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ORIGINAL ARTICLE

Microspore embryogenesis in barley: anther pre-treatment stimulates plant defence gene expression

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Abstract Microspore embryogenesis (ME) is a process in which the gametophytic pollen programme of the microspore is reorientated towards a new embryo sporophytic programme. This process requires a stress treatment, usually performed in the anther or isolated microspores for several days. Despite the universal use of stress to induce ME, very few studies have addressed the physiological processes that occur in the anther during this step. To further understand the processes triggered by stress treatment, we followed the response of anthers by measuring the expression of stress-related genes in two barley (*Hordeum vulgare* L.) cultivars differing in their ME response. Genes encoding enzymes involved in oxidative stress (*glutathione-S-transferase*, *GST*; *oxalate oxidase*, *OxO*), in the synthesis of jasmonic acid (*13-lipoxygenase*, *Lox*; *allene oxide cyclase*, *AOC*; *allene oxide synthase*, *AOS*) and in the phenylpropanoid pathway (*phenylalanine ammonia lyase*, *PAL*), as well as those encoding PR proteins (*Barwin*, *chitinase 2b*, *Chit 2b*; *glucanase*, *Gluc*; *basic pathogenesis-related protein 1*, *PR1*; *pathogenesis-related protein 10, PR10*) were up-regulated in whole anthers upon stress treatment, indicating that anther perceives stress and reacts by triggering general plant defence mechanisms. In particular, both *OxO* and *Chit 2b* genes are good markers of anther reactivity owing to their high level of induction during the stress treatment. The effect of copper sulphate appeared to limit the expression of defence-related genes, which may be correlated with its positive effect on the yield of microspore embryos.

Keywords Anther culture · Barley · Cold pre-treatment · Copper · Defence mechanisms · Starvation · Stress

Abbreviations

AGPs	Arabinogalactan proteins
cv.	Cultivar
DH	Doubled haploid
JA	Jasmonic acid
ME	Microspore embryogenesis
NBT	Nitroblue tetrazolium
PR	Pathogenesis related
ROS	Reactive oxygen species

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Introduction

Microspores are initially destined to develop into pollen grains but can be reprogrammed to behave like a zygote, thus, developing into a haploid embryo (Kimber and Riley 1963). The production of doubled-haploid (DH) plants is a useful tool for breeding in many crop species (Devaux and Pickering 2005), and has generated a number of commercial cultivars (Devaux et al. 1996; Szarejko 2003). The most commonly used system for large-scale DH production in barley is microspore embryogenesis (ME), through either anther culture or isolated microspore culture (Szarejko 2003).

A stress treatment is necessary to switch the gametophytic pollen programme of the microspore towards a new sporophytic embryo developmental pathway (Touraev et al. 1996; Maraschin et al. 2005; Shariatpanahi et al. 2006). The success of DH production from the male gametophytes depends on the quality of the stress treatment (Heberle-Bors 1985). Several abiotic stresses, alone or in combination, are used to trigger ME. These include cold shock (Gonzalez and Jouve 2005; Labbani et al. 2005), heat-shock (Custers et al. 1994; Touraev et al. 1996; Rimberia et al. 2005), osmotic shock (Caredda et al. 2000; Touraev et al. 2001; Li and Devaux 2003; Wojnarowiez et al. 2004), pH variation (Barinova et al. 2004), nutrient starvation (Touraev et al. 1996), exposure to heavy metals, such as lithium (Zonia and Tupy 1995) or copper sulphate (Wojnarowiez et al. 2002). In barley anthers, ME is induced using a combined cold- and osmotic shock treatment (Caredda et al. 2000).

Many species remain recalcitrant to ME or regenerate albino plantlets (Caredda and Clément 1999). For example, in barley, the winter cv. Igri regenerates approximately 90% green plantlets, whereas the spring cv. Cork produces exclusively albino plantlets (Caredda and Clément 1999). This problem may be practically overcome by the addition of copper sulphate during anther pre-treatment and culture (Wojnarowiez et al. 2002), although the mechanism underlying the beneficial effect of copper is not understood.

In plants, abiotic stresses induce physiological changes, as revealed by modifications in cellular and molecular processes (Verslues et al. 2006; Talame et al. 2007). Stress promotes adaptive mechanisms including water uptake, accumulation of solutes, modifications of cells properties or accumulation of protective proteins, allowing cells to restore their initial state and to limit tissue damage (Netting 2002; Verslues et al. 2006). Several signalling molecules are involved in stress perception, including calcium, abscissic acid, ethylene, jasmonic acid, salicylic acid or reactive oxygen species (Netting 2002; Verslues et al. 2006) and induce significant changes in gene expression.

The transcriptome changes in barley microspores following mannitol/cold stress treatment of anthers was recently studied in detail. It was shown that the stress treatment blocks the expression of pollen-related genes, whereas the expression of new sets of genes related to sugar metabolism, stress response and proteolysis is stimulated (Maraschin et al. 2006; Munoz-Amatriain et al. 2006). These results lead to the identification of molecular markers associated with the induction of ME in barley. However, these studies only dealt with microspores collected after the stress treatment, i.e. microspores that have already been induced to undergo ME. The different events that the whole anther undergoes during the early steps of stress treatment, and thus those involved in the initial induction of ME remain poorly documented.

