Plants are considered to biosynthesize specialized (traditionally called secondary) metabolites to adapt to environmental stresses such as biotic and abiotic stresses. The majority of specialized metabolites induced by abiotic stress characteristically exhibit antioxidative activity *in vitro*, but their function *in vivo* is largely yet to be experimentally confirmed. In this review, we highlight recent advances in the identification of the role of abiotic stress-responsive specialized metabolites with an emphasis on flavonoids. Integrated ‘omics’ analysis, centered on metabolomics with a series of plant resources differing in their flavonoid accumulation, showed experimentally that flavonoids play a major role in antioxidation *in vivo*. In addition, the results also suggest the role of flavonoids in the vacuole. To obtain more in-depth insights, chemical and biological challenges need to be addressed for the identification of unknown specialized metabolites and their *in vivo* functions.
Introduction

Environmental stresses such as biotic and abiotic stresses are serious threats to agricultural production [1]. Most notably, abiotic stresses, such as drought, salinity, cold, high light/UV-B, heat, air pollution, heavy metal, mechanical wounding and nutritional deficiency [2,3], result in a global reduction of crops, which leads to worldwide economic costs [4].

To understand and improve crops’ stress responses and tolerances, researchers have focused on the signaling perception, transcriptional regulation and expression of functional proteins in the stress response mechanisms used by plants against abiotic stresses [5]. In addition, posttranslational, posttranscriptional and epigenetic regulations have been studied. The accumulation of small molecules with antioxidative activity in vitro has often been discussed with respect to the role they play in mitigating the accumulation of reactive oxygen species (ROS) induced by abiotic stresses. This discussion has progressed under the conjecture that the reaction in vitro may occur in vivo.

Integrated ‘omics’ analysis centered on metabolomics (integrated metabolomics) can be a powerful technique to identify the functions of genes involved in the metabolic processes of plants [6]. Analytical methodologies for narrowing down potential genes and identifying their functions are relatively mature; transcriptome coexpression analysis and (un)targeted analysis in metabolomics using mutant lines have become commonplace [7]. The next step is to identify the function of specialized metabolites, which is a demanding task. This review highlights recent advances in understanding the function of abiotic stress-responsive specialized metabolites with an emphasis on flavonoids as a representative example of such compounds.

The mystery of abiotic stress-responsive specialized metabolites: why so many? The evolution of aerobic organisms, including plants, has been dependent on the development of efficient mechanisms to mitigate the damage of highly reactive and toxic ROS induced by abiotic stresses, i.e. singlet oxygen, superoxide anion radicals, hydrogen peroxide (H₂O₂), and hydroxyl radicals (*OH) [8]. Given that plant cells and the organelles they contain (e.g. chloroplasts, peroxisomes, cytosol, mitochondria and vacuoles) are exposed to ROS [9], plants have evolved two different biological processes to cope with ROS: prevention or avoidance of ROS formation and scavenging ROS by enzymatic and non-enzymatic processes such as the accumulation of low molecular weight antioxidants. Ascorbic acid (AsA), glutathione (GSH), α-tocopherols, amino acids (e.g. proline [10–12]), sugars [13], carotenoids [14] and quinic acid derivatives (e.g. chlorogenic acid [15]) are antioxidants presumed to function in planta; however, it is unclear why plants produce such a wide variety of antioxidants. Along with the accumulation of metabolites exhibiting antioxidative activity, abiotic stresses also induce the production of various kinds of specialized metabolites (Supplementary Table 1). Of these, it has also been suggested that saponins...
glucosinolates [17], phenolamides [18], phenylpropanoids [19,20] and flavonoids [21,22] act as antioxidants in vivo on the basis of their in vitro antioxidative activity.

Integrated metabolomics experimentally identifies flavonoids as antioxidants in planta
Flavonoids that are common in the plant kingdom [23] are responsive to almost all abiotic stresses (Supplementary Table 1). Given that flavonoid aglycones (e.g. phenylchroman- and flavilium-based structures) generally exhibit antioxidative activity in vitro, flavonoids are assumed to function as antioxidants in vivo. It has been suggested that there is a spatio-temporal correlation between flavonoid accumulation and oxidative stress [24].

In comparisons of natural varieties and mutant lines, integrated metabolomics is useful for identifying the functions of genes performing the biosynthesis of target specialized metabolites. The utilization of single gene knock-out/-down lines and overexpressing lines clarify the correlation between gene expression and metabolite accumulation, enabling the identification of the gene’s function in the integrated analysis of transcriptomic and metabolomic data [6,25,26]. These genetic lines can be also used in identifying the in vivo functions of target metabolites by investigating the phenotypes of plants.

