growth-promoting hormone IAA, was in some instances also active as a growth hormone (Jones et al., 1952). It is therefore no surprise that the interest in plant nitrilases was highly focused on whether these enzymes were able to convert the natural occurring IAN into the auxin IAA. This interest still persists since auxin biosynthesis is not yet fully understood (Pollmann et al., 2006; Woodward and Bartel, 2005).

Presently, only two groups of nitrilases that convert IAN to IAA with significant velocities are known, the NIT1 homologs of the Brassicaceae and the NIT4A homologs of maize (*Zea mays*) and sorghum (*Sorghum bicolor*). Even these enzymes hydrolyze IAN very slowly (~5 nkat [mg protein]$^{-1}$ or 0.2 s^{-1}) (Ishikawa et al., 2007; Jenrich et al., 2007; Kriechbaumer et al., 2007; Osswald et al., 2002; Park et al., 2003), and, more importantly, for each of them, other natural substrates have been identified that were processed at least 100 times faster (Jenrich et al., 2007; Vorwerk et al., 2001) (see also below). Nevertheless, this slow hydrolysis of IAN was still sufficient to produce an auxin-overproduction phenotype in Arabidopsis plants if IAN was added exogenously (Normanly et al., 1997; Schmidt et al., 1996). This phenotype was caused by the activity of NIT1, because *nit1* mutants were resistant to the effects of IAN (Normanly et al., 1997). Also, ectopic expression of the Arabidopsis nitrilases NIT1, NIT2 and NIT3 in tobacco caused sensitivity of the transgenic plants to IAN, while wild-type plants were resistant (Dohmoto et al., 2000b, 2000a; Schmidt et al., 1996). On the other hand, in the absence of exogenous IAN, neither the *nit1* mutant nor Arabidopsis plants that overexpress the various *NIT* genes showed an auxin-related phenotype, and they also had no clear differences in the content of endogenous IAN and IAA (Normanly et al., 1997). Some changes in the content of free IAA and IAN were found in a different set of experiments with Arabidopsis plants that overexpress the 4 *NIT* genes in sense and antisense, but these plants also did not show typical phenotypes related to auxin biosynthesis (Grsic et al., 1998).

Based on the current knowledge, auxin biosynthesis in Arabidopsis proceeds partially via the intermediate indole-3-acetaldoxime (IAOx), which is also an intermediate in the biosynthesis of indole glucosinolates and camalexin in this plant (Fig. 3). It was suggested that IAN is an intermediate in the conversion of IAOx to IAA (reviewed in Ljung et al., 2002; Woodward and Bartel, 2005). However, the *sur*2 mutant, which has reduced flux of IAOx into glucosinolates and increased levels of IAA has rather lower than higher levels of IAN (Barlier et al., 2000). Also, *sur*2/*nit*1 double mutants still have the *sur*2 phenotype, arguing against a role of IAN and nitrilases in IAA biosynthesis from IAOx in *A. thaliana* (Bak et al., 2001). Interestingly, IAOx can be converted to IAN by the cytochrome P450 monooxygenase 71A13, which is involved in biosynthesis of the phytoalexin camalexin and the role of IAN as an intermediate in this pathway has been established (Nafisi et al., 2007). Expression of camalexin biosynthesis genes is highly induced by pathogens at the infection site but remains very low in uninfected plants (Glawischnig, 2007); it therefore seems unlikely that CYP71A13 plays a major role in producing IAN under normal conditions.

The most likely source of free IAN in Arabidopsis (and other Brassicaceae species) is the indole glucosinolate glucobrassicin. Glucobrassicin can be metabolized to IAN, and a positive correlation between glucobrassicin and IAN levels in Arabidopsis has been reported (Müller and Weiler, 2000; Reintanz et al., 2001; Zhao et al., 2002). Thus, in situations where increased release of IAN from glucobrassicin occurs, nitrilases may play a role in IAA biosynthesis. Such a case was reported for Arabidopsis plants suffering from sulfur starvation, in which expression of the *NIT3* gene was strongly induced while at the same time glucosinolate levels dropped (Hirai et al., 2005; Kutz et al., 2002; Nikiforova et al., 2003). Since glucobrassicin was found to be degraded faster than the other glucosinolates in roots, a model was devised in which release of IAN from glucobrassicin and further conversion of IAN to IAA by the induced NIT3 enzyme stimulates root growth and lateral root formation. This would allow the plant to make new soil areas accessible (Kutz et al., 2002).

A second example in which IAA biosynthesis from glucobrassicin-derived IAN by nitrilases may play a role is during infection by *Plasmodiophora brassicae*. Infection with this pathogen results in formation of root galls that are dependent on increased levels of

the two phytohormones cytokinine and IAA. Several publications supported a model in which IAA biosynthesis in root galls proceeds via glucobrassicin, IAN and nitrilases (Grsic et al., 1999; Grsic-Rausch et al., 2000; Ishikawa et al., 2007; Ludwig-Müller et al., 1999; Neuhaus et al., 2000; Rausch et al., 1981). However, a recent report demonstrated that Arabidopsis *cyp79b2/b3* mutants, which are deficient in glucobrassicin and have strongly reduced levels of IAN (Zhao et al., 2002), are not affected in infection rates and root gall development (Siemens et al., 2008).

IAN was also identified as an endogenous compound in maize (Park et al., 2003). Its concentration is an order of magnitude below the levels found in Arabidopsis, and its biosynthetic origin is unknown. Coleoptiles of maize react very weakly, if at all, to exogenously applied IAN, but root growth is clearly inhibited by the same treatment (Kriechbaumer et al., 2007; Thimann, 1953). Maize plants with a mutated *NIT4A* gene are less sensitive to IAN, have delayed root growth shortly after imbibition, and contain significantly lower amounts of total IAA in kernels and roots of young seedlings (75% of wild-type plants). In contrast, mutants of the second maize nitrilase, *Zm*NIT4B, do not differ from the wild-type (Kriechbaumer et al., 2007). These data suggest that *Zm*NIT4A is involved in IAA biosynthesis from IAN within the first few days of germination.

Taken together, nitrilases may contribute to IAA biosynthesis in some plants under certain conditions, but it has become clear that they are not key enzymes in IAA biosynthesis. Nitrilase mutants or nitrilase overexpressors lack severe phenotypes, which would be expected if auxin biosynthesis is significantly disturbed. In addition, IAN is a very poor substrate for plant nitrilases; indeed, it seems that evolution in plants has selected against nitrilases with "better" IAN-hydrolyzing activity, since the NIT1 homologs in Arabidopsis display high activities with a broad range of different nitriles except IAN (see Section 4.4). This makes sense with respect to the Brassicaceae species, in which IAN levels may exceed levels of free IAA by several fold (Müller and Weiler, 2000; Normanly et al., 1993; Zhao et al., 2002). An exception to this rule is the NIT4B2 isoform of sorghum, which, as part of a NIT4A/B2 heterocomplex, shows very high IAN hydrolysis (see Section 4.3). It is unknown if sorghum contains IAN, but the preferred substrate of this enzyme complex is 4-hydroxyphenylacetonitrile, which is also the proposed natural substrate (Jenrich et al., 2007). Interestingly, maize does contain IAN but has (most likely) lost its NIT4B2 isoform, which fits the idea that nitrilases with high IAN-hydrolyzing activity are counterselected in IAN-containing plants.

4.2. Nitrilases in cyanide detoxification

Conversion of IAN by Arabidopsis NIT4 and other NIT4 homologs *in vitro* is hardly, if at all, detectable (e.g., Ishikawa et al., 2007; Piotrowski et al., 2001; Xu et al., 1998). This is in agreement with the observation of Thimann and Mahadevan (1958) that IAN-hydrolyzing nitrilase activity is not common in the plant kingdom, although, as we know today, NIT4 homologs are ubiquitous in higher plants (Fig. 2). Even tobacco plants that overexpress NIT4 isoforms from Arabidopsis and tobacco did not display an IAN-sensitive phenotype (Dohmoto et al., 2000b, 2000a). An examination of the substrate profile of Arabidopsis NIT4 revealed that this enzyme possesses high specificity for the nitrile 3-cyano-L-alanine (β-cyanoalanine) (Piotrowski et al., 2001). Conversion of other substrates was below 1%, and IAN hydrolysis was not detected under the conditions applied. β-Cyanoalanine is an endogenous plant compound that was identified for the first time in *Vicia sativa* (Ressler, 1962). Shortly afterwards, its role as an intermediate in the plant's cyanide metabolism became clear in feeding experiments with $H^{14}CN$, in which the radiolabel of cyanide was recovered in β-cyanoalanine and/or asparagine (Blumenthal-Goldschmidt et al., 1963; Tschiersch, 1964). Subsequent experiments confirmed that β-cyanoalanine is the primary fixation product of cyanide and is further converted to asparagine (Fowden and Bell, 1965; Ressler et al., 1963). Since cyanide was not known to be a general metabolic product in plants, the identification of a widespread cyanide detoxification pathway in plants astonished the scientists of that time (Blumenthal-Goldschmidt et al., 1963; Floss et al., 1965). Possible endogenous sources of cyanide in plants are cyanogenic glycosides, which are present in many plants (approx. 2500 species) but are not ubiquitous (for review see Bak et al. (2006)). However, 20 years later, it was shown that cyanide in higher plants is also produced as a by-product in the biosynthesis of the plant hormone ethylene (Peiser et al., 1984; Pirrung, 1985). In the last step of this biosynthesis, ethylene is released from its immediate precursor 1-aminocyclopropane-1-carboxylic acid with the concomitant formation of cyanoformic acid, which spontaneously decomposes into CO_2 and HCN. Thus, cyanide is produced in equimolar amounts to ethylene, which explains the necessity for a general cyanide detoxification pathway in plants.

