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Constitutive salicylic acid defences do not compromise seed yield, drought tolerance and water productivity in the *Arabidopsis* accession C24

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ABSTRACT

Plants that constitutively express otherwise inducible disease resistance traits often suffer a depressed seed yield in the absence of a challenge by pathogens. This has led to the view that inducible disease resistance is indispensable, ensuring that minimal resources are diverted from growth, reproduction and abiotic stress tolerance. The *Arabidopsis* genotype C24 has enhanced basal resistance, which was shown to be caused by permanent expression of normally inducible salicylic acid (SA)-regulated defences. However, the seed yield of C24 was greatly enhanced in comparison to disease-resistant mutants that display identical expression of SA defences. Under both water-replete and -limited conditions, C24 showed no difference and increased seed yield, respectively, in comparison with pathogen-susceptible genotypes. C24 was the most drought-tolerant genotype and showed elevated water productivity, defined as seed yield per plant per millilitre water consumed, and achieved this by displaying adjustments to both its development and transpiration efficiency (TE). Therefore, constitutive high levels of disease resistance in C24 do not affect drought tolerance, seed yield and seed viability. This study demonstrates that it will be possible to combine traits that elevate basal disease resistance and improve water productivity in crop species, and such traits need not be mutually exclusive.

Key-words: Abiotic stress; basal disease resistance; biotic stress; plant fitness; transpiration efficiency; water limitation.

INTRODUCTION

Plants live and grow in a variable environment, and are continuously challenged with combinations of adverse biotic and abiotic factors. Consequently, plants have evolved multiple mechanisms of perception, reaction and resistance to external challenges. The interaction of external challenges, such as pathogens and limitation of water availability, is a major driving force in the evolution of adaptive and defensive mechanisms to improve plant reproductive fitness (Dudley 1996; Heschel *et al*. 2002; Brown 2003; Morison *et al*. 2008).

Nearly all terrestrial plants are exposed to water limitation at some point throughout their lives (Morison *et al*. 2008). Strategies to cope with water limitations have evolved including developmental and physiological changes, which seek to maximize exploitation of water resources, avoid water loss, maintain tissue osmotic potential and minimize tissue damage (Schulze 1986a; Chaves *et al*. 2002; Passioura 2002; Condon *et al*. 2004). Survival of dehydration stress has often been used to define drought tolerance, especially in molecular genetic studies employing *Arabidopsis thaliana* L. Heynh. (*Arabidopsis*; Liu *et al*. 1998; Passioura 2007). However, such terms do not address the impact of water limitation on plant productivity. Even moderate limitation of water availability not only diverts resources away from growth and into protective responses, but brings about reduced stomatal conductance, which can limit CO_2 uptake, consequently limiting photosynthesis and plant growth (Boyer 1970; Schulze 1986a,b; Condon *et al*. 2004; Morison *et al*. 2008).

In this study, we employ the term *water productivity*, which describes the relationship between yield of the harvestable product and water loss (Steduto, Hsiao & Fereres 2007). In addition, the term *biomass water ratio* (*BWR*) is used to describe total plant biomass per unit volume water supplied, and is part of a yield equation, which describes water productivity (Passioura 1977). These two definitions are important when looking at plant productivity in a water-limiting environment (Monteith 1984, 1993; Condon *et al*. 2004; Morison *et al*. 2008). Because yield depends on the water available for transpiration, *BWR* can also be regarded as equivalent to the transpiration efficiency (TE) of a plant (Passioura 1977; Morison *et al*. 2008). In this

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context, quantitative trait loci (QTL) that influence TE have been uncovered in *Arabidopsis* genotypes (Juenger *et al*. 2005; Masle, Gilmore & Farquhar 2005; McKay *et al*. 2008), and an improved TE trait was also used to select for wheat cultivars with a higher yield under water-limiting conditions (Gregory 2004), establishing that this approach is valuable for both model and crop species. However, to our knowledge, in *Arabidopsis*, changes in TE have never been reported in the context of altered water productivity.

The direct effect of drought stress on plant performance can also be exacerbated by the promotion of disease susceptibility (Mattson & Haack 1987; Desprez-Loustau *et al*. 2006). Plant pathogen defence strategies frequently rely on inducible defence mechanisms that can be classified into three types: non-host resistance (Ellis 2006), race-specific or vertical resistance and basal or horizontal resistance (Agrios 1997). It is widely accepted that various modes of pathogen resistance are costly to plants (Baldwin 1998; Brown 2002, 2003; Tian *et al*. 2003), and that the evolution of inducible pathogen resistance has occurred to save energy (Heil & Baldwin 2002; Heidel & Dong 2006; van Hulten *et al*. 2006). As a result, breeding for or engineering constitutive expression of normally inducible resistance is not straightforward and can lead to yield penalties in the absence of a challenge (Brown 2002; Damgaard & Jensen 2002). For example, *Arabidopsis* plants harbouring alleles of the resistance (*R*) gene *RPM1*, which confers resistance to *Pseudomonas syringae* pv. *maculicola*, showed smaller shoots and reduced seed yield per plant (Tian *et al*. 2003). In particular, *RPM1*-mediated resistance seems to incur a high associated cost, although costs may differ for individual *R* genes (Brown 2003). The overall impression that constitutive expression of pathogen resistance in *Arabidopsis* leads to reduced plant fitness is also echoed in crop-based studies where yield has been shown to be depressed in genotypes with enhanced disease resistance when not infected by pathogens (reviewed by Brown 2002).

Besides drought, other abiotic factors also influence disease progression and/or resistance mechanisms. For example, light intensity and quality impact on the salicylic acid (SA) signalling pathway important in the induction of resistance to biotrophic pathogens (Zeier *et al*. 2004; Bechtold, Karpinski & Mullineaux 2005; Chandra-Shekara *et al*. 2006). More recently, abscisic acid (ABA) has been shown to be exploited by some pathogens by increasing its biosynthesis to negatively regulate SA signalling (de Torres-Zabala *et al*. 2007; Yasuda *et al*. 2008) and promoting jasmonic acid (JA) signalling (Fan *et al*. 2009). The interaction between ABA and SA signalling may also explain the sensitivity of pathogen defences to prevailing humidity (May, Hammond-Kosack & Jones 1996; Jambunathan, Siani & McNellis 2001; Zhou *et al*. 2004; Noutoshi *et al*. 2005).

The aim of this study was to identify *Arabidopsis* genotypes that have elevated basal resistance to pathogens, but also improved tolerance to abiotic stresses. In addition to SA, it has previously been shown that the levels of the thiol antioxidant reduced glutathione (GSH) correlate with the degree of basal resistance in *Arabidopsis* (Ball *et al*. 2004; Senda & Ogawa 2004; Parisy *et al*. 2006), and increased foliar levels of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2), most likely associated with the apoplast, have also been implicated in promoting basal resistance (Parker 2003; Custers *et al*. 2004; Torres, Jones & Dangl 2005). Additionally, both GSH and H_2O_2 are strongly implicated in signalling in response and tolerance to abiotic stress (Apel & Hirt 2004; Foyer & Noctor 2005; Mullineaux & Rausch 2005; Mullineaux, Karpinski & Baker 2006). Therefore, we reasoned that genotypes with elevated levels of GSH and H_2O_2 could have altered responses to biotic and abiotic stress. From this survey, we identified the genotype C24, which has been previously shown to be resistant to the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*; Holub & Beynon 1997) and the virulent strain of *Pseudomonas syringae* pv. tomato DC3000 (Ton, Pieterse & Van Loon 1999), as having elevated H_2O_2 and GSH levels. However, to our surprise, the reproductive fitness of C24 was not compromised, and therefore our observations question general assumptions regarding the impact of disease resistance on yield. Furthermore, C24 was also shown to not only have a high degree of drought tolerance, but also a greatly elevated water productivity compared with reference genotypes, indicating that elevated basal disease resistance need not compromise the expression of traits associated with seed yield under water-limiting conditions.

