MS to quantify individually the different flavonols. In addition, PA contents were assessed using acid-catalysed hydrolysis (Porter et al., 1986) both on the extract (hereafter called soluble PAs) and on the remaining pellet (hereafter called insoluble PAs). The first observation was that, among the accessions tested, essentially quantitative rather than qualitative variations were observed (Fig. 2; Supplementary Fig. S1 and Supplementary Table S1 at JXB online). All the flavonoids previously characterized in Col-0, Ws-4, or Ler were found in all the accessions. Five accessions illustrated the range of changes observed, namely Col-0, Cvi-0, Nok-1, Sp-0, and Sha (Fig. 2A; Supplementary Fig. S1). Col-0 contained the least kaempferol derivatives and was clearly different from other accessions. Cvi-0 had low quercetin 3-O-rhamnoside content and, consequently, low levels of the derived biflavonols (Pourcel et al., 2005), whereas PAs were 3-fold higher. Interestingly, these three compounds are mainly accumulated in the seed coat (Routaboul et al., 2006). Flavonols and PAs accumulated to the highest levels in the Nok-1 accession in which flavonoids account for 1.7% of dry weight (DW). Sp-0 had the highest quercetin 3-O-rhamnoside content with a concomitant increase in PAs. It should be noted that the largest variations in flavonoids were obtained for some seed coat-specific flavonols, such as quercetin 3-O-rhamnoside (from 0.008% in Cvi-0 up to 0.6% of DW in Sp-0) or PAs (from 0.2% in Sav-0 up to 0.9% DW in Gre-0), or kaempferol derivatives (from 0.04% in Col-0 to 0.3% DW in Nok-1).

Analysis of Sha accessions uncovers new biosynthetic step

The Shahdara accession contained three novel flavonol-hexoside-rhamnoside derivatives. They possessed the same glycosylations but a different quercetin, kaempferol, or isorhamnetin aglycone ([M+H]⁺=611, 595, and 625; [M+H-hexose]⁺=449, 433, and 463; and [M+H-hexose-rhamnose]⁺=303, 287, and 317, respectively). These compounds had a retention time of ~1 min before the corresponding aglycone-3-O-glucoside-7-O-rhamnoside isomers and are thus different from these previously characterized flavonols (Kerhoas et al., 2006; Supplementary Fig. S5 at JXB online). Nevertheless, Sha was also able to synthesize all the flavonols detected in Bay-0. This result suggested that a novel and specific glycosyl transferase that catalyses the production of flavonol-hexoside-rhamnoside is active in Shahdara but not in Bay-0.

Relationships between the contents of different flavonoids in mature seeds

One could expect to observe some correlations between the accumulations of different flavonoids that belong to various subpathways or represent related quantitative traits. Alternatively, the lack of correlation may reveal regulatory steps for which specific QTLs should be detected. These correlations were measured and are depicted as a tree (Fig. 2A; Supplementary Tables S2–S4 at JXB online). Some of these statistically significant correlations could be foreseen, such as the one between the accumulation of precursor quercetin-3-O-rhamnoside and its derived biflavonol products (Pourcel et al., 2005) (r=0.71, P<0.0001) or between soluble and insoluble PAs (r=0.84, P<0.0001). This also shows that some accessions such as Cvi-0 or Bur-0 display a more contrasted quercetin-3-O-rhamnoside/biflavonol ratio relative to that of Sp-0 or Edi-0. However, the correlation between kaempferols and PAs was unexpected (r=0.52, P<0.0001 and r=0.33, P=0.048 between soluble PA and kaempferol-3,7-di-rhamnoside or kaempferol-3-O-glucoside-7-O-rhamnoside, respectively).

A clustering of accessions based on their flavonoid profile was carried out (Fig. 2A). Five major groups could be distinguished. In Arabidopsis, it is often difficult to associate specific genotypes with geographic origin (Anastasio et al., 2011) since human activities tend to homogenize variation among populations, especially in Europe and North America, and recolonization events from circum Mediterranean glacial refuges have also been proposed to occur (Mitchell-Olds and Schmitt, 2006). The accessions of cluster 1 contained a higher level of phenotypic variation than other clusters (these accessions could be considered as separate clusters) and were characterized by less kaempferol-3-O-rhamnoside. Cluster 2 represented western European accessions (Fig. 3) that accumulated less quercetin derivatives and more PAs and kaempferol (such as Cvi-0; see Supplementary Fig. S1 at JXB online). Cluster 3 contained a single accession from The Netherlands, Nok-1, that stood alone away from the other accessions due to its overall high levels of flavonoids. Accessions of cluster 4 included many central European accessions that had, on average, more quercetin derivatives
but less PAs (such as Sp-0), whereas cluster 5 is the only group containing Asian and North American accessions that appeared to contain more PAs (such as Shahdara).

Extended flavonoid variation in two recombinant inbred line sets

To dissect these natural variations genetically, selected progeny of two RIL populations, Cvi-0×Col-0 and Bay-0×Shahdara (Loudet et al., 2002; Simon et al., 2008), were analysed. Correlations between the different flavonoid contents in the Cvi-0×Col-0 RIL set were similar to those observed among the accessions (Fig. 4A; Supplementary Table S3 at JXB online). In contrast, correlations were generally weaker or no longer existent in the Bay-0×Shahdara RIL population, such as that between quercetin-3-O-rhamnoside and one of its products the biflavonols ($r = -0.07, P > 0.5$), between soluble and insoluble PAs ($r = 0.48, P < 0.0001$) or between PAs and kaempferols (Fig. 4B; Fig. 4A; Supplementary Table S3). Variations in flavonoid content in both RIL populations are presented in Fig. 4C and D. The two RIL populations showed transgressive segregation from their parents for most traits, especially for diglycosylated quercetins andisorhamnetin derivatives that displayed small differences among the parents. This should indicate that all four parents have positive-effect alleles for these compounds and that numerous QTLs are likely to be detected.

Developing seeds from the four parental accessions were also analysed (Fig. 5) to uncover additional compounds that are not detected in mature seed. All four accessions contained a novel diglycosylated quercetin, namely quercetin-rhamnoside-glucoside, which differs from the two quercetin-glucoside-rhamnosides described above (quercetin-3-O-glucoside-7-O-rhamnoside and quercetin-hexoside-rhamnoside from Shahdara). The accumulation of this new compound could be associated with that of quercetin 3-O-rhamnoside since both compounds were lower in Cvi-0 and

Fig. 4. Natural variation among recombinant inbred lines (RILs) derived from the Cvi-0×Col-0 and Bay-0×Shahdara crosses. Relationships between mature seed flavonoid contents in two RIL populations Cvi-0×Col-0 (A) and Bay-0×Shahdara (B). log2 % of Col-0 or Bay-0 and boxplot analysis for each flavonoid giving the minimum, lower quartile, median, upper quartile, and outlier, from the bottom to the top (C and D). G, glucoside; H, hexoside; I, isorhamnetin; insol., insoluble; K, kaempferol; Q, quercetin; PA, proanthocyanidin; R, rhamnoside; sol., soluble.
Shahdara compared with Col-0 and Bay-0. Additionally, quercetin-hexoside-rhamnoside 2, detected only in Shahdara, accumulated steadily during seed development but at very low levels (Fig. 5F).

QTL analysis uncovers 22 flavonoid QTLs, of which only one is common to the two populations.

A total of 22 significant QTLs involved in flavonoid variation (termed ‘FLA’) were detected in the two RIL populations. The chromosome location of each QTL is presented in Table 1 together with its significance (LOD score), additive effects (a), and the percentage of total variance explained for the given flavonoid ($R^2$). These QTLs represent from 11% to 61% of the flavonoid variation. Most QTLs were detected in only one of the two mapping populations. Nevertheless, one locus involved in kaempferol changes could be common to both RIL populations (FLA5/FLA15, Table 1, located at ~3 Mb on chromosome 5). The co-localization of QTLs for quercetin 3-O-rhamnoside and...
biflavonols (FLA1/FLA3 and FLA12/FLA14), PAs and quercetin-3-O-rhamnoside (FLA2/FLA9 and FLA11/FLA21), and PAs (FLA20/FLA22), as well as the direction of their predicted allelic effects are consistent with the phenotypic correlations observed between some flavonoids in parental accessions and RIL populations.

**Sixteen FLA loci are confirmed using HIF lines**

HIFs, generated from the residual heterozygosity still segregating in some F₆ RILs (Loudet et al., 2005), were used for further characterization (mapping and analysis) of the QTLs. Each HIF contains a short region fixed for one or other parental allele in an otherwise identical genetic background. From the 22 QTLs characterized using the two RIL populations, 16 were confirmed in HIFs that showed the expected variation (for both the direction and amplitude of the variations) (Table 2; Supplementary Figs S2, S3 at JXB online). FLA1, 3, 5, 11, 13, 15, 19, and 21 were validated with at least two independent HIF lines. Metabolite changes within the HIFs provided additional information about the flavonoid phenotypes and, in several cases, explained the occurrence of suggestive loci (1 < LOD < 2.5) detected with the RILs. This validates the quality of the data and the conservative nature of the QTL thresholds. The flavonoid contents of selected lines of the two RIL sets are given in Supplementary Tables S5 and S6.

Flavonoid analysis of the myb12 mutant provides a candidate gene for the FLA2 locus and also shows that MYB12 controls flavonol accumulation in the seed coat.
compounds are essentially accumulated in the seed coat and were also controlled by the FLA2 QTL (the QTL for biflavonol was only marginally suggestive with a LOD of 1.1). In addition, myb11 and myb111 mutants and double or triple mutant with myb11 and myb111 alleles had lower diglycosylated flavonol contents, that were essentially accumulated in the embryo. Interestingly, the triple mutant contained more soluble PAs (as previously observed in the fsl1 mutant; Routaboul et al., 2006). This specific pattern of accumulation is consistent with a role for these closely related R2R3-MYBs in the control of flavonol accumulation through the early biosynthesis genes, in distinct parts of the seed, as previously observed in seedlings (Stracke et al., 2007, 2010a, b; Dubos et al., 2010). The changes observed in the myb12 mutant suggested that this MYB12 gene is a strong candidate for FLA2. However, the HIF at the FLA2 locus also showed modifications of diglycosylated flavonols (see Supplementary Fig. S2C), and suggestive QTLs (1 < LOD < 2) for these compounds were also detected. These results may thus reveal an additional QTL at the end of chromosome 2. Alternatively, the genetic modification at the FLA2 QTL could be more complex than a simple loss of function of the myb12 gene or the genetic background of the RILs/HIFs could modify its output through epistasis.

Neither ANL2 nor 72B1 glycosyltransferase are involved in PA accumulation

ANTHOCYANINLESS (ANL2) is a homeobox gene that affects anthocyanin distribution in vegetative tissues (Kubo et al., 1999). GT72B1 is a glycosyltransferase which is the most closely related gene to UGT72L1 that is involved in epicatechin-3’-glucoside synthesis in Medicago.
Both genes co-localized with the FLA19 QTL (Table 2; Supplementary Fig. S3B at *JXB* online). Nevertheless, neither *anl2* (Ler) nor *gt72b1* (Col-0) mutants showed significant variation in seed PAs, suggesting that this variation cannot be explained by a loss-of-function allele of any of these genes in Shahdara.

**Fig. 6.** Flavonoid analysis of mutants for genes located near the FLA loci. G, glucoside; H, hexoside; I, isorhamnetin; K, kaempferol; Q, quercetin; PA, proanthocyanidin; R, rhamnoside; sol., soluble Significance in *t*-test compared with the wild type at the *5%, *1%, and **0.1% level.
78D2 glycosyltransferase are implicated in seed flavonol glucosylation

A cluster of three highly homologous glycosyltransferases, namely 78D2, 78D3, and At5g17040, that could be involved in the accumulation of a new flavonol-hexoside-rhamnoside found in Shahdara (Supplementary Fig. S5 at JXB online) is located in the region of the FLA13 locus (Supplementary Fig. S3D). Interestingly, 78D2 has been shown to be involved in anthocyanidin and flavonol glucosylation in leaves (Tohge et al., 2005; Kubo et al., 2007) and 78D3 is a flavonol arabinosyltransferase in leaves (Yonekura-Sakakibara et al., 2008), whereas the At5g17040 product has not yet been functionally characterized. Unfortunately, neither wild-type Col-0 nor the corresponding Col-0 mutants accumulate the additional quercetin derivative, so their involvement could not be tested (Fig. 6).