The first step in cyanide detoxification is formation of β-cyanoalanine from cyanide and cysteine, which is catalyzed by β-cyanoalanine synthase, a mitochondrial localized enzyme (Fig. 4) (Blumenthal et al., 1968; Hendrickson and Conn, 1969). The second step is conversion of β-cyanoalanine to asparagine. The enzyme catalyzing this step was enriched and characterized from extracts of blue and white lupine (Castric et al., 1972; Galoian et al., 1982). This reaction is formally a nitrile-hydratase reaction (see Fig. 1B); as a consequence, the enzyme was entitled β-cyanoalanine hydratase or β-cyanoalanine hydrolase. Interestingly, it was found that the cloned NIT4 homologs from Arabidopsis and tobacco converted β-cyanoalanine not only to aspartic acid and ammonia, which would be the typical nitrilase reaction (see Fig. 1A), but also to asparagine (Fig. 4) (Piotrowski et al., 2001). Thus, the question arose whether NIT4 and β-cyanoalanine hydratase are identical. Indeed, recombinant NIT4 from Arabidopsis and β-cyanoalanine-hydratase activity of blue lupine extracts were very

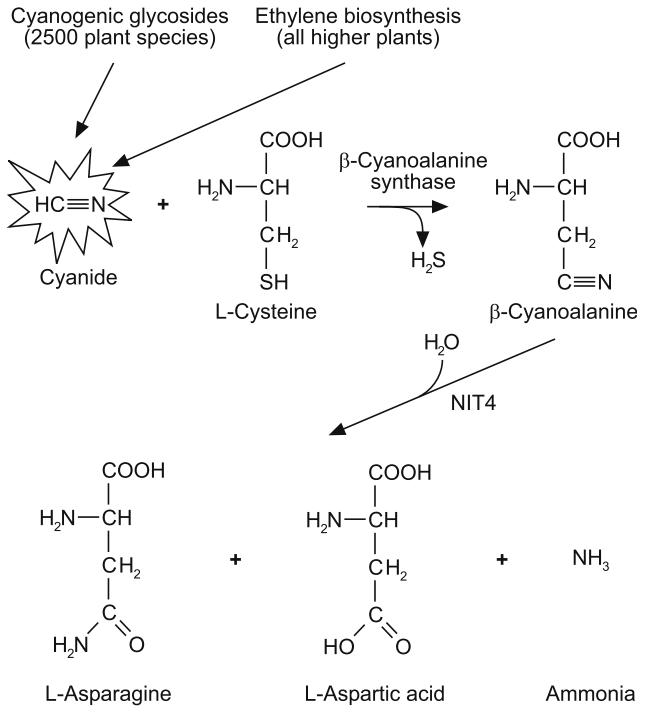

Fig. 4. Generation of cyanide in higher plants and its detoxification and recycling by β-cyanoalanine synthase and nitrilase 4.

similar with respect to substrate specificity, K_M, pH optimum, precipitation in ammonium sulfate and native molecular mass (Castric et al., 1972; Piotrowski et al., 2001). To uncover the enzyme responsible for the β-cyanoalanine hydratase activity *in vivo*, it was purified from blue lupine extracts and identified as a NIT4 homolog (Piotrowski and Volmer, 2006). The recombinant blue lupine NIT4 displays β-cyanoalanine-hydratase activity that is 4 times higher than its nitrilase activity, which may explain why nitrilase activity with β-cyanoalanine had remained unrecognized for so long.

Homologs of NIT4 are ancient within higher plants (Fig. 2), and the phylogenetically oldest enzyme, the NIT4 homolog from the moss *Physcomitrella patens*, already possesses β-cyanoalanine-hydrolyzing activity (our unpublished data). It can therefore be concluded that the primary and general function of plant nitrilases lies in the process of cyanide detoxification. The role of NIT4 in this process may be twofold: One role is detoxification of β-cyanoalanine. Application of this compound to germinating Arabidopsis seeds is toxic, and *nit*4 mutants are more sensitive, while NIT4 overexpressors have increased resistance (our unpublished data). The toxic effect of β-cyanoalanine in plants is very likely due to its structural similarity to the proteinogenic amino acid asparagine because it has been shown to inhibit the asparagine:tRNA synthetase of *Phaseolus aureus* (Lea and Fowden, 1973). However, an Arabidopsis *nit4* mutant, despite having increased β-cyanoalanine levels, is still viable and does not show an aberrant phenotype (our unpublished data); thus, NIT4 is not essential for the plant under laboratory and greenhouse conditions. β-Cyanoalanine may also be conjugated to give the dipeptide γ-glutamyl-β-cyanoalanine. This compound has been found in large quantities in *Vicia sativa*, a plant that lacks NIT4 activity (Castric et al., 1972; Fowden and Bell, 1965; Peiser et al., 1984), but it is also present in Arabidopsis (Watanabe et al., 2008). Nevertheless, the fact that the *NIT4* gene has not been lost during evolution from mosses to higher plants clearly indicates that it plays an important role for the plant. A second function may be the recovery and recycling of nitrogen, which is usually the limiting macronutrient for plant development. Since β-cyanoalanine is not an intermediate in other metabolic processes, the nitrile nitrogen (originally derived from an amino acid) as well as the amino nitrogen would be lost for the plant. Conversion of β-cyanoalanine by NIT4 recovers these nitrogen molecules as ammonia, aspartic acid and asparagine, which are typical plant metabolites.

4.3. Nitrilases in catabolism of cyanogenic glycosides

As mentioned earlier, members of the Poaceae possess at least two *NIT4* genes (designated *NIT4A* and *NIT4B*) due to an ancient polyploidization event in this family (Fig. 2). The isolated NIT4A homologs of maize and sorghum have very low activity with some nitriles (including IAN), but not with β-cyanoalanine (Jenrich et al., 2007; Kriechbaumer et al., 2007; Park et al., 2003). The NIT4B isoforms of these plants are more or less inactive with a range of different nitriles. However, if both isoform types are combined to give NIT4A/B heterocomplexes, high β-cyanoalanine hydrolysis in the range of 100 to 400 nkat (mg protein)$^{-1}$ is restored while conversion of the other substrates remains low (our unpublished data and Jenrich et al., 2007). For the sorghum nitrilases, it has been shown that conversion of β-cyanoalanine in the heterocomplexes occurs at the NIT4A isoform only. Interestingly, sorghum possesses two NIT4B homologs, and the NIT4B2 isoform, when present in the NIT4A/B2 heterocomplex, displays high activity with several other nitriles (except β-cyanoalanine) (Jenrich et al., 2007). One of these substrates is IAN, which is converted by this complex with a velocity that exceeds those of other plant nitrilases dramatically (approx. 300 nkat [mg protein]$^{-1}$). An activity three times higher was observed with the two arylnitriles phenylacetonitrile and 4-hydroxyphenylacetonitrile. The latter compound is an intermediate in the biosynthesis of the cyanogenic glycoside dhurrin, which is present in high amounts in young seedlings of sorghum (Akazawa et al., 1960; Busk and Møller, 2002; Halkier and Møller, 1989). Cyanogenic glycosides are plant defense compounds, which, upon tissue disruption by a herbivore or pathogen, are hydrolyzed by β-glucosidases and α-hydroxynitrile lyases to release an oxo compound and cyanide (for review see Bak et al. (2006), Morant et al. (2008b) and Zagrobelny et al. (2008)). In the case of dhurrin, the intermediate degradation product is 4-hydroxymandelonitrile, which is further converted to 4-hydroxybenzaldehyde and cyanide (Fig. 5). However, dhurrin may also be metabolized to 4-hydroxyphenylacetonitrile without the release of cyanide (Jenrich et al., 2007). Sorghum seedlings catabolize a large amount of its dhurrin beginning 3 days after germination, and it has been suggested that 4-hydroxyphenylacetonitrile is the main intermediate in this pathway, which is further converted by the NIT4A/B2 complex to 4-hydroxyphenylacetic acid and ammonia. Dhurrin constitutes up to 6% of the dry weight of young seedlings and thus represents a significant pool of the plant's total nitrogen. The nitrile pathway of dhurrin catabolism allows for recovery of this nitrogen as ammonia without the intermediate release of the highly toxic cyanide (Fig. 5).

Many Poaceae species possess cyanogenic glycosides; this provokes the question of whether all NIT4B homologs in this family are involved in the endogenous catabolism of these compounds. An ortholog of sorghum NIT4B2 is present in sugarcane, a plant that also contains dhurrin. All available data indicate that maize has lost its NIT4B2 gene (Jenrich et al., 2007) coincident with the fact that maize is not cyanogenic. On the basis of the observations that (i) the sorghum NIT4A/B2 complex shows high activity with

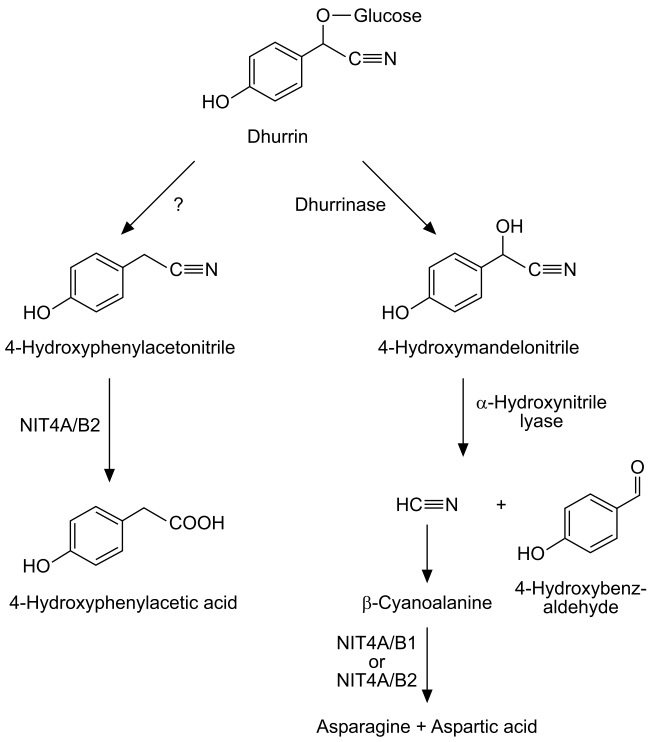

Fig. 5. Dhurrin metabolism in *Sorghum bicolor*. Upon wounding, dhurrin is catabolized by dhurrinase and α-hydroxynitrile lyase to release cyanide as a defense compound. Self-intoxication is prevented by the plants own cyanide detoxification pathway (right). Endogenous turnover of dhurrin may proceed via 4-hydroxyphenylacetonitrile, which is formed by a yet unknown mechanism and is further metabolized by the nitrilase 4A/B2 heterocomplex (left pathway).

several substrates including IAN and (ii) oat and barley have high IAN-hydrolyzing activity, while maize has only very weak IAN-converting activity (Thimann and Mahadevan, 1958), it may be speculated that oat and barley possess a NIT4B isoform capable of converting nitriles other than β-cyanoalanine.