MATERIALS AND METHODS

Plant material

Plants were grown in two different environments stated for each experiment. Plants were grown in compost (Levington F2+S, The Scotts Company, Ipswich, UK). In the controlled environment room, plants were kept in an 8/16 h light/dark cycle at a photosynthetically active photon flux density (PPFD) of 120 μmol m^{-2} s^{-1} at 60% RH and 23 °C. Glasshouse conditions were deemed variable as temperatures fluctuated during the experimental period (February–April 2007) with a mean temperature of 18.1 ± 7.5 °C. Lighting was maintained at a minimum threshold PPFD of 1800 μmol m^{-2} s^{-1} for a 12 h day, and supplementary lighting was switched on if light intensity fell below this threshold. The PPFD at plant level was ~200 μmol m^{-2} s^{-1}. Plants in the glasshouse were watered and positions changed daily. Plants were used at different ages as stated during individual stress experiments.

GSH, H_2O_2, SA measurements and transmission electron microscopy (TEM)

In vitro measurements of GSH in total leaf extract were carried out on 100 mg fresh leaf tissue of 5-week-old plants. For the measurements shown in Supporting Information Fig. S1b, the method described by Creissen *et al*. (1999) was used. For measurements shown in Fig. 1b, leaves were ground in ice-cold 1% metaphosphoric acid. Extracts were subjected to centrifugation at 4 °C, 13 000 rpm, and the

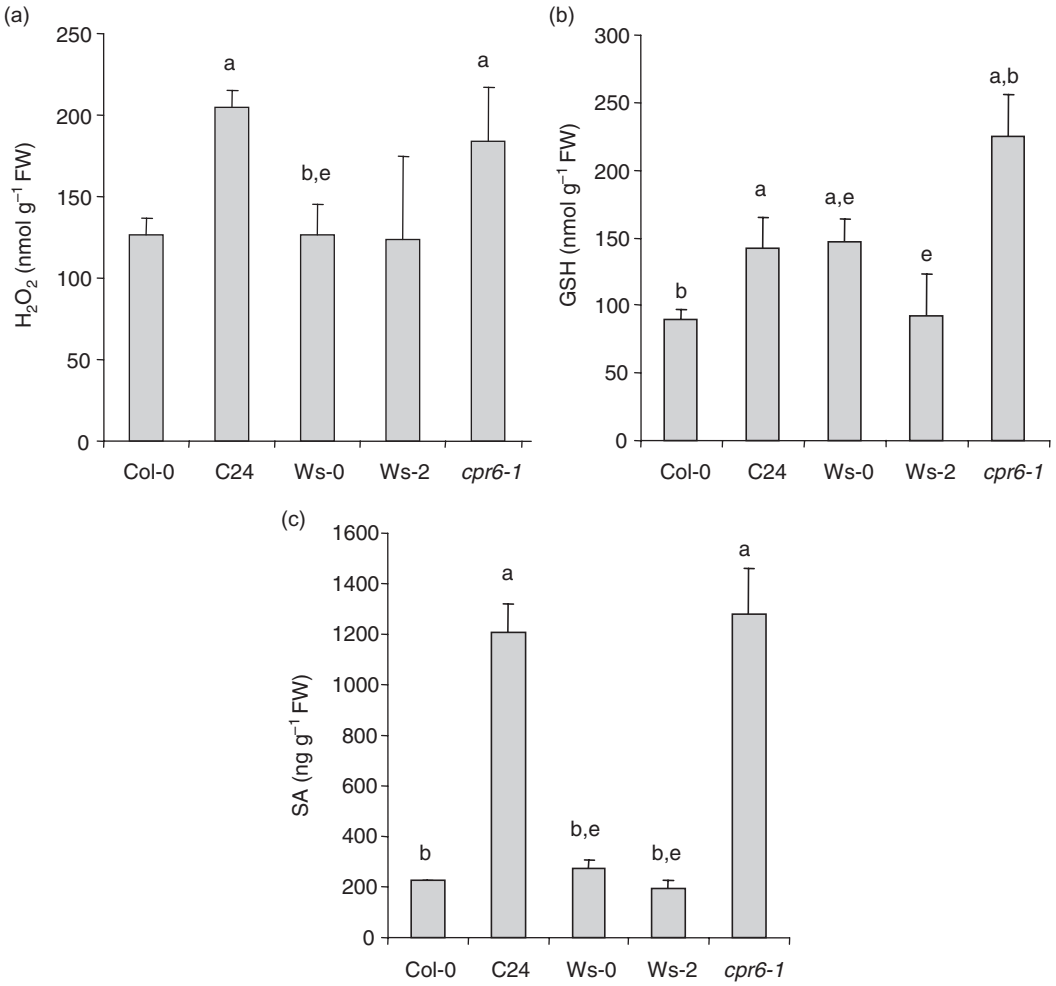

Figure 1. Measurements of foliar defence- and stress-associated small molecules. (a) Total foliar H_2O_2 concentration. (b) Total foliar reduced glutathione (GSH) concentration. (c) Total foliar salicylic acid (SA) concentration in leaves of 5-week-old plants. Error bars indicate standard error ($n = 5$). Letters indicate significant differences ($P \leq 0.05$) between genotypes. a, Col-0; b, C24; c, Ws-0; d, Ws-2; e, *cpr6-1*.

supernatant was treated for 1 h at room temperature in the dark with 10 mM iodoacetic acid in 10 mM ammonium hydroxide buffer pH 11 (Loughlin *et al*. 2001) in order to methylate the free thiol group of GSH. *S*-carboxymethyl glutathione (GSH-CM) and oxidized glutathione (GSSG) were separated by ultra-performance liquid chromatography (Acquity UPLC®; Waters UK, Manchester, UK), and analysed using tandem mass spectrometry (Quattro Premier XE; Waters). Ten microlitres of the extracts was separated by reversed-phase chromatography on an Acquity UPLC BEH C18 100 × 1 mm column at 50 °C. The solvent linear gradient used was 100% A (94.5% H_2O, 5% acetonitrile, 0.5% formic acid) to 95% B (99.5% acetonitrile, 0.5% formic acid) and 5% A over 1.4 min. The solvent flow rate was 0.6 mL min^{-1}. Electrospray ionization mass spectrometry in positive ion mode was used to detect the following transitions: GSH-CM *m*/*z* 366→237, cone voltage: 30 V, collision energy: 15 eV; and GSSG *m*/*z* 613→355, cone voltage: 40 V, collision energy: 24 eV. A standard curve of known concentrations for both GSH and GSSG was used for quantification and calculations of recovery rates. The variation in total glutathione levels between the two methods used in this study can be explained by different plant growth conditions (2 years apart in two different laboratories), extraction and derivatization procedures.

In vitro measurements of H_2O_2 in total leaf extracts were carried out on 100 mg fresh leaf tissue of 5-week-old plants. Leaves were ground in ice-cold 0.1 M HCl, and extracts were analysed as described previously (Bechtold *et al*. 2009).

The detection of electron dense cerium perhydroxide deposits by TEM, formed from the reaction of cerium trichloride with H_2O_2, was carried out according to Bestwick *et al*. (2002). Briefly, three leaves per line were infiltrated with $CeCl_3$ essentially as described by Galvez-Valdivieso *et al*. (2009). Controls included samples that were incubated with 2.2 U mL^{-1} catalase for 30 min prior to $CeCl_3$ infiltration and a buffer control. No deposits were detected in these controls as found previously (Solyu, Brown & Mansfield 2005).