To evaluate the effect of the stress treatment on barley anthers, we followed several processes, including oxidative burst through the production of $O_2^{\cdot-}$ and expression of genes encoding (a) enzymes involved in protection against oxidative stress (*glutathione-S-transferase*, *GST*; *oxalate oxidase*, *OxO*), (b) PR proteins involved in pathogen defence (*Barwin*, *chitinase 2b*, *Chit 2b*; *glucanase*, *GLUC*; *basic pathogenesis-related protein 1*, *PR1*, *pathogenesis-related protein 10; PR10*), (c) enzymes involved in the phenylpropanoid pathway (*phenylalanine ammonia lyase*, *PAL*) and (d) enzymes involved in JA biosynthesis (*13-lipoxygenase*, *Lox; allene oxide cyclase*, *AOC*; *allene oxide synthase*, *AOS*). None of these 11 genes have been previously examined during ME in barley or in other species. The work was performed (a) in both the winter cv. Igri and the spring cv. Cork, which are also models for investigating the relationship between ME and albinism in barley (Caredda et al. 2000) and (b) in the presence/absence of copper sulphate, owing to its positive effect on ME (Wojnarowiez et al. 2002).

Materials and methods

Plant material, growth conditions and anther pre-treatment

We used two two-rowed barley (*Hordeum vulgare* L.) cvs., namely, the winter cv. Igri and the spring cv. Cork, both from Florimond Desprez Ind. (Cappelle en Pévèle, France). Plants were grown as described earlier (Jacquard et al. 2003, 2006). Spikes were collected when the microspores were at the mid-uninucleate stage (Szarejko 2003), which was determined using Hoechst 33342 nuclear staining according to Varnier et al. (2005).

We followed expression of stress-related genes during in vivo development and in vitro during the stress treatment. For in vivo development, we collected anthers at the mid-uninucleate stage (T0) and 48 h (uninucleate stage) and 96 h later (early binucleate stage). For in vitro culture, anthers were sterilised in 70% ethanol and rinsed in sterile distilled water. Anthers from proximal and distal flowers were removed from each floral spike as these flowers do not develop synchronously with others in the spike (Szarejko 2003). Thirty anthers from the same spike were collected and incubated in a 5.5-cm diameter Petri dish in 10 ml of a medium containing mannitol (62 g/l), providing an osmotic pressure of 180 mosm/l, with or without copper sulphate (10 μM). Anthers were then cultured at 4°C in the dark at 80% relative humidity and collected 3, 6, 9, 15, 24, 36, 48, 72 and 96 h later. Harvested anthers were immediately frozen in liquid nitrogen and stored at −80°C until the extraction of total RNAs.

Nitroblue tetrazolium (NBT) staining

$O_2^{\cdot-}$ was detected in situ as described by Doke (1983) with some modifications. Anthers collected at T0, 48 h and 96 h were immersed in 0.1% NBT (yellow nitroblue tetrazolium), 10 mM NaN_3 and 50 μM NADPH. Anthers were stained for 20 min at room temperature in the light, and then placed in 95% (v/v) ethanol to stop the reaction, remove pigments, and preserve the tissue integrity.

RNA extraction and quantitative real-time RT-PCR analysis

A total of 180 anthers (approximately 50 mg), from six different spikes, were collected for each timepoint and ground in liquid nitrogen. Total RNA was isolated using Extract All® (Eurobio) according to the manufacturer's instructions.

RNA was reverse transcribed with SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. We used the barley *actin* and *ef1α* genes to normalise different samples for differences in the amount of plant RNA (Gjetting et al. 2004). We followed the expression of 11 genes involved in different plant stress-response pathways (Table 1).

PCRs were performed as described by Bézier et al. (2002) on a GeneAmp 5700 Sequence Detection System (PE Biosystems). Transcript levels were calculated using the standard curve method and normalised against barley *actin* and *ef1α* genes as internal controls (Gjetting et al. 2004). The T0 anther sample was used as a reference sample for each cultivar. Experiments were repeated five times in duplicate.

Results

In vivo pollen development

Pollen development was synchronous in the Igri and Cork cvs. At the time of collection (T0), microspores were at the mid-uninucleate stage, as revealed by the round shaped nucleus. Most of the microspores reached the late uninucleate stage 48 h later, and the bicellular stage 96 h later.

Superoxide radical detection

Cells that accumulate $O_2^{\cdot-}$ stain dark blue in the presence of NBT. At T0, $O_2^{\cdot-}$ accumulation was only visible in the upper part of filament of both cvs. (Fig. 1a–c). During the stress treatment, a dark blue staining was observed in the anther wall layers (Fig. 1d, e, g, h) and in the pollen wall (Fig. 1f, i), suggesting that the treatment conditions induced oxidative stress. After 48 h of stress treatment, a characteristic dark blue staining was observed in anther wall, in the connective tissue and in the pollen walls (Fig. 1d–f) of both cv. Cork and cv. Igri, whereas no staining was observed in anthers developing in vivo. After 96 h of stress treatment the intensity of staining in the anther wall increased (Fig. 1g, h), especially in the cv. Cork (Fig. 1g).