Besides the Poaceae, cyanogenic glycosides are widespread in the plant kingdom. *Lotus japonicus*, for example, contains not only two cyanogenic α-hydroxynitrile glycosides (linamarin and lotaustralin) but also β- and γ-hydroxynitrile glycosides (rhodiocyanoside A and D), which are not cyanogenic but release β- and γ-hydroxynitriles (Bjarnholt et al., 2008; Bjarnholt and Møller, 2008; Forslund et al., 2004; Morant et al., 2008a). We have cloned two nitrilases from *L. japonicus*, one of which is a typical β-cyanoalanine-hydrolyzing enzyme, while the second enzyme shows only very low activity with this substrate (our unpublished data). It remains to be seen whether the second enzyme is involved in the metabolism of either of these hydroxynitrile glycosides. Nitrilases may also be involved in the catabolism of the cyanogenic glycoside prunasin in sweet almonds, which, in contrast to the bitter varieties, degrade their prunasin in the seed (Sanchez-Perez et al., 2008).

4.4. Nitrilases in catabolism of glucosinolates

The fact that plant nitrilases convert other nitriles at much higher rates than IAN was reported quite early (Mahadevan and Thimann, 1964). Rausch and Hilgenberg (1980) and Bestwick and colleagues (1993) used 3-cyanopyridine and 3-phenylpropionitrile to follow nitrilase activity in Chinese cabbage and *Brassica napus*, respectively, indicating that the nitrilases in Brassicaceae are also not specific for IAN. A detailed analysis of the substrate specificities of NIT1 and its homologs NIT2 and NIT3 from Arabidopsis (Vorwerk et al., 2001) as well as of the NIT1 homologs NIT-T1 and NIT-T2 from *Brassica rapa* (Ishikawa et al., 2007) clearly demonstrated that these enzymes display broad substrate profiles, accepting aromatic nitriles like 3-phenylpropionitrile and 4-phenylbutyronitrile but also aliphatic nitriles like 3-butenenitrile (allylcyanide) and heptanenitrile. In comparison to the best substrates found, conversion of IAN by the purified enzymes was below 1% (Ishikawa et al., 2007; Osswald et al., 2002). Also, β-cyanoalanine, the physiological substrate for the NIT4 homologs, was not accepted by the NIT1 homologs, demonstrating a clear neofunctionalization of these enzymes.

Interestingly, the best substrates identified in these experiments were either identical or very similar to the side chains of glucosinolates, which are the main secondary metabolites in these plants (Fig. 6). Glucosinolates are nearly restricted to the order Brassicales and act as plant defense compounds (for a recent review, see Halkier and Gershenzon (2006)). More than 120 different glucosinolates have been identified so far and for Arabidopsis the presence of 23 different glucosinolates in the same plant has been described (Hogge et al., 1988). Upon wounding, glucosinolates are metabolized by the thioglucosidase myrosinase and further break down to give predominantly isothiocyanates, nitriles and epithionitriles, or thiocyanates (Fig. 6A). The nature of the final product depends on several factors, but, *in vivo*, it is mostly regulated by the presence or absence of proteins with modifying properties, like the epithiospecifier protein, the thiocyanate-forming protein or the epithiospecifier-modifier protein (Bernardi et al., 2000; Burow et al., 2007; Foo et al., 2000; Lambrix et al., 2001; Zhang et al., 2006). In addition to the wounding-dependent glucosinolate breakdown, endogenous catabolism of these compounds has been described in several instances, especially during the early stages of seedling development (Brown et al., 2003; James and Rossiter, 1991; Petersen et al., 2002). The mechanism of this endogenous catabolism and its products are, however, unknown so far.

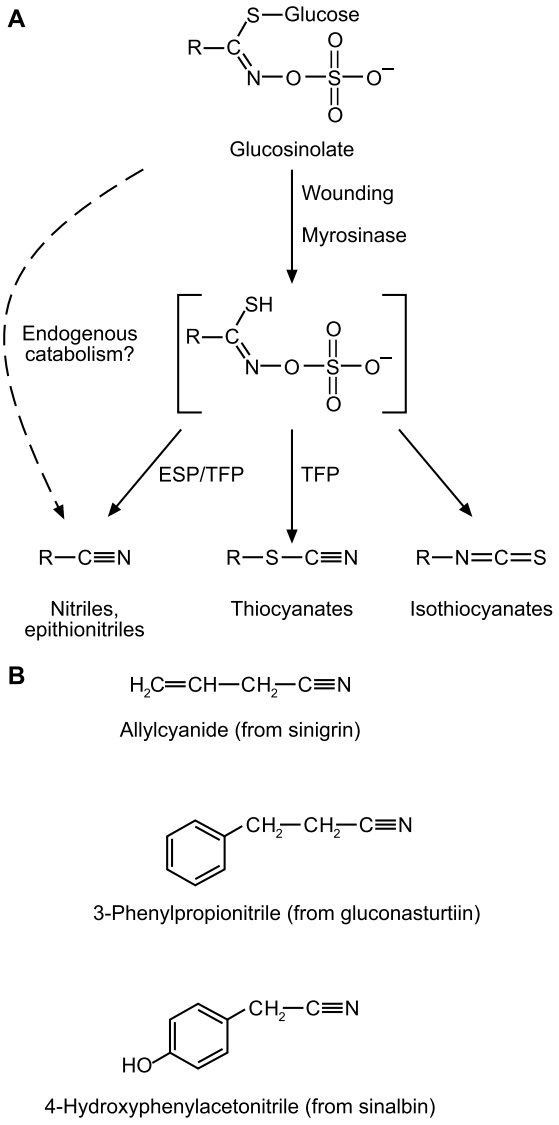

Fig. 6. Glucosinolate metabolism in Brassicales. (A) After wounding, glucosinolates are broken down by the enzyme myrosinase and yield mainly isothiocyanates. In the presence of the modifying proteins epithiospecifier protein (ESP) or thiocyanate-forming protein (TFP), breakdown is directed towards the formation of nitriles and epithionitriles or thiocyanates, respectively. The mechanism and products of the endogenous catabolism (indicated on the left) are not known yet. (B) Examples of glucosinolate-derived nitriles that have been identified as good substrates for nitrilase 1 homologs.

Comparing the properties of the NIT1 homologs and glucosinolates side by side reveals many matching properties: (i) NIT1 homologs are restricted to the glucosinolate-containing Brassicaceae, (ii) NIT1 homologs have broad substrate profiles and glucosinolates have diverse side chains, and (iii) glucosinolates can release nitriles that are among the preferred substrates for the NIT1 enzymes. We have also obtained hints for a co-evolutionary adaptation of NIT1 substrate specificity to the glucosinolates spectrum of their plants (our unpublished data). These are strong indications that the NIT1-homologous enzymes are involved in the catabolism of glucosinolates.

In Arabidopsis, NIT1 is the predominantly expressed isoform (Bartel and Fink, 1994) and may thus be responsible for the general turnover of glucosinolates. In contrast, expression of the *NIT2* gene is strongly induced by wounding and pathogens (Almeras et al., 2003; Bartel and Fink, 1994; Grsic-Rausch et al., 2000; O'Donnell et al., 2003; Stintzi et al., 2001; Weber et al., 2004). In these

situations, an increased release of nitriles from glucosinolates may be expected, and the function of NIT2 may be to detoxify these compounds in the tissues adjacent to the wound site. Wounding also induces aggregation of NIT1 in cells abutting the wound site within minutes, but it is unknown if this aggregation affects nitrilase activity (Cutler and Somerville, 2005).

Glucosinolates contain at least two sulfur atoms and one nitrogen atom and are thus potential storage compounds for these macronutrients. Sulfur deprivation not only results in degradation of glucosinolates in Arabidopsis (e.g., Hirai et al., 2005; Kutz et al., 2002) but also in a strong induction of *NIT3* gene expression (Kutz et al., 2002; Nikiforova et al., 2003). *NIT3* expression is localized to the vascular tissue (Bartel and Fink, 1994; Kutz et al., 2002), which coincides with the localization of the glucosinolate-containing S cells (Koroleva et al., 2000) and the myrosinase-containing myrosin cells (Andreasson et al., 2001; Husebye et al., 2002). Taken together, these data indicate that NIT3 is involved in glucosinolate catabolism under sulfur-limiting conditions. It has also been suggested that NIT3 is involved in auxin biosynthesis under these conditions (see Section 4.1, Kutz et al., 2002). It should be mentioned that the NIT3 homologs from Arabidopsis (Vorwerk et al., 2001) and *Capsella rubella* (our unpublished data) have clearly reduced enzymatic activities in comparison to the respective NIT1 homologs; thus, it is also possible that NIT3 homologs have (a) different, yet unknown, substrate(s) *in vivo*.

5. Cellular localization and posttranslational modifications of plant nitrilases

Nitrilases do not contain predictable transmembrane regions or targeting signals and are thus proposed to be soluble cytoplasmic proteins. However, NIT1 from Arabidopsis was first identified as a putative plasma membrane localized protein by screening a cDNA expression library with antibodies against plasma membranes (Bartling et al., 1992). An independent, nearly identical screen also identified NIT2 as putative plasma membrane protein (Galaud et al., 1999). Plasma membrane localization of NIT1 is supported by some proteomic studies (Alexandersson et al., 2004; Benschop et al., 2007), while others localized NIT1 to the soluble fraction (Dixon et al., 2005; Giavalisco et al., 2005; Sarry et al., 2006) or even to the chloroplast's stroma (Zybailov et al., 2008). From these data, it is hard to judge if NIT1/NIT2 localization in plasma membrane preparations and chloroplast fractions reflects the situation *in vivo* or results from contamination with cytoplasm. A fusion protein consisting of green fluorescent protein (GFP) coupled to NIT1 is soluble in the cytoplasm (and nucleus), but it forms organelle-associated aggregates after wounding (Cutler and Somerville, 2005). Application of the benzonitrile herbicides bromoxynil and chloroxynil had the same effect, suggesting that GFP-NIT1 aggregation near wounding sites may be induced by release of nitriles from glucosinolates.

