A polyhedral approach for understanding flavonoid biosynthesis in Arabidopsis

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Abstract

The sequencing of the entire genome of model plants has made it possible to use these plants for producing metabolites that are beneficial to humans. Plants produce a large number of metabolites, which are potentially valuable sources of novel pharmaceutically active agents that benefit human health. The industrial use of these beneficial metabolites from plants, known as secondary metabolites or specialized metabolites, necessitates the systematic understanding of the biosynthetic mechanisms from the genetic to the metabolic level. Here, we review the recent developments on flavonoid biosynthesis in *Arabidopsis thaliana* (Arabidopsis) as a model plant. These developments have been made in the fields of metabolomics, transcriptomics, natural products chemistry, genetics, and biochemistry. We focused on natural products chemistry in this polyhedral approach, which proved to be indispensable for elucidating the secondary metabolism in not only Arabidopsis but also other model-like and crop plants.

Keywords: *Arabidopsis thaliana*, metabolomics, transcriptomics, natural products chemistry, forward/reverse genetics, biochemistry, secondary metabolites, flavonoid, flavonol, anthocyanin

Introduction

Plants produce a large number of metabolites that are an abundant source of novel pharmaceutically active agents [1]. The sequencing of the entire genome of model plants [2–8] has made it possible to use these plants for producing novel secondary metabolites, or alternatively referred as ‘specialized metabolites’, that are beneficial to humans [9]. For the industrial use of plants as biosynthetic machinery, we first have to understand their biosynthetic mechanisms from the genetic to the metabolic level (Fig. 1). We then need to optimize the machinery using overexpression, knockout, or knockdown methods of the targeted genes for high-producing secondary metabolites in the most efficient way [10, 11]. Elucidating the biological roles of metabolites and the process of their evolution under various environmental conditions can likely help to increase the manufacture yield [12–15]. For the stable production of secondary metabolites, we finally need the construct of large-scale cell or hairy root cultures [16, 17]. Our knowledge of the biosynthetic mechanisms of secondary metabolites in *Arabidopsis thaliana* (Arabidopsis) and many other plants is continuously expanding at various points [18–23].

Flavonoids constitute a large group of secondary metabolites that play important roles in plants such as attracting insects, UV-B protection, phytoalexins, allelochemicals, and antioxidants [24–29]. Flavonoids are also useful for humans because of their antioxidant, antiinflammatory, anticancer, antifungal, and antiviral properties [30–34]. The biosynthesis of flavonols and anthocyanins involves the

construction of the basic skeleton, followed by the addition of the modifying moiety. Flavonoid biosynthesis that requires metabolites and genes encoding structural enzymes, modification enzymes, and transcription factors (TFs) is still not fully understood even in *Arabidopsis*. This fact could be attributed to the chemical diversity of flavonoids and the complexity of the associated mechanisms [35–38].

Integrated analysis of metabolomics and transcriptomics has recently provided an improved understanding of plant secondary metabolism from the genetic to the metabolic level [39–49]. Metabolomics involves the comprehensive identification and quantification of metabolites. However, all metabolites, especially secondary metabolites, have not been identified with this method because of the lack of standard compounds. Unequivocal identification of metabolites by natural products chemistry, which provides detailed structure information on chemical compounds, is crucial for the development of metabolomics [50, 51]. Recently, a polyhedral approach has been shown to be a powerful tool for decoding gene function (functional genomics) and understanding the biosynthetic mechanisms of flavonoids [9]. The polyhedral approach encompasses omics-guided fusion approaches from the genetic to the metabolic level (Fig. 1). For example, the combination of metabolomics and natural products chemistry could be referred to as a metabolomics-guided fusion approach, whereas the combination of transcriptomics, biochemistry, and genetics could be regarded as a transcriptomics-guided fusion approach. These fusion approaches enable clear identification of metabolites and the functions of genes of interest. The polyhedral approach is required for an accurate understanding of cellular function and, hence,

furthering the applications of metabolites in plant biotechnology.

Here, we review recent studies on the use of the polyhedral approach including natural products chemistry for comprehensively understanding the flavonoid biosynthesis.