Free SA was extracted twice in 400 μL extraction buffer containing 10% methanol and 1% acetic acid, and was analysed as described by Forcat *et al*. (2008) using UPLC coupled to tandem mass spectrometry. Separation on UPLC was carried out using the same column as that for GSH separation. The solvent linear gradient used was 100% A (94.9% H_2O, 5% acetonitrile, 0.1% methanol) to 80% B (5 % H_2O, 94.9% acetonitrile, 0.1% methanol) over 0.3 min. Solvent B was maintained at 80% for 1.4 min before returned to 100% A in 0.3 min. The solvent flow rate was 0.6 mL min^{-1}. Electrospray ionization mass spectrometry in negative ion mode was used to detect the following transitions: SA m/z 136.9 $\rightarrow$ 92.8, cone voltage: 30 V and collision energy: 20 eV.

Drought conditions and determination of *BWR*s

The plants were transferred into individual pots (filled with identical amount of well-mixed compost) 2 weeks after the sowing date and were kept well watered until the beginning of the drying episode. At the same time, control pots were used to determine 100 and 0% soil water content. Five weeks after sowing, half the plants were maintained at well-watered conditions, while for the remaining half, water was withdrawn and pot weight was determined daily. Relative soil water content (*rSWC*) was calculated for each day, and pots were left to dry until 20% *rSWC* was reached, at which point pots were rewatered. The plants were bagged once the final flower had opened, and seed yield was determined once the plants had dried out.

For the water productivity experiments, 8-cm-diameter pots were filled with exactly 76 g of well-mixed compost. A 5 mL plastic pipette tip was inserted into the soil for watering, and plastic beads were placed on top of the soil to reduce evaporation. Control pots for the different *rSWC*s (40 and 80%) were set up to determine the water loss through evaporation. The plants were transferred into the centre of each pot 2 weeks after sowing and were kept well watered in the controlled environment room until 4 weeks after sowing. At this time, the plants were transferred into the glasshouse and placed in a random block design. Pot weight was measured daily and water added individually to each pot according to their requirements to achieve either 40 or 80% *rSWC*. The daily amount of water used was recorded, as well as flowering time and number of leaves at floral initiation. Once the final flower had opened, watering ceased, plants were bagged and left to dry out before harvesting. At harvesting, rosettes, stalks and seeds were separated, and seed weight and dry weight of rosettes and stalks/pods were determined. At least 10 plants per line per watering regime were measured.

Relative leaf water content (*rLWC*) was calculated using the formula: $rLWC\ (\%) = (FW - DW)/(SW - DW) \times 100$, where *FW* is the rosette fresh weight on day of measurement, *SW* is the fully saturated rosette weight and *DW* is the dry weight of the rosette.

Thermal imaging, analysis of rosette area and stomatal conductance measurements

Five weeks after emergence, thermal images of individual plants were captured with a NEC thermal camera (320×240 pixels; San-ei Instruments, Tokyo, Japan) under controlled environment conditions as described above. Digital photographs of rosettes were analysed using ImageJ (Abramoff, Magalhaes & Ram 2004). Measurements of stomatal conductance under growth conditions were determined using a Porometer AP4 from Delta-T Devices (Cambridge, UK) according to the manufacturer's instructions.

RNA extraction, qRT-PCR and micro-array analysis

RNA was extracted from leaf material using TriReagent (Ambion, Austin, TX, USA) according to the manufacturer's instructions. For the micro-array analysis, RNA was additionally purified using the RNAeasy kit (Qiagen, Crawley, UK), and then purity and lack of degradation were analysed using a micofluidics-based separation system (Bioanalyser; Agilent Technologies, Stockport, UK). Extraction of RNA, synthesis of cDNA, PCR reactions and conditions for qRT-PCR analysis were as described previously (Galvez-Valdivieso *et al*. 2009). Primer sequences for qRT-PCR experiments can be found in Supporting Information Table S1.

Agilent *Arabidopsis* 4x44k whole genome arrays (http://www.chem.agilent.com) were used. Micro-array analysis was performed according to the manufacturer's processing protocols (G4140-90051) available from their website (http://www.genomics.agilent.com). Briefly, 500 ng of total RNA was used in Cy3 and Cy5 labelling reactions using the Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies) according to instructions. After hybridization and washing, arrays were scanned using the GenePix 4000B scanner. Images were normalized and analysed using Acuity 3.1 software (Molecular Devices, Workingham, UK). In total, three independent experiments using individual rosettes per experiment and one dye swap were carried out. Up-regulated genes were determined as >2-fold in C24 at a 5% false discovery rate (FDR) using the program Rank Products (Breitling & Herzyk 2005). Raw data from these experiments can be found on the ArrayExpress database (at http://www.ebi.ac.uk/microarray-as/ae, ID: A-MEXP1847; array express accession: E-MEXP-2732). Raw fluorescence data from the *cpr5-1* (ID: E-GEOD-5745) arrays were downloaded from the Array Express database, normalized and subsequently analysed using the R based packages Harshlight (Suarez-Farinas *et al*. 2005), gcrma (Wu *et al*. 2004) and Simpleaffy and Annaffy available from Bioconductor (http://www.bioconductor.org). Confirmation of the transcriptome analysis was carried out by qRT-PCR as part of a separate experiment using three biological replicates of a different set of plants. The Biological Network Gene

Ontology tool (BINGO; Maere, Heymans & Kuiper 2005) was used in conjunction with Cytoscape v2.6.3 to generate a network of over-represented GO terms (Cline *et al*. 2007).

Glucose (reticuline) oxidase activity

To obtain a cell wall fraction, 0.5 g plant material was frozen in liquid nitrogen and extracted on ice in 1 mL of extraction buffer containing 50 mM potassium acetate (pH 5.2), 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM ascorbic acid and 0.1% Triton X-100. Samples were kept on ice for 15 min and subjected to centrifugation for 30 min at 4 °C and 13 000 rpm. The supernatant was then dialysed twice in 5 mM sodium acetate (pH 5.5) and 0.5 mM $CaCl_2$. Glucose oxidase activity was measured as the formation of H_2O_2 over time using 100 mM glucose as substrate. The reaction mix contained 10 mM sodium acetate and 25 μM flavin adenine dinucleotide (FAD). Reaction mix, substrate and plant extract were mixed together; samples were taken at 20 min intervals for up to 1 h; and the amount of H_2O_2 formed was determined using Amplex Red (Invitrogen, Carlsbad, CA, USA) as described previously (Bechtold *et al*. 2009).

Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (Chicago, IL, USA; http://www.spss.com/). Parameter differences between genotypes were determined using one-way analysis of variance (ANOVA) with appropriate post-hoc analysis. TukeyHSD test was used if variances of means were homogenous, and Games Howell test, if variances were not homogenous.

RESULTS

Genotypes with elevated levels of GSH, H_2O_2 and SA

Our studies were initiated when 13 *Arabidopsis* genotypes were screened for elevated levels of H_2O_2 and GSH. Whereas some genotypes had either elevated levels of GSH or H_2O_2, C24 had elevated levels of both compounds (Supporting Information Fig. S1a,b). From this survey, we chose C24 and compared it to commonly used laboratory genotypes. In a repeat analysis of C24, under different growth conditions (see Materials and methods), the increased H_2O_2 and GSH levels, relative to the genotypes Col-0, Ws-0 and Ws-2 (Fig. 1a,b), were confirmed. Elevated levels of GSH and H_2O_2 are also often coincident with elevated levels of SA (Karpinski *et al*. 2003; Mateo *et al*. 2006), indicating the induction of resistance to biotrophic pathogens (Ball *et al*. 2004; Bechtold *et al*. 2005). C24 plants showed between a 4.5- and 6.3-fold increase in foliar free SA levels relative to the control genotypes and is similar to the mutant *constitutive expresser of PR1-6* (*cpr6-1*, Fig. 1c). *cpr6-1* Permanently expresses SA-mediated defences and consequently has high levels of resistance to biotrophic pathogens (Clarke *et al*. 1998), similar to biotrophic pathogen resistance reported previously for C24 (Holub & Beynon 1997; Ton *et al*. 1999).