Arabidopsis NIT2 was found by proteomic studies in soluble fractions as well as in a tonoplast fraction (Sarry et al., 2006; Shimaoka et al., 2004). A tonoplastic localization of NIT2 would be interesting considering that glucosinolates are stored within the vacuole. Arabidopsis NIT3 was also found in the soluble fraction in several proteomic studies (Dixon et al., 2005; Galaud et al., 1999; Sarry et al., 2006; Wienkoop et al., 2004). Notably, NIT3 was identified in trichomes together with proteins involved in methionine metabolism, again indicating a connection between NIT3 and sulfur metabolism (Wienkoop et al., 2004).

Arabidopsis NIT4-GPF fusion proteins are localized in the cytoplasm (and the nucleus, our unpublished data). Interestingly, tobacco NIT4 was found to interact with ethylene-responsive element-binding proteins (EREBPs) in yeast two-hybrid screens (Xu et al., 1998). EREBPs bind to an ethylene-responsive element present in promoters of genes for basic pathogenesis-related proteins (PR proteins). It was suggested that interaction with NIT4 serves to sequester EREBPs in the cytoplasm and signaling by a pathogen would result in dissociation of both proteins and subsequent translocation of the EREBPs into the nucleus to active expression of defense genes (Xu et al., 1998). At that time, the function of NIT4 in cyanide detoxification was not known. Today, it would be interesting to analyze if β-cyanoalanine, which is produced in consequence to pathogen-induced ethylene (and cyanide) biosynthesis, is involved in this process.

So far, naturally occurring post-translational modifications of plant nitrilases have not been described. However, incubation of an Arabidopsis crude extract with biotinylated oxidized glutathione and subsequent purification of the labeled proteins by streptavidin-agarose led to the identification of NIT1 and NIT3, amongst others, as putative glutathionylated proteins (Dixon et al., 2005). It is unknown, whether glutathionylation would occur at the active-site cysteine of the nitrilases and would thus inhibit enzyme function. Incubation of purified recombinant NIT1 with oxidized glutathione did not result in a substantial glutathionylation, and *in vivo* feeding experiments also did not result in the identification of glutathionylated nitrilases (Dixon et al., 2005). Thus, at the moment, it is not clear if glutathionylation is of physiological relevance for plant nitrilases.

6. Biochemistry of plant nitrilases

Nitrilases are phylogenetically related to a large range of other C–N bond hydrolyzing enzymes summarized as the superfamily of C–N hydrolases or the nitrilase superfamily (Bork and Koonin, 1994; Pace and Brenner, 2001). Crystal structures of several members of this family have been solved and led to the identification of a conserved catalytic triad consisting of glutamic acid, lysine and cysteine (Hung et al., 2007; Kimani et al., 2007; Kumaran et al., 2003; Lundgren et al., 2008; Nakai et al., 2000; Pace et al., 2000; Sakai et al., 2004; Wang et al., 2001) (Fig. 7). The Glu residue is thought to act as a general base by accepting the thiol hydrogen of the Cys residue. The activated Cys then carries out a nucleophilic attack on the C–N carbon, forming a covalently bound enzyme–substrate complex. The Lys residue should stabilize a tetrahedral intermediate of the reaction (Nakai et al., 2000). Mahadevan and Thimann had already proposed in 1964 that a thiol group of the nitrilase performs a nucleophilic attack on the nitrile and binds the substrate covalently. Later, such a covalently bound enzyme–substrate complex was verified for a nitrilase from *Rhodococcus* (Stevenson et al., 1990). Kobayashi and colleagues were then the first to identify the essential cysteine residue in two different bacterial nitrilases (Kobayashi et al., 1992a, 1993). Exchange of the catalytically active cysteine to alanine by site-directed mutagenesis in all four Arabidopsis nitrilases also resulted in inactive enzymes (Piotrowski et al., 2001; Vorwerk et al., 2001). The essential nature of the remaining two residues (Glu and Lys) of the catalytic triad has been proved for Arabidopsis NIT4 (our unpublished data).

A proposed intermediate in the conversion of nitriles to their corresponding carboxylic acids is the respective amide. Since hydrolysis of IAN by the barley nitrilase did not give detectable amounts of indoleacetamide and conversion of indoleacetamide by the enzyme was negligible, it was suggested that the intermediates of the reaction remain tightly bound to the enzyme (Thimann and Mahadevan, 1964). In some cases, low amounts of amides were found as a nitrilase product (2–10%), and it was proposed that the reaction proceeds via a tetrahedral intermediate and that amide formation is the result of a premature release of the

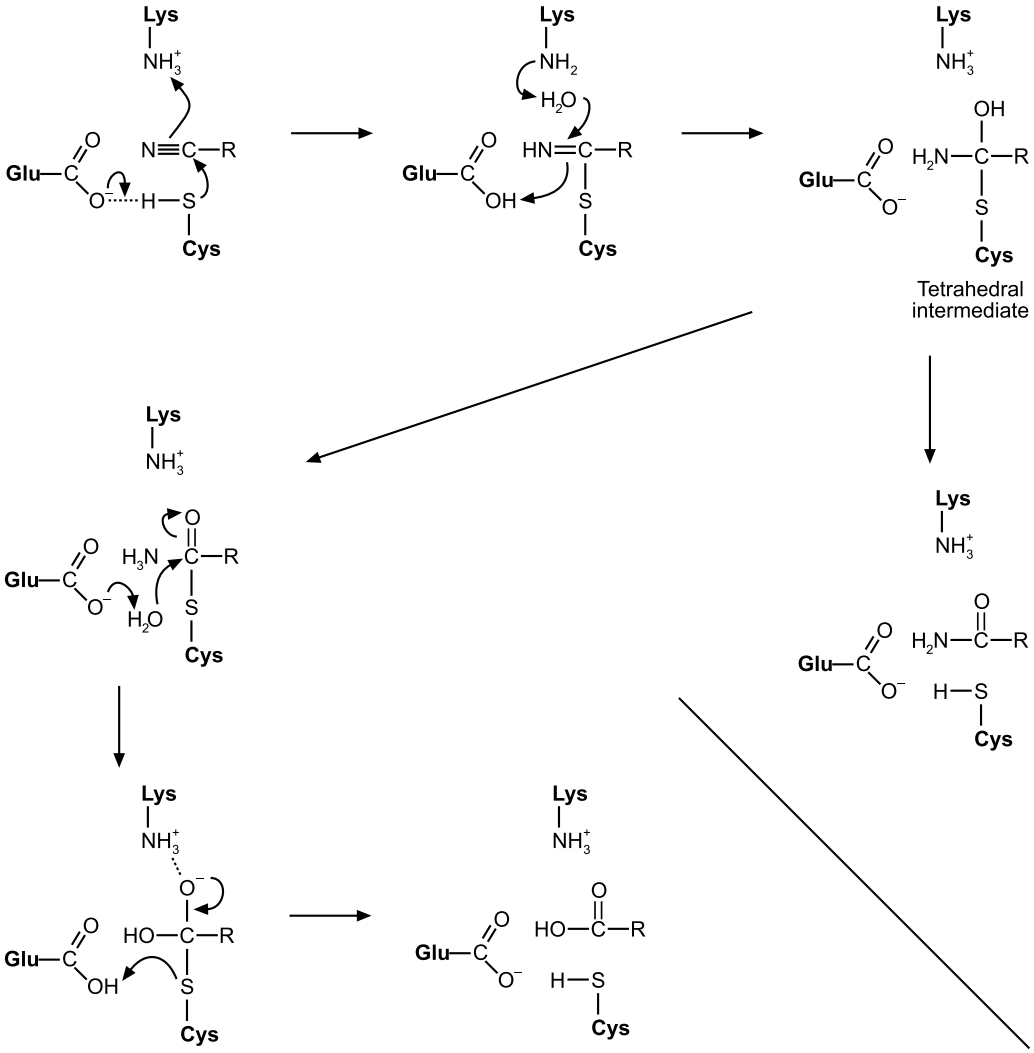

Fig. 7. Proposed mechanism of nitrilases. The three conserved amino acids of the catalytic center (cystein, glutamic acid and lysine) are printed in bold. After formation of the tetrahedral intermediate, the reaction may proceed either to the formation of a carboxylic acid (left) or an amide (right). Modified from Jandhyala et al. (2005).

substrate from the tetrahedral intermediate (Hook and Robinson, 1964; Robinson and Hook, 1964). In striking contrast, hydrolysis of β-cyanoalanine by NIT4 homologs results in the formation of 50–80% of the amide, which is asparagine (Jenrich et al., 2007; Kriechbaumer et al., 2007; Piotrowski et al., 2001; Piotrowski and Volmer, 2006). Thus, NIT4 homologs were the first nitrilases described that display a higher nitrile-hydratase activity (formation of amide) than nitrilase activity (formation of carboxylic acid plus ammonia). This high nitrile-hydratase activity is not unique to NIT4 homologs but could also be observed, to some extent, for the other three nitrilases from Arabidopsis (Bartel and Fink, 1994), which produce between 15% and 35% indoleacetamide from IAN (Pollmann et al., 2002). It has been shown that formation of amides by Arabidopsis NIT1 is favored by substrates with strong electron-withdrawing substituents, which are thought to stabilize the tetrahedral intermediate (Osswald et al., 2002). This cannot, however, explain why different NIT4 homologs show defined amide/acid ratios of approx. 1:1 (Arabidopsis, tobacco) to 4:1(lupine, sorghum) with the same substrate β-cyanoalanine. We have identified a single amino acid position whose state is responsible for these different ratios; exchange of the different states also switches the amide/acid ratios of the mutated enzymes (our unpublished data). The formation of asparagine from β-cyanoalanine instead of aspartic acid and ammonia could, in a physiological view, make sense, because ammonia is toxic to the cell while asparagine is a typical plant metabolite that also acts as a form of storage and transport for nitrogen. Thus, energy-requiring re-fixation of ammonia into glutamine could be diminished.