Genes-encoding enzymes protecting against oxidative stress

Both *OxO* and *GST* genes were studied (Fig. 2). The transcript level of both genes did not change significantly in the anther during in vivo development. In Igri,

Table 1 List of primers used for gene expression analysis

Genes	Accession number	Primer sequence forward 5′–3′	Primer sequence reverse 5′–3′
Actin	U21907	TGGGTCAACTATGTTCCCGG	GTGCCACCACCTTGATCTTCA
Elongation factor 1 alpha (ef1 α)	L11740	GATAGTTGTTTTAGTCGCTTGGGTTATT	CACCAACACAACCGAACGATAC
13-Lipoxygenase (Lox)	AJ507212	CTGCACGGATTATGCACTGG	TACAAGGCGTCGATGGAGG
Allene oxide cyclase (AOC)	AJ308488	ATCTGCATCCTGATCCAGCAC	CTGATGTGGCCGTAGTCGC
Allene oxide synthase (AOS)	AJ250864	GCATGCATCTTGTTCGGTTCT	GGCTTATTACACTGGGCAAGATTG
Barwin	Y10814	ACTGGGACACCGTCTTCGC	AATCTAGTCGCGGCAGTCGA
Chitinase (Chit2b)	X78672	CGATCGGGAAGGACCTTGT	GCCGTCATCCAGAACCAAA
Glucanase (Gluc)	M62907	GCCATGTTCAACGAGAACCAG	CGACTTGTCCGGGTTGAAGA
Glutathion-S-transferase (GST)	AF109194	CAGTGCGTGCGTTTGGATATT	GCCGCAAGCCTACCAACA
Oxalate oxidase (OxO)	Y14203	CGAGATCGGCATGGTGATG	CACGCACCACCCTGGAGTA
Phenylalanine ammonia lyase (PAL)	X97313	CGCAAACCGAGCTTGGACTA	TCACTGGGTTGGCAAGGTATT
PR1	X74939	GTCTGCAACAACAACGGCG	TTCTGTCCAACAACATTCCCG
PR10	AY220734.1	ATGAGCAAGTAGCCCGGTGTT	GAAAAACTCAATACACACCCCAAAC

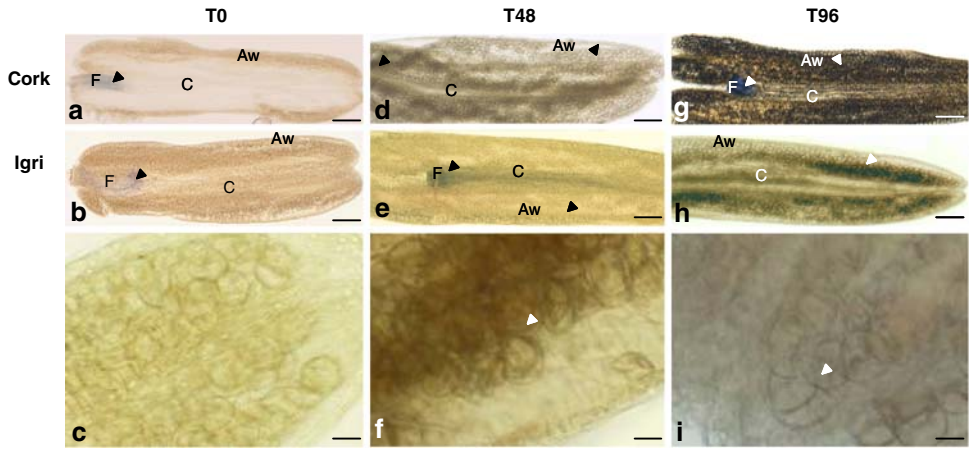

Fig. 1 Histochemical localisation of $O_2^{\cdot-}$ generation in anthers by nitroblue tetrazolium (NBT) staining in freshly collected anthers (T0), and after 48 h (T48) and 96 h (T96) of pretreatment. NBT forms a *blue-dark* formazan precipitate upon reduction by superoxide. **a** Cork anther at T0. Blue staining was detected in the filament (*arrowhead*). *Bar* 250 μm. **b** Igri at T0. Blue staining was visible in the filament (*arrowhead*). *Bar* 250 μm. **c** Detailed view of NBT stained Igri microspores at T0. No staining was visible in vacuolated microspores. *Bar* 25 μm. **d** Cork anthers at T48. The anther wall exhibited moderate blue NBT staining (*arrowhead*). *Bar* 200 μm. **e** Igri anthers at T48. Dark blue NBT staining was visible in the upper part of filament, in the connective tissue and to a lesser extent in the anther wall (*arrowheads*). *Bar* 200 μm. **f** Detailed view of NBT-stained Cork microspore at T48. Dark blue staining was observed in the microspore wall (*arrowhead*). *Bar* 25 μm. **g** NBT-stained Cork anthers at T96. Dark blue staining was visible in the filament and anther wall (*arrowhead*). *Bar* 200 μm. **h** NBT-stained Igri anthers at T96. The anther wall exhibits dark blue staining (*arrowhead*). *Bar* 200 μm. **i** Detailed view of NBT-stained Igri microspore at T96. Blue staining was detected in the microspore wall (*arrowhead*). *Bar* 25 μm. *F* filament, *Aw* anther wall, *C* connective tissue