Reproductive fitness is not compromised in C24 under fluctuating environments

Knowing that C24 possesses a high level of constitutive basal disease resistance, we ascertained the reproductive fitness of this genotype in comparison with disease-susceptible genotypes by measuring lifetime seed yield, seed weight and germination efficiency in a fluctuating glasshouse environment (see Materials and methods). Out of all the genotypes used in the study, C24 was one of the smallest in terms of rosette area (Fig. 2a). Importantly, total seed biomass of C24 from unstressed plants was equal or better compared to other genotypes, and revealed that the negative effects observed on vegetative growth were not translated into diminished reproductive fitness for C24 (Fig. 2b).

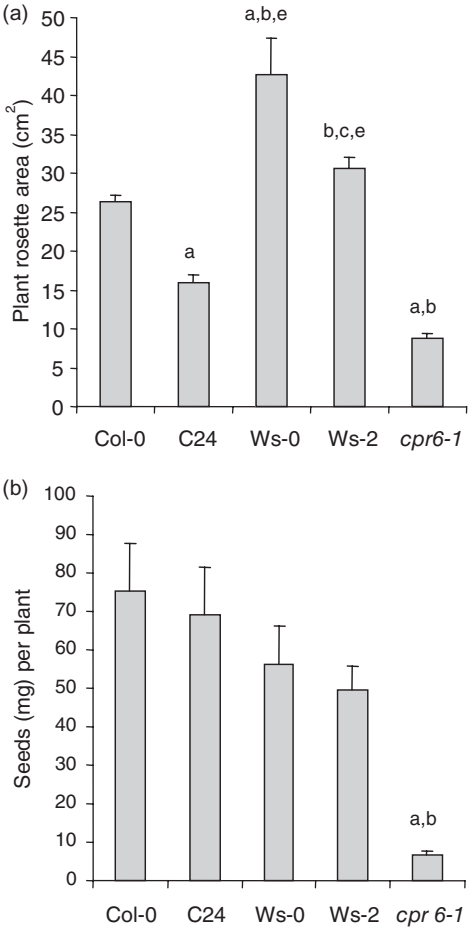

Figure 2. Plant growth of *Arabidopsis* genotypes. (a) Total rosette area of 4-week-old *Arabidopsis* genotypes. (b) Total seed biomass per plant of *Arabidopsis* genotypes. Data are means (±SE) of eight replicates. Letters indicate significant differences ($P \leq 0.05$) between genotypes: a, Col-0; b, C24; c, Ws-0; d, Ws-2; e, *cpr6-1*.

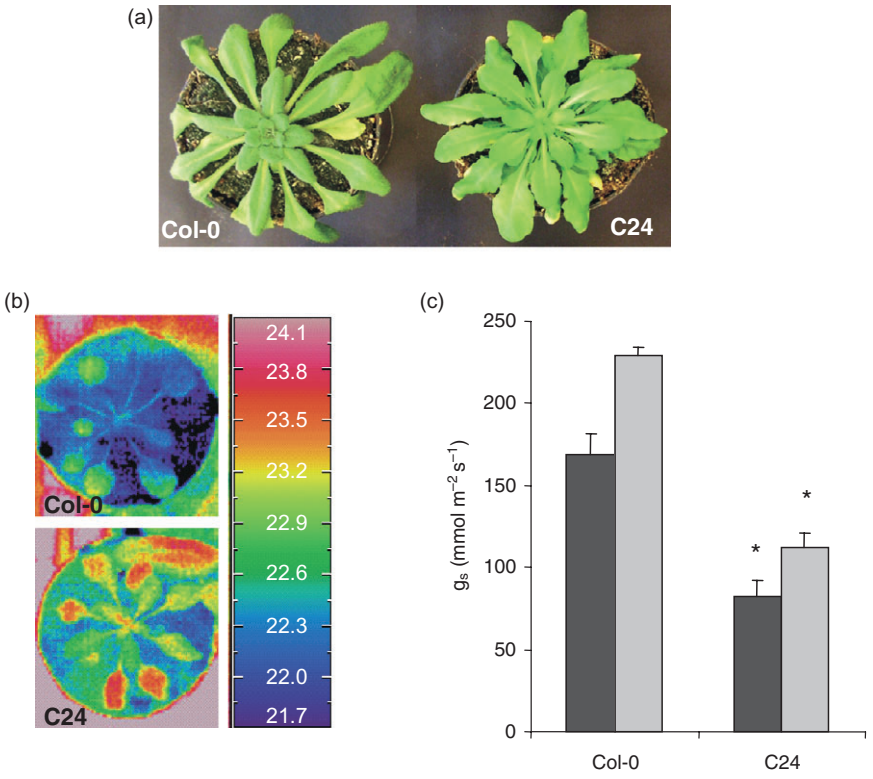

Figure 3. Enhanced drought resistance in C24 and stomatal conductance measurements. (a) Col-0 and C24 plants were left to dry for 12 d after which a clear wilting response could be observed in Col-0. (b) Infrared thermal image of Col-0 and C24 plants indicating a raised leaf temperature in C24 compared with Col-0. Images were taken under growth conditions 2 h after onset of the photoperiod. (c) Stomatal conductance (g_s) measurements taken at growth conditions at 2 h (black bars) and 6 h (grey bars) into the photoperiod. Steady-state rates of g_s at atmospheric [CO_2] were recorded using a porometer as described in Materials and methods. Data are means (±SE) of a minimum of 10 replicates. *Significant differences between C24 and Col-0 ($P \leq 0.05$ from *t*-test).

Drought tolerance is not compromised in C24 plants

The maintenance of constitutive defences could prove costly to C24 in the face of abiotic stress (Asselbergh, Vleesschauwer & Höfte 2008). In support of this argument, C24 has been reported to be more freezing sensitive than Col-0 (Rohde, Hincha & Heyer 2004; Hannah *et al*. 2006). Conversely, C24 is among the most ozone tolerant of genotypes (Brosché *et al*. 2010), and in response to high light stress revealed no difference to other genotypes studied (Supporting Information Fig. S2). Additionally, rosettes of C24 showed a greater degree of drought tolerance in comparison to Col-0, Ws-0 and Ws-2. After 12 d of water withdrawal, C24 plants still remained green, while Col-0 had begun to wilt (Fig. 3a). Associated with the enhanced drought tolerance was an increase in leaf temperature (Fig. 3b), which may be caused by a significant reduction in stomatal conductance (Fig. 3c).

In a controlled drying experiment in which *rSWC* was determined, C24 lost water at a much reduced rate in comparison to Col-0, Ws-0 and Ws-2 (Fig. 4a). A contributing factor to reduced water loss could have been the differences observed in vegetative growth in which C24 showed a 40% reduction compared with Col-0 (Fig. 2a). Ws-0, on the other hand, had the largest vegetative area and also showed one of the highest water losses, supporting a correlation between rosette area and water loss (Fig. 4a). *cpr6-1* Also exhibited a strong drought tolerance with greatly reduced drying rates (Fig. 4a).