Members of the superfamily of C–N hydrolases usually form homomeric complexes ranging from dimers to high-molecular mass complexes with 18 subunits (Hung et al., 2007; Jandhyala et al., 2003; Kimani et al., 2007; Kumaran et al., 2003; Lundgren et al., 2008; Nagasawa et al., 2000; Pace et al., 2000; Piotrowski et al., 2003; Sakai et al., 2004; Sewell et al., 2003; Thuku et al., 2007). Monomers of plant nitrilases have molecular masses of 36 to 39 kDa, but the native enzymes have been found to exist as multimeric complexes (Bestwick et al., 1993; Castric et al., 1972; Piotrowski et al., 2001; Rausch and Hilgenberg, 1980). The native mass of recombinant NIT1 from Arabidopsis was determined by gel-filtration to be ~450 kDa, which would correspond to a dodecamer (Osswald et al., 2002; Xie et al., 2006). Formation of these high-molecular mass complexes seems to be necessary for activity, since a natural "mutant" of a NIT4 homolog, which predominantly forms tetramers, has very little activity with β-cyanoalanine (our unpublished data). Activity could be restored by a single amino acid substitution, and the re-activated enzyme formed multimeric complexes exceeding 440 kDa.

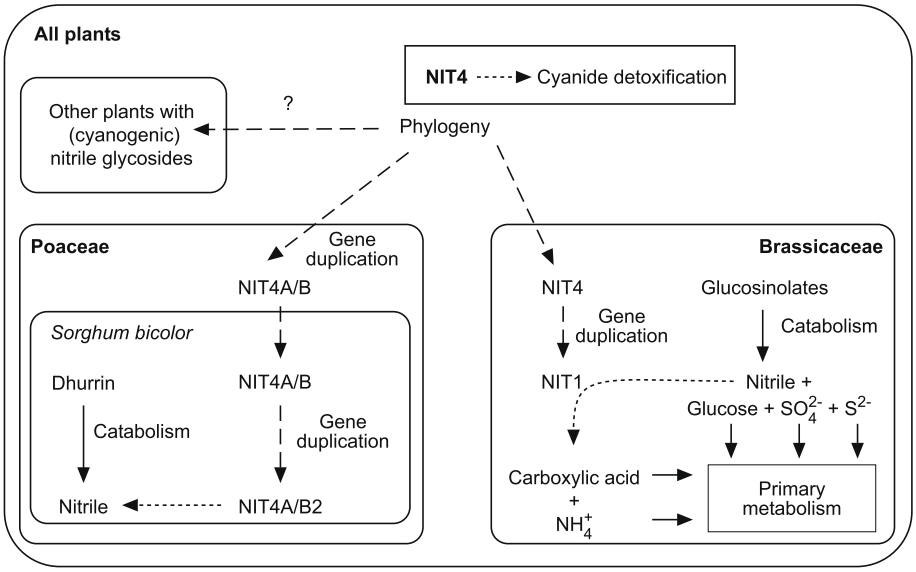

Fig. 8. Evolution of nitrilase function in plants.

An unexpected finding was that the NIT4 homologs of Poaceae species, although they are able to form homomeric complex, were only active upon formation of heterocomplexes of NIT4A and NIT4B isoforms, both present in the same plant (Jenrich et al., 2007; Kriechbaumer et al., 2007). The homomeric enzymes showed little or no activity with different nitriles in the range of 0.5–30 nkat (mg protein)$^{-1}$, while the NIT4A/B heterocomplexes exhibited high activities of up to 900 nkat (mg protein)$^{-1}$ (Jenrich et al., 2007; Park et al., 2003). This principle seems to be conserved within the Poaceae, since the NIT4A and NIT4B isoforms could also form active complexes when they were derived from different species (e.g., NIT4A from rice and NIT4B from barley). Mixtures of the same orthologs from different species, e.g., NIT4A from rice and sorghum, remained inactive. Thus, the possibility and necessity to form heterocomplexes seems to have developed early in the phylogeny of the Poaceae. By site-directed mutagenesis of the catalytically active cysteine in the different isoforms, it could be shown that β-cyanoalanine hydrolysis takes place only in the NIT4A subunit. Sorghum has two NIT4B isoforms, and both can interact with and consequently activate the NIT4A isoform. While the NIT4B1 isoform seems to have no activity, the NIT4B2 enzyme, when present as a NIT4A/B2 complex, also has catalytic activity, but with substrates other than β-cyanoalanine (see Section 4.3). Actually, the NIT4A/B2 complex of sorghum can hydrolyze two different substrates simultaneously (Jenrich et al., 2007).

Having two different isoforms that interact to gain activity seems to complicate things unnecessarily. Even if both isoforms can use different substrates (as in the NIT4A/B2 complex of sorghum) having two different enzymes that are active on their own, like the NIT1 and NIT4 isoforms of Arabidopsis, would provide more flexibility. It has been suggested that the situation in Poaceae is the result of an evolutionary process similar to that described by the duplication–degeneration–complementation model. This model states that two gene copies may accumulate deleterious mutations in different elements as long as both copies are still able to complement one another (Force et al., 1999). The result is that both copies are retained. Thus, the Poaceae NIT4 homologs may have lost their ability to self-activate because they were still able to form active heterocomplexes. Once this situation has been reached, it was not longer possible to lose one of the *NIT4* gene copies without losing all NIT4 activity. One of the *NIT4* genes (*NIT4A*) retained the β-cyanoalanine-hydrolyzing activity while the other copy (*NIT4B*) could neofunctionalize (Jenrich et al., 2007).

7. Summary

Once thought to be key enzymes in auxin biosynthesis, plant nitrilases are now known to fulfill several functions in plant primary and secondary metabolism (Fig. 8). All plants contain one NIT4 homolog, which is involved in the process of cyanide detoxification. At least twice, nitrilases with new functions have evolved from this ancestor, and, in both cases, the new enzymes seem to be involved in endogenous catabolism pathways of secondary metabolites: the NIT4B2 enzyme in sorghum, which is involved in catabolism of the cyanogenic glycoside dhurrin, and the NIT1 homologs, which are very likely involved in catabolism of glucosinolates. Cyanogenic and non-cyanogenic nitrile glycosides are widespread in the plant kingdom, and it remains to be seen whether other plants have independently adapted their nitrilase system for recycling their secondary metabolites.

Acknowledgements

I want to thank the students who, at different stages of their scientific careers, worked in my laboratory and contributed to new insight into the world of plant nitrilases. I am also very grateful to the German Research Foundation for funding my nitrilase projects.

References

Akazawa, T., Miljanich, P., Conn, E.E., 1960. Studies on cyanogenic glycoside of *Sorghum vulgare*. Plant Physiol. 35, 535–538.

Alexandersson, E., Saalbach, G., Larsson, C., Kjellbom, P., 2004. Arabidopsis plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. Plant Cell Physiol. 45, 1543–1556.

Almeras, E., Stolz, S., Vollenweider, S., Reymond, P., Mene-Saffrane, L., Farmer, E.E., 2003. Reactive electrophile species activate defense gene expression in Arabidopsis. Plant J. 34, 205–216.

Andreasson, E., Bolt, J.L., Hoglund, A.S., Rask, L., Meijer, J., 2001. Different myrosinase and idioblast distribution in Arabidopsis and *Brassica napus*. Plant Physiol. 127, 1750–1763.

Bak, S., Paquette, S.M., Morant, M., Morant, A.V., Saito, S., Bjarnholt, N., Zagrobelny, M., Jørgensen, K., Osmani, S., Hamann, T., Simonsen, H.T., Pérez, R.S., van Heeswijck, T.B., Jørgensen, B., Møller, B.L., 2006. Cyanogenic glycosides: a case study for evolution and application of cytochromes P450. Phytochem. Rev. 5, 309–329.

Bak, S., Tax, F.E., Feldmann, K.A., Galbraith, D.W., Feyereisen, R., 2001. CYP83B1, a cytochrome P450 at the metabolic branch point in auxin and indole glucosinolate biosynthesis in Arabidopsis. Plant Cell 13, 101–111.

Banerjee, A., Sharma, R., Banerjee, U.C., 2002. The nitrile-degrading enzymes: current status and future prospects. Appl. Microbiol. Biotechnol. 60, 33–44.

Barlier, I., Kowalczyk, M., Marchant, A., Ljung, K., Bhalerao, R., Bennett, M., Sandberg, G., Bellini, C., 2000. The SUR2 gene of *Arabidopsis thaliana* encodes the cytochrome P450 CYP83B1, a modulator of auxin homeostasis. Proc. Natl. Acad. Sci. USA 97, 14819–14824.

Bartel, B., Fink, G.R., 1994. Differential regulation of an auxin-producing nitrilase gene family in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 91, 6649–6653.

Bartling, D., Seedorf, M., Mithofer, A., Weiler, E.W., 1992. Cloning and expression of an Arabidopsis nitrilase which can convert indole-3-acetonitrile to the plant hormone, indole-3-acetic acid. Eur. J. Biochem. 205, 417–424.

Bartling, D., Seedorf, M., Schmidt, R.C., Weiler, E.W., 1994. Molecular characterization of two cloned nitrilases from *Arabidopsis thaliana*: key enzymes in biosynthesis of the plant hormone indole-3-acetic acid. Proc. Natl. Acad. Sci. USA 91, 6021–6025.

Benschop, J.J., Mohammed, S., O'Flaherty, M., Heck, A.J., Slijper, M., Menke, F.L., 2007. Quantitative phosphoproteomics of early elicitor signaling in Arabidopsis. Mol. Cell. Proteomics 6, 1198–1214.

Bentley, J.A., Bickle, A.S., 1952. Studies on plant growth hormones: II. Further biological properties of 3-indolacetonitrile. J. Exp. Bot 3, 406–423.

Bentley, J.A., Housley, S., 1952. Studies on Plant Growth Hormones: I. Biological activities of 3-indolacetaldehyde and 3-indolacetonitrile. J. Exp. Bot 3, 393–405.

Bernardi, R., Negri, A., Ronchi, S., Palmieri, S., 2000. Isolation of the epithiospecifier protein from oil-rape (*Brassica napus* ssp. *oleifera*) seed and its characterization. FEBS Lett 467, 296–298.

Bestwick, L.A., Gronning, L.M., James, D.C., Bones, A., Rossiter, J.T., 1993. Purification and characterization of a nitrilase from *Brassica napus*. Physiol. Plant. 89, 811–816.