However, the 78D2 mutant still contained isorhamnetin 3-O-glucoside-7-O-rhamnoside when kaempferol or quercetin 3-O-glucoside-7-O-rhamnoside was absent. This showed that the 78D2 flavonol-3-glycosyltransferase solely catalyses the addition of a glucose moiety on kaempferol and quercetin aglycone but not on isorhamnetin. This also means that another, still unknown, glycosyltransferase transfers a glucose onto the isorhamnetins. Flavonol-arabinoside could not be detected in the seed, and the 78D3 glycosyltransferase mutant did not show any significant flavonoid changes. Other genes involved in flavonoid synthesis are located close to the FLA13 locus, such as the Bsister MADS domain TT16, the glutathione-s-transferase TT19, or chalcone synthase (CHS); however, their modifications are unlikely to produce such specific variation in a single flavonol.

HIF analysis around the loci FLA12, 14, 20, and 22 suggests a complex genetic basis for the observed variation in flavonoids

The QTLs explaining the variation of quercetin-3-O-rhamnoside, biflavonols, and soluble PA located at the end of chromosome 5 (FLA12, 14, 20 and 22) could be related to the LAC15/TT10 gene. TT10 encodes a laccase-like enzyme involved in oxidation of quercetin-3-O-rhamnoside to biflavonols and of epicatechin monomer and oligomers to oxidized procyanidins in the Arabidopsis seed coat (Poutre et al., 2005). Indeed, quercetin-3-O-rhamnoside and soluble PA contents were higher in plants fixed for the Bay-0 fixed allele [see additive effect (a) in Table 1] when biflavonols are more abundant in plants fixed for the Shahdara allele. However, HIF410, heterozygous around FLA12, only showed an accumulation of biflavonol with the Shahdara allele (Table 2; Supplementary Fig. S3E at JXB online).

HIF108 on the lower arm of chromosome 5 displayed higher soluble PA content (and perhaps quercetin-3-O-rhamnoside), whereas HIF093 segregated for higher quercetin-3-O-rhamnoside content with the Bay-0 allele (and possibly less biflavonols as observed for HIF410). Finally, these results suggested that the metabolic variations observed for the FLA12, 14, 20, and 22 loci are probably not explained by TT10 (LAC15) polymorphism and that biflavonol and PA variations could be controlled by different loci or are subjected to complex epistatic interactions.

HIFs fixed for the Cvi-0 or Shahdara alleles at the FLA5 or FLA15 locus contained more kaempferol derivatives than those fixed for the Col-0 or Bay-0 alleles, respectively, both in seeds and in leaves (Supplementary Fig. S6 at JXB online). Around FLA5, several genes belong to the flavonoid pathway, namely F3’H (TT7), FLS, and CHS. However, CHS alteration should affect the accumulation of all flavonoids (Routaboul et al., 2006). On the same line, the selective reduction of kaempferol derivatives observed both in seeds and in leaves (see Supplementary Fig. S6 at JXB online) is unlikely to be related to a modification of the FLS enzyme that uses both dihydroquercetin and dihydrokaempferol as substrates for quercetin and kaempferol production, respectively. A putative candidate for the FLA5 and FLA15 QTL was the F3’H enzyme that converts dihydrokaempferol into dihydroquercetin, the inhibition of which produces an increase in dihydrokaempferol and a decrease in quercetin derivatives, in the tt7-4 mutant (Routaboul et al., 2006). Finally, TT15 (DeBolt et al., 2009) is involved in PA accumulation and the corresponding gene is located near FLA10 and FLA18. The two tt15 mutant alleles (in the Col-0 and Ws-4 background) had reduced amounts of quercetin-3-O-rhamnoside and PAs (Fig. 6) that could match the observed variation linked to the FLA10 and FLA18 loci, respectively.

Discussion

Large quantitative variations for flavonoids are observed in Arabidopsis seed

The seed flavonoids of 41 accessions grown in controlled conditions have been analysed to gain a first insight into the naturally occurring variation in Arabidopsis. They were chosen among 265 worldwide accessions to maximize genetic diversity (McKhann et al., 2004). These secondary metabolites, at first sight, appear to be mostly dispensable in Arabidopsis, because the CHS mutants (tt4) that lack flavonoids showed limited adverse effects (Ylstra et al., 1996; Brown et al., 2001; Buer and Muday, 2004) at least under laboratory conditions. Nevertheless, all flavonoids, flavonols, and procyanidins were detected in all the accessions that were analysed. However, large quantitative variations were observed for seed flavonoids that were mainly due to quercetin-3-O-rhamnoside and PAs that accumulate in the seed coat. For instance, in Cvi-0, the amount of quercetin-3-O-rhamnoside was ~1% of that found in the Sp-0 accession. These quantitative variations were amplified, probably due to transgression, in the two RIL populations. Finally, the correlation between the
accumulation of different flavonoids observed in accessions or in the two RIL populations were usually conserved. These observations confirmed that this metabolism is highly regulated in Arabidopsis. A notable exception to these quantitative changes are three new flavonol-hexoside-rhamnosides found in the Shahdara accession that are presumably isomers of the known flavonol-3-O-glucoside-7-O-rhamnoside accumulated in the other accessions. This result suggested that a novel and specific glycosyl transferase that catalyses the production of flavonol-hexoside-rhamnoside isomers is active in Shahdara but not in Bay-0.

A limitation of the chemical analysis using quadrupole mass spectrometry [rather than a time-of-flight (TOF); Keurentjes et al., 2006] is that the characterization is limited to major UV-detected peaks and their derivatives, and thus minor compounds may be overlooked. Nevertheless, in Arabidopsis seedlings, a wider LC-MS untargeted screening of accumulated metabolites has been previously performed, revealing six different flavonols present in the two studied accessions (Ler and Cvi-0). Comparative analysis of seven oilseed rape genotypes (Auger et al., 2010), almond (Frison and Sporns, 2002), or fruit such as apples (Wojdylo et al., 2008), strawberries (Almeida et al., 2007), or grapes (Mane et al., 2007) also revealed essentially quantitative rather than qualitative changes.

Flavonoid accumulation is significantly controlled by a limited number of additive loci, of which only one seems common to both RIL sets.

Detected QTLs account for 11–61% of the observed phenotypic variation, suggesting that flavonoid accumulation in seeds is under the genetic control of a few additive loci, similarly to anthocyanin content in grape berry (Fournier-Level et al., 2009). Most loci were validated with two or more independent HIF lines with consistent phenotypic variation related to the segregating alleles at a given locus in different genetic backgrounds. This suggests that epistasis is usually not decisive in determining seed flavonoid content in the materials and conditions used here. In contrast, analysis of isoflavones in soybean seeds revealed QTLs that account for <5% of allelic differences (Melchinger et al., 1998; Gutierrez-Gonzalez et al., 2010). In the present analyses, only one QTL could be common to the two populations. In seedlings of a Cvi-0×Ler population, a QTL for flavonol content was also detected at ~90 cM on chromosome 1 that was not detected in the populations examined here (Keurentjes et al., 2006). This shows that the studied accessions have retained different genetic variations for shaping flavonoid accumulation (McMullen et al., 1998).

MYB12, TT15, and TT7 genes are candidates for the control of the observed natural flavonoid variations.

In total, three QTLs could be associated with a known candidate gene, MYB12 (R2R3 domain transcription factor), TT15 (UDP glucose:sterol-glucosyltransferase), and TT7 (F3′H, flavonoid-3′-hydroxylase). Further molecular characterization of these candidates, including quantitative expression analysis in HIF lines, promoter GUS reporter gene analysis, and allelic complementation will be needed to assess the mechanisms involved in natural variation.

The most promising candidate for controlling kaempferol contents (around FLA5 and FLA15) was the F3′H gene, which encodes the enzyme converting dihydrokaempferol into dihydroquercetin. Mutations at F3′H led to the accumulation of kaempferol derivatives (Routaboul et al., 2006; Routaboul et al., 2006). Col-0 compared with Cvi-0 accessions and the two independent HIF lines fixed for the Col allele showed a similar decrease in all kaempferol derivatives, suggesting that the Cvi F3′H allele could be limiting. The FLA5 locus was mapped between 0.0 Mb and 5.3 Mb, and the FLA15 QTL around marker NGA249 at 2.8 Mb, close to the F3′H gene position (2.5 Mb). In maize, the prl locus was recently characterized and shown to correspond to a F3′H gene (Sharma et al., 2011). This prl locus was detected as a major QTL for the synthesis of C-glycosyl flavones that have insecticidal activity against corn earworm (Lee et al., 1998; Cortes-Cruz et al., 2003).

Most QTLs that have been characterized showed genetic variation in Myb factors regulating transcription. For instance, MYB12 in the present study is possibly involved in the control of flavonol content. The white grape phenotype is also caused by the insertion of a transposable element in the promoter of the VvMYBA transcription factor that regulates a VvUFGT glycosyltransferase needed for anthocyanin accumulation (Kobayashi et al., 2004; Fournier-Level et al., 2009; This et al., 2007). Elsewhere, the PI locus in maize was governed by two duplicated Myb genes (Zhang et al., 2003). Additional experiments measuring the level of expression—rather than metabolites—in leaves detected PAPI, TTGI, and TTG2 as candidate genes in eQTL studies (Kliebenstein et al., 2006).

Most of the characterized QTLs may correspond to novel functions.

Interestingly, although >60 genes involved in flavonoid metabolism have already been characterized, most of the FLA QTLs may correspond to new functions, directly (i.e. new regulators, transporters, etc.) or indirectly (i.e. developmental genes or regulatory genes of higher hierarchical order) involved in this metabolic pathway. This rather unexpected number of new loci involved in the natural variation of flavonoids may be due to the fact that QTL analysis can reveal subtle quantitative and/or additive changes that have been overlooked in previous visual screens (Trontin et al., 2011). Co-localization of different QTLs might also be a first indication that some loci have a pleiotropic effect, due to a common mechanistic basis.

FLA5 and FLA15 co-localize with Flowering Locus C that encodes a transcription factor involved in the repression of flowering (Michaels and Amasino, 1999). Nevertheless, although HIF157 segregated for both (i.e.
flowering and flowering time) phenotypes, the HIF216 segregated only for flavonoid variations. This indicated that the flavonoid and flowering time changes around the FLC locus have independent genetic bases.

Flavonol and PAs have been proposed to be important for seed quality (i.e. germination, dormancy, and longevity; Debeaujon et al., 2000; Thompson et al., 2010). The variation in flavonoid identified in this study may be indirectly related to previously identified QTLs for seed quality. CDG3 and CDG6 that account for germination at low temperature in the dark in the Bay-0×Shahdara population (Meng et al., 2008) may correspond to FLA4, 17, and 19. DOG4 and 5 (Bentsink et al., 2007, 2010) that are related to a delay in germination co-localize with FLA11/21 and FLAS/15. Other loci (e.g. GW1/SSR2, 0SR1, and GW2) involved in the control of germination under moderate osmotic and salt stresses co-localize with FLA12, 17, and 21, respectively (Vallejo et al., 2010). GRS, an enhancer of abi-3-5, that affects seed longevity (Clerkx et al., 2003), co-localized with FLA19 responsible for increased PA accumulation in Sha relative to Bay-0. The flavonoid content of the two RIL sets given in Supplementary Tables S5 and S6 at JXB online will allow a finer comparison of the data with previous QTL analysis for the above flavonoid-related traits or others.

In summary, the metabolic analysis of 41 accessions and two RIL populations revealed the broad variation of seed flavonoid accumulation in Arabidopsis (and three new flavonol derivatives). The characterization of 22 QTLs in the two RIL populations dissected the genetic architecture underlying this natural variation. Most of the traits are controlled by a few additive loci with relatively broad effects. Further studies with the genotypes described here will be required to confirm candidate loci such as TT7, TT15, or MYB12. This work also paves the way for identifying novel genes that correspond to the other QTLs. More broadly, this study shows the potential of combining metabolomics and quantitative genetic for the characterization of new genes and novel markers for crop improvement that have not been revealed by previous qualitative screen.

**Supplementary data**

Supplementary data are available at JXB online.