The *rLWC* measured throughout a drying episode is shown in Fig. 4b. Fully watered C24 plants had significantly higher *rLWC* than Col-0, Ws-0 and Ws-2, which was maintained throughout the drying episode (Fig. 4b). The genotype with largest rosette area and drying rate, Ws-0, showed a marked decrease in leaf water content at ~20% *rSWC* (Fig. 4b). After a drought episode to 20% *rSWC*, the plants were rewatered and allowed to flower and set seed. C24 had slightly, but not significantly, increased seed biomass in comparison to Col-0, and showed a significantly improved performance in comparison to Ws-0, Ws-2 and *cpr6-1* (Fig. 4c).

C24 plants require less water to maintain seed yield

The slower water loss of C24 combined with its maintenance of seed biomass led us to hypothesize that C24 may also exhibit improved water productivity. To determine water productivity, Col-0, C24, Ws-0 and Ws-2 plants were maintained at two different *rSWC*s (80 and 40%), and water use was monitored from rosette growth stage 3 (Boyes *et al*. 2001) through to silique yellowing (see Materials and methods). Flowering time in C24 was not delayed in comparison to Col-0 (Supporting Information Fig. S3a), while rosette leaf number at time of flowering was much greater in C24 than in the other genotypes (Fig. 5a). However, rosette dry biomass was greatly reduced in C24 compared with Col-0 (Fig. 5b), showing that an increase in leaf number did not lead to increased biomass. In this experiment, total seed biomass remained similar between Col-0 and C24, as previously observed (Figs 2b & 4c), while total water use was greatly reduced in the C24 plants under both watering regimes (Supporting Information Fig. S3b).

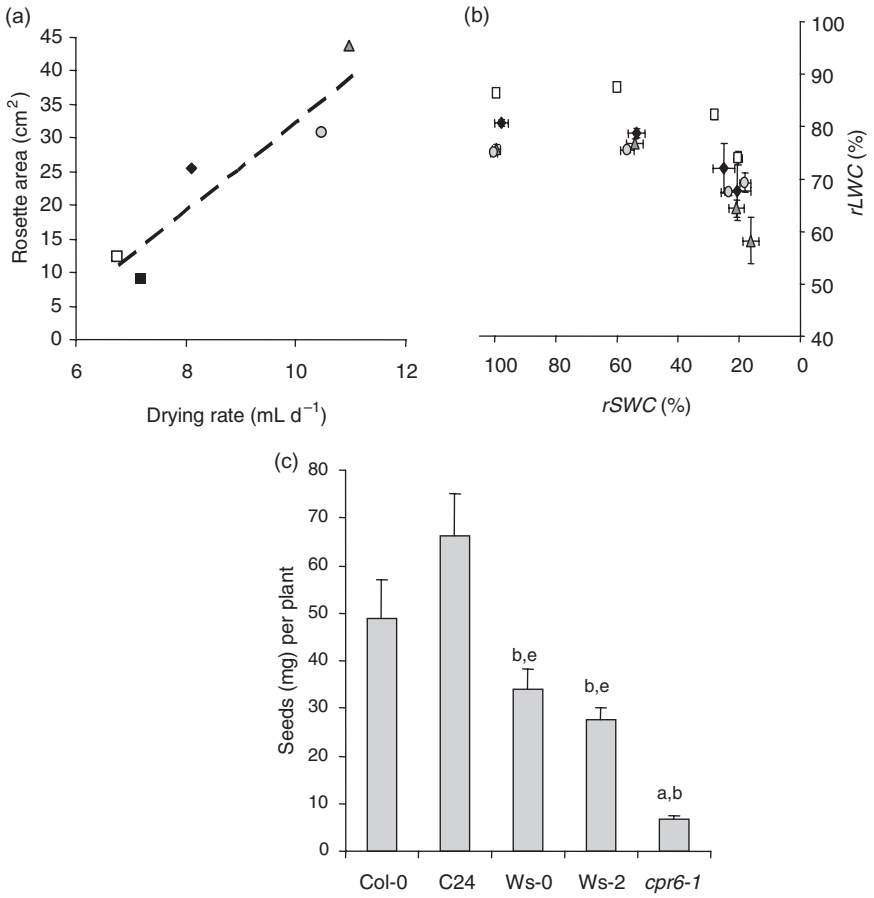

Figure 4. Biomass measurements. (a) Correlation between rosette area and drying rate in different genotypes. Open squares, C24; closed squares, *cpr6-1*; closed diamond, Col-0; closed circle, Ws-2; closed triangle, Ws-0. (b) Relative leaf water content (*rLWC*) in relation to relative soil water content (*rSWC*) throughout a drying episode. Open squares, C24; closed diamond, Col-0; closed circle, Ws-2; closed triangle, Ws-0. Data are means (±SE) of five replicates. (c) Seed yield determination after a drying episode to 20% *rSWC* in different genotypes. Data are means (±SE) of eight replicates. Letters indicate significant differences ($P \leq 0.05$) between genotypes: a, Col-0; b, C24; c, Ws-0; d, Ws-2; e, *cpr6-1*.

Analyzing total above ground biomass showed that Col-0 had the greatest biomass (Fig. 5c), but when expressed in relation to water used (*BWR* – total dry biomass per unit volume water consumed), C24 had the highest value (Fig. 5d). This ratio improved markedly when calculated as water productivity (Fig. 5e). The water productivity, calculated as total seed biomass (mg per plant) per millilitre water used during the period of the experiment, was 3.3–3.5 times higher in C24 in comparison to Col-0, Ws-0 and twice as high as Ws-2 (Fig. 5e). Additionally, the distribution of above-ground biomass differed greatly between the genotypes. In contrast to Col-0, Ws-0 and Ws-2, C24 had increased the biomass of its reproductive structures (flowers, stalks, siliques and seed) at the expense of vegetative (rosette) biomass (Fig. 5f), thereby increasing harvest index (seed biomass as a proportion of total above-ground dry biomass). There was twice as much seed biomass in relation to the total above-ground biomass (Fig. 5f). Seed weight and germination frequency were not significantly different across all genotypes (Supporting Information Fig. S3c,d).

Micro-array comparison of the genotypes C24 and Col-0

To help uncover possible explanations for improved water productivity, rosette drought tolerance and enhanced basal resistance to biotrophic pathogens, a micro-array experiment was carried out using RNA prepared from 5-week-old non-stressed C24 plants compared with that from Col-0 plants. The expression of 693 genes was up-regulated >2-fold ($P < 0.05$) in C24 compared with Col-0 (Supporting Information Table S2), of which 18% were annotated as stress- and/or SA-responsive genes. On the other hand, there was no elevation of genes associated with drought tolerance. For example, increased expression of ABA-regulated genes, implicated in drought responses (Yamaguchi-Shinozaki & Shinozaki 1994; Sakuma *et al.* 2006), were not significantly elevated (Supporting Information Table S2).