Recently, the antioxidative function of flavonoids in planta was experimentally identified using a series of transgenic and mutant Arabidopsis lines [27**]. Wild-type Col-0 (Columbia-0), single overexpressors of MYB12/PFG1 (PRODUCTION OF FLAVONOL GLYCOSIDES1) or MYB75/PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1), double overexpressors of MYB12 and PAP1, and flavonoid-deficient MYB12 or PAP1 overexpressing lines – obtained by crossing tt4 (transparent testa4) and the individual MYB overexpressor – were subjected to an extensive integrated analysis using transcriptomics, hormonomics and metabolomics. The results showed that the overaccumulation of flavonoids with strong antioxidative activity in vitro and in vivo enhanced the plants’ oxidative- and drought-stress tolerance. This study excluded the possible effects of the overexpression of the MYBs, the expression of stress-related genes, and the alteration of phytohormones and additional metabolites other than flavonoids on enhancing stress tolerances. The enhanced stress tolerance in this report was solely due to the antioxidative chemical character of overaccumulated flavonoids. It is inferred that the flavonoid accumulation in accordance with abiotic stress exposure is a late response implemented to protect plants [28].

A hypothesis on the role of flavonoids in the vacuole
The hypothetical insights regarding the role of antioxidative metabolites, particularly flavonoids, may be further extended to understanding their role in the vacuole. H$_2$O$_2$ in the cytosol can easily enter into the vacuole [29]. H$_2$O$_2$-dependent class III peroxidase near the inner side of tonoplasts catalyzes the reaction that converts H$_2$O$_2$ to *OH, which is known to
react with almost all metabolites. AsA and GSH present in the vacuole (e.g. 4.19 mM AsA in *Catharanthus roseus* [30] and 0.03–0.70 mM GSH in *Arabidopsis* [31,32]) are general scavengers of H$_2$O$_2$ and *OH* [10] (Figure 1).

In addition, sugars and water-soluble specialized metabolites that are stored in the vacuole are utilized as important antioxidants in plants [30,33]. The *in vitro* research on the *OH*-scavenging capacity indicates that sugars and water-soluble specialized metabolites may play a role in radical reactions occurring near the inner side of the tonoplast and in the vacuolar rumen [33]. Interestingly, in *Arabidopsis*, the overaccumulation of galactinol and raffinose, which are abiotic stress-responsive vacuole components [34] and display *OH*-scavenging activity *in vitro*, enhanced oxidative stress tolerance *in vivo* [13,35]. It has been hypothesized that flavonoids mediate the previously unknown key role of the vacuole in maintaining cellular H$_2$O$_2$ homeostasis [36], despite the concentration of H$_2$O$_2$ in the vacuole being much lower than in other cell components [21]. It is estimated that the concentration of rutin (100 μM) in the vacuole is capable of reducing H$_2$O$_2$ at a rate of 0.045 mM s$^{-1}$, which is close to the rate of H$_2$O$_2$ generation [37].

Specialized metabolites in the vacuole have been partially qualified and quantified – 1.39 mM total chlorogenic acid (caffeoyl quinic acids) and 1.57 mM total phenolics in *C. roseus* [30] – suggesting that these highly accumulated compounds play an ROS-mitigating role in the vacuole. Reports of the *in vitro* antioxidative activity of chlorogenic acid, flavonol glycosides, and anthocyanins against H$_2$O$_2$ and *OH* also suggest their role as antioxidants in the vacuole [22,36,38,39]. To evaluate the participation of flavonoids in the vacuolar antioxidative system in plants, however, further experimental analysis would require concurrent qualification and quantification of vacuole metabolites, because the number of vacuole metabolites discussed in the existing literature remains limited. Additionally, the evaluation of the antioxidative role of flavonoids and other vacuole metabolites would require comparative analysis of the metabolism of a series of transgenic or mutant lines.

**Toward identifying the role of flavonoids in the vacuole**

*Chemical challenges*

Theoretically, metabolomics can concurrently qualify and quantify vacuole metabolites. Recently, metabolic profiling using gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) allowed the concurrent detection of 259 putative metabolites, including primary and specialized metabolites, in the vacuoles of barley (*Hordeum vulgare*) [40**]. By taking advantage of the giant cells in alga *Chara australis*, a study using isolated vacuoles determined the metabolic dynamism between vacuolar and extra-vacuolar compartments [41,42]. These research studies indicate that combining metabolomics with cutting-edge technologies can characterize the vacuole metabolome, gaining further insights into its functionality. However, they also indicate the
difficulties associated with the qualification and quantification of the metabolome, especially regarding specialized metabolites, because, in many cases, reference materials (i.e. standard compounds or MS/MS spectra) for chemical assignment are often unavailable because of the specialization of their metabolites.