Metabolomics and natural products chemistry combination approach to identify flavonoids in Arabidopsis

Targeted analyses of flavonoids in Arabidopsis have previously been conducted [41, 42, 52]. Flavonoid-targeted analysis by high performance liquid chromatography–mass spectrometry (LC-MS) was conducted using samples collected from different plant organs and extracted with a specific solvent, MeOH-H₂O-CH₃COOH (9:10:1). Extraction from each organ (flower, leaf, stem, and root) yielded a higher flavonoid concentration than extraction from whole plants and resulted in a high detection rate in LC-MS analysis. Flavonoid-targeted analysis by LC-MS has resulted in the detection of 32 flavonols and 11 anthocyanins derived from 4 aglycones namely kaempferol, quercetin, isorhamnetin, and cyanidine. This method has revealed their distribution in each organ (flower, leaf, stem, and root) in a wild-type plant (Col-0) and in a mutant (*pap1-D*) overexpressing an MYB transcription factor (Table 1). Through the use of this analysis along with flavonoid-defective mutants, the positions at which hydroxylation, methylation, and glycosylation occur were identified. In addition, all the detected flavonoids were examined for their retention time, possible fragment ions, UV spectrum, and presence/absence in mutants [41, 42, 53–55].

The predicted structures of flavonoids have been confirmed by metabolomics-oriented isolation and structure elucidation [52]. Seven flavonols (f1, f2, f3, f5, f6, f8, and f24) and 7 anthocyanins (A3, A5, A6, A8, A9, A10, and A11) have been identified using authentic standards isolated from Arabidopsis (Table 1). After the

fragment ions, retention time, and UV spectrum were annotated using LC-MS, new structures (A8 and A10) were elucidated using the approaches of natural products chemistry. Further, biosynthetic pathways were delineated on the basis of previous research wherein the structures of flavonoids and novel isolated compounds were identified by targeted analysis with flavonol-defective mutants. After obtaining accurate structural information was obtained, a possible biosynthetic pathway from cyanidin to A11, which can be referred to as the “Anthocyanin biosynthetic grid of Arabidopsis” was proposed. This pathway involves acylation and glycosylation reactions (Fig. 2) [56, 57].

The exact information from the combination of metabolomics and natural products chemistry is useful for narrowing down candidate genes in the conduct of functional genomics [51]. For example, organ specific flavonoid with a new sugar likely indicates the existence of a new gene encoding glycosyltransferase and the expression part of the gene. The gene expression should be done at the almost same part where the flavonoid is accumulated. This focusing to metabolite information, metabolite accumulation, and gene expression will be surely applied to identification of other unknown genes involved in flavonoid biosynthetic pathway.

Natural products chemistry and biochemistry combination approach to identify the function of flavonoid-related genes in Arabidopsis

Flavonoid can be biosynthesized via the conversion of aglycone to glycoside by the action of genes involved in early and late biosynthesis [58]. Genes involved in early and late biosynthetic processes, ie., *CHS/tt4*, *CHI/tt5*, *F3H/tt6*, *F3'H/tt7*, *DFR/tt3*, and *ANS/tt15*, which encode biosynthetic enzymes from *p*-coumarate to flavonol and anthocyanin aglycones, are well known. On the other hand, the function of genes encoding enzymes involved in the glycosylation of flavonols is only partially understood, even in Arabidopsis. In fact, the primary biosynthetic pathway in the Anthocyanin biosynthetic grid of Arabidopsis is also ambiguous. This biosynthetic pathway is thought to be controlled by MYB TFs together with the proteins bHLH and WD40. Recently, several flavonoid-related MYB TFs have been characterized. MYB11/PFG2, MYB12/PFG1, and MYB111/PFG3 regulate flavonol biosynthesis, and MYB75/PAP1, MYB90/PAP2, MYB113, MYB114, and MYBL2 regulate anthocyanin biosynthesis. Each TF rigidly and independently regulates the expression of target genes [59–64] (Fig. 2).