**Figure S1.** Natural variation of seed flavonoid content in five contrasted accessions of Arabidopsis.

**Figure S2.** Confirmation of the major QTLs of the recombinant population Cvi-0×Col-0 by comparison of the phenotypes of heterogeneous inbred families (HIFs).

**Figure S3.** Confirmation of the major QTLs of the recombinant population Bay-0 and Shahdara by comparison of the phenotypes of heterogeneous inbred families (HIFs).

**Figure S4.** Mutation in 72B1 and ANL2, or CPC cannot explain natural variation corresponding to QTL FLA16 and FLA2, respectively.

**Figure S5.** Three additional glycosylated flavonols in the Shahdara genotype.

**Figure S6.** QTLs 5, 13, and 15 are also confirmed in leaves using HIF lines (HIF223 and 301, HIF157 and 216, and HIF157 and 214, respectively).

**Table S1.** Flavonoid content (mg g⁻¹) in accessions.

**Table S2.** Correlations (r and P-values) between the different flavonoids in selected accessions.

**Table S3.** Correlations (r and P-values) between the different flavonoids in selected recombinant inbred lines of Cvi-0×Col-0.

**Table S4.** Correlations (r and P-values) between the different flavonoids in selected recombinant inbred lines of Bay-0×Shahdara.

**Table S5.** Flavonoid content in selected Cvi-0×Col-0 RIL lines.

**Table S6.** Flavonoid content in selected Bay-0×Shahdara. RIL lines.

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Three different mechanisms of energy dissipation of a desiccation-tolerant moss serve one common purpose: to protect reaction centres against photo-oxidation

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* Dedicated to Professor Otto Ludwig Lange, pioneer of plant ecophysiology, on the occasion of his 83rd birthday
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Abstract

Three different types of non-photochemical de-excitation of absorbed light energy protect photosystem II of the sun- and desiccation-tolerant moss Rhytidium rugosum against photo-oxidation. The first mechanism, which is light-induced in hydrated thalli, is sensitive to inhibition by dithiothreitol. It is controlled by the protonation of a thylakoid protein. Other mechanisms are activated by desiccation. One of them permits exciton migration towards a far-red band in the antenna pigments where fast thermal deactivation takes place. This mechanism appears to be similar to a mechanism detected before in desiccated lichens. A third mechanism is based on the reversible photo-accumulation of a radical that acts as a quencher of excitation energy in reaction centres of photosystem II. On the basis of absorption changes around 800 nm, the quencher is suggested to be an oxidized chlorophyll. The data show that desiccated moss is better protected against photo-oxidative damage than hydrated moss. Slow drying of moss thalli in the light increases photo-protection more than slow drying in darkness.

Key words: Chlorophyll fluorescence, energy conservation, energy dissipation, photoprotection, photosystem II, reaction centre.

Introduction

After light has been absorbed in the pigment system of photosynthetic organisms, energy conservation is initiated by charge separation and electron transfer reactions within specialized reaction centres (RCs) of the photosynthetic apparatus. Within the RCs of photosystem II (PSII), primary photo-induced electron transfer from an electron donor termed P680 to the electron acceptor pheophytin creates a strong oxidant, P680+, and a reduced pheophytin, Pheo-. The former is capable of oxidizing water. The latter reduces the primary quinone electron acceptor QA within the RC. Further electron transfer reactions, which include photosystem I (PSI), result finally in the reduction of CO2. Problems arise when either water or electron acceptors, or both, are unavailable and light intensity is high. In that case, light damages the photosynthetic apparatus. The recombination of charges on P680+ and Pheo- activates oxygen (Krieger-Liszkay, 2005; Asada, 2006; Krieger-Liszkay et al., 2008). Singlet oxygen, 1O2, is highly oxidative. Damage by 1O2 or other oxidants formed in strong light can be prevented or minimized if excess light energy is rapidly converted into heat by the action of non-photochemical quenching mechanisms. In hydrated plants, a main mechanism of thermal energy dissipation is inactive in low light. It is activated under excess light. For activation, it requires...
the presence of the xanthophyll zeaxanthin and the light-dependent protonation of a thylakoid protein (Demmig-Adams, 1990; Niyogi, 1990; Björkman and Demmig-Adams, 1994; Ma et al., 2003; Li et al., 2004; Takizawa et al., 2007).

Light intensity controls this energy dissipation mechanism preventing competition with RCs, which remain active for photosynthesis even under strong light.

In desiccated photoautotrophs, persistence of normal RC activity under strong sunlight would cause serious photooxidative damage. It would endanger survival. Many mosses (more than 15,000 species in total) and most lichens (more than 13,000 species) are desiccation-tolerant (Lakatos, 2011). Full photo-protection in the desiccated state requires mechanisms of energy dissipation, which are more effective in dissipating light energy thermally than the zeaxanthin-dependent photo-protection, which operates in hydrated photoautotrophs.

Recently, picosecond measurements of fluorescence lifetimes have revealed a new mechanism of photoprotection in desiccated lichens (Veerman et al., 2007; Komura et al., 2010; Miyake et al., 2011). Migration of excitation energy to a far-red emitting pigment protein permits de-excitation of the bulk pool of excited chlorophyll, which is faster in the desiccated than in the hydrated state. Energy transfer with short time constants of 0.31, 23, and 112 ps appeared to drain excitation energy from PSII RCs (Komura et al., 2010; Miyake et al., 2011). This protects PSII RCs against photo-inactivation.

An earlier work with a shade-adapted moss, *Rhytidiadelphus squarrosus*, observed activation of zeaxanthin-dependent energy dissipation while the moss was hydrated (Heber et al., 2006a). On desiccation of the moss, photo-protective energy dissipation increased. This was attributed to the formation of a radical in PSII RCs, which is capable of quenching excitation energy. It is not yet known whether the energy dissipation mechanism that operates in the moss is related to a mechanism reported for desiccated lichens that exhibits picosecond fluorescence decay (Veerman et al., 2007; Komura et al., 2010; Miyake et al., 2011).

The present study used a closely related desiccation-tolerant moss species, *Rhytidium rugosum*, which survives full exposure to sunlight in contrast to shade-adapted *Rhytidiadelphus squarrosus*. The following questions were addressed: (1) Is *Rhytidium rugosum*, when desiccated, protected also by a mechanism of thermal energy dissipation, which is similar to that shown to prevent photooxidative damage to desiccated lichens? (2) What is the role of the radical observed earlier in *Rhytidiadelphus squarrosus* in desiccated *Rhytidium rugosum*, and is it stable? (3) Is the recombination of unstable radical pairs such as P680\(^+\) and Pheo\(^-\) non-toxic or potentially toxic by giving rise to the activation of oxygen in desiccated *Rhytidium rugosum*? (4) Does the effectiveness of photo-protection of hydrated moss differ from that of desiccated moss? To answer these questions, modulated fluorescence and fluorescence lifetimes were measured. Measurements of absorption changes in the far-red served to identify radicals in desiccated moss thalli.

### Materials and methods

The moss *Rhytidium rugosum* (Ehrh.) Kindb., family *Rhytiadaceae*, was collected either in the dry or the hydrated state from a sun-exposed location on calcareous soil near Leinach, 25 km from Würzburg, Bavaria, Germany. This site served repeatedly as a source of material during different seasons in the years 2006–2010. After collection, hydrated moss was slowly dried in dim light (<4 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) photosynthetically active photon flux density, PPFD) or in complete darkness and stored in darkness at a relative humidity below 65\% or a water potential below ~70 MPa before being used for experiments. Prolonged dark adaptation (usually 36 or 48 h) of hydrated thalli was intended to decrease zeaxanthin levels. Zeaxanthin is converted to violaxanthin in the dark or in low light (Björkman and Demmig-Adams, 1994).

The yield of chlorophyll fluorescence was measured beyond 700 nm after excitation with 650 nm light in a pulse amplitude modulation fluorometer (model 101, Walz, Effeltrich, Germany) (Schreiber et al., 1986). A modulated measuring beam of low intensity (average PPFD of 0.04 or 2.5 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) served to elicit fluorescence. Halogen lamps (KL 1500, Schott, Mainz, Germany) provided strong short light pulses (usually 1 s) and continuous illumination of white light through heat- and far-red-absorbing filters (Callflex c and DT-Cyan, Balzers, Liechtenstein) and fibre optics. The PPFD of the light pulses was 10,000 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) unless otherwise stated. During prolonged illumination with strong continuous light, the temperature of moss samples was monitored by a thermocouple and regulated by a cooling system. PPFDs were measured by a LI-COR189 quantum sensor (Fa Walz, Effeltrich, Germany).

Experiments with gas mixtures of different CO\(_2\) concentrations were performed using a sandwich-type cuvette, which permitted controlled gas flow over hydrated moss thalli. Light-dependent absorption changes were measured in reflection using the pulse amplitude modulation fluorometer in combination with ED800 T emitter/detector units (Walz) equipped with different LEDs. Peak emissions of the LEDs were at 802, 835, 875, and 950 nm.

Fluorescence lifetimes were measured as reported in Komura and Itoh (2009) and Komura et al. (2010). Fluorescence of dry and wet moss thalli was excited by a 430-nm laser pulse. The excitation light was obtained from a Ti:Sapphire laser (Mai Tai; Spectra-Physics, Newport Corporation, Irvine, CA, USA). The frequency-doubled light at 430 nm was generated by a type-I BBO crystal from an 860-nm laser pulse with a pulse duration of 150 fs and a repetition rate of 80 MHz. Fluorescence was focused onto the entrance slit of a 50-cm polychromator and was detected by a streak camera detector (50 cm, Chromex 2501-S, 100 g/mm, entrance slit of a 50-cm polychromator and was detected by a streak camera system (Hamamatsu Photonics, Hamamatsu, Japan) as described previously. The streak camera system was operated in the photon-counting mode to give 640 (wavelength) \(\times\) 480 (time) pixel 2D images for a 636–778 nm fluorescence emission range with 1-nm resolution and 1100- or 5530-ps time range. The signal was accumulated for approximately 0.5–1 h in each measurement or as described elsewhere in detail (Komura and Itoh, 2009; Komura et al., 2010).

The following parameters are described: \(F_m\), minimum yield of modulated fluorescence at the dark-adapted level, indicating that \(Q_A\) of PSII is oxidized; \(F_o\), stationary yield above \(F_o\), indicating that \(Q_A\) is partially reduced; \(F_m/F_o\) dessicated – 1 because \(F_m'\) is very close to \(F_o\) dessicated; then, \(F_m/F_o\) dessicated \(- 1\) because \(F_m'\) is very close to \(F_o\) dessicated; \(F_m/F_o\) dessicated \(- 1\) because \(F_m'\) is very close to \(F_o\) dessicated; \(F_m/F_o\) dessicated \(- 1\) because \(F_m'\) is very close to \(F_o\) dessicated; \(F_m/F_o\) dessicated \(- 1\) because \(F_m'\) is very close to \(F_o\) dessicated.

Glutaraldehyde, dithiothreitol (DTT), and nigericin were obtained from Sigma-Aldrich/Fluka (Seelze, Germany).
Results

Fluorescence emission spectra of hydrated and desiccated thalli

Fig. 1 shows fluorescence emission spectra of *Rhytidium rugosum* measured at room temperature. Emission around 680 nm originates mainly from PSII. Emission above 700 nm contains contributions of PSI, which is less fluorescent than PSII at room temperature. Desiccation suppressed fluorescence at 680 nm more strongly than above 700 nm. Moss desiccated in darkness had bands with maxima close to 680 and 710 nm (middle spectrum). Desiccation in the light decreased fluorescence more than desiccation in darkness. It shifted the peak of the far-red band to about 720 nm (lower spectrum).

The colour of the moss was not changed much by desiccation. Therefore, the large desiccation-induced loss of fluorescence cannot be ascribed to altered light absorption. It can be explained by effective competition between the radiation-less energy dissipation processes induced by desiccation and the fluorescence emission process in PSII.