In order to investigate further the link to enhanced basal resistance, the C24 micro-array data set was also compared with a *cpr5-1* data set available from the ArrayExpress database (see Material and methods). Both the *cpr5-1* and *cpr6-1* mutants are phenotypically similar to C24 in terms of constitutive expression of SA-mediated pathogen resistance genes (Clarke *et al.* 1998), elevated levels of H_2O_2 (Fig. 1a), GSH (Fig. 1b), SA (Fig. 1c) and increased basal resistance to infection by biotrophic pathogens (Bowling *et al.* 1997; Karpinski *et al.* 2003; Mateo *et al.* 2006). Two hundred sixty-eight significant differentially up-regulated genes (>2fold, $P < 0.01$) were identified in *cpr5-1* compared with Col-0. The expression of these genes was compared with the 693 genes found to be differentially expressed in

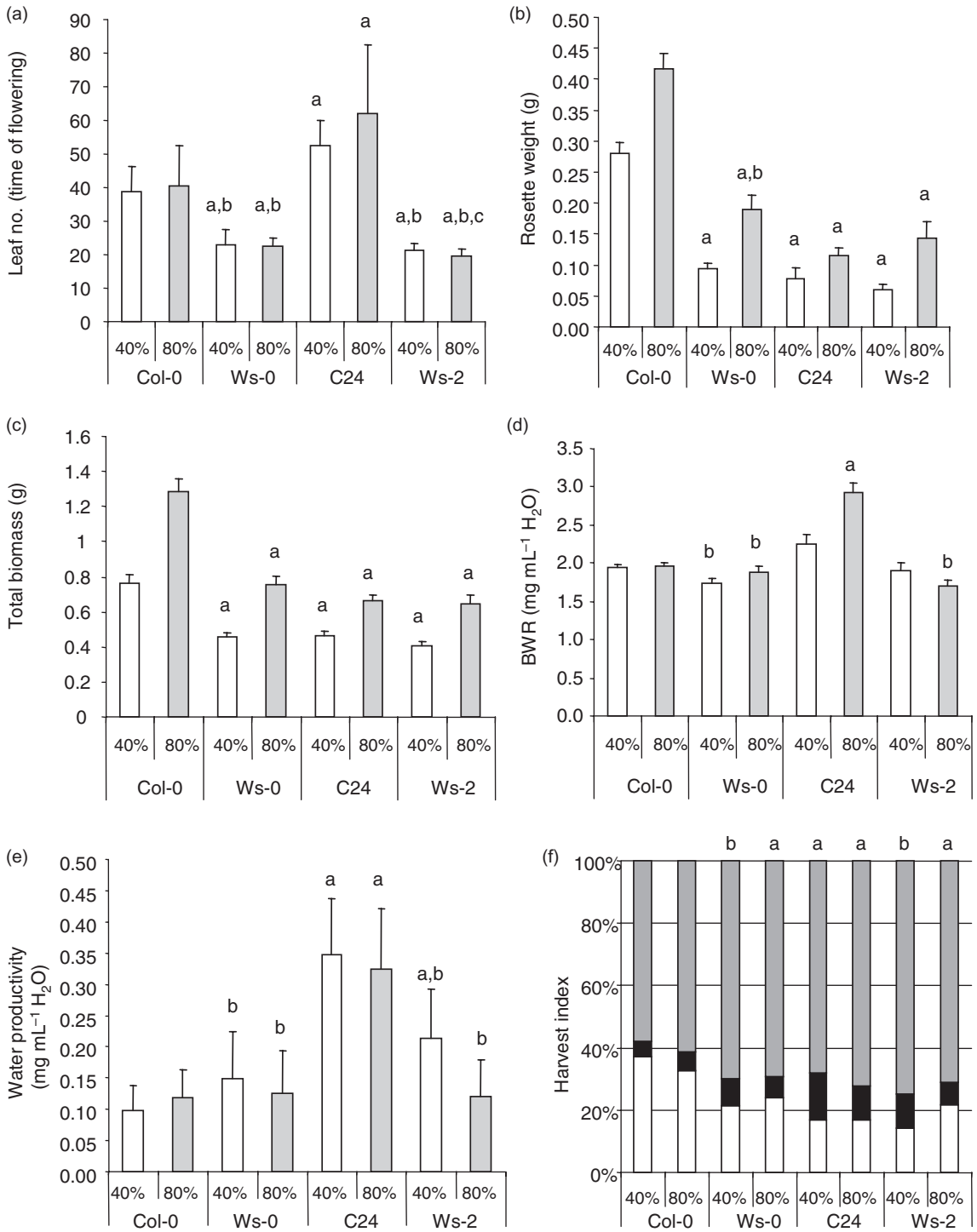

Figure 5. Enhanced biomass water ratio (*BWR*) and water productivity in C24. (a) Rosette leaf number at time of flowering of different genotypes. (b) Dry weight of rosette biomass. Rosettes were harvested and dried at 80 °C until a stable weight was reached. (c) Total above-ground biomass calculated as grams (g) per plant. (d) *BWR*, calculated as total above-ground biomass per plant per volume water used. (e) Water productivity calculated as seeds per volume water used at 40 and 80% relative soil water content. (f) Harvest index calculated as biomass distribution of the reproductive and vegetative biomass relative to the total above-ground biomass. White, rosette; black, seeds; grey, stalks/pots. Data are means (±SE) of 10 replicates. Letters indicate significant differences ($P \leq 0.05$) between genotypes: a, Col-0; b, C24; c, Ws-0; d, Ws-2; e, *cpr6-1*.

Table 1. Relative gene expression of stress-associated genes was analysed by qRT-PCR in *Arabidopsis* genotypes and *cpr6-1*

Relative gene expression (fold induction)					
Gene name	Locus no.	C24/Col-0	Ws-0/Col-0	Ws-2/Col-0	*cpr6-1*/Col-0
PR-1	At2g14610	160.5[a]	1.1	0.2	26.9[a]
WRKY26	At5g07100	4.5[a]	0.2	0.5	7.9[a]
WRKY38	At5g22570	11.9[a]	0.7	1.0	6.8[a]
WRKY51	At5g64810	21.6[a]	0.6	1.5	11.2[a]
WRKY53	At4g23810	18.7[a]	1.6	1.6	4.6[a]
HSFA7a	At4g18880	5.3[a]	1.1	1.1	1.8[a]
MBF1c	At3g24500	8.6[a]	0.9	1.4	31.2[a]
HSFA4a	At4g18880	5.4[a]	1.2	1.2	1.8[a]
HSFA2	At2g26150	1.2	0.7	0.6	0.8
HSFB1	At4g36990	3.6[a]	0.8	0.9	2.9[a]
HSFB2a	At5g62020	1.5[a]	1.2	1.2	1.76[a]
MYB15	At3g23250	28.3[a]	1.1	1.3	26.9[a]
MYB95	At1g74430	4.2[a]	0.7	1.1	2.8[a]

[a]Significant difference of the ratios with $P \leq 0.05$.
The fold difference represents the mean of the ratio of three biological replicates.

C24. In total, up-regulated expression of 36 genes overlapped between the two experiments (Supporting Information Table S3). A significant over-representation of GO terms in the overlapping group was analysed and resulted in mainly cell death and stress-associated genes (Supporting Information Table S4). To test the significance of the occurrence of this group of 36 genes up-regulated in both C24 and *cpr5-1*, a hypergeometric distribution was calculated to determine the probability of this overlap being significant in relation to the total number of genes in the *Arabidopsis* genome. The test resulted in a *P* value of $2.34e^{-15}$, indicating a significant overlap between the two experiments.

From the stress-responsive group of genes, qRT-PCR analysis of 14 genes was also carried out on cDNA prepared from a separate experiment using the different genotypes and *cpr6-1*. The results confirmed the micro-array data (Table 1), but also showed that the pattern of stress-dependent gene expression in *cpr6-1* mirrored that of C24. In the pathogen-susceptible Ws-0 and Ws-2 genotypes, no increase or even reduced gene expression was observed (Table 1). These data strongly suggest that C24 has a pre-activated defence mechanism, comparable to *cpr5-1* plants (Jing *et al*. 2008).

No attempt was made to analyse down-regulated genes because we could not rule out that lower fluorescence signals from micro-array probes, designed from Col-0 genome sequence data, could be caused by their mismatch when hybridized to C24 cDNA.