Metabolite biosynthesis generally occurs in coordination with the expression of genes involved in the biosynthesis of individual metabolites at individual organs. Thus, a large amount of information for mining genes involved in the biosynthesis of secondary metabolites can be obtained from DNA microarray data, which depict snapshots of gene expression at a certain point in time and an organ [65, 66]. The

database ATTED-II [67] provides coexpression sets of microarray data collected according to the developmental stage, stress induced, and hormone treatment given to the plants [68]. Using known genes as guide-genes proved useful for predicting new candidate genes—not only biosynthetic genes but also genes encoding TFs involved in flavonoid biosynthesis (guide-gene approach) [69]. Transcriptome coexpression analysis in ATTED-II was performed using known flavonoid-related genes that encode biosynthetic enzymes or TFs and 107 UDP glycosyltransferase (*UGT*) genes in order to identify new candidate genes that encode flavonol glycosyltransferase [41, 55].

Natural products chemistry has shown the importance of characterizing the functions of genes that encode anthocyanin 5-*O*-glucoside:6'''-*O*-malonyltransferase in the grid as well as flavonol 3-*O*-arabinosyltransferase (F3AraT/UGT78D3) and flavonol 7-*O*-rhamnosyltransferase (F7RhaT/UGT89C1) in the flavonoid-glycoside biosynthetic pathway (Fig. 2) [41, 55, 57]. Using natural products chemistry and biochemistry, Luo *et al.* [57] found that the most efficient reaction of malonyltransferase was via the conversion of A3 to A5 as well as bypaths from A7 to A9 and A6 to A8 by using *in vitro* recombinant proteins and standard compounds. In addition, these approaches suggest that the end products of Arabidopsis anthocyanin are A10 and A11. Through conventional approaches for the characterization of UGT78D3 as F3AraT, 2 flavonols (f19 and f25) were synthesized by the enzymatic reaction of F7RhaT with kaempferol 3-*O*-arabinoside and quercetin 3-*O*-arabinoside and identified by the approaches of natural products chemistry, because these 2 flavonols (f19 and f25) were not commercially available (Table 1) [41]. Another flavonol (f1) also helped to characterize

the function of UGT89C1 as F7RhaT and led to the discovery of a new biosynthetic pathway [55].

It is difficult to obtain standard compounds with complicated structure. The combination of natural products chemistry and biochemistry is necessary in the conduct of more high-level research on flavonoid biosynthesis.

Current situation: Delay in the incorporation of natural products chemistry into omics approaches

In 1999, Veit and Pauli found it surprising that the structures of the phenolics accumulating in *A. thaliana* had not been studied in detail [70]. Only a few papers described Arabidopsis flavonoids, possibly because researchers in natural products chemistry find studying a non-medicinal plant such as Arabidopsis less beneficial [71–74]. In the genomic era, natural products chemistry is necessary for omics approaches. However, the integration of these 2 methods is a very slow process, for 3 possible reasons: (1) the potential of natural products chemistry in phytochemical genomics is unrecognized; (2) all metabolome elements, i.e., all cell metabolites, are not systematically isolated; and (3) a scientific community effort that combines natural products chemistry and metabolomics or conventional approaches is lacking. For the promotion of the entry of natural products chemistry into omics approaches, the improvements are required below.

Recognition of the potential of natural products chemistry in functional genomics

Using novel or known compounds can lead to the discovery of structural information, biosynthetic pathways, and the functions of new genes. Such advancements are initiated using natural products chemistry to identify secondary metabolites. Therefore, collaboration between researchers in natural products chemistry and those in modern omics is very important [9, 75].

Systematic isolation approach to metabolomics

It is believed that >200,000 metabolites are involved in plant metabolism. These metabolites have a broad spectrum of chemical diversity and low to high polarity [76, 77]. We have presented a list of the secondary metabolites found in Arabidopsis (Table 2) [52]. It is extremely difficult for one research group to elucidate an entire metabolome by using conventional approaches for compound isolation because of the wide chemical diversity of metabolome. Consequently, systematic approaches should be developed for the targeted isolation of compounds with the same polarity or of the same metabolite group.