Fluorescence lifetime measurements of hydrated and desiccated thalli

Fig. 2 shows 2D images of fluorescence decay (wavelength on the X-axis and delay time with respect to the 430-nm excitation laser flash on the Y-axis) of desiccated (A, dry) and hydrated (B, wet) thalli of *Rhytidium rugosum* measured at room temperature (25 °C). Excitation of fluorescence was at 430 nm. Each photon emitted as fluorescence emission from moss was detected by a charge-coupled device in a streak camera system as reported by Komura and Itoh (2009) and Komura et al. (2010). Each dot on the image indicates the trace of a photon accumulated in the photon-counting mode in the apparatus. As can be seen from the very short tail along the Y-axis of fluorescence at 680 nm of the image, which disappeared at 0.5 ns after the flash excitation, the decay of fluorescence was very fast in the dry thalli at all wavelengths (Fig. 2A). However, within a few minutes after re-wetting the thalli, the Y-axis tail of the fluorescence became longer (Fig. 2B). It should also be noted that the range of emission wavelengths did not change at all, although lifetime changed significantly.

Fig. 2C compares the fluorescence emission spectra of dry and wet thalli, calculated as the integration of all counts over the measurement times in the two images of Fig. 2A and B. A dotted line also indicates the expanded spectrum of the dry thalli after normalization of the peak heights. As already shown in Fig. 1, the spectrum of dry thalli showed smaller peak height than that of the wet thalli, especially in the 650–700 nm region, indicating the decrease of PSI fluoroscence. The smaller integrated intensities in the dry thalli in Fig. 2C, therefore, come mainly from the faster decay of PSII fluorescence after desiccation as shown in Fig. 2A and 2B.

Fig. 2D shows the decay kinetics of fluorescence at different wavelengths with respect to the laser excitation time. The apparent decay times (1/e) at 670, 690, 720, and 750 nm were 487, 596, 354, and 403 ps in the wet thalli (Table 1). They were 105, 137, 113, and 137 ps in the dry thalli. The acceleration ratios at 670 and 690 nm were 4.6 and 4.4, respectively, and larger than those of 3.1 and 2.9 at 720 and 750 nm. The time courses at 750 nm also indicated some rising phase immediately after the excitation. This suggests energy transfer from the shorter wavelength bands in the dry moss. The result indicates acceleration by desiccation of
energy migration from PSII antenna (at 670 nm) and the reaction centre (680–690 nm bands) towards the far-red lower energy bands, which dissipate energy as heat and do not transfer energy back to the shorter bands in an uphill process.

The result of measurements of fluorescence lifetime also indicates that desiccation of moss accelerates the decay of chlorophyll fluorescence. The effect is similar to that reported recently for desiccated lichens (Veerman et al., 2007; Komura et al., 2010; Miyake et al., 2011). The extent of acceleration in Rhytidium under the present condition was a little smaller compared to that reported for lichens. The results suggest the induction by desiccation of a new energy dissipation pathway in PSII that quenches excitation energy.

**Effects of consecutive hydration/desiccation cycles on modulated chlorophyll fluorescence emission**

Fig. 3A–C shows changes of modulated fluorescence of *Rhytidium rugosum* during three consecutive hydration–desiccation cycles that lasted about 24 h in total. Excitation was done with a PPFD of 0.04 μmol m$^{-2}$ s$^{-1}$ (during the first and third hydration cycles) to establish near-darkness conditions. Strong light pulses (6000 μmol m$^{-2}$ s$^{-1}$) of 1 s duration interrupted near-darkness conditions once every 500 s. PPFD was 2 μmol m$^{-2}$ s$^{-1}$ during the second hydration cycle where additional actinic light was given to decrease noise levels. The light pulses served to monitor the maximum level of fluorescence yield ($F_m$). Before the start of the first cycle, hydrated moss had been dark-adapted for 36 h. It was then dried in darkness to decrease zeaxanthin levels.

In the first hydration–desiccation cycle in the desiccated moss (Fig. 3A), two strong light pulses elicited small transient fluorescence responses, which indicated some charge separation in the PSII RCs followed by the reduction of the primary quinone electron acceptor $Q_A$ (Duyzens and Sweers, 1963). Fluorescence decreased slightly immediately upon hydration. This is probably caused by the oxidation of $Q_A$ (see also Fig. 1B in Heber et al., 2006a for the moss *Rhytidadelphus*). Subsequently, fluorescence increased from the minimum level slowly towards a maximum. Pulse-induced fluorescence responses increased strongly towards $F_m$. The ratio of variable to maximum fluorescence $[(F_m-F_o)/F_m = F_v/F_m]$ is known to be a measure of the quantum efficiency of energy conversion in PSII RCs (Genty et al., 1989). $F_v/F_m$ increased to 0.58 during the first hydration phase (Fig. 3A). This value is lower than maximum values of $F_v/F_o$ (about 0.8), which are reported for unstressed higher plants (Björkman and Demmig, 1987). $F_v/F_m$ ratios of hydrated thalli observed in the field after darkening were variable depending on previous exposure to strong light.

Both the steady-state fluorescence yield and fluorescence responses towards $F_m$ decreased as the moss dried out. This is direct evidence of the activation of photo-protective energy dissipation mechanisms because energy dissipation, fluorescence and photosynthetic reactions are competitive to one another. Non-photochemical fluorescence quenching (NPQ), a conventional measure of the efficiency of thermal
energy dissipation, increased from zero to 4 during the first dehydration phase (Fig. 3A) while the moss dried out.

A second hydration cycle increased fluorescence again (Fig. 3B). Illumination at approximately 50% of bright sunlight (600 μmol m⁻² s⁻¹) decreased both steady-state fluorescence and pulse-induced fluorescence responses suggesting activation of photo-protective thermal energy dissipation. NPQ increased to 1.9 in the hydrated condition. Desiccation then increased NPQ to 5.6. Strong light pulses given after complete desiccation failed to elicit fluorescence responses. Illumination was turned off before the third hydration phase.

In the third cycle (Fig. 3C), addition of water once again increased steady-state fluorescence. Pulse-induced fluorescence responses increased to a level smaller than that observed in the first hydration phase (Fig. 3A). The peak Fₐ/Fₘ value in the third cycle was 0.42 compared to 0.58 during the first hydration. This shows that the stable charge separation in PSII RCs, indicated by the Fₐ/Fₘ values, had decreased by more than 25%. This is seen as a consequence of the illumination given during the second hydration phase (Fig. 3B). Apparently, the activation of energy dissipation that produced an NPQ of 1.9 during the preceding illumination period in the second cycle (Fig. 3B) had not been sufficient for full photo-protection of PSII RCs. NPQ returned to 4.3 after the third hydration phase (Fig. 3C).

The ratios of Fₐ hydrated/Fₐ desiccated were also calculated to reveal how much faster excitation energy is trapped in active dissipation centres of desiccated thalli than in open RCs of hydrated thalli. Fₐ/Fₐ was 2.3 after the first hydration–dehydration cycle. It became 3.9 under the influence of illumination during the second hydration–dehydration cycle. When illumination was absent during the third hydration–dehydration cycle, Fₐ/Fₐ returned to 2.8.

In a number of additional experiments similar to those shown in Fig. 3, in which hydrated dark-adapted moss was exposed to 600 μmol m⁻² s⁻¹ for a few hours, loss of stable charge separation was as large as or larger than that detected in the experiment of Fig. 3. It is therefore concluded that strong illumination of hydrated sun-tolerant moss cannot prevent appreciable photo-damage to PSII RCs. The observations appear to explain the considerable variation in Fₐ/Fₘ values of hydrated Rhytidium in the field.

Energy dissipation in hydrated moss: activation by protonation and inhibition by DTT

Increased NPQ under strong light during the second hydration cycle in Fig. 3B (NPQ 1.9) suggests involvement of zeaxanthin in energy dissipation (Demmig-Adams, 1990). Zeaxanthin-dependent energy dissipation is known to be controlled by the protonation of the PsbS protein (Li et al., 2004) or a similar thylakoid protein (Benente et al., 2010). In low light or darkness, zeaxanthin is reconverted to violaxanthin. Fig. 4 shows a fluorescence experiment in which hydrated thalli of Rhytidium rugosum, which had been dark adapted for 48 h, received strong light pulses given 1 min apart. In Fig. 4A, replacement of air by 20% CO₂ in air decreased not only Fₘ but also steady-state fluorescence, Fₛ, that was slightly above F₀. As shown by Bukhov et al. (2001), the potential acid CO₂ is capable of replacing light as a source of protons for the activation of energy dissipation in hydrated mosses and lichens provided zeaxanthin is present. Removal of CO₂ largely reversed fluorescence quenching. The observation of CO₂-dependent quenching in Fig. 4A suggests that thalli of dark-adapted Rhytidium rugosum still contained zeaxanthin.

DTT is known to be an inhibitor of the light-dependent de-epoxidation of violaxanthin to zeaxanthin (Yamamoto and Kamite, 1972). The experiment of Fig. 4B shows that DTT is also an inhibitor of the activation of energy dissipation by protonation. After dark-adapted moss thalli were incubated for 5 h with 5 mM DTT, 20% CO₂ failed to decrease steady-state fluorescence. As DTT cannot prevent protonation reactions caused by CO₂, it is concluded that inhibition of the activation of energy dissipation by DTT occurs at a step in the signal transduction chain of zeaxanthin-dependent energy dissipation, which is beyond the protonation reaction.

Moss thalli, which had been pre-incubated for 5 h in 5 mM DTT, lost fluorescence during desiccation as much as that in hydrated controls (data not shown). This shows that DTT does not inhibit desiccation-induced energy dissipation. The protonophore nigericin, another inhibitor of zeaxanthin-dependent energy dissipation, was also ineffective to inhibit desiccation-induced energy dissipation (data not shown).

Effects of glutaraldehyde on desiccation-induced energy dissipation

Glutaraldehyde possesses two aldehyde groups that can react with proteins (Coughlan and Schreiber, 1984). In Fig. 5A,
fluorescence was measured immediately after hydration of dried moss in 0.2% aqueous solution of glutaraldehyde. Fluorescence increased slowly. It did not decrease while the moss dried. In Fig. 5B, fluorescence was measured after hydrated thalli had been pre-incubated for 1 h with 0.2% aqueous glutaraldehyde. During drying, fluorescence decreased, but much less than that in the control experiment (Fig. 5C), in which dry thalli had been hydrated in water for 1 h before fluorescence was measured.

A comparison of the three experiments of Fig. 5 shows that 0.2% aqueous glutaraldehyde decreased, but did not fully inhibit, pulse-induced charge separation in PSII RCs. Drying caused complete or strong loss of charge separation. Importantly, drying resulted in strong loss of fluorescence only in the control experiment (Fig. 5C) but not in the glutaraldehyde experiments (Fig. 5A and B). The data suggest involvement of glutaraldehyde-sensitive proteins in desiccation-induced energy dissipation.

Desiccation-induced energy dissipation is highly effective in providing phototolerance

Fig. 6 shows modulated chlorophyll fluorescence during two consecutive hydration/desiccation cycles of Rhytidium rugosum. In Fig. 6A, the moss had been dried slowly immediately after collection in the sun. In Fig. 6B, the moss, collected from the same place, had been dark-adapted in the hydrated state for 36 h. It was then dried in the dark.

Initial NPQ in the dry state was 6 in Fig. 6A. After hydration in near-darkness and subsequent desiccation it was reduced to 3.5 (not shown in Fig. 6A). This is a result of partial relaxation of desiccation-induced energy dissipation during hydration in darkness. The maximum Fv/Fm ratio was 0.28 during hydration. The ratio Fv/F’o, a measure of the suppression of Fv by desiccation, was about 5 before and 3.3 after the hydration. This also reveals relaxation of desiccation-induced energy dissipation. A 60 min exposure of the dry moss to 10,000 μmol m⁻² s⁻¹, i.e. to almost eight times maximum sunlight, decreased fluorescence of the desiccated moss. Most of this decrease was not reversed by darkening. A subsequent hydration increased fluorescence to give the maximum Fv/Fm ratio of 0.3. This shows that exposure of the desiccated moss to extremely strong irradiation for 1 h had not damaged the PSII RCs.

The only difference between the experiments in Fig. 6A and B was dark adaptation of the moss used for Fig. 6B. Fv/F’o was 2.7 in Fig. 6A and 1.9 in Fig. 6B. This reveals loss of photo-protection during the dark adaptation. Extremely strong illumination for 60 min (10,000 μmol m⁻² s⁻¹) for 60 min decreased fluorescence of the dry thalli as shown in Fig. 6A. The subsequent hydration increased fluorescence again, but pulse-induced fluorescence responses were now decreased compared to those in the first hydration/desiccation cycle. Maximum Fv/Fm was 0.18, which is lower than the value of 0.28 observed before strong illumination of the dry moss. The result confirms that dark-adaptation of hydrated moss had increased the sensitivity to photo-inactivation after drying.