Sources and localization of elevated H_2O_2 levels in C24

No contribution of chloroplasts to the elevated levels of H_2O_2 could be discerned, as dark-grown plants showed no reduction of H_2O_2 levels (Supporting Information Fig. S1c). Furthermore, the involvement of NADPH oxidases in the production of the ROS superoxide under both abiotic and biotic stress conditions, which would lead to increased H_2O_2 production (Torres *et al*. 2005; Miller *et al*. 2009), was ruled out because the expression of NADPH oxidase genes (*AtrbohC,D* and *F*) was unaltered in C24 plants compared with Col-0 (data not shown). Likewise, treatment of C24 leaves with the flavin oxidase inhibitor diphenyl iodinium (Cross & Jones 1986) had no significant effect on H_2O_2 levels (Supporting Information Fig. S1c). However, micro-array analysis revealed a group of genes up-regulated in C24 compared with Col-0, annotated as coding for isoforms of berberine bridge-containing reticuline oxidase. These enzymes use hexose sugars as substrates to generate H_2O_2, and when over-expressed in transgenic plants have been shown to increase resistance to infection by bacterial pathogens (Custers *et al*. 2004). Nine out of 28 reticuline oxidase genes in *Arabidopsis* were up-regulated in the C24 background compared with Col-0. We identified the reticuline oxidase gene *At1g30720* as the highest expressed with a 13-fold induction, the remaining eight genes were two- to eightfold up-regulated (Fig. 6a). The micro-array data were also confirmed by qRT-PCR for eight of the nine genes (Fig. 6a). The up-regulation of gene expression was also reflected in a two fold increase in enzyme activity in an anionic cell wall fraction from C24 compared with Col-0, using glucose as the substrate (Fig. 6b). $CeCl_3$ staining followed by TEM revealed an apoplastic location for the enhanced levels of foliar H_2O_2 in C24 (Fig. 6c), confirming the *in vitro* measurements made on total leaf extracts (Fig. 1a) and agrees with the apoplastic location of reticuline oxidase activity (Custers *et al*. 2004).

DISCUSSION

High levels of basal resistance in C24 are mediated via SA, but do not compromise reproductive fitness

SA-associated defences are normally induced in response to infection by diverse biotrophic pathogens in many *Arabidopsis* genotypes (van Leeuwen *et al*. 2007). In contrast,

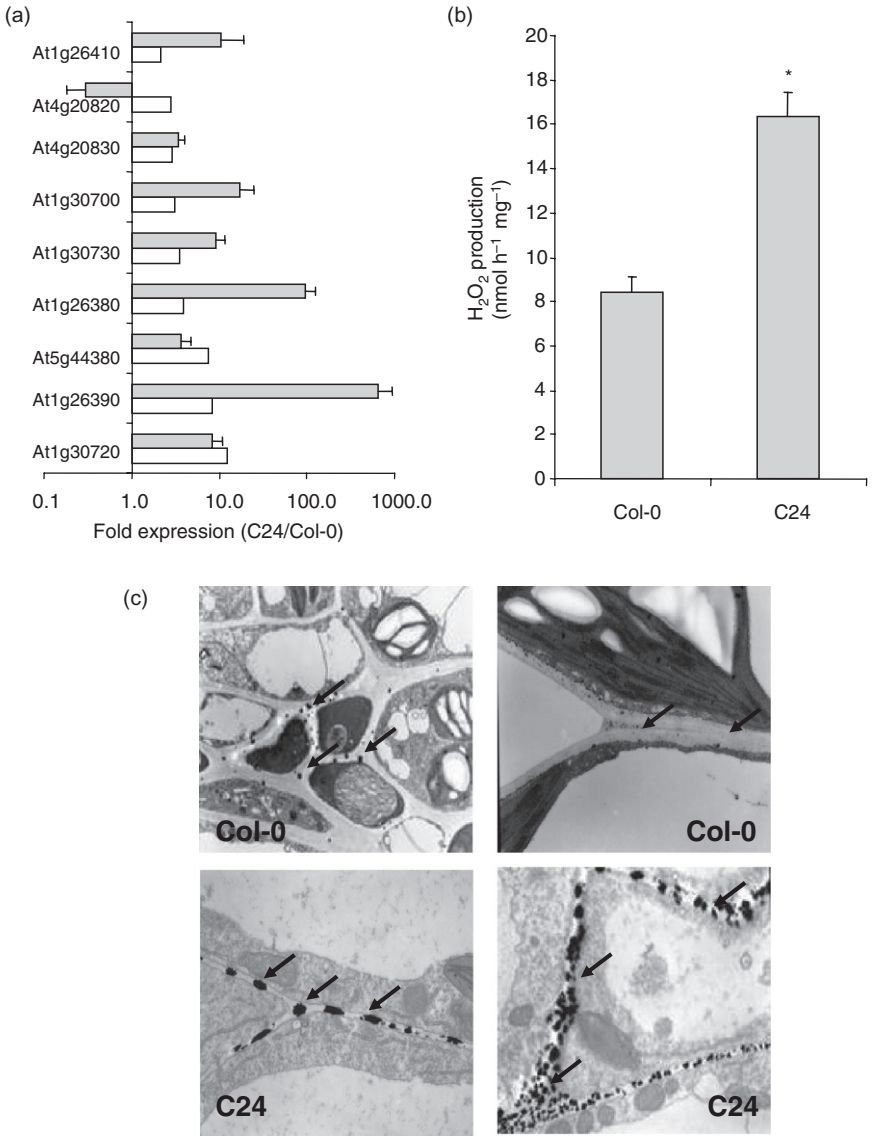

Figure 6. Source of and localization of elevated H_2O_2 levels in C24. (a) Relative gene expression analysis of reticuline oxidase genes determined by micro-array (white bars) and qRT-PCR (grey bars). (b) Glucose oxidase activity of a total cell wall protein extract. Enzyme activity was measured as the production of H_2O_2 h^{-1} mg^{-1} protein. Data are means (±SE) of four replicates. (c) Localization of H_2O_2 by $CeCl_3$ staining. The black arrows indicate deposition of electron-dense cerium perhydroxide, formed by reaction of $CeCl_3$ with H_2O_2 within the apoplast, detected by transmission electron microscopy (TEM). The cerium perhydroxide in C24 leaves is formed in regular patches across the apoplast. Magnifications: top left: ×7000, top right: ×20 000, bottom left: ×17 000 and bottom right: ×15 000. *Significant difference between C24 and Col-0 ($P \leq 0.05$ from *t*-test).

mutants such as *cpr6-1* and *cpr5-1* display constitutive biotrophic pathogen resistance associated with elevated expression of SA-associated defences, and consequently suffer depressed seed yields and reduced vegetative growth (Heil & Baldwin 2002; Heidel & Dong 2006; Mateo *et al.* 2006).

C24 displays high resistance to *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* pv. tomato infection (Holub & Beynon 1997; Ton *et al.* 1999), and a reduced vegetative growth phenotype similar to *cpr6-1* (Figs 2a & 5b,f), which is associated with elevated levels of SA (Fig. 1c). However, unlike *cpr6-1*, these traits in C24 do not translate into reduced seed yield, seed weight or viability under water-replete or -limited conditions (Figs 2b & 4c; Supporting Information Fig. S3c,d). The difference observed in reproductive fitness is most likely caused by genotypic variations between *cpr* mutants (Col-0 background) and C24. Therefore, we conclude that the constitutive expression of SA-associated defences need not lead to a loss of reproductive fitness under pathogen-unchallenged conditions. We speculate that this is most likely caused by a genetic component(s) alleviating negative effects of the expression of constitutive SA defences. This does not mean that there is no cost associated with the expression of high

levels of basal resistance in C24. For example, the constitutive elevated apoplastic H_2O_2 levels (Figs 1a & 6c; Supporting Information Fig. S1a) could conceivably have a negative effect on reproductive fitness.