AnalytiCon Discovery (<http://www.ac-discovery.com/>), which commercially supplies secondary metabolites, has established a systematic method for compound isolation. This method includes high-performance liquid chromatography-diode array detection (HPLC-DAD) for profiling, HPLC-evaporative light scattering detection (LC-ELSD) for quantification, LC-MS for structure annotation, automated HPLC for purification, and high-throughput structure identification by using in-house and natural products databases. The structure of unidentified compounds is then elucidated using 1D and 2D nuclear magnetic resonance (NMR) spectroscopy [^1H , ^{13}C , heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond connectivity (HMBC), and correlation spectroscopy (COSY)] [78]. Using these methods, AnalytiCon Discovery has been a pioneer in providing a vast collection of secondary plant metabolites of plants (3,674 compounds) for many years.

In the academic field, however, manpower is required instead of a

well-constructed isolation system. Researchers who are skilled at targeted isolation of a particular crude fraction and metabolite group (e.g., flavonoids) can experiment with combinations of individual approaches to overcome the difficulties associated with the chemical diversity of metabolome. The use of several public NMR databases that are currently under development will increase the throughput of identification or structure elucidation (Table 3). It is possible to develop different systematic isolation approaches for the metabolome.

A scientific community effort that combines natural products chemistry and metabolomics or conventional approaches

Organized scientific community efforts that combine transcriptomics and conventional approaches, such as biochemistry and genetics, are quite well established [79, 80]. Full-length Arabidopsis cDNAs are distributed through the Arabidopsis Biological Resource Center (ABRC) (<http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrchome.htm>), which is a publicly available DNA resource. Several resources for obtaining mutants for reverse genetics are provided by a number of organizations [T-DNA-tagged mutants: SALK (<http://signal.salk.edu/>), SAIL (<http://arabidopsis.info/students/paaras/sail.htm>), and GABI-Kat (<http://www.gabi-kat.de/>); transposon-tagged lines: Cold Spring Harbor Laboratory (<http://genetrap.cshl.edu/>) and RIKEN PSC (<http://www.brc.riken.jp/lab/epd/catalog/transposon.html>); and activation-tagged lines: RIKEN PSC (<http://amber.gsc.riken.jp/act/top.php>) and TILLING mutants (<http://tilling.fhcrc.org/>)] [9].

An organized scientific community effort that combines natural products chemistry and metabolomics or conventional approaches for the collection and distribution of standard compounds is also required. Researchers in natural products chemistry, biochemistry, or genetics can use these resources and promote donor-user collaboration for mutual benefit (Fig. 1). Standards can be obtained only by purchase from companies (Table 4), transfer by collaboration, or free transfer. Because it is difficult to discuss the structure of metabolites only based on the information from LC-DAD and molecular ions on mass spectrometry [KNApSAcK (<http://kanaya.naist.jp/KNApSAcK/>) combined with MS/MS spectral tag (<http://prime.psc.riken.jp/>) [50, 51, 79, 80], the construction of the science community for exchange and stock of standard compounds is definitely required.

Future outlook: Use of polyhedral approaches leading to great advancements in plant biotechnology

Model organisms, including plants, whose entire genomes have been sequenced by next generation sequencers, are increasingly being focused [GOLD (<http://genomesonline.org/index2.htm>)] [81]. Because of its small genome size, Arabidopsis does not biosynthesize compounds with complex structures and relatively large molecular weights [52]. However, the genome size of model-like plants or crop plants is predicted to be much larger than that of Arabidopsis. The number of structurally complex metabolites with structural isomers will in turn increase according to the genome size [82]. In the future, the use of natural products chemistry in the polyhedral approach will be increasingly useful for identifying these complex metabolites, in functional genomics, and in plant biotechnology. Overcoming the present problems associated with omics approaches will enable us to efficiently use plants for producing secondary metabolites according to our needs [83– 85].

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Figure legends

Figure 1. A polyhedral approach for understanding of biosynthetic mechanisms of secondary metabolites

The combination of omics and conventional approach could be regarded as omics-guided fusion approach and that of omics-guided fusion approaches could be regarded as a polyhedral approach.

Figure 2. A proposed flavonoid biosynthetic pathway.

The biosynthesis of flavonol glycoside has almost characterized [41, 42, 54, 55]. ‘f number’ and ‘A number’ compounds were annotated and partly identified (Table 1) [41, 42]. Red arrows indicate *in vitro* malonyltransferase reactions with substrates (A3, A6, A7 and A10) [57]. The thickest arrow shows the most efficient reaction.