Bischoff, M., Low, R., Grsic, S., Rausch, T., Hilgenberg, W., Ludwig-Müller, J., 1995. Infection with the obligate biotroph *Plasmodiophora brassicae*, the causal agent of the clubroot disease, does not affect expression of NIT1/NIT2-related nitrilases in roots of Chinese cabbage. J. Plant Physiol. 147, 341–345.

Bjarnholt, N., Møller, B.L., 2008. Hydroxynitrile glucosides. Phytochemistry 69, 1947–1961.

Bjarnholt, N., Rook, F., Motawia, M.S., Cornett, C., Jørgensen, C., Olsen, C.E., Jaroszewski, J.W., Bak, S., Møller, B.L., 2008. Diversification of an ancient theme: Hydroxynitrile glucosides. Phytochemistry 69, 1507–1516.

Blumenthal, S.G., Hendrickson, H.R., Abrol, Y.P., Conn, E.E., 1968. Cyanide metabolism in higher plants. III. The biosynthesis of β-cyanolanine. J. Biol. Chem. 243, 5302–5307.

Blumenthal-Goldschmidt, S., Butler, G.W., Conn, E.E., 1963. Incorporation of hydrocyanic acid labelled with carbon-14 into asparagine in seedlings. Nature 197, 718–719.

Bork, P., Koonin, E.V., 1994. A new family of carbon–nitrogen hydrolases. Protein Sci. 3, 1344–1346.

Brown, P.D., Tokuhisa, J.G., Reichelt, M., Gershenzon, J., 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry 62, 471–481.

Burow, M., Bergner, A., Gershenzon, J., Wittstock, U., 2007. Glucosinolate hydrolysis in *Lepidium sativum* - identification of the thiocyanate-forming protein. Plant Mol. Biol. 63, 49–61.

Busk, P.K., Møller, B.L., 2002. Dhurrin synthesis in sorghum is regulated at the transcriptional level and induced by nitrogen fertilization in older plants. Plant Physiol. 129, 1222–1231.

Castric, P.A., Farnden, K.J., Conn, E.E., 1972. Cyanide metabolism in higher plants. V. The formation of asparagine from β-cyanoalanine. Arch. Biochem. Biophys. 152, 62–69.

Chiba, R., Dohmoto, M., Yamaguchi, K., 1999. *Oryza sativa* (japonica cultivar-group) ONIT4 mRNA for nitrilase-like protein. GenBank Accession Number AB027054.

Cutler, S.R., Somerville, C.R., 2005. Imaging plant cell death: GFP-Nit1 aggregation marks an early step of wound and herbicide induced cell death. BMC Plant Biol. 5, 4.

D'Haese, D., Horemans, N., De Coen, W., Guisez, Y., 2006. Identification of late O_3-responsive genes in *Arabidopsis thaliana* by cDNA microarray analysis. Physiol. Plant. 128, 70–79.

Dixon, D.P., Skipsey, M., Grundy, N.M., Edwards, R., 2005. Stress-induced protein *S*-glutathionylation in Arabidopsis. Plant Physiol. 138, 2233–2244.

Dohmoto, M., Sano, J., Isaji, G., Yamaguchi, K., 2000a. Indole acetonitrile-sensitivity of transgenic tobacco containing *Arabidopsis thaliana* nitrilase genes. Plant Cell Rep. 19, 1027–1032.

Dohmoto, M., Tsunoda, H., Isaji, G., Chiba, R., Yamaguchi, K., 2000b. Genes encoding nitrilase-like proteins from tobacco. DNA Res. 7, 283–289.

Floss, H.G., Hadwiger, L., Conn, E.E., 1965. Enzymatic formation of β-cyanoalanine from cyanide. Nature 208, 1207–1208.

Foo, H.L., Gronning, L.M., Goodenough, L., Bones, A.M., Danielsen, B., Whiting, D.A., Rossiter, J.T., 2000. Purification and characterisation of epithiospecifier protein from Brassica napus: enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis. FEBS Lett. 468, 243–246.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y.L., Postlethwait, J., 1999. Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151, 1531–1545.

Forslund, K., Morant, M., Jørgensen, B., Olsen, C.E., Asamizu, E., Sato, S., Tabata, S., Bak, S., 2004. Biosynthesis of the nitrile glucosides rhodiocyanoside A and D and the cyanogenic glucosides lotaustralin and linamarin in *Lotus japonicus*. Plant Physiol. 135, 71–84.

Fowden, L., Bell, E.A., 1965. Cyanide metabolism by seedlings. Nature 206, 110–112.

Galaud, J.P., Carriere, M., Pauly, N., Canut, H., Chalon, P., Caput, D., Pont-Lezica, R.F., 1999. Construction of two ordered cDNA libraries enriched in genes encoding plasmalemma and tonoplast proteins from a high-efficiency expression library. Plant J. 17, 111–118.

Galoian, S.M., Tolosa, E.A., Goriachenkova, E.V., 1982. Role of β-cyanoalanine hydratase in the synthesis of asparagine in white lupine. Biokhimiia. 47, 1949–1953.

Giavalisco, P., Nordhoff, E., Kreitler, T., Kloppel, K.D., Lehrach, H., Klose, J., Gobom, J., 2005. Proteome analysis of *Arabidopsis thaliana* by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry. Proteomics 5, 1902–1913.

Glawischnig, E., 2007. Camalexin. Phytochemistry 68, 401–406.

Grsic, S., Kirchheim, B., Pieper, K., Fritsch, M., Hilgenberg, W., Ludwig-Müller, J., 1999. Induction of auxin biosynthetic enzymes by jasmonic acid and in clubroot diseased Chinese cabbage plants. Physiol. Plant. 105, 521–531.

Grsic, S., Sauerteig, S., Neuhaus, K., Albrecht, M., Rossiter, J., Ludwig-Müller, J., 1998. Physiological analysis of transgenic *Arabidopsis thaliana* plants expressing one nitrilase isoform in sense or antisense direction. J. Plant Physiol. 153, 446–456.

Grsic-Rausch, S., Kobelt, P., Siemens, J.M., Bischoff, M., Ludwig-Müller, J., 2000. Expression and localization of nitrilase during symptom development of the clubroot disease in Arabidopsis. Plant Physiol. 122, 369–378.

Halkier, B.A., Gershenzon, J., 2006. Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. 57, 303–333.

Halkier, B.A., Møller, B.L., 1989. Biosynthesis of the cyanogenic glucoside dhurrin in seedlings of *Sorghum bicolor* (L.) Moench and partial purification of the enzyme system involved. Plant Physiol 90, 1552–1559.

Hendrickson, H.R., Conn, E.E., 1969. Cyanide metabolism in higher plants. IV. Purification and properties of the β-cyanolanine synthase of blue lupine. J. Biol. Chem. 244, 2632–2640.

Hillebrand, H., Bartling, D., Weiler, E.W., 1998. Structural analysis of the *nit2*/*nit1*/*nit3* gene cluster encoding nitrilases, enzymes catalyzing the terminal activation step in indole-acetic acid biosynthesis in *Arabidopsis thaliana*. Plant Mol. Biol. 36, 89–99.

Hillebrand, H., Tiemann, B., Hell, R., Bartling, D., Weiler, E.W., 1996. Structure of the gene encoding nitrilase 1 from *Arabidopsis thaliana*. Gene 170, 197–200.

Hirai, M.Y., Klein, M., Fujikawa, Y., Yano, M., Goodenowe, D.B., Yamazaki, Y., Kanaya, S., Nakamura, Y., Kitayama, M., Suzuki, H., Sakurai, N., Shibata, D., Tokuhisa, J., Reichelt, M., Gershenzon, J., Papenbrock, J., Saito, K., 2005. Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J. Biol. Chem. 280, 25590–25595.

Hogge, L.R., Reed, D.W., Underhill, E.W., Haughn, G.W., 1988. HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid-chromatography mass-spectrometry. J. Chromatogr. Sci. 26, 551–556.

Hook, R.H., Robinson, W.G., 1964. Ricinine nitrilase. II. Purification and properties. J. Biol. Chem. 239, 4263–4267.

Hung, C.L., Liu, J.H., Chiu, W.C., Huang, S.W., Hwang, J.K., Wang, W.C., 2007. Crystal structure of *Helicobacter pylori* formamidase AmiF reveals a cysteine–glutamate–lysine catalytic triad. J. Biol. Chem. 282, 12220–12229.

Husebye, H., Chadchawan, S., Winge, P., Thangstad, O.P., Bones, A.M., 2002. Guard cell- and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in Arabidopsis. Plant Physiol. 128, 1180–1188.

Ishikawa, T., Okazaki, K., Kuroda, H., Itoh, K., Mitsui, T., Hori, H., 2007. Molecular cloning of *Brassica rapa* nitrilases and their expression during clubroot development. Mol. Plant Pathol. 8, 623–637.

James, D.C., Rossiter, J.T., 1991. Development and characteristics of myrosinase in *Brassica napus* during early seedling growth. Physiol. Plant. 82, 163–170.

Jandhyala, D., Berman, M., Meyers, P.R., Sewell, B.T., Willson, R.C., Benedik, M.J., 2003. CynD, the cyanide dihydratase from *Bacillus pumilus*: gene cloning and structural studies. Appl. Environ. Microbiol. 69, 4794–4805.

Jandhyala, D.M., Willson, R.C., Sewell, B.T., Benedik, M.J., 2005. Comparison of cyanide-degrading nitrilases. Appl. Microbiol. Biotechnol. 68, 327–335.

Jenrich, R., Trompetter, I., Bak, S., Olsen, C.E., Møller, B.L., Piotrowski, M., 2007. Evolution of heteromeric nitrilase complexes in Poaceae with new functions in nitrile metabolism. Proc. Natl. Acad. Sci. USA 104, 18848–18853.

Jones, E.R., Henbest, H.B., Smith, G.F., Bentley, J.A., 1952. 3-Indolylacetonitrile: a naturally occurring plant growth hormone. Nature 169, 485–487.

Kimani, S.W., Agarkar, V.B., Cowan, D.A., Sayed, M.F., Sewell, B.T., 2007. Structure of an aliphatic amidase from *Geobacillus pallidus* RAPc8. Acta Crystallogr. D Biol. Crystallogr. 63, 1048–1058.