To overcome the situation, metabolomic strategies are being improved to assign putative structural information to detected metabolites using a combination of the retention times, exact masses, UV spectra and MS/MS patterns of known metabolites, according to the guidelines of the alphanumeric metrics of identification [43], the plant science community [44] or the Metabolomics Standards Initiative (MSI) [45]. According to levels 1 and 2 of the MSI guideline, chemically assigned metabolites are often used in integrated metabolomics studies (Figure 2A). For unknown metabolites, MS/MS, UV and isotope analysis are performed to characterize metabolite features such as substructure, metabolite group or elemental composition (Figure 2B). Utilizing the patterns of electrospray ionization-based MS/MS spectra on known metabolites in the positive and negative ion modes allows the chemical assignment of unknown metabolites [46]. The large amount of data on flavonoid O-glycosides [47,48], flavone C-glycosides [49], phenolamides [50,51], saponins [52,53] and glucosinolates [54] yield a certain pattern of MS/MS fragmentation for each group of compounds. Characteristic UV spectra distinguish these metabolite groups [25,49,50,55,56]. Applying the information of exact mass and natural abundance to isotopes also allows the chemical assignment of elemental composition to unknown metabolites. Using the differences in exact mass and natural abundance of $^{32}$S and $^{34}$S, S-containing metabolites were profiled in *Arabidopsis* [57] and onion (*Allium cepa*) [58]. The unambiguous elemental compositions determined using the sulfur and carbon numbers in stable isotope labeling is powerful information that can be utilized in further experiments on chemical assignment.

The precise information concerning the generally accepted metabolites assignments can be the precursor of their isolation and structure identification/elucidation to obtain standard compounds for quantification and qualification using general or recent technologies [59] (Figure 2C). So far, metabolomics- and phytochemical genomics-oriented studies have revealed 78 metabolites in *Arabidopsis* and rice (*Oryza sativa*) [49,60–63]. Recently, the preparative LC-MS and LC-solid phase extraction-nuclear magnetic resonance-MS (LC-SPE-NMR-MS) systems have been made available for high-throughput analysis [49,64]. The combination of empirical and computational approaches is expected to streamline the identification of metabolite structures [59].

**Biological challenge**

Genome sequencing using next-generation sequencers holds great promise for generation of mutants involved in biosynthesis and roles of vacuolar metabolites. Recently, the gene
Virus-induced gene silencing (VIGS) techniques are used for mutants of biosynthetic genes that define the metabolic pathways of \( \gamma \)-aminobutyric acid in tomatoes (\textit{Solanum lycopersicum}) [65\textsuperscript{*}] and vindoline in \textit{C. roseus} [66\textsuperscript{*}]. Transcription activator-like effector nuclease (TALEN) technology was applied to disrupting a cholesterol biosynthetic gene, sterol side chain reductase 2, in potatoes (\textit{S. tuberosum}) [67\textsuperscript{**}]. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated protein 9 (Cas9) technology [68] was utilized in plants and crops and was used for editing the \textit{tt4} gene in \textit{Arabidopsis} [69]. Fusion proteins consisting of nuclease-dead Cas9 and activator/repressor domains are expected to regulate targeted gene expression in primary and specialized metabolisms [70\textsuperscript{*}]. A series of mutants are required to allow the identification of the roles of not only flavonoids in the vacuole but also other specialized metabolites. In addition, consideration of pathway evolution would yield increasingly interesting information, particularly with regards to gene clusters of certain specialized metabolites [71].

**Conclusions**

The limitations to the identification of specialized metabolites’ functions in plants are shifting from the development of methodologies to the collection of both chemical and biological resources. Specialized metabolites have been discussed as a waste of primary metabolites since the 1950s [72]. Cutting-edge technologies utilizing integrated metabolomics can be used to conclude this lengthy discussion. Because a common factor among abiotic stresses is oxidative stress, the abilities of antioxidants to counteract or prevent the effects of ROS, which is necessary for plants’ survival, can be applied to breeding plants to improve multiple tolerances against all abiotic stresses. Interestingly, the overaccumulation of anthocyanins and flavonol glycosides in \textit{Arabidopsis} seems not to cause significant growth inhibition, at least when under experimental conditions [27\textsuperscript{**}]. Given that the maximum capacity of the vacuole to accept primary and specialized metabolites has never been investigated to our knowledge, it is interesting that future studies using the new technologies are focused on the concurrent overaccumulation of various antioxidants to the vacuole.