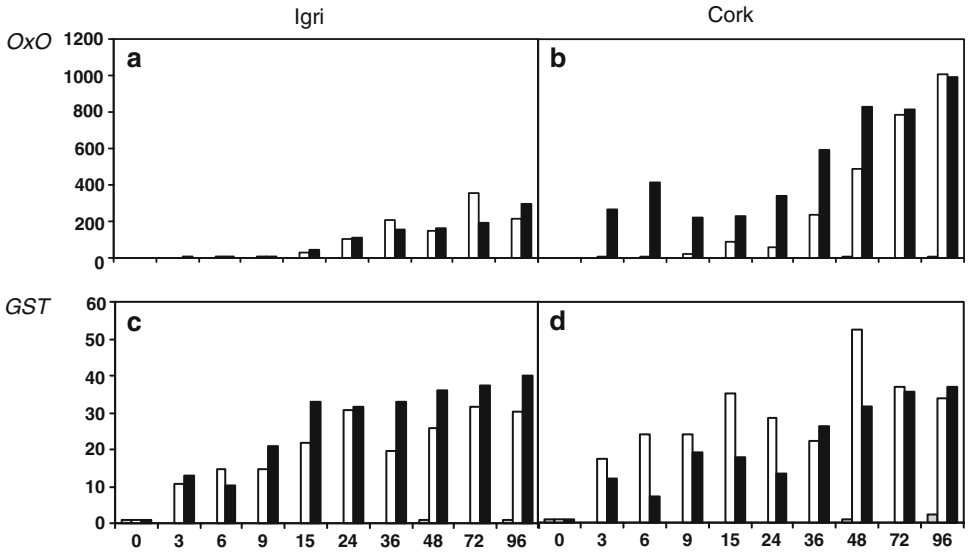

Fig. 2 The expression levels of *OxO* and *GST* genes were quantified by real-time RT-PCR in cvs. Igri and Cork during anther development in vivo (*light-shaded bar*) and during the stress pretreatment in vitro, with (*dark-shaded bar*) and without (*white bar*) addition copper sulphate to the medium. The *x* axis indicates the time of analysis (hours after collection). The *y* axis indicates the fold change in gene expression relative to 0 h. **a**, **c** cv. Igri; **b**, **d** cv. Cork. The data presented are representative of five independent repeats

expression of *OxO* was similar in anthers that were stress treated, both with and without copper sulphate (Fig. 2a). *OxO* gene expression was stimulated after 15 h of stress treatment and slowly increased thereafter until the end of treatment period, where it was expressed at a 200-fold higher level than in untreated anthers. *OxO* transcript accumulation was higher in cv. Cork than in cv. Igri (Fig. 2b), reaching a 1,000 fold higher expression level at 96 h. In contrast to cv. Igri, *OxO* gene expression in cv. Cork anthers was influenced by the addition of copper sulphate to the medium. In the absence of copper sulphate, there was a regular increase in *OxO* expression between 24 and 96 h of stress treatment; however, in the presence of copper sulphate, a strong (approximately 400-fold) induction was observed after 6 h of pre-treatment, followed by a progressive twofold increase in expression until 48 h and then a slight increase until the end of stress treatment.

GST gene expression during the in vitro stress treatment was similar in the presence or absence of copper sulphate in cv. Igri (Fig. 2c), increasing gradually from 3 h. In cv.

Cork, *GST* transcript levels increased in time, although somewhat erratically, starting 3 h after the onset of the stress treatment, both with and without copper sulphate addition (Fig. 2d). The relative *GST* transcript level was higher when copper sulphate was included in the medium.

Genes encoding enzymes involved in JA biosynthesis

Expression of the *Lox*, *AOS* and *AOC* genes was either not detected or expressed at a low level in the anthers during in vivo pollen development (Fig. 3). *Lox* gene expression was induced in cv. Igri and cv. Cork anthers cultured in medium lacking copper sulphate within 6 h after the onset of the stress treatment (Fig. 3a, b). In both cvs. *Lox* transcript levels continued to increase during the remaining culture period; however *Lox* gene expression in cv. Igri was higher than that in cv. Cork.

In cv. Igri anthers, expression of the *AOS* gene progressively increased during the stress treatment, regardless of whether the medium contained copper sulphate (Fig. 3c). In cv. Cork anthers, the peak in *AOS* expression was delayed when copper sulphate was added to the medium (Fig. 3d).