Kobayashi, M., Izui, H., Nagasawa, T., Yamada, H., 1993. Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine residues. Proc. Natl. Acad. Sci. USA 90, 247–251.

Kobayashi, M., Komeda, H., Yanaka, N., Nagasawa, T., Yamada, H., 1992a. Nitrilase from *Rhodococcus rhodochrous* J1. Sequencing and overexpression of the gene and identification of an essential cysteine residue. J. Biol. Chem. 267, 20746–20751.

Kobayashi, M., Shimizu, S., 2000. Nitrile hydrolases. Curr. Opin. Chem. Biol. 4, 95–102.

Kobayashi, M., Yanaka, N., Nagasawa, T., Yamada, H., 1992b. Primary structure of an aliphatic nitrile-degrading enzyme, aliphatic nitrilase, from *Rhodococcus rhodochrous* K22 and expression of its gene and identification of its active site residue. Biochemistry 31, 9000–9007.

Koroleva, O.A., Davies, A., Deeken, R., Thorpe, M.R., Tomos, A.D., Hedrich, R., 2000. Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. Plant Physiol. 124, 599–608.

Kriechbaumer, V., Park, W.J., Piotrowski, M., Meeley, R.B., Gierl, A., Glawischnig, E., 2007. Maize nitrilases have a dual role in auxin homeostasis and β-cyanoalanine hydrolysis. J. Exp. Bot. 58, 4225–4233.

Kumaran, D., Eswaramoorthy, S., Gerchman, S.E., Kycia, H., Studier, F.W., Swaminathan, S., 2003. Crystal structure of a putative CN hydrolase from yeast. Proteins 52, 283–291.

Kutz, A., Müller, A., Hennig, P., Kaiser, W.M., Piotrowski, M., Weiler, E.W., 2002. A role for nitrilase 3 in the regulation of root morphology in sulphur-starving *Arabidopsis thaliana*. Plant J. 30, 95–106.

Lambrix, V., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J., Gershenzon, J., 2001. The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. Plant Cell 13, 2793–2807.

Lea, P.J., Fowden, L., 1973. Amino acid substrate specificity of asparaginyl-, aspartyl- and glutaminyl-tRNA synthetase isolated from higher plants. Phytochemistry 12, 1903–1916.

Ljung, K., Hull, A.K., Kowalczyk, M., Marchant, A., Celenza, J., Cohen, J.D., Sandberg, G., 2002. Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol. Biol. 49, 249–272.

Ludwig-Müller, J., Pieper, K., Ruppel, M., Cohen, J.D., Epstein, E., Kiddle, G., Bennett, R., 1999. Indole glucosinolate and auxin biosynthesis in *Arabidopsis thaliana* (L.) Heynh. glucosinolate mutants and the development of clubroot disease. Planta 208, 409–419.

Lundgren, S., Lohkamp, B., Andersen, B., Piskur, J., Dobritzsch, D., 2008. The crystal structure of β-alanine synthase from *Drosophila melanogaster* reveals a homooctameric helical turn-like assembly. J. Mol. Biol. 377, 1544–1559.

Mahadevan, S., Thimann, K.V., 1964. Nitrilase. II. Substrate specificity and possible mode of action. Arch. Biochem. Biophys. 107, 62–68.

McBride, K.E., Kenny, J.W., Stalker, D.M., 1986. Metabolism of the herbicide bromoxynil by Klebsiella pneumoniae subsp. Ozaenae. Appl. Environ. Microbiol. 52, 325–330.

Morant, A.V., Bjarnholt, N., Kragh, M.E., Kjaergaard, C.H., Jørgensen, K., Paquette, S.M., Piotrowski, M., Imberty, A., Olsen, C.E., Møller, B.L., Bak, S., 2008a. The β-glucosidases responsible for bioactivation of hydroxynitrile glucosides in *Lotus japonicus*. Plant Physiol. 147, 1072–1091.

Morant, A.V., Jørgensen, K., Jørgensen, C., Paquette, S.M., Sanchez-Perez, R., Møller, B.L., Bak, S., 2008b. Β-Glucosidases as detonators of plant chemical defense. Phytochemistry 69, 1795–1813.

Müller, A., Weiler, E.W., 2000. Indolic constituents and indole-3-acetic acid biosynthesis in the wild-type and a tryptophan auxotroph mutant of *Arabidopsis thaliana*. Planta 211, 855–863.

Nafisi, M., Goregaoker, S., Botanga, C.J., Glawischnig, E., Olsen, C.E., Halkier, B.A., Glazebrook, J., 2007. Arabidopsis cytochrome P450 monooxygenase 71A13 catalyzes the conversion of indole-3-acetaldoxime in camalexin synthesis. Plant Cell 19, 2039–2052.

Nagasawa, T., Wieser, M., Nakamura, T., Iwahara, H., Yoshida, T., Gekko, K., 2000. Nitrilase of *Rhodococcus rhodochrous* J1. Conversion into the active form by subunit association. Eur. J. Biochem. 267, 138–144.

Nakai, T., Hasegawa, T., Yamashita, E., Yamamoto, M., Kumasaka, T., Ueki, T., Nanba, H., Ikenaka, Y., Takahashi, S., Sato, M., Tsukihara, T., 2000. Crystal structure of *N*-carbamyl-D-amino acid amidohydrolase with a novel catalytic framework common to amidohydrolases. Structure 8, 729–737.

Neuhaus, K., Grsic-Rausch, S., Sauerteig, S., Ludwig-Müller, J., 2000. Arabidopsis plants transformed with nitrilase 1 or 2 in antisense direction are delayed in clubroot development. J. Plant Physiol. 156, 756–761.

Nikiforova, V., Freitag, J., Kempa, S., Adamik, M., Hesse, H., Hoefgen, R., 2003. Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. Plant J. 33, 633–650.

Normanly, J., Cohen, J.D., Fink, G.R., 1993. *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. Proc. Natl. Acad. Sci. USA 90, 10355–10359.

Normanly, J., Grisafi, P., Fink, G.R., Bartel, B., 1997. Arabidopsis mutants resistant to the auxin effects of indole-3-acetonitrile are defective in the nitrilase encoded by the *NIT*1 gene. Plant Cell 9, 1781–1790.

O'Donnell, P.J., Schmelz, E.A., Moussatche, P., Lund, S.T., Jones, J.B., Klee, H.J., 2003. Susceptible to intolerance - a range of hormonal actions in a susceptible Arabidopsis pathogen response. Plant J. 33, 245–257.

O'Reilly, C., Turner, P.D., 2003. The nitrilase family of CN hydrolysing enzymes - a comparative study. J. Appl. Microbiol. 95, 1161–1174.

Osswald, S., Wajant, H., Effenberger, F., 2002. Characterization and synthetic applications of recombinant AtNIT1 from *Arabidopsis thaliana*. Eur. J. Biochem. 269, 680–687.

Pace, H.C., Brenner, C., 2001. The nitrilase superfamily: classification, structure and function. Genome Biol. 2 (REVIEWS0001).

Pace, H.C., Hodawadekar, S.C., Draganescu, A., Huang, J., Bieganowski, P., Pekarsky, Y., Croce, C.M., Brenner, C., 2000. Crystal structure of the worm NitFhit Rosetta Stone protein reveals a Nit tetramer binding two Fhit dimers. Curr. Biol. 10, 907–917.

Park, W.J., Kriechbaumer, V., Müller, A., Piotrowski, M., Meeley, R.B., Gierl, A., Glawischnig, E., 2003. The nitrilase ZmNIT2 converts indole-3-acetonitrile to indole-3-acetic acid. Plant Physiol. 133, 794–802.

Paterson, A.H., Bowers, J.E., Chapman, B.A., 2004. Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. Proc. Natl. Acad. Sci. USA 101, 9903–9908.

Peiser, G.D., Wang, T.-T., Hoffman, N.E., Yang, S.F., Liu, H.-W., Walsh, C.T., 1984. Formation of cyanide from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during its conversion to ehtylene. Proc. Natl. Acad. Sci. USA 81, 3059–3063.

Petersen, B.L., Chen, S., Hansen, C.H., Olsen, C.E., Halkier, B.A., 2002. Composition and content of glucosinolates in developing *Arabidopsis thaliana*. Planta 214, 562–571.

Piotrowski, M., Janowitz, T., Kneifel, H., 2003. Plant C–N hydrolases and the identification of a plant *N*-carbamoylputrescine amidohydrolase involved in polyamine biosynthesis. J. Biol. Chem. 278, 1708–1712.

Piotrowski, M., Schönfelder, S., Weiler, E.W., 2001. The *Arabidopsis thaliana* isogene NIT4 and its orthologs in tobacco encode β-cyano-L-alanine hydratase/nitrilase. J. Biol. Chem. 276, 2616–2621.

Piotrowski, M., Volmer, J.J., 2006. Cyanide metabolism in higher plants: cyanoalanine hydratase is a NIT4 homolog. Plant Mol. Biol. 61, 111–122.

Pirrung, M.C., 1985. Ethylene biosynthesis. 3. Evidence concerning the fate of C1–N1 of 1-aminocyclopropane carboxylic-acid. Bioorgan. Chem 13, 219–226.

Pollmann, S., Müller, A., Piotrowski, M., Weiler, E.W., 2002. Occurrence and formation of indole-3-acetamide in *Arabidopsis thaliana*. Planta 216, 155–161.

Pollmann, S., Müller, A., Weiler, E.W., 2006. Many roads lead to "auxin": of nitrilases, synthases, and amidases. Plant Biol. 8, 326–333.

Poole, R.J., Thimann, K.V., 1964. Uptake of indole-3-acetic acid and indole-3-acetonitrile by Avena coleoptile sections. Plant Physiol. 39, 98–103.

Purkarthofer, T., Skranc, W., Schuster, C., Griengl, H., 2007. Potential and capabilities of hydroxynitrile lyases as biocatalysts in the chemical industry. Appl. Microbiol. Biotechnol. 76, 309–320.

Rausch, T., Butcher, D.N., Hilgenberg, W., 1981. Nitrilase activity in clubroot diseased plants. Physiol. Plant. 52, 467–470.