Fig. 5. Changes in modulated chlorophyll fluorescence in hydrated Rhytidium rugosum during slow desiccation. (A) Desiccated moss thalli were hydrated in 0.2% aqueous glutaraldehyde shortly before the measuring beam was turned on. (B) Rehydrated thalli were exposed for 1 h to 0.2% glutaraldehyde before the measuring beam was turned on. (C) Control experiment without glutaraldehyde, otherwise as in B. Saturating light pulses were given every 500 s to probe for stable charge separation in PSII RCs. Measuring beam (m. b.) was turned on and off as shown by up and down arrows, respectively.

Fig. 6. Modulated chlorophyll fluorescence during two consecutive hydration/desiccation cycles of Rhytidium rugosum interspaced by a 60 min illumination period (PPFD of 10,000 μmol m⁻² s⁻¹) of the dry moss. (A) Thalli exposed for 4 days to full sunshine during a high-pressure period in early October and dried. (B) Thalli obtained as in A hydrated in near-darkness for 2 days in the laboratory and then dried in darkness. Strong light pulses (1 s) were given every 500 s. For explanation, see text.
In Fig. 7, the kinetics of light-induced changes of the fluorescence yield is compared with those of light-induced absorption changes at 802 and 950 nm. In Fig. 7A, the thalli used had been dark-adapted in the hydrated state for 36 h before they were dried in the dark. Upon strong illumination with white light, fluorescence increased briefly showing charge separation in PSII RCs and rapid reduction of QA. Subsequently, fluorescence declined slowly during the illumination. It increased again on darkening. The initial brief increase in fluorescence seen in Fig. 7A was always absent when light-adapted desiccated thalli were illuminated (Fig. 7B). A semi-logarithmic plot of fluorescence intensity against recovery time in the dark revealed two decay phases. The slowest phase obeyed first order kinetics with a half time of 6 s (apparent reaction rate constant of 0.12 s\(^{-1}\)). The faster recovery phases could not be resolved well with the present experimental set up.

This study always detected reversible light-dependent absorption changes in the far-red region when desiccated thalli were strongly illuminated with white light. Strong far-red illumination did not elicit appreciable absorption changes or fluorescence responses. It is therefore concluded that the fluorescence changes shown in Fig. 7A and B originated mainly from PSII and not from PSI (see also data in Heber et al., 2006a). The time course of the absorption change at 802 nm (Fig. 7D) or 832 nm (not shown) resembled that of the change in fluorescence yield (Fig. 7B). Strong illumination increased absorption of far-red measuring light, and darkening decreased it.

Light-induced increases in absorption were also observed at 950 nm (Fig. 7C) and 875 nm (not shown). They may be attributed to the formation of carotenoid radicals. Their kinetics were different from those observed at 802 and 835 nm. The dark recovery was too fast to be resolved.

**Discussion**

Mosses occupy a broad spectrum of ecological niches. Within the family Rhytidiaceae, *Rhytidiadelphus squarrosus* tolerates full exposure to the sun, whereas a closely related species, *Rhytidium rugosum* (Heber et al., 2006a) is restricted to shaded environments. Whereas both species tolerate desiccation, *Rhytidium rugosum* bleaches slowly when left desiccated in the sun. In contrast, desiccated thalli of *Rhytidiadelphus squarrosus* were not damaged even by very high photon fluxes. Figs. 3 and 6 show that slow desiccation severely depresses the \(F_m\) and \(F_o\) levels of PSII fluorescence of *Rhytidium rugosum*. This is accompanied by the acceleration of fluorescence decays (Fig. 2). Hydrated thalli proved to be sensitive to strong light (Fig. 3). From the data, it is concluded that three different mechanisms of NPQ protect PSII RCs of *Rhytidium rugosum*. One of them is active in hydrated thalli. It appears to involve zeaxanthin and is activated by a protonation reaction (Fig. 4). Two other mechanisms are induced by desiccation. One of them is similar to a mechanism detected before in desiccated lichens (Fig. 2; Veerman et al., 2007; Komura et al., 2010; Miyake et al., 2011). The other is based on a photoreaction. This effect can be observed not only in hydrated but also in desiccated moss thalli (Fig. 7; Heber et al., 2006a). It is characterized by light-dependent absorption changes around 800 nm and is thought to represent the formation of a chlorophyll radical which acts as a quencher of excitation energy in RCs of PSII.

**Effectiveness of photoprotection**

Values of NPQ, commonly used to describe the extent of photo-protective energy dissipation, are derived from experimentally observed changes in \(F_{aw}\). They fail to describe sufficiently the competition between different fates of absorbed light energy in photosynthesis. Therefore, \(F_o/F_{aw}\) ratios were calculated in an attempt to understand competition between radiationless energy dissipation and fluorescence when photochemical use of light for photosynthesis becomes negligible as thalli dry out (Figs. 3 and 6). In hydrated moss thalli, \(F_o\) describes a fluorescence situation, in which photochemical light use approaches 100% and thermal energy dissipation is negligible. After desiccation, \(F_o\) describes a very different situation. Thermal energy dissipation has been activated and photochemical light use is essentially absent. This situation lowers fluorescence...
below the original $F_o$ level. High $F_o/F_o'$ ratios suggest energy dissipation to be so fast in desiccated thalli as to compete successfully with open PSII RCs for trapping excitation energy. This protects the RCs from photodamage.

Further protection is not necessarily required, but can be provided, if needed, by the formation of a quencher of excitation energy within the RCs. The light-dependent formation of such a quencher is demonstrated in Fig. 7. In Fig. 3, desiccation resulted in NPQ values of 4 and 4.3 when thalli were dried in darkness. Under these conditions, the corresponding $F_o/F_o'$ ratios were 2.3 and 2.8, respectively. Charge separation in PSII RCs was strongly, but not completely, suppressed as shown by residual fluorescence responses to strong light pulses. When the thalli were dried in the light, NPQ was 5.6 and $F_o/F_o'$ was 3.9. At such ratios, charge separation in PSII RCs was fully suppressed. Apparently, excitation energy bypassed RCs. It was rapidly converted to thermal energy.

In hydrated plants, charge separation in PSII RCs is known to take 3–5 ps (Zinth and Kaiser, 1993; Holzwarth et al., 2006). Although it is not known whether desiccation changes these values, the high $F_o/F_o'$ ratios in Fig. 3 suggest that the bulk of excitons is rapidly deactivated and converted to thermal energy in competition with the trapping of excitons by functional PSII RCs. In fact, fluorescence decays in the picosecond time scale for desiccated moss reveal very fast energy transfer from the major PSII antenna bands to a far-red band (Fig. 2). In lichens, the quencher is an unidentified far-red absorbing molecule (Veerman et al., 2007; Komura et al., 2010; Miyake et al., 2011). In contrast, Slavov et al. (2011) proposed that desiccation had increased spillover of excitation energy from PSII to PSI, and that fluorescence is quenched by P700$^+$. The activation of energy dissipation in hydrated moss thalli by CO$_2$ in the experiment of Fig. 4 resulted in an NPQ of 1.2. $F_o/F_o'$ could not be measured because the $F_o$ level measured in Fig. 4 was above the $F_o$ level. The NPQ of 1.9 induced by the actinic illumination during the second hydration in the experiment of Fig. 3B can, therefore, be attributed to zeaxanthin-dependent energy dissipation.

A comparison of NPQ values shows that activation of energy dissipation by protonation or by light cannot increase NPQ as much as desiccation does. Thus, some photo-damage to RCs under excess light appears to be unavoidable in hydrated thalli (Fig. 3) and metabolic repair of damage is required (Aro et al., 1993). Desiccation-induced fluorescence quenching provides stronger photoprotection to the moss Rhytidium (Figs. 3 and 6) and to lichens (Heber et al., 2007; Veerman et al., 2007; Heber, 2008; Komura et al., 2010; Slavov et al., 2011) than the protonation-regulated energy dissipation mechanism.

**Picosecond fluorescence decay**

Desiccation of Rhytidium decreased fluorescence intensity of the main band at 687 nm more strongly than above 700 nm (Fig. 1). More fluorescence was lost by desiccation under light than in darkness. This observation suggested migration of excitons from the PSII major antenna chlorophylls towards the far-red bands where they are trapped in desiccation centres (Heber and Shuvalov, 2005). For lichens, time-resolved fluorescence analysis has led to the discovery of a long-wavelength quencher, which is coupled to the pigment system of PSII (Veerman et al., 2007; Komura et al., 2010; Miyake et al., 2011).

For the moss Rhytidium, acceleration of the decay of fluorescence by desiccation is shown in Fig. 2. Fluorescence decay of hydrated thalli was faster at 670 nm than at other wavelengths. This fluorescence is thought to be mainly emitted by antenna chlorophylls in PSI. The decay was 4.6-fold accelerated by desiccation (Table 1). At 680–690 nm of PSI range, decay was 4.4-fold accelerated by desiccation. At 720 and 750 nm, where fluorescence is thought to come also from PSI that decays faster, desiccation accelerated the decays only about 3-fold, which is less compared to the acceleration in the PSI range. It is, therefore, concluded that fluorescence of PSI was specifically accelerated by desiccation (Fig. 2A).

In a previous lichen study, it was proposed that a newly activated energy-accepting molecule, which emits fluorescence around 740 nm, accepts excitation energy from PSI bands of shorter wavelengths (Veerman et al., 2007; Komura et al., 2010). The acceleration of the 720 and 750 nm fluorescence decay by desiccation in Fig. 2 suggests that the quencher activated in Rhytidium rugosum is also connected to the PSI antenna. It, therefore, appears that the mechanism of fluorescence quenching in the near far-red detected in the desiccated moss in this study is very similar to that in desiccated lichens. The acceleration of fluorescence decay in the moss is less marked compared to that in lichens.

**Co-operation of desiccation-induced energy dissipation centres**

Results of Figs. 2, 3, and 7 are interpreted to show cooperation of two different mechanisms of energy dissipation in desiccated Rhytidium. One is characterized by the accelerated decay of 690 nm emission and the light-dependent formation of a quencher presumably in PSI RCs (Fig. 7), the other one by rapid fluorescence decay in the near far-red. Fast loss of 750 nm fluorescence is interpreted to be the result of fast migration of excitons from the PSI antenna to an as yet unknown pigment protein in or near the PSI antenna. There, thermal de-excitation to the ground state takes place.

Special proteins such as the pH-sensitive PsbS protein of higher plants (Li et al., 2004) and the LhCSR3 protein of lower plants (Benente et al., 2010) are known to be essential components of the zeaxanthin-dependent mechanism of photo-protection. Glutaraldehyde, an agent capable of fixing protein structures (Coughlan and Schreiber, 1984), inhibits loss of fluorescence during desiccation of Rhytidium (Fig. 5A and B) and of lichens (Heber, 2008). Inhibition of desiccation-induced energy dissipation by glutaraldehyde
demonstrates the involvement of a pigment protein in the mechanism of energy dissipation. The far-red emission observed at room temperature in Figs. 1 and 2 is, therefore, assumed to partially originate from a special pigment protein, which undergoes conformational changes during desiccation, thereby activating dissipation centres.

Energy, not trapped in these centres, can be quenched in PSII RCs that are transformed into dissipation centres (Fig. 7). Fast decay of fluorescence at 690 nm, which is accelerated by desiccation, may originate from the PSII RC core complex (Fig. 2). It is thought to reflect the quenching of excitation energy in PSII RCs. Fig. 7A shows that strong illumination first increased and then decreased fluorescence of dark-adapted desiccated moss. The initial increase, which indicates reduction of QA in PSII RCs (Duysens and Sweers, 1963), was never observed in desiccated moss thalli that had been dried in the light (Fig. 7B). The slow light-dependent loss of fluorescence (Figs. 7A and B) shows formation of a quencher, which appears from its optical properties to be a chlorophyll radical (Fig. 7D; Borg et al., 1970; Fujita et al., 1978). Such radicals can act as quenchers (Faller et al., 2006).