AOC gene expression was slightly stimulated within the first hours of stress treatment in both cvs. and then decreased to a basal level (Fig. 3e, f).

Genes encoding PR proteins

The *PR1*, *Gluc*, *PR10*, *Barwin* and *Chit 2b* genes were expressed at a low level during in vivo pollen development, but were highly expressed in response to the stress treatment (Fig. 4). The anthers of the two cvs. reacted differently with respect to PR-gene expression during the stress treatment (Fig. 4). In cv. Igri, the transcript levels of the five genes increased during anther stress treatment. This increase was steady for the *PR1* and *Gluc* genes (Fig. 4a, c). In the case of *PR10*, *Barwin* and *Chit 2b*, expression only started to increase at 24 h and continued to increase until the end of culture period (Fig. 4e, g, h). Despite a similar global expression profile, some differences could be noticed between the expression patterns of the different genes. First, the increase in expression of the *PR1*, *Gluc* or *PR10* genes was in the same range (20–30-fold) after 4 days of treatment, while that of *Barwin* was higher (270-fold). The most spectacular increase of induction was observed for the *Chit 2b* gene (5,300-fold) at the end of the stress treatment in medium lacking copper sulphate. Second, the presence of copper sulphate did not alter *PR1* or *Gluc* transcript levels, but it negatively affected expression of the *PR10*, *Barwin* and *Chit 2b* genes.

Gene expression profiles in anthers treated without copper were the same in cvs. Cork and Igri for all PR genes

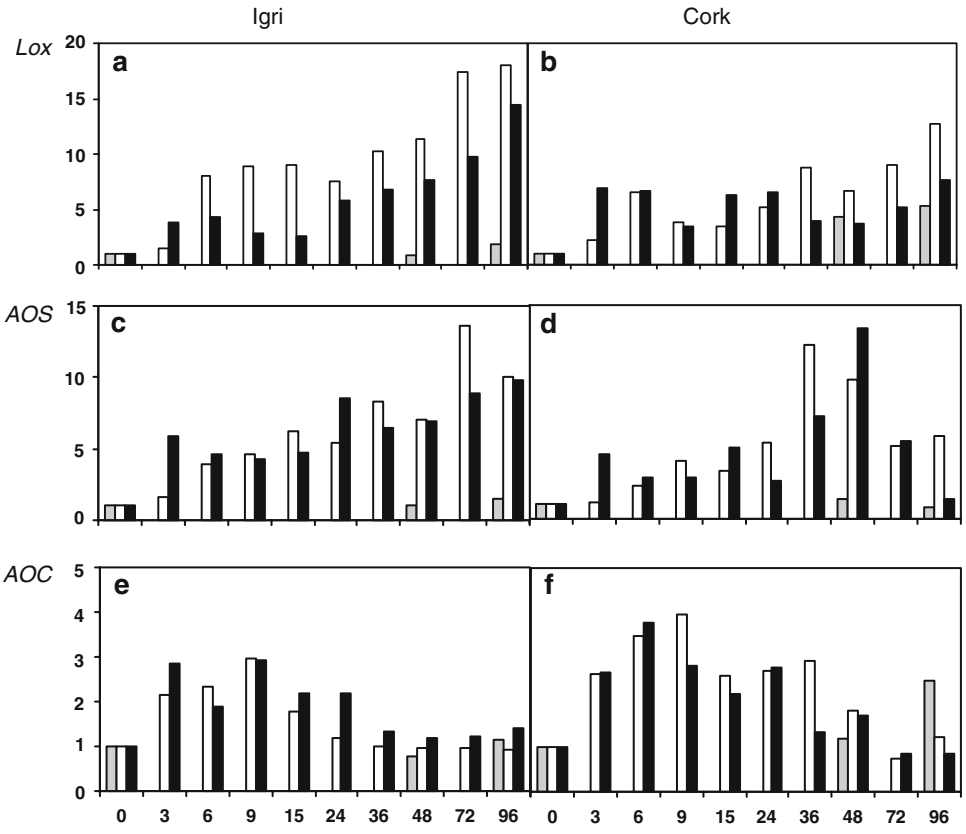

Fig. 3 The expression levels of *Lox*, *AOS* and *AOC* genes were quantified by real-time RTPCR in cvs. Igri and Cork during anther development in vivo (*light-shaded bar*) and during the stress treatment in vitro, with (*dark-shaded bar*) and without (*white bar*) addition copper sulphate to the medium. The *x* axis indicates the time of analysis (hours after collection). The *y* axis indicates the fold change in gene expression relative to 0 h. **a**, **c**, **e** cv. Igri; **b**, **d**, **f** cv. Cork. The data presented are representative of five independent repeats

except *Chit 2b* and *Barwin,* for which the maximum induction in cv. Cork was first detected at 48 h and remained stable thereafter (Fig. 4f, h). When copper sulphate was added to the medium, *PR1* gene expression in cv. Cork anthers dropped below the level observed in the in vivo growing anthers (Fig. 4b), while *Gluc* and *PR10* gene expression strongly decreased (Fig. 4d, j). The *Barwin* transcript level (Fig. 4h) in cv. Cork anthers cultured in the presence of copper sulphate was similar to that in cv. Igri. No significant difference in the *Chit 2b* transcript level was observed when copper sulphate was added to the medium (Fig. 4f).