CHS; chalcon synthase, CHI; chalcon isomerase, F3H; flavanone 3-hydroxylase, F3'H; flavonoid 3'-hydroxylase, FLS1; flavonol synthase1, OMT1; caffeic acid/5-hydroxyferulic acid *O*-methyltransferase, flavonol 3'-*O*-methyltransferase, Fd3GlcT; flavonoid 3-*O*-glucosyltransferase, F3AraT; flavonol 3-*O*-arabinosyltransferase, F3RhaT; flavonol 3-*O*-rhamnosyltransferase, F7RhaT; flavonol 7-*O*-rhamnosyltransferase, F7GlcT; flavonol 7-*O*-glucosyltransferase, XylT: xylosyltransferase (unknown), *p*-CouT; Anthocyanin 3-*O*-glucoside:6''-*O*-*p*-coumaroyltransferase, GlcT; glucosyltransferase (unknown), 5GlcT; Anthocyanin 5-*O*-glucosyltransferase, MaT; Anthocyanin

5-*O*-glucoside:6'''-*O*-malonyltransferase, SAT; sinapoylglucose:anthocyanin acyltransferase.

Cy; cyanidin, ia; intermediate of anthocyanin of A1 to A11, ia1; cyanidin

5-*O*-β-D-glucopyranoside, ia2; cyanidin 5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside], ia3;

cyanidin 3-*O*-β-D-glucopyranoside, ia4; cyanidin

3-*O*-β-D-glucopyranoside-5-*O*-β-D-glucopyranoside, ia5; cyanidin

3-*O*-β-D-glucopyranoside-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside], ia6; cyanidin

3-*O*-[2-*O*-(β-D-xylopyranosyl)-β-D-glucopyranoside], ia7; cyanidin

3-*O*-[2-*O*-(2-*O*-*E*-sinapoyl-β-D-xylopyranosyl)-β-D-glucopyranoside], ia8; cyanidin

3-*O*-[2-*O*-(2-*O*-(*E*-sinapoyl-β-D-xylopyranosyl)-5-*O*-[6-*O*-(malonyl)-β-D-glucopyranoside], ia9; cyanidin

3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(*E*-*p*-coumaroyl)-β-D-glucopyranoside], ia10;

cyanidin

3-*O*-[2-*O*-(2-*O*-*E*-sinapoyl-β-D-xylopyranosyl)-6-*O*-(*E*-*p*-coumaroyl)-β-D-glucopyranoside], ia11; cyanidin

3-*O*-[2-*O*-(β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*E*-*p*-coumaroyl)-β-D-glucopyranoside], ia12; cyanidin

3-*O*-[2-*O*-(2-*O*-*E*-sinapoyl-β-D-xylopyranosyl)-6-*O*-(4-*O*-(β-D-glucopyranosyl)-*E*-*p*-coumaroyl)-β-D-glucopyranoside].