It should be noted that the quencher is unlikely to be P700+ in PSI RC because strong far-red illumination, which oxidizes P700, did not quench fluorescence in desiccated thalli. PSII RCs contain six chlorophylls, two pheophytins, and two β-carotenes in the core D1/D2 subunit moiety. In desiccated plant leaves and in desiccated moss, carotene was oxidized in the light with very low quantum efficiency (Shuvalov and Heber, 2003; Heber et al., 2006b). When oxidized carotene reacts with a neighbouring chlorophyll in a PSII RC, Chl+ is formed. This chlorophyll radical could be the quencher shown in the experiments of Fig. 7B and D (Faller et al., 2006).

Presence of light during desiccation increases phototolerance

In some chlorolichens, the presence of light during desiccation has been shown to increase fluorescence quenching (Heber et al., 2007). In Fig. 1, illumination of thalli of Rhytidium rugosum during desiccation increased the loss of fluorescence. In Fig. 3, NPQ increased from 4 during the desiccation in darkness to 5.6 after desiccation in the light. The F/F’ ratio increased from 2.3 to 3.9. Apparently, the presence of light during the desiccation increased phototolerance considerably.

Levels of zeaxanthin are known to increase under strong illumination when violaxanthin is de-epoxidized in a light-dependent reaction of the xanthophyll cycle (Demmig-Adams, 1990). In a similar de-epoxidation reaction, lutein is synthesized in the light (Matsubara et al., 2007). The photo-protective effect of light during desiccation may suggest a role of zeaxanthin or other carotenoids in the mechanisms of desiccation-induced photoprotection, although inhibitors such as dithiothreitol (Fig. 4) or nigericin (not shown), known to inhibit zeaxanthin-dependent energy dissipation, failed to inhibit desiccation-induced energy dissipation in Rhytidium and in chlorolichens (Heber, 2008).

Molecular mechanisms of energy dissipation

The discussion of molecular mechanisms of energy dissipation is still controversial. In higher plants, energy dissipation is thought to occur in the major light harvesting complex LHC2 of PSII (Pascal et al., 2005) or in the minor antenna proteins such as CP24 or CP29 (de Bianchi et al., 2011). It has been proposed to be the result of aggregation of LHC2 proteins (Horton et al., 1996; Pascal et al., 2005) or of energy transfer from chlorophyll to low-lying electronic states of xanthophylls such as zeaxanthin or lutein (Ruban et al., 2007; Liao et al., 2010). Other proposals envisage the rapid reversible charge transfer from chlorophyll to a xanthophyll (Holt et al., 2005; Avenson et al., 2008) or to chlorophyll (Müller et al., 2010) followed by an energy-dissipating recombination reaction. On the other hand, Slavov et al. (2011) proposed desiccation-induced spillover of excitation energy from PSII to PSI and quenching by P700+ as the main mechanisms of photoprotection in the lichen Parmelia sulcata. A minor role in photo-protection was assigned to desiccation-induced Chl–Chl charge transfer in the antenna of PSI followed by dissipative recombination.

Concluding remarks

The initial aim of the present study was to answer several questions:

(1) Are desiccated thalli of the moss Rhytidium rugosum protected by a mechanism of thermal energy dissipation similar to that previously shown to prevent photo-oxidative damage to desiccated lichens? The answer is yes (Fig. 2).

(2) What is the role of a previously observed radical in the photoprotection of desiccated Rhytidium rugosum and is it stable? The answer is that the radical is a quencher of excitation energy. It is tentatively identified as a chlorophyll radical in PSII RCs, which is produced in a reversible photoreaction even in desiccated moss thalli (Fig. 7).

(3) Is the recombination of unstable radicals such as P680+ with pheo non-toxic or potentially toxic by giving rise to the activation of oxygen? The answer is that recombination reactions of radical pairs, when they occur, are non-toxic in desiccated thalli of Rhytidium rugosum as shown by full photo-protection of thalli after exposure to extremely strong illumination (Fig. 6). Similar observations reported for desiccated cyanobacteria are explained by a pheophytin-independent recombination pathway (Ohad et al., 2011). Oxygen activation is not observed.

(4) Does the effectiveness of photo-protection of hydrated moss differ from that of desiccated moss? The answer is yes. Hydrated thalli are far more sensitive to damage by strong light than desiccated thalli (Fig. 3).

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Ammonium-induced loss of root gravitropism is related to auxin distribution and TRH1 function, and is uncoupled from the inhibition of root elongation in *Arabidopsis*

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Abstract

Root gravitropism is affected by many environmental stresses, including salinity, drought, and nutrient deficiency. One significant environmental stress, excess ammonium (NH₄⁺), is well documented to inhibit root elongation and lateral root formation, yet little is known about its effects on the direction of root growth. We show here that inhibition of root elongation upon elevation of external NH₄⁺ is accompanied by a loss in root gravitropism (agravitropism) in *Arabidopsis*. Addition of potassium (K⁺) to the treatment medium partially rescued the inhibition of root elongation by high NH₄⁺ but did not improve gravitropic root curvature. Expression analysis of the auxin-responsive reporter gene *DR5::GUS* revealed that NH₄⁺ treatment delayed the development of gravity-induced auxin gradients across the root cap but extended their duration once initiated. Moreover, the β-glucuronidase (GUS) signal intensity in root tip cells was significantly reduced under high NH₄⁺ treatment over time. The potassium carrier mutant *trh1* displayed different patterns of root gravitropism and *DR5::GUS* signal intensity in root apex cells compared with the wild type in response to NH₄⁺. Together, the results demonstrate that the effects of NH₄⁺ on root gravitropism are related to delayed lateral auxin redistribution and the TRH1 pathway, and are largely independent of inhibitory effects on root elongation.

Key words: Auxin, gravitropism, K⁺, NH₄⁺ stress, root elongation, TRH1.

Introduction

Root gravitropism is the process that dictates the growth of roots along a specific angle relative to gravity, also known as the gravitational set point angle (GSPA) (Blancaflor and Masson, 2003). Gravitropism is a major determinant in the distribution of root systems in soil and thus influences critical activities such as anchorage and uptake of water and nutrients from the soil (Forde and Lorenzo, 2001; Perrin *et al.*, 2005). Upon gravistimulation, the gravitropic response of roots has been conceptually separated into four phases: gravity perception, signal transduction, signal transmission, and curvature response (Perrin *et al.*, 2005). Gravity sensing occurs mainly in the root cap and through the sedimentation of amyloplasts within the columella cells (Chen *et al.*, 2002; Masson *et al.*, 2009). Although the receptors and molecular mechanisms that sense amyloplast motion are still unknown, there have been observations suggesting that cytosolic ions such as Ca²⁺ and the rapid changes of cytoplasmic pH within columella cells may participate in gravity signal transduction in roots (Scott and Allen, 1999; Fasano *et al.*, 2001, 2002; Hou *et al.*, 2004; Monshausen *et al.*, 2011). Gravity perception and signal transduction promote the formation of a lateral auxin gradient across the stimulated organs, leading to differential cell expansion on opposite flanks of elongation zone tissues.
responsible for tip curvature (Swarup et al., 2005; Masson et al., 2009). However, the root cap and elongation zone, the locations of stimulus perception and growth response, respectively, are spatially separated. Therefore, the root gravitropic response requires the transmission of a gravitational signal, which occurs via a lateral auxin gradient, toward the elongation zone (Chen et al., 2002; Swarup et al., 2005). In Arabidopsis roots, auxin influx carriers of the AUX1/LAX family, the PIN family of auxin efflux facilitators, ABC transporters, and the putative potassium transporter TRH1 all participate in a complex network that mediates polar auxin transport and regulates morphogenesis and growth of roots (Perrin et al., 2005).

In addition to gravity, an omnipresent environmental signal, root growth direction, is also affected by various environmental stresses, such as nutrient status, water availability, gradients in temperature, salinity, and mechanical impedance (Bonser et al., 1996; Liao et al., 2001; Forde and Lorenzo, 2001; Vicente-Agullo et al., 1996; Liao et al., 2002; Takahashi et al., 2003; Vicente-Agullo et al., 2004; Sun et al., 2008; Shibasaki et al., 2009). In all these cases, a complex mix of signals must be sensed by roots and integrated into an appropriate developmental response in order to overcome the signal from gravity and reorient root growth to navigate past barriers or toward favourable conditions (Fasano et al., 2002). For example, the availability of phosphorus can regulate the root configuration of leguminous plants by altering the growth angle of the basal roots so as to better take up phosphorus from soil (Bonser et al., 1996; Liao et al., 2001). Likewise, reductions in external potassium trigger agravitropic root growth so that roots can grow away from potassium-impaired regions, which may well represent a mechanism by which plants respond to mineral deficiencies in general (Vicente-Agullo et al., 2004). In addition, moisture gradients or water stress cause immediate degradation of amyloplasts in columella cells of the root cap, so as to reduce the response of roots to gravity and allow them to exhibit hydrotropism (Takahashi et al., 2003). These studies show that plants have evolved highly adaptive regulatory mechanisms in the control of root-directional growth and are capable of perceiving and responding to a variety of external stimuli so as to maintain optimal development.

Ammonium (NH₄⁺), an important source of nitrogen for many species (Kronzucker et al., 1997), is frequently present in soil environments in excessive quantities and leads to growth retardation (Britto and Kronzucker, 2002). This is especially so when NH₄⁺ is supplied as the sole nitrogen source or in combination with a low availability of potassium (K⁺) (Kronzucker et al., 2003; Qin et al., 2008; Balkos et al., 2010; ten Hoopen et al., 2010). In recent years, significant advances have been made in the study of the mechanisms of ammonium toxicity (Gerendás et al., 1997; Britto and Kronzucker, 2002; Qin et al., 2008; Li et al., 2010; Kempinski et al., 2011; Li et al., 2011a, b). However, very little is known about the root gravitropic response to excess ammonium.

Root tips act as sensors for different stimuli such as gravity and moisture gradients, and can integrate multiple tropic responses essential for root navigation (Takahashi et al., 2009). Laser ablation of defined columnella cell layers in the cap of Arabidopsis primary roots inhibited root curvature (Blancaflor et al., 1998). Previous research found an alteration in the distal organizer pattern of the primary root tip in Arabidopsis with NH₄⁺ treatment, although this was not the ion’s primary inhibitory effect on root elongation. Cell elongation was the major target in the suppression of primary root growth by NH₄⁺ (Li et al., 2010). Whether NH₄⁺ may affect other processes that require the participation of root tips, such as root gravitropism, is still unknown. As explained above, a differential cellular elongation on opposite flanks of the elongation zone is responsible for gravitropic curvature. This study was intended to explore whether NH₄⁺ influences root gravitropism in Arabidopsis and its relationship with the retardation of root growth. In addition, it was interesting to test whether exogenous K⁺ can alleviate root growth inhibition and/or effects on gravitropism. Furthermore, it was determined whether influences of NH₄⁺ on gravitropism arise from changes in auxin redistribution and auxin signal intensity in root tips.

Materials and methods

Plant material and growth conditions

Columbia-0 ecotype Arabidopsis thaliana (Col-0), the Arabidopsis mutant trh1 (SALK_086060), and the transgenic Arabidopsis DR5::GUS (β-glucuronidase; Ulmasov et al., 1997) in the Col-0 background were used. trh1 plants carrying the DR5::GUS construct were derived from crosses between trh1 and DR5::GUS-transformed plants, and homozygous plants for both trh1 and the DR5::GUS insertion were used.

After being surface sterilized and cold treated at 4 °C for 2 d, the seeds were sown on Arabidopsis normal growth medium. The composition of the normal growth medium was as described by Li et al. (2010): 2 mM KH₂PO₄, 5 mM NaNO₃, 2 mM MgSO₄, 1 mM CaCl₂, 0.1 mM Fe-EDTA, 50 μM H₂BO₃, 12 μM MnSO₄, 1 μM ZnCl₂, 1 μM CuSO₄, 0.2 μM Na₂MoO₄, 1% (w/v) sucrose, 0.5 g 1⁻¹ MES, and 0.8% (w/v) agar (adjusted to pH 5.7 with 1 M NaOH).

The culture plates were placed vertically in a growth chamber at 23 ± 1 °C, under a light intensity of 100 μmol photons m⁻² s⁻¹, with a 16 h light/8 h dark cycle. Five-day-old seedlings germinated on normal growth medium, with relatively straight root tips and ~1.5 cm in length, were selected for gravity stimulation experiments.