Gene encoding an enzyme involved in the phenylpropanoid biosynthesis pathway

The *PAL* gene expression profile remained constant in both cvs., during in vivo pollen development (Fig. 5). During in vitro development in the absence of copper sulphate, *PAL* transcript levels remained stable during the first 24 h and then increased fivefold in cv. Igri (Fig. 5a) and up to tenfold in cv. Cork (Fig. 5b) by the end of the stress treatment. The presence of copper sulphate reduced *PAL* gene expression in the anthers of both cvs. relative to anthers cultured in medium lacking copper sulphate.

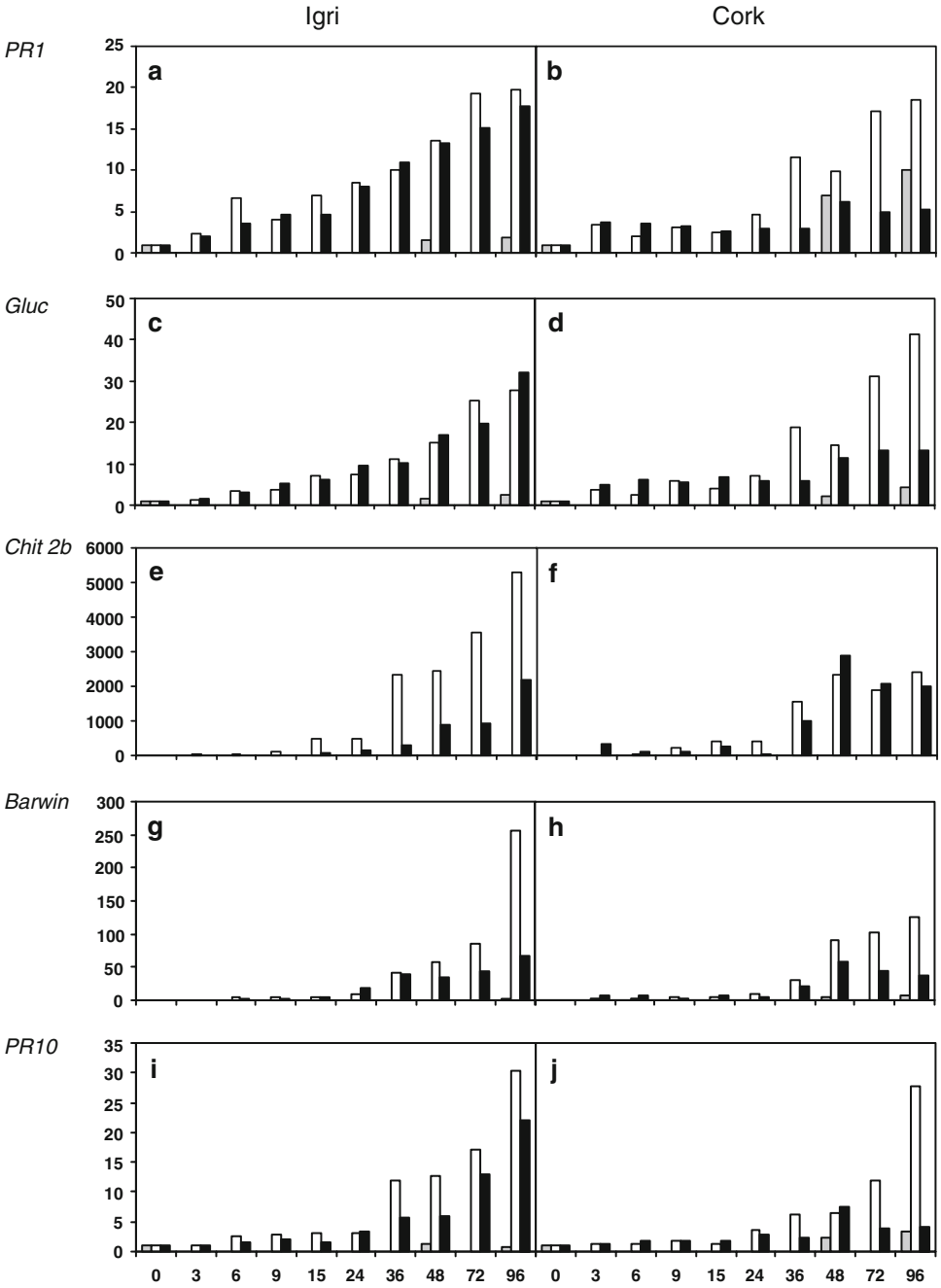

Fig. 4 The expression levels of five PR-genes were quantified by real-time RT-PCR in cvs. Igri and Cork during anther development in vivo (*light-shaded bar*) and during the stress pre-treatment in vitro, with (*dark-shaded bar*) and without (*white bar*) addition copper sulphate to the medium. The *x* axis indicates the time of analysis (hours after collection). The *y* axis indicates the fold change in gene expression relative to 0 h. **a**, **c**, **e**, **g**, **i** cv. Igri; **b**, **d**, **f**, **h**, **j** cv. Cork. The data presented are representative of five independent repeats