Rausch, T., Hilgenberg, W., 1980. Partial purification of nitrilase from Chinese Cabbage. Phytochemistry 19, 747–750.

Reintanz, B., Lehnen, M., Reichelt, M., Gershenzon, J., Kowalczyk, M., Sandberg, G., Godde, M., Uhl, R., Palme, K., 2001. *Bus*, a bushy Arabidopsis CYP79F1 knockout mutant with abolished synthesis of short-chain aliphatic glucosinolates. Plant Cell 13, 351–367.

Ressler, C., 1962. Isolation and identification from common vetch of neurotoxin β-cyano-L-alanine, a possible factor in neurolathyrism. J. Biol. Chem. 237, 733–735.

Ressler, C., Giza, Y.H., Nigam, S.N., 1963. Biosynthesis and metabolism in species of vetch and Lathyrus of γ-glutamyl-β-cyanoalanine: Relation to the biosynthesis of asparagine. J. Am. Chem. Soc. 85, 2874–2875.

Robinson, W.G., Hook, R.H., 1964. Ricinine nitrilase. I. Reaction product and substrate specificity. J. Biol. Chem. 239, 4257–4262.

Rosenthaler, L., 1908. Durch Enzyme bewirkte asymmetrische Synthesen. Biochem. Z. 14, 238–253.

Sakai, N., Tajika, Y., Yao, M., Watanabe, N., Tanaka, I., 2004. Crystal structure of hypothetical protein PH0642 from *Pyrococcus horikoshii* at 1.6Å resolution. Proteins 57, 869–873.

Sanchez-Perez, R., Jørgensen, K., Olsen, C.E., Dicenta, F., Møller, B.L., 2008. Bitterness in almonds. Plant Physiol. 146, 1040–1052.

Sarry, J.E., Kuhn, L., Le Lay, P., Garin, J., Bourguignon, J., 2006. Dynamics of *Arabidopsis thaliana* soluble proteome in response to different nutrient culture conditions. Electrophoresis 27, 495–507.

Schmidt, R.C., Müller, A., Hain, R., Bartling, D., Weiler, E.W., 1996. Transgenic tobacco plants expressing the *Arabidopsis thaliana* nitrilase II enzyme. Plant J. 9, 683–691.

Schranz, M.E., Mitchell-Olds, T., 2006. Independent ancient polyploidy events in the sister families Brassicaceae and Cleomaceae. Plant Cell 18, 1152–1165.

Sewell, B.T., Berman, M.N., Meyers, P.R., Jandhyala, D., Benedik, M.J., 2003. The cyanide degrading nitrilase from *Pseudomonas stutzeri* AK61 is a two-fold symmetric, 14-subunit spiral. Structure 11, 1413–1422.

Shimaoka, T., Ohnishi, M., Sazuka, T., Mitsuhashi, N., Hara-Nishimura, I., Shimazaki, K., Maeshima, M., Yokota, A., Tomizawa, K., Mimura, T., 2004. Isolation of intact vacuoles and proteomic analysis of tonoplast from suspension-cultured cells of *Arabidopsis thaliana*. Plant Cell Physiol. 45, 672–683.

Siemens, J., Glawischnig, E., Ludwig-Müller, J., 2008. Indole glucosinolates and camalexin do not influence the development of the clubroot disease in *Arabidopsis thaliana*. J. Phytopathol. 156, 332–337.

Stalker, D.M., Malyj, L.D., McBride, K.E., 1988a. Purification and properties of a nitrilase specific for the herbicide bromoxynil and corresponding nucleotide sequence analysis of the *bxn* gene. J. Biol. Chem. 263, 6310–6314.

Stalker, D.M., McBride, K.E., 1987. Cloning and expression in *Escherichia coli* of a *Klebsiella ozaenae* plasmid-borne gene encoding a nitrilase specific for the herbicide bromoxynil. J. Bacteriol. 169, 955–960.

Stalker, D.M., McBride, K.E., Malyj, L.D., 1988b. Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science 242, 419–423.

Steiner, M., 1948. Ein Beitrag zur Konstitutionsspezifität der Heteroauxinwirkung. Planta 36, 131–153.

Stevenson, D.E., Feng, R., Storer, A.C., 1990. Detection of covalent enzyme–substrate complexes of nitrilase by ion-spray mass spectroscopy. FEBS Lett. 277, 112–114.

Stintzi, A., Weber, H., Reymond, P., Browse, J., Farmer, E.E., 2001. Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc. Natl. Acad. Sci. USA 98, 12837–12842.

Stowe, B.B., Thimann, K.V., 1954. The paper chromatography of indole compounds and some indole-containing auxins of plant tissues. Arch. Biochem. 51, 499–516.
Thimann, K.V., 1953. Hydrolysis of indoleacetonitrile in plants. Arch. Biochem. Biophys. 44, 242–243.
Thimann, K.V., Mahadevan, S., 1958. Enzymatic hydrolysis of indoleacetonitrile. Nature 181, 1466–1467.
Thimann, K.V., Mahadevan, S., 1964. Nitrilase. I. Occurrence, preparation, and general properties of the enzyme. Arch. Biochem. Biophys 105, 133–141.
Thuku, R.N., Weber, B.W., Varsani, A., Sewell, B.T., 2007. Post-translational cleavage of recombinantly expressed nitrilase from Rhodococcus rhodochrous J1 yields a stable, active helical form. FEBS J. 274, 2099–2108.
Tschiersch, B., 1964. Metabolism of hydrocyanic acid. III. Assimilation of $H^{14}CN$ by *Lathyrus odoratus* L., *Vicia sativa* L., and *Ricinus communis* L. Phytochemistry 3, 365–367.
Tsunoda, H., Yamaguchi, K., 1995. The cDNA sequence of an auxin-producing nitrilase homolog in tobacco.. Plant Physiol 109, 339.
Vorwerk, S., Biernacki, S., Hillebrand, H., Janzik, I., Müller, A., Weiler, E.W., Piotrowski, M., 2001. Enzymatic characterization of the recombinant *Arabidopsis thaliana* nitrilase subfamily encoded by the *NIT*2/*NIT*1/*NIT*3-gene cluster. Planta 212, 508–516.
Wajant, H., Effenberger, F., 1996. Hydroxynitrile lyases of higher plants. Biol. Chem. 377, 611–617.
Wang, W.C., Hsu, W.H., Chien, F.T., Chen, C.Y., 2001. Crystal structure and site-directed mutagenesis studies of *N*-carbamoyl-D-amino-acid amidohydrolase from *Agrobacterium radiobacter* reveals a homotetramer and insight into a catalytic cleft. J. Mol. Biol. 306, 251–261.
Watanabe, M., Kusano, M., Oikawa, A., Fukushima, A., Noji, M., Saito, K., 2008. Physiological roles of the β-substituted alanine synthase gene family in Arabidopsis. Plant Physiol. 146, 310–320.
Weber, H., Chetelat, A., Reymond, P., Farmer, E.E., 2004. Selective and powerful stress gene expression in Arabidopsis in response to malondialdehyde. Plant J. 37, 877–888.
Wienkoop, S., Zoeller, D., Ebert, B., Simon-Rosin, U., Fisahn, J., Glinski, M., Weckwerth, W., 2004. Cell-specific protein profiling in *Arabidopsis thaliana* trichomes: identification of trichome-located proteins involved in sulfur metabolism and detoxification. Phytochemistry 65, 1641–1649.
Woodward, A.W., Bartel, B., 2005. Auxin: regulation, action, and interaction. Ann. Bot. (Lond) 95, 707–735.
Xie, Z.Y., Feng, J.L., Garcia, E., Bernett, M., Yazbeck, D., Tao, J.H., 2006. Cloning and optimization of a nitrilase for the synthesis of (3S)-3-cyano-5-methyl hexanoic acid. J. Mol. Catal. B – Enzym. 41, 75–80.
Xu, P., Narasimhan, M.L., Samson, T., Coca, M.A., Huh, G.H., Zhou, J., Martin, G.B., Hasegawa, P.M., Bressan, R.A., 1998. A nitrilase-like protein interacts with GCC box DNA-binding proteins involved in ethylene and defense responses. Plant Physiol. 118, 867–874.
Yamada, H., Shimizu, S., Kobayashi, M., 2001. Hydratases involved in nitrile conversion: screening, characterization and application. Chem. Rec. 1, 152–161.
Zagrobelny, M., Bak, S., Møller, B.L., 2008. Cyanogenesis in plants and arthropods. Phytochemistry 69, 1457–1468.
Zhang, Z., Ober, J.A., Kliebenstein, D.J., 2006. The gene controlling the quantitative trait locus EPITHIOSPECIFIER MODIFIER1 alters glucosinolate hydrolysis and insect resistance in Arabidopsis. Plant Cell 18, 1524–1536.
Zhao, Y., Hull, A.K., Gupta, N.R., Goss, K.A., Alonso, J., Ecker, J.R., Normanly, J., Chory, J., Celenza, J.L., 2002. Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev. 16, 3100–3112.
Zimmerman, P.W., Wilcoxon, F., 1935. Several chemical growth substances which cause initiation of roots and other responses in plants. Contrib. Boyce Thompson Inst. 7, 209–229.
Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., van Wijk, K.J., 2008. Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS ONE. 3, e1994.

Markus Piotrowski received both his Diploma in Biology and his Ph.D from the Ruhr-Universität Bochum in 1994 and 1998, respectively. His diploma thesis was focused on phosphorylation of membrane proteins in coiling tendrils of red-berried Bryony. In his PhD studies he identified, cloned and characterized 14-3-3 proteins from tobacco and was involved in the elucidation of the molecular architecture of the plant's fusicoccin receptor. Following the Ph.D work he started his research on plant nitrilases. In 2005 he worked as visiting professor in Birger L. Møller's laboratory, Faculty of Life Sciences, University of Copenhagen (formerly Royal Veterinary and Agricultural University). He is now heading the C-N Hydrolase Group within the Department of Plant Physiology of the Ruhr-Universität Bochum. Besides nitrilases, he is interested in metabolism of glucosinolates and cyanogenic glycosides and in polyamine biosynthesis. In 2007 he habilitated in botany with his thesis "C-N hydrolases in primary and secondary metabolism of plants". In 2008 he was selected as editorial board member of the Plant Metabolic Network (www.plantcyc.org).