Ammonium treatment and gravity stimulation

The ammonium treatment medium consisted of normal growth medium supplemented by varying concentrations of (NH₄)₂SO₄. Ion effects were analysed by using NH₄Cl with the same NH₄⁺ concentration, Na₂SO₄ and K₂SO₄ with the same SO₄²⁻ concentration, and KNO₃ with the same N concentration as substitutes for (NH₄)₂SO₄. Exogenous K⁺ experiments were performed by adding KNO₃ (NaNO₃) or KCl (NaCl) of appropriate concentrations to the 30 mM (NH₄)₂SO₄ medium. It is important to note that in agar media, unlike in soil or hydroponic culture, diffusion limitation for nutrients necessitates the application of higher than normal concentrations of nutrients, including those of the toxicant NH₄⁺ (Li et al., 2010; Barth et al., 2010; Li et al., 2011a). To achieve growth suppressions and tissue NH₄⁺ contents (Barth et al.,
Ammonium disruption of root gravitropism

Results

Influence of ammonium on Arabidopsis root growth and gravitropism

With an increase in ammonium concentration, growth of Arabidopsis roots was inhibited and their orientation of growth was changed (Fig. 1A–D). The effects of ammonium on root growth and root gravitropism angle were quantified (Fig. 1E). The results showed that all of the (NH₄)₂SO₄ treatments impeded root growth. Primary root lengths of 5-day-old seedlings germinated on treatment medium with 5, 10, 20, and 30 mM (NH₄)₂SO₄ were shortened to 81.10, 68.66, 39.25, and 13.24%, respectively (Table 1). Unlike the concentration-dependent inhibition of root growth, root gravitropic angles first decreased and then increased with the elevation of ammonium concentration. Root gravitropic angles of 5-day-old seedlings on media with 5 mM and 10 mM (NH₄)₂SO₄ were 4.53° and 5.87°, respectively, much smaller relative to the control (10.45°), and closer to the gravity vector. However, with an additional increase of (NH₄)₂SO₄ in the culture medium, Arabidopsis roots gradually deviated from the direction of gravity and lost gravitropism. The loss of gravitropism manifested in horizontal growth, curling, and occasionally upward growth of roots. This latter condition was observed in 30 mM (NH₄)₂SO₄ treatments (Fig. 1D). Given the influence of root length on the gravitropic angle during growth, comparisons were performed of changes in the angle between plants of similar root lengths. For similar root lengths, the average gravitropic angle of 30 mM (NH₄)₂SO₄-treated plants (5-day-old seedlings) was much greater than that of 2-day-old control plants (Fig. 1C, D; Table 1). These results imply that intermediate levels of (NH₄)₂SO₄ can promote positive gravitropism while excess levels can cause roots to deviate from gravity; that is, lead to agravitropism. Moreover, the influence of excess NH₄⁺ on the gravitropic angle may not be caused by the inhibitory effect of ammonium on root growth.

To better understand the influence of ammonium on root growth and gravitropism, an additional experiment was carried out to study the effect of (NH₄)₂SO₄ on the root gravitropic response (Fig. 2A). After 5-day-old seedlings were transferred to ammonium medium, initiation of root elongation and gravitropic bending were both highly delayed relative to controls. For this reason, data were collected from the sixth

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**Table 1.** Effect of different concentrations of (NH₄)₂SO₄ on root growth and gravitropism angle

<table>
<thead>
<tr>
<th>(NH₄)₂SO₄ (mM)</th>
<th>Days after germination</th>
<th>Root length (mm)</th>
<th>Gravitropic angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>15.57±0.25 a</td>
<td>10.45±0.43 b</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>12.63±0.24 b</td>
<td>4.53±0.27 d</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>10.69±0.15 c</td>
<td>5.87±0.34 d</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>6.11±0.22 d</td>
<td>9.53±0.73 bc</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>2.06±0.09 e</td>
<td>24.29±3.34 a</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>2.31±0.10 e</td>
<td>6.99±0.54 cd</td>
</tr>
</tbody>
</table>

Values of root length and angle are means ± SE (n > 60) from two independent experiments. Letters after the SE indicate whether the different treatments have a significant influence (P < 0.05) by one-way ANOVA followed by least significance difference (LSD) post-hoc tests.
hour of treatment imposition. The results (Fig. 2B, C) showed that inhibition brought about by ammonium increased with both ammonium dose and time. For example, average root elongation on the third day was reduced to 73.0% and 33.0%, at 10 mM (NH₄)₂SO₄ and 30 mM (NH₄)₂SO₄, respectively. On the other hand, ammonium affected root gravitropism in two ways: in the short term (<12 h), (NH₄)₂SO₄ at all concentrations inhibited root gravitropism; but with extended time (24 h and beyond) the gravitropic angle became increasingly smaller. The ammonium-treated roots continued to bend after control roots had attained their final gravitropic angle of ∼20°. Consequently, 10 mM (NH₄)₂SO₄ increased the maximum curvature of treated roots (displaying a smaller gravitropic angle) in contrast to controls after 48/72 h of gravistimulation, which might be a result of the postponement of the gravitropic bending period (Fig. 2D). Although 30 mM (NH₄)₂SO₄ also postponed the bending period, the bending capability of plants at this ammonium level was much lower relative to those in the 10 mM (NH₄)₂SO₄ treatment, and, moreover, the gravitropic angles between the two treatments diverged increasingly over time (Fig. 2B, D).

Further analyses revealed that elongation of roots with 30 mM (NH₄)₂SO₄ treatment at 12 h was not significantly different from that of control at 6 h (P > 0.05); corresponding gravitropic angles were 46.4° and 40.0° (P < 0.05). For seedlings under 10 mM (NH₄)₂SO₄ treatment, the root gravitropic angle was not distinguishable from that of the
control at 24 h, but root elongation was significantly smaller than in controls. These findings demonstrated that the influence of ammonium on the gravitropic angle was not directly linked to its inhibition of root elongation.

**Specificity of the influence of ammonium on root elongation and gravitropic response**

As ammonium application in the present study was in the form of (NH₄)₂SO₄, it is possible that effects on root elongation and gravitropism may have occurred through the actions of either the NH₄⁺ or SO₄²⁻ ions, or due the fact that highly concentrated levels were typically applied. To address these possibilities, NH₄Cl, with the same concentration of NH₄⁺, Na₂SO₄ and K₂SO₄, with the same concentration of SO₄²⁻, and KNO₃, with the same concentration of N, were substituted for (NH₄)₂SO₄ in the treatment medium. Because the gravity of roots in control seedling was also affected by light (compared with Figs 2 and 3, also in Supplementary Table S1 available at JXB online), to exclude interference by light, experiments were carried out in the dark. It was observed that NH₄Cl had very similar effects on the root gravitropic angle to (NH₄)₂SO₄, while Na₂SO₄ had very little effect. After 6 h of gravistimulation, K₂SO₄ and KNO₃ reduced the root gravitropic angle, but to a much smaller extent relative to (NH₄)₂SO₄. Thus, it was clear that the influence of ammonium on root gravitropic angle was largely caused by NH₄⁺, and was not duplicated upon exposure to SO₄²⁻, Cl⁻, Na⁺, N, or high ionic concentration per se. Moreover, based on the data (compared with Figs 2 and 3, and Supplementary Table S1), the gravitropic angle under NH₄⁺ stress seemed to be independent of light, but this aspect will need further experiments for confirmation. Nevertheless, in the early stage, K₂SO₄ and KNO₃ showed some influence on root gravitropism, probably through the influence of K⁺, but the alteration was far less than that caused by NH₄⁺ of the same concentration.

The inhibition intensity of root growth by these substances was as follows: (NH₄)₂SO₄ > NH₄Cl > Na₂SO₄ > K₂SO₄ > KNO₃, and elongation of roots was reduced to 39.8, 48.5, 73.6, 88.4, and 93.8%, respectively, after 3 d treatments with each of these salts (Fig. 3B). Although (NH₄)₂SO₄ caused inhibition primarily via NH₄⁺, SO₄²⁻ at high concentration also showed some effect on root growth, which was demonstrated by the fact that inhibition by 30 mM (NH₄)₂SO₄ was significantly greater than that by 60 mM NH₄Cl (P < 0.05) based on the 3 d treatment.

**Influence of exogenous K⁺ on ammonium-induced root elongation and gravitropic response**

Because of their similarity in ionic radius and electric charge, K⁺ and NH₄⁺ compete with each other for absorption, and excess NH₄⁺ can inhibit the absorption and accumulation of K⁺ (Szczesba, 2008; ten Hoopen et al., 2010). Research has shown that low K⁺ not only inhibits root growth of Arabidopsis but also weakens its gravitropic response (Vicente-Agullo et al., 2004). This raised the question of whether the influence of ammonium on Arabidopsis roots in this study was caused by a K⁺ deficiency induced by highly concentrated NH₄⁺. The results (Fig. 4A) showed that addition of KNO₃ at different concentrations to 30 mM (NH₄)₂SO₄ treatment media significantly reversed the inhibition of root elongation, recovering the root elongation from 27.3% to 36.6–43.4% following 3 d treatments. However, KNO₃ did not affect rescue of the root gravitropic response, and the addition of 20 mM KNO₃, in fact, slightly enhanced the disruption in gravitropism, following gravistimulation treatments for ≥12 h (Fig. 4B). In order to determine further the influence of exogenous KNO₃ on root growth and gravitropism and the relationship between KNO₃ and the role of the K⁺ ion in this, experiments were carried out with 20 mM NaNO₃, 20 mM KCl, or 20 mM NaCl substituted for 20 mM KNO₃. KCl addition relieved ammonium’s inhibition of root elongation, while NaNO₃ and NaCl accentuated the inhibition (Fig. 4C). In terms of the gravitropic response,
neither KCl nor NaCl reduced the effects of ammonium, while NaNO$_3$ accentuated agravitropism (data not shown). That exogenously supplied K$^+$ partially relieved ammonium’s inhibition of root elongation but not the gravitropic response underscores that the influence of ammonium on gravitropism was not due to K$^+$ deficiency. This was consistent with research on bean by Bonser et al. (1996), who found that, with the exception of Pi, the deficiencies of minerals such as N, K, S, Ca, or Mg had no effect on root angle.

Influence of ammonium on DR5::GUS expression in the root tip

In Arabidopsis roots, the expression of DR5::GUS, an auxin-responsive promoter (Ulmasov et al., 1997), has been used to infer the development of a lateral auxin gradient during the gravitropic process (Hou et al., 2004). Histochemical methods were used to test the influence of ammonium on auxin signals in root tips during the gravitropic response. The results showed that in vertically grown roots (0 h), DR5::GUS was expressed mainly in the quiescent centre, columella initial cells, and columella cells of the root cap. In control roots, after 3 h of gravistimulation, 69% of plants (29/42) activated the expression of DR5::GUS on the lower side of the root tips, and extending basipetally along the lateral root caps and epidermal cells to the meristematic zone. Thereafter, the lateral auxin gradient was gradually weakened with the passage of time. At 24 h, it disappeared in 80% (20/25) of plants, and the expression of DR5::GUS returned to pre-treatment states (Fig. 5). In contrast, (NH$_4$)$_2$SO$_4$ treatment delayed the lateral redistribution of auxin in root tips. After 3 h of gravistimulation, only 23.7% (9/38) of the roots displayed asymmetric expression of DR5::GUS upon treatment with 30 mM (NH$_4$)$_2$SO$_4$. At 6 h, the asymmetric auxin gradient was observed in 68.4% (26/38) of roots treated with (NH$_4$)$_2$SO$_4$. Furthermore, after 24 h of gravistimulation, it was still observed in 56.7% (17/30) of roots, while disappearing at 72 h. Moreover, in contrast to controls and roots treated with 10 mM (NH$_4$)$_2$SO$_4$, the expression of DR5::GUS in apical cells of roots treated with 30 mM (NH$_4$)$_2$SO$_4$ decreased over time (>1 d), but auxin signals in stele cells increased (Fig. 5). Expression of DR5::GUS was also observed in stele cells of controls, with results fluctuating slightly.