Figure 1

Figure 2

Table 1

TABLE 1. Arabidopsis flavonoids

Flavonol		R ₁	R ₂	R ₃	Anthocyanin		R ₁	R ₂	R ₃
f1	○	H	rhamnoside	rhamnoside	A1	△	H	H	H
f2	○	H	glucoside	rhamnoside	A2		H	H	malonyl
f3	○	H	rhamnosyl (1→2) glucoside	rhamnoside	A3	○	H	<i>p</i> -coumaroyl	H
f4		H	glucosyl (1→6) glucoside	rhamnoside	A4		H	<i>p</i> -coumaroyl-glucose	H
f5	○	OH	rhamnoside	rhamnoside	A5	○	sinapoyl	<i>p</i> -coumaroyl	H
f6	○	OH	glucoside	rhamnoside	A6	○	H	<i>p</i> -coumaroyl-glucose	malonyl
f7	△	OH	rhamnoside	glucoside	A7		sinapoyl	<i>p</i> -coumaroyl	H
f8	○	OH	rhamnosyl (1→2) glucoside	rhamnoside	A8	○	H	<i>p</i> -coumaroyl-glucose	malonyl
f9		H	conjugate		A9	○	sinapoyl	<i>p</i> -coumaroyl	malonyl
f10		H	hexose-deoxyhexose	—	A10	○	sinapoyl	<i>p</i> -coumaroyl-glucose	H
f11		H	conjugate		A11	○	sinapoyl	<i>p</i> -coumaroyl-glucose	malonyl
f12		H	rhamnoside conjugate	—					
f13		OH	hexosyl deoxyhexose	—	Flavonol				
f14		OCH ₃	glucoside	rhamnoside					
f15		OCH ₃	rhamnoside	glucoside					
f16	△	OH	glucoside	H					
f17	△	H	rhamnoside	H					
f18	△	H	glucoside	H					
f19	□	H	arabinoside	rhamnoside					
f20		H	rhamnoside	glucoside					
f21		H	hexose glucoside	rhamnoside					
f22		H	pentose, deoxyhexose	rhamnoside	Anthocyanin				
f23		OH	arabinoside	H					
f24	○	OH	rhamnoside	H					
f25	□	OH	arabinoside	rhamnoside					
f26		OH	glucose, hexose	—					
f27		OCH ₃	rhamnoside	H					
f28	△	OCH ₃	glucoside	H					
f29		OCH ₃	arabinoside	rhamnoside					
f30		OCH ₃	rhamnoside	rhamnoside					
f31		OCH ₃	glucose, hexose	—					
f32		OCH ₃	rhamnosyl (1→2) glucoside	rhamnoside					

○: Isolated standard from Arabidopsis, △: Purchased standard from company, □: standard synthesized by enzymatic reaction

TABLE 2. Actual and expected distribution of Arabidopsis metabolites.

Fraction	Group
<i>n</i> -Hexane	Fatty acid, Terpenoid
EtOAc	Apocarotenoid, Carotenoid, Chlorophyll, Flavonol (mono and di)glycoside, Galactolipid, Indole, Phenylpropanoid
<i>n</i> -BuOH	Acylated anthocyanin, Flavonol (di and tri)glycoside, Phenylpropanoid
H ₂ O	Amino acid, Flavonol triglycoside, Glucosinolate, Nucleoside, Phenylpropanoid, Sugar

TABLE 3. Useful databases for structure elucidation

Database	URL
<i>Chemical-shift-based prediction</i>	
Biological Magnetic Resonance Data Bank	http://www.bmrb.wisc.edu/metabolomics/query_metab.php
C-13 NMR Chemical Shifts	http://www.chem.wisc.edu/areas/reich/Handouts/nmr-c13/cdata.htm
NMR Data Libraries	http://www.acdlabs.com/products/spec_lab/predict_nmr/data_libraries.html
NMRshiftDB	http://www.ebi.ac.uk/nmrshiftdb/
Proton NMR Chemical Shift	http://www.chem.wisc.edu/areas/reich/Handouts/nmr-h/hdata.htm
SDBS	http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi
Spectral Similarity Search	http://nmrpredict.orc.univie.ac.at/case/propose.php
SpinAssign	http://prime.psc.riken.jp/?action=nmr_search
<i>Structure-based prediction</i>	
CSEARCHlite	http://nmrpredict.orc.univie.ac.at/csearchlite/index.php
NMR Database of Lignin and Cell Wall Model Compounds	http://ars.usda.gov/Services/docs.htm?docid=10491
nmrdb.org	http://www.nmrdb.org/predictor?smiles=O%3dC4c5c
NPedia Compounds Search	http://npd.riken.jp/npedia/keywords.php
Web Version of SciFinder	http://www.cas.org/products/scifindr/sfweb/

TABLE 4. Companies provide standards of secondary metabolites

Company	URL
Advanced Technology & Industrial Co, Ltd	http://www.advtechind.com/
Analyticon Discovery	http://www.ac-discovery.com/
Apin Chemicals Ltd.	http://www.apinchemicals.com/
ChromaDex, Inc.	http://www.chromadex.com/index2.html
Herb Standard Company	http://herbstandard.com/
PhytoLab	http://www.phytolab.com/phytolab/
Polyphenols	http://www.polyphenols.com/
TOKIWA PHYTOCHEMICAL CO, LTD	http://www.tokiwapl.co.jp/en/
TransMIT	http://www.transmit.de/en/index.htm