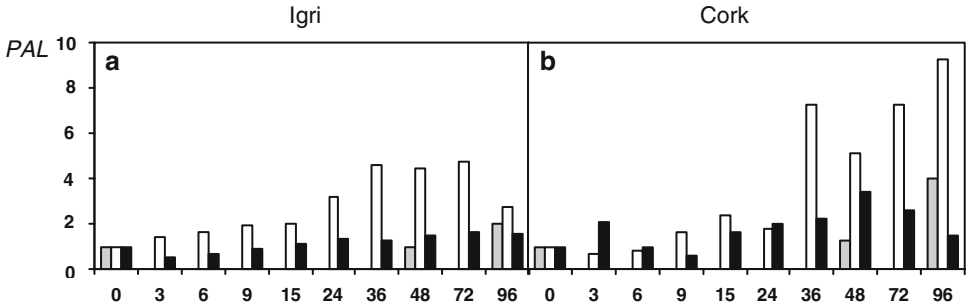

Fig. 5 The expression levels of *PAL* were quantified by real-time RT-PCR in cvs. Igri and Cork during anther development in vivo (*light-shaded bar*) and during the stress pre-treatment in vitro, with (*dark-shaded bar*) and without (*white bar*) addition copper sulphate to the medium. The *x* axis indicates the time of analysis (hours after collection). The *y* axis indicates the fold change in gene expression relative to 0 h. **a** cv. Igri; **b** cv. Cork. The data presented are representative of five independent repeats

Discussion

Our results show that barley anthers are able to perceive abiotic stress conditions early during the stress treatment used to induce ME and respond by triggering various aspects of stress-related physiology.

An oxidative burst occurs in anthers during pre-treatment

Stress treatment induces an oxidative burst in anthers, as revealed by the formation of $O_2^{\cdot-}$ that gives rise to hydrogen peroxide (H_2O_2). Oxidative burst is one of the earliest responses of plant cells to various types of stress (Bolwell et al. 2002; Rentel et al. 2004) and leads to reactive oxygen species (ROS) accumulation (Bhattacharjee 2005). Among ROS, H_2O_2 may have a central role in anthers as a diffusible signal molecule allowing selective induction of defence-related genes (Varnova et al. 2002). Since an NBT positive reaction was mainly detected in the anther wall layers and only poorly in the microspores, the question remains whether the microspores, which represent the targets of the process, also experience an oxidative burst and thus react directly to the stress treatment.

The stress treatment also stimulates *OxO* expression. Oxalate oxidase is a germin-like protein that degrades oxalate into H_2O_2 and CO_2 (Woo et al. 1998). Its expression is constitutive in some reproductive tissues (Dunwell 1998), or is induced following pathogen attack (Dunwell 1998) or in response to environmental fluctuations (Zhou et al. 1998), especially in barley (Hurkman and Tanaka 1996). In our system, the $O_2^{\cdot-}$ production observed during the stress treatment correlates with *OxO* expression.

Our results also show that *GST* expression is induced in the anther during the stress treatment. *GST* expression may be correlated with three processes that occur in the anther. First, GSTs are involved in cell protection against the harmful effects of ROS (Kampranis et al. 2000). The promoter region of many plant *GST* genes contains ROS-responsive elements (Chen and Singh 1999; Garretón et al. 2002). It should be noted that *GST* expression is also stimulated under stress conditions that do not lead to ME (Maraschin et al. 2006), suggesting that its expression is stimulated under stress conditions regardless of the developmental fate of the microspores. Second, it has been reported that *GST* gene expression is spatially and temporarily regulated during plant development (Bailly et al. 2004). In barley *GST* gene expression is temporally regulated during in vivo microspore development and, as indicated earlier, in response to the in vitro stress treatment used to induce ME (Maraschin et al. 2006). Third, *GST* gene expression is associated with in vitro morphogenesis (Holmberg and Bülow 1998; Gong et al. 2005) although the mechanism whereby stress enhances morphogenesis is not clear.

The pre-treatment stimulates defence and stress-related gene expression

Expression of three genes encoding the enzymes involved in the first step of JA biosynthesis is stimulated in anthers during the stress treatment. This is in accordance with the literature, since JA is known to be involved in the plant's reaction to biotic or abiotic stresses (Ozturk et al. 2002). Low temperature and high osmotic pressure are the abiotic stresses used to induce barley ME. The presence of copper sulphate in some experiments may also be considered as an additional abiotic (chemical) stress. Our data show that *AOC* expression peaks after 24 h in cv. Igri and 36 h in cv. Cork, while levels of both *Lox* and *AOS* transcripts continued to increase. We propose that the stress treatment used to induce ME stimulates expression of *Lox*, *AOS* and *AOC* genes within a few hours and leads to JA synthesis, which in turn activates a negative feedback loop leading to the reduction of *AOC* expression.