TRH1 participates in ammonium inhibition of root gravitropism and auxin signals in root tip cells

The potassium transporter TRH1 (AtKT3/AtKUP4), which is strongly expressed in the root cap, is required for auxin transport in Arabidopsis roots (Rigas et al., 2001; Vicente-Agullo et al., 2004). Disruption of this gene in the trh1 (tiny root hair 1) mutant not only blocks the translocation of auxin in the root cap but also weakens root gravitropism (Vicente-Agullo et al., 2004). In order to examine whether the alteration of root gravitropism and auxin signalling in root caps was related to TRH1, we tested the gravitropic response of the trh1 mutant under high ammonium exposure. If (NH$_4$)$_2$SO$_4$ inhibits Arabidopsis root gravitropism via TRH1-mediated auxin transport in the root cap, the gravitropism of trh1 is expected to show at least partial resistance to ammonium stress relative to Col-0. The results...
showed that, in the early stages (6 h), the gravitropic curvature of the trh1 mutant root was less than that of Col-0, but, with time (48/72 h), trh1 mutants displayed similar gravitropism to the wild type under ammonium (Fig. 6). In controls, trh1 showed clearly weakened gravitropism compared with the wild type. These data indicate that TRH1 participates in the ammonium-induced gravity response in roots. Nevertheless, the reduction of root elongation in trh1 by ammonium was similar to that observed in Col-0. Under a 3 d treatment with 10 mM and 30 mM (NH₄)₂SO₄ separately, root elongation of trh1 and Col-0 was 75.74% and 67.81%, and 30.42% and 29.93% of their controls, respectively.

With the DR5:GUS construct in the trh1 mutant, the influence of ammonium on the expression of DR5:GUS in trh1 root tips was analysed in the context of the gravitropic response. No significant differences were found in auxin signals between trh1 and Col-0 under control conditions (Fig. 7A, B). After 1 d and 3 d of treatments under 30 mM (NH₄)₂SO₄, auxin signals decreased noticeably in root tip cells but increased in central tissues of Col-0. Similar to Col-0, ammonium treatment caused auxin accumulation in stele cells of trh1 roots. However, the auxin signal intensity in the root apex cells did not decrease over time (Fig. 7B). These findings indicate that TRH1 regulates the reduction of auxin signal intensity in root tip cells under ammonium exposure.

**Discussion**

The relationship of ammonium to root gravitropism and elongation

Root gravitropism responses always depend on root elongation. However, it is not known whether these two
biological processes are always synchronized. This study provides compelling evidence that responses of root gravitropism and elongation to ammonium stress are regulated independently. Firstly, with the rise of ammonium concentration, root length shortened while gravitropism initially strengthened and only later weakened (Table 1). Secondly, when a root length similar to that of the control was attained, roots under 30 mM (NH4)2SO4 treatment deviated from the gravity vector to a greater degree. Thirdly, the dynamic results obtained with variations in time and concentration also showed that ammonium’s influence on the gravitropic bending angle was not a secondary effect of its inhibition of root elongation (Fig. 2; see also Fig. 3). Furthermore, added KNO3 relieved NH4+-induced inhibition of root elongation by partial restoration of root length, but failed to rescue the gravitropic angle (Fig. 4). These results suggest that root gravitropism and elongation in response to ammonium stress are regulated by two at least partially distinct pathways.

As plants have evolved an elaborate and sophisticated set of growth responses to environment cues (Cassab, 2008), it is hypothesized that the alteration of root gravitropism with increased ammonium supply may be a key process by which the plant acclimates to a changed soil condition. When at a low concentration, NH4+ is an important N fertilizer, especially when nitrate is also present in solution (Kronzucker et al., 1999). Therefore, under the experimental conditions in this study (2 mM K+, 5 mM NO3), and concentrations of (NH4)2SO4 <10 mM, ammonium may be an important source of N and, under such conditions, the gravitropic angle becoming smaller might be due to the fertilizer tropism of roots. In fact, when supplied via the roots, 10 mM (NH4)2SO4 had no inhibitory effect on the aerial parts of Arabidopsis; moreover, it increased the number of lateral roots (Li et al., 2010). However, excess ammonium increased the angle of the root tip from the gravity vector, and this may be a mechanism of risk aversion by the root system. That the NH4+ inhibition of root growth was partially relieved by exogenous K+ can probably be explained as follows: supplied K+ can effectively alleviate the futile ammonium cycling at the plasma membrane and reduce the ion’s entry and accumulation (Nielsen and Schjoerring, 1998; Szczerba et al., 2008; Balkos et al., 2010; ten Hoopen et al., 2010).

Effect of ammonium on root gravitropism relates to the redistribution of auxin in root tips

The asymmetric distribution of the plant hormone auxin in root tips has long been regarded as an important factor in the regulation of root gravitropism (Ottenschläger et al., 2003). The present results showed that in the early stages of the root gravitropic response, ammonium treatment delayed the gravity-induced development of asymmetric DR5::GUS expression across the root caps, but, once the asymmetry was established, ammonium further prolonged
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its expression on the lower side (Fig. 5). This was consistent with the observation that 10 mM (NH$_4$)$_2$SO$_4$ treatment prolonged the period of gravitropic response of the treated root and consequently increased its maximum curvature (Figs 2D, 6A). The continuous asymmetric auxin signal as a consequence of ammonium exposure is clearly related to the increased root gravitropic curvature, in particular at 10 mM (NH$_4$)$_2$SO$_4$. From these observations, it can be concluded that the weakening of root gravitropism by ammonium in *Arabidopsis* relates to the redistribution of auxin in root tips. A close relationship between gravity-induced cytoplasmic alkalinization in columella cells and lateral auxin redistribution in root caps has been demonstrated (Fasano et al., 2001; Boonsirichai et al., 2003; Wolverton et al., 2011). Ammonium absorption, especially under high external supply conditions, can induce alkalinization of the cytoplasm in root cells (Britto and Kronzucker, 2005; Monshausen et al., 2011). Whether the persistent auxin gradient is related to the alkalinization of cytoplasm in root tip cells under ammonium treatments needs further study.

In addition to delaying and prolonging the formation of a lateral auxin gradient, 30 mM (NH$_4$)$_2$SO$_4$ treatment in this experiment notably decreased the auxin signal in apical cells of root tips (Fig. 5). With time further prolonged (5 d after treatment), the auxin distribution in the root cap was altered, with the maximum shifted upward encompassing the quiescent centre and vascular initials (Li et al., 2010). However, the gravitropism of seedling roots in 30 mM (NH$_4$)$_2$SO$_4$ treatments at the later stage was recovered to control levels; that is, the reduction of the auxin response in the root apex should not be the primary reason for the ammonium-induced loss of root gravitropism.

**TRH1 is related to root gravity response and auxin signals in the root apex under ammonium treatment**

It has been shown that the potassium transporter TRH1 affects the gravity response of *Arabidopsis* roots and is involved in auxin transport in the root apex (Vicente-Agullo et al., 2004). In this study, it was also observed that the mutation of TRH1 was associated with weakened gravitropism compared with the wild type. Under 30 mM (NH$_4$)$_2$SO$_4$, the *trhl* mutant showed more weakened gravitropism than the wild type at the early stage, and recovered gravitropism in later stages. Thus, it was clear that TRH1, while not sufficient to explain the phenomenon, was nevertheless associated with the weakening of root gravitropism by excess ammonium in *Arabidopsis*. Due to the fact that external potassium failed to recover the root gravity response under ammonium in the wild type, the data suggest the involvement of TRH1-mediated auxin transport rather than potassium transport in the ammonium-induced weakening of the gravitropism response. However, it is not known at this time whether the potassium carrier TRH1 transports auxin directly or is involved indirectly, through the generation of ionic and electric gradients across the plasma membrane that favour auxin efflux via other auxin transporters, as suggested by Vicente-Agullo et al. (2004). This warrants further investigation.

In addition, it was interesting to find that the auxin signal in apical cells in the *trh1* mutant did not significantly change in response to ammonium (Fig. 7). This result demonstrates that TRH1-mediated auxin translocation is likely to be involved in this process. However, this study did not confirm that the TRH1-mediated auxin response in the root apex is related to the ammonium-induced loss of gravitropism, because the reduction of *DR5::GUS* expression in the root apex was not always coupled with loss of root gravitropism.

**Fig. 7. Influence of ammonium on *DR5::GUS* expression in root tips of Col-0 and *trhl* during the gravitropic response.**

(A) Expression of the auxin reporter *DR5::GUS* in Col-0 and *trhl* seedlings germinated on normal growth medium for 5 d. (B) Effect of 30 mM (NH$_4$)$_2$SO$_4$ on the expression of *DR5::GUS* in Col-0 and *trhl* seedlings following 24 h and 72 h of gravistimulation. GUS staining was conducted for 4 h. One representative image for each experiment is shown. Scale bar=100 µm in A and 50 µm in B. The arrow indicates the direction of the gravity vector. (This figure is available in colour at *JXB* online.)
In plants, auxin is synthesized mainly in young shoot tissues, and transported through the central tissues to the roots. There, auxin is transported acropetally toward the tip and adds to a pool of locally synthesized auxin, forming an auxin-maximal centre that overlaps with the quiescent centre and upper cap, and is then redistributed toward the flanks (lateral cap) and transported basipetally through the lateral root cap and epidermal cells toward the elongation zone (Massion et al., 2009). Research has shown that auxin transport is sufficient to generate a maximum as well as a gradient to guide root growth (Grieneisen et al., 2007). Therefore, for the reduction of auxin signal intensity within the root apex under excess ammonium treatment, it is probable that NH$_4^+$ inhibits acropetal translocation of auxin towards the root tip, such as the functions of the auxin influx carrier AUX1, but has no effect on the auxin translocation away from the root cap (Li et al., 2011a,b), so that the auxin signal within apex cells is reduced. Yet, in the trh1 mutant, the disruption of TRH1-mediated auxin export resulted in an accumulation of auxin in the root cap (Vicente-Agullo et al., 2004; this study), which can compensate for the reduction of acropetal auxin transport. This is consistent with the observation that there was an increased auxin signal in stele tissues in trh1 and Col-0 roots under ammonium treatment (Fig. 7), indicating that the auxin whose transport was inhibited accumulated in the stele, and activated the expression of DR5::GUS. However, it is also plausible that the translocation of auxin through the root cap by TRH1 was promoted and thus the auxin signal accumulation in the root cap of Col-0 was reduced under excess ammonium. Results have shown that ammonium accumulation can increase ethylene evolution from leaf tissues (Barker, 1999), while ethylene can promote transport-dependent auxin distribution (Ruzicka et al., 2007). In addition, under excess ammonium, cell membranes in root tips can be induced to depolarize rapidly, and thereafter efflux of K$^+$ will increase (Higinbotham et al., 1964; Wang et al., 1994; Nocito et al., 2002; Coskun et al., 2010). As the molecular mechanism of TRH1-dependent auxin transport is not yet clear, whether ammonium regulates TRH1 activity via ethylene and/or ion and electro-chemical potential gradients to promote TRH1 or other carriers mediating auxin translocation warrants future study.

Interestingly, it was found that the oscillating growth pattern for roots was inhibited at elevated (NH$_4$)$_2$SO$_4$ implying that ammonium did act on the auxin transport systems, because the oscillating growth was disturbed in mutants of auxin transporters such as aux1 and agrl (allelic with eir1/pin2/law6) or NPA (naphthylphthalamic acid)-treated Col-0 roots (Rashotte et al., 2000; Migliaccio and Piconese, 2001), all of which related to polar auxin transport. Evidence has been obtained that the ammonium-induced reduction of the auxin signal in root tips may not be due to the lack of auxin content (Qin et al., 2011). Nevertheless, the possibility that the alteration of the auxin maximum was a consequence of changes in metabolism and synthesis of auxin within the root apex under ammonium stress cannot be excluded, because mutants relevant to auxin synthesis, transport, and response have been shown to affect gravitropism (Masson et al., 2009). Nor can it be excluded that there may be other auxin carriers in addition to TRH1 taking part in the ammonium-induced gravitropic response and auxin distribution.

In summary, although root gravitropism and root growth were both greatly influenced by excessive ammonium, they seemed to be under the influence of independent mechanisms. The latter was partially relieved by exogenously supplied K$^+$, while the former was related to the alteration of auxin redistribution in the root apex. The data suggest an involvement of TRH1 in the ammonium-induced loss of root gravitropism in Arabidopsis. In addition, it was interesting to observe that the reduction of auxin signal intensity within the root apex caused by prolonged ammonium exposure was eliminated by the mutation in TRH1.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Table S1.** Comparison of the effects of NH$_4^+$ on root gravitropism, affected by light and dark

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