**Acknowledgements**

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:
● of special interest
●● of outstanding interest

65. Silencing of three genes involved in the biosynthetic pathway of γ-aminobutyric acid (GABA) revealed the role of GABA and the pathway in salt stress in tomato. This result showed that the VIGS approach can be applied to biosynthetic genes involved in primary metabolism.

66. A VIGS approach was used to identify tabersonine 16-hydroxylase (CYP71D351) in vindoline synthesis in young leaves of C. roseus. The CYP71D351-silencing construct caused a decrease of approximately 80% of the corresponding transcript in young C. roseus leaves.

70. This review article discusses the current questions, potential improvements and engineering platforms of TALEN and CRISPR/Cas9 in plants.

27. Transcriptomics, hormonomics and metabolomics with the series of flavonoid mutant lines in Arabidopsis were applied to the identification of biological function of flavonoids as antioxidants in vivo.

40. Extensive metabolome analysis using GC-MS and LC-MS was conducted to understand the distribution of primary and secondary metabolites in the barley vacuole. The coexpression analysis of barley vacuolar probe sets was performed to classify gene expression clusters.

46. A new chemical assignment approach using the algorithm candidate substrate–product pairs (CSPP) enables the assigning of 145 metabolites to glucosinolates, flavonoids, benzenoids, phenylpropanoids, (neo)lignans/oligolignols, indolics and apocarotenoids.

67. This article describes the identification of sterol side chain reductase 2 (SSR2), which is a key enzyme in the biosynthesis of cholesterol and steroidal glycoalkaloids, by using TALEN technology.


64. Sturm S, Seger C: Liquid chromatography-nuclear magnetic resonance coupling as alternative to liquid chromatography-mass spectrometry hyphenations: Curious option or powerful and complementary routine tool? *J Chromatogr A* 2012.


Supplementary data
Supplementary Table 1
Abiotic stress-responsive metabolites.

Figure legends

Figure 1
Possible vacuolar antioxidative system in plants.
Abbreviations: \( \text{H}_2\text{O}_2 \), hydrogen peroxide; \( \text{OH}^* \), hydroxyl radical.

Figure 2
Overview of integrated metabolomics.

A. Integrated transcriptomic and metabolomic analysis of chemically assigned metabolites. Metabolite function is identified through both this type of analysis and mutant analysis. B. MS/MS UV, isotope analysis to identify metabolite features, substructures, metabolite groups, and elemental composition. C. General (left-hand side) and recent isolation (right-hand side) approaches to characterize metabolites.

Abbreviations: C.C., column chromatography; FTICR-MS, Fourier transform ion cyclotron-mass spectrometry; HCA, hierarchical cluster analysis; LC-MS, liquid chromatography-mass spectrometry; LC-SPE-NMR-MS, liquid chromatography-solid phase extraction-nuclear magnetic resonance-mass spectrometry; MS/MS, tandem mass spectrometry; prep. HPLC, preparative high performance liquid chromatography; prep. LC-MS, preparative LC-MS; RNA Seq., RNA sequence; TLC, thin layer chromatography; UV, ultraviolet.

Picture of LC-SPE-NMR-MS courtesy of Bruker BioSpin (Rheinstetten, Germany).
Figure 1

- **H$_2$O$_2$**
- **H$_2$O$_2$**
- **•OH**
- **H$_2$O**

- anthocyanins
- rutin
- chlorogenic acid
- glutathione
- ascorbic acid
- proline
- galactinol
- raffinose
**Figure 2**

Extraction

Metabolome analysis

LC-MS

FTICR-MS

Chemical assignment

Database search

**A**

**B**

Unknown metabolites

Structure characterization

MS/MS analysis

UV analysis

Isotope analysis

**C**

Isolation of putatively assigned metabolites

TLC

C. C.

prep. HPLC

prep. LC-MS

LC-SPE-NMR-MS

Structure identification/elucidation

**Assigned metabolites**

**Transcriptome analysis**

micro array / RNA Seq.

integrated analysis

HCA etc.

mutant analysis

comparative analysis etc.

Metabolite function