Genes encoding PR proteins are known to be expressed at the end of both flower and pollen development (Van Loon et al. 2006), also in barley anthers (Liljeroth et al. 2005). We also detected PR gene expression during in vivo pollen development.

Besides their developmental regulation, the expression of genes encoding PR proteins is also stimulated by pathogens and by abiotic stresses such as drought, salinity, wounding or elicitor treatments (Thomma et al. 1998; Yeh et al. 2000). Our study confirms that abiotically stressed anthers of barley also react by inducing PR gene expression. *PR1*, *Gluc* and *PR10* gene expression is stimulated during the entire stress treatment. These genes are known to respond to fungal infection in barley (Gjetting et al. 2004); however, no information is available on their involvement in abiotic stress responses. Similarly, *Barwin*, which encodes a protein similar to the antifungal chitin-binding protein hevein isolated from rubber tree latex, is expressed in response to ethylene or viral infection (Pritsch et al. 2000; Wu et al. 2004). These data suggest that the increase in expression of *Barwin* observed in the in vitro cultured anthers is due to the abiotic stress treatment.

The *Chit 2b* gene is of particular interest in the context of ME for several reasons. First, it is the most highly upregulated gene among the 11 tested genes. Second, chitinases are known to play a nursing role in carrot somatic embryogenesis by providing somatic embryogenesis-promoting factors (Van Hengel et al. 1998) and are required for the process of carrot embryogenesis itself (Kragh et al. 1996). Third, chitinases catalyse the hydrolysis of arabinogalactan proteins (Van Hengel et al. 2001), which themselves are believed to promote ME in maize and other species (Paire et al. 2003; Borderies et al. 2004). Fourth, in addition to its proposed role in the plant's reaction to pathogen attack (Gjetting et al. 2004), *Chit 2b* gene also seems to play a role in cold resistance (Yeh et al. 2000). We suggest that induction of *Chit 2b* gene expression is correlated with both the anther's reaction to the stress treatment and the initiation of ME through AGP production.

As with *PR1*, expression of the *PAL* gene, which encodes the first enzyme in phenylpropanoid synthesis (Kervinen et al. 1998), is slightly stimulated in anthers developing in vivo. This is in agreement with previous findings demonstrating a requirement for phenylpropanoids during normal pollen development (Matsuda et al. 1996).

Copper sulphate limits induction of defence-related gene expression

The cvs. Igri and Cork were followed in this study because they exhibit opposite behaviour during ME, in the sense that without copper sulphate in the stress treatment medium, albino plantlets are regenerated exclusively from cv. Cork, whereas mostly green plantlets are regenerated from cv. Igri (Caredda et al. 2000). In cv. Igri, the presence of copper sulphate in the stress treatment medium increases the total number of regenerated plantlets, but has little effect on the production of green plantlets (Wojnarowiez et al. 2002). In cv. Cork, the presence of copper sulphate in the stress treatment medium significantly increases the number of green plantlets that are produced (data not shown).

The presence of copper in the stress treatment medium leads to a global reduction in the stimulation of stress-related gene expression in both cvs. However, this effect is of particular importance in cv. Cork when considering the *PR1*, *Gluc* and *PR10* genes. Our results suggest that copper sulphate limits induction of defence-related gene expression in cv. Cork during the stress treatment. The modulation of stress-related gene expression may thus be correlated with a beneficial effect of copper on both quantitative and qualitative aspects of anther response for ME in cv. Cork. The mechanisms underlying this phenomenon remain to be elucidated.

High copper concentrations are known to generate stress leading to ROS production in a number of plant species, including wheat (Navari-Izzo et al. 1998) and *Arabidopsis thaliana* (Drazkiewicz et al. 2004). In the barley cv. Nure, it has been shown that copper exposure alone stimulates expression of defence genes to a greater extent than when copper is supplied together with a cold shock (Atienza et al. 2004), which is in agreement with our results. Our stress treatment medium also contains high amounts of mannitol causing a supplementary osmotic stress that may interact with the cold/copper stress reaction.

The expression of defence-related genes was similar in the two cvs. except for *Chit 2b* expression, which was expressed at a higher level in cv. Igri anthers versus cv. Cork anthers cultured without copper sulphate, and for *OxO* expression, which was more highly expressed in cv. Cork than cv. Igri under both in vitro conditions. These two differences are not sufficient to explain the different ME responses of the two cvs. This point could be partially elucidated by global transcriptome analysis of the anther expression profiles of the two cvs.

In barley, whole anthers containing microspores are generally used for DH production; thus any attempt to understand the mechanisms underlying stressed-induced ME in this system must take the whole anther and the various physiological processes that occur in the anther into account. The mRNAs analysed in this study were extracted from whole anthers, which include anther wall layers and the target microspores. It is therefore difficult to determine whether the changes in gene expression that we observed correspond to gene expression changes in the

anther wall, in microspores, or in both. This point should be further examined, using either in situ hybridization or gene expression analyses on separated anther walls and microspores.

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