Vegetative growth phenotype and performance under water-replete and -limited conditions

Reduced vegetative area and dry biomass (Figs 2a & 5b) in C24 negatively correlate with leaf number (correlation coefficient: –0.87), suggesting a smaller rosette area increases the number of leaves. A slower rate of rosette development, increased rosette leaf number and vegetative dry matter have been associated previously with increased seed yield (Redei 1962), and could account for maintained seed biomass in C24 despite the elevated levels of basal disease resistance. A positive correlation between rosette area and soil drying rate was also observed (Fig. 4a), suggesting that plants with fewer, but larger, rosette leaves and greater vegetative biomass suffer an elevated water loss (Figs 3a & 4a). This is also reflected in the *rLWC* of the different genotypes, which was higher in C24 with a larger number of small leaves (Fig. 4b). These observations suggest that in *Arabidopsis*, increasing the leaf number with a concomitant reduction of individual leaf area is not only beneficial to water management, but also to overall plant productivity.

A combined physiological and developmental basis for the high water productivity of C24

C24 displayed a superior water productivity compared to all other genotypes tested (Fig. 5e). The partitioning of biomass into the main harvestable product (harvest index) was greatly increased, seen here as changes in biomass distribution (Fig. 5f), which suggests that a developmental component may play an important role in maintaining yields despite the high constitutive expression of resistance to biotrophic pathogens. Many traits associated with cellular adaptation to drought are constitutive, determining plant development and shape (Passioura 2002). The developmental component of biomass distribution may involve the repressor of floral induction *FLOWERING LOCUS C* (*FLC*), which is 6.6-fold up-regulated in C24 in comparison to Col-0 (Supporting Information Table S2), and leads to a vernalization-sensitive late-flowering phenotype (Sheldon *et al*. 2000). However, despite increased leaf number, C24 showed no difference in flowering time compared with Col-0 under the fluctuating conditions in our glasshouse (Supporting Information Fig. S3a; see Materials and methods), suggesting that timing of flowering induction was not a critical component in our experiments. Nevertheless, a number of QTL have been identified that associate improved TE, with flowering loci in *Arabidopsis* accessions (Loudet *et al*. 2003; Juenger *et al*. 2005). *FLC* can also control the circadian rhythm of leaf movement and therefore impact on the regulation of stomatal transpiration (Swarup *et al*. 1999; Edwards *et al*. 2006). This would be consistent with our observations on drying rates, *rLWC* and stomatal conductance in C24 (Figs 4a,b & 3c). Similarly, allelic variations in the gene *ERECTA*, which can influence stomatal and mesophyll cell density and TE, also link leaf and rosette development with plant–water relations (Masle *et al*. 2005). In an ecological context, it is predicted that drought-tolerant plants maximize fitness by decreasing leaf size and stomatal conductance (Dudley 1996), which also depends on the growing season length and plant lifespan (Donovan & Ehleringer 1992). This means that longer-lived plants have a fitness advantage by lowering stomatal conductance to achieve better *BWR* in a long growing season, which could include short periods of drought. In comparison, shorter-lived plants compensate high stomatal conductance with rapid growth prior to the onset of any drought stress (Geber & Dawson 1997; Pimentel, Laffray & Louguet 1999). Thus, C24 may be a longer-lived *Arabidopsis* genotype that achieves its high *BWR* because of a reduced leaf size, increased leaf number and lowered stomatal conductance, which in some circumstances may also be linked to the late-flowering phenotype (Sanda & Amasino 1995).

While the geographical origin of C24 is ambiguous, recent polymorphism analysis indicates that C24 is closely related to at least one of the Coimbra accessions from Portugal (Schmid *et al*. 2006), and was originally selected from M. Jacobs's *Arabidopsis* collection by the laboratories of M. van Montague and J. Schell in the early 1970s at the Free University of Brussels (Belgium). However, it has also been suggested that C24, because of its glabrous phenotype, may have arisen out of a mutagenesis experiment (Michaels *et al*. 2003).

The relevance of the observations on C24 traits of enhanced basal disease resistance and *BWR* for crop improvement

In the context of plant breeding, the improvement of water productivity is of major agronomical importance to increase water productivity, which is the amount of crop yield for a defined amount of water consumed (Chaves *et al*. 2002). Improved biomass production in relation to water used and increasing the harvest index are a number of requirements that have been realized in C24 to improve its performance. Greatly enhanced water productivity means that C24 requires substantially less water than other genotypes to produce similar seed yields. Importantly, C24 achieves constitutive expression of pathogen defences and drought tolerance without incurring a yield penalty. Despite the ambiguity of its origin, these resistance and productivity traits have combined in C24 to achieve a superior genotype. This opens up new possibilities for the development of plants combining a number of resistance traits without compromising yield.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Analysis of total foliar H_2O_2 and reduced glutathione (GSH) levels in 13 *Arabidopsis* genotypes. Plants were grown in a special custom mix compost with Intercept 5GR (The Scotts Company Ltd, Ipswich, UK; 280 g m^{-3} of compost) at 85 μmol m^{-2} s^{-1}, 22 °C and a relative humidity of 55%, under short-day conditions of 8 h light and 16 h darkness. (a) H_2O_2 measurements were carried out on 100 mg leaf tissue and was analysed based on a method adapted from (Jimenez *et al*. 2002). (b) Total leaf GSH was analysed using the monobromobimane derivatization assay as described in (Creissen *et al*. 1999). (c) Total leaf H_2O_2 measurements in C24 plants during dark, light and after DPI treatment. No significant difference was observed between different treatments.

Figure S2. High-light stress of *Arabidopsis* genotypes. Maximum efficiency of photosystem II (F_v/F_m, see Materials and methods) of 5-week-old rosettes was measured after 1 h at low light (LL), high light (HL) and 1 d post-stress (post). White bars, Col-0; black bars, C24; light grey bars, Ws-0. Data are means (±SE) of three replicates.

Figure S3. Flowering time, water use, seed weight and germination frequency. (a) Days to flowering of different genotypes. (b) Water use (mL) during an 8 week growth period. (c) Seed weight determined as weight/1000 seeds. A known weight of seeds was placed onto filter paper and a photograph was taken. The images were analysed using ImageJ (http://rsb.info.nih.gov/ij/) to count the number of seeds. (d) Germination frequency of seeds was analysed for four

genotypes. The filter paper containing the seeds was soaked with 5 mL of water, and seeds were left to germinate in the controlled environment room. Successful germination was scored as radicle emergence after 3–4 d. Data are means (±SE) of 10 replicates. Letters indicate significant differences ($P \leq 0.05$) between genotypes: a, Col-0; b, C24; c, Ws-0; d, Ws-2.

Table S1. Primer sequences used in qRT-PCR experiments; 3 μg of total RNA was used to synthesize random-primed cDNA, which was then used at appropriate dilutions in qRT-PCR.

Table S2. Significantly up-regulated genes in C24 versus Col-0. Details of the micro-array experiment can be found in experimental procedures.

Table S3. Significantly up-regulated genes in both the C24 and *cpr5-1* micro-array experiments. The *cpr-5-1* raw data were downloaded from the array express database (http://www.ebi.ac.uk/microarray-as/ae/) and analysed using R-packages Harshlight and GCRMA available from the Bioconductor Website.

Table S4. Over-representation of GO terms and cluster frequencies of overlapping genes between C24 and *cpr5-1*. The Biological Network Gene Ontology tool (BINGO; Maere *et al*. 2005) was used in conjunction with Cytoscape v 2.6.3 to generate a network of GO terms (Cline *et al*. 2007).

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