Theor Appl Genet (1993) 86:843–850

Identification of a 32-kDa anther marker protein for androgenic response in maize, *Zea mays* L.

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Received: 21 September 1992 / Accepted: 3 November 1992

Abstract. Variations in the whole anther protein pattern have been investigated in a highly androgenic maize hybrid during the inductive pretreatment for androgenesis. It was found that a 32-kDa protein (MAR32) is induced and accumulates in the anthers during cold pretreatment of the tassel. A positive correlation between the rate of embryo formation via anther culture and the level of this protein after 7 days of cold treatment was observed. In addition, the in vivo synthesis of this protein by cold-pretreated anthers was demonstrated. Different responsive and non-responsive genotypes were also evaluated, and the accumulation of MAR32-like protein was only observed in certain responsive genotypes. The results suggest that the protein MAR32 is a marker for a form of androgenic responsiveness in maize.

Key words: Maize – Marker protein – Cold pretreatment – Androgenesis – Embryogenesis

Introduction

Recent success in cereal transformation has indicated the key importance of efficient regeneration systems (Vasil et al. 1992). Androgenesis, i.e. the regeneration of haploid or doubled haploid plants from single immature microspores, represents a promising method for potent regeneration. To this end, embryogenic suspension cultures and totipotent protoplasts have been obtained via androgenesis in several cereal species (Datta et al. 1990; Jähne et al. 1991; Mitchell and Petolino 1991).

Since the original discovery of androgenesis (Guha and Maheshwari 1964), little progress has been made in elucidating the cellular and molecular mechanisms of androgenic induction. Much effort is currently being expended in this field, and promising data have been already obtained (Dunwell 1992, and references therein). In maize, in vitro anther culturability is strongly genetically determined (Petolino 1990). Despite the development of highly androgenic germplasm in several genetic backgrounds (Petolino et al. 1988; Barloy et al. 1989), the utilization of in vitro androgenesis in breeding and biotechnological applications still requires an increased responsiveness in germplasm of agronomic value. Clearly, a better knowledge of the mechanisms of androgenic induction is necessary in order to address the problem of recalcitrant genotypes.

It has been demonstrated that cold pretreatment of maize tassels significantly enhances the androgenic response of the anther (Genovesi and Collins 1982). There is also some evidence that anther responsiveness in maize is partially sporophytically determined (Barloy et al. 1989; Cowen et al. 1992), i.e. that the anther wall plays an important role in the induction of microspore embryogenesis. Studies in carrot somatic embryogenesis have demonstrated the functional importance of extra-cellular proteins in the initiation of embryogenesis (Van Engelen and De Vries 1992). Furthermore, pilot studies using maize plants from the synthetic population NS (Barloy et al. 1989) have shown that genotype-specific proteins can be detected in the anthers of some plants after cold pretreatment (Delvallée 1987). These data led us to investigate variations in the whole anther protein pattern during

Communicated by G. Wenzel
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cold pretreatment, and preliminary results on the detection of putative androgenesis-related maize proteins have been reported (Vergne et al. 1990). In the investigation reported here we have extended these experiments and characterized the kinetics of synthesis and accumulation of these marker polypeptides. Moreover, we show that the accumulation of a 32-kDa anther protein at a particular stage of the cold treatment is positively correlated with the intensity of the androgenic response.

Materials and methods

Plant material

Several responsive and non-responsive maize genotypes were used in this study. Most of the experiments were carried out on the highly androgenic hybrid line DH5 × DH7 (Barloy et al. 1989). DH5 and DH7 are doubled haploid lines derived through in vitro androgenesis from essentially Chinese material; separately, they display approximately 30% embryogenic anthers, while their cross reveals considerable heterosis with yields as high as 70–80% embryogenic anthers (Barloy et al. 1989). The inbred line H99, which originates from Illinois synthetic 60C (Purdue University), has consistently appeared as a parent of responsive crosses in previous studies (Petolino and Jones 1986; Petolino and Thompson 1987). Four non-responsive inbred lines (A188, B73, F1656 and Oh43) were also compared with the androgenic genotypes. A188 and B73 have been found to be non-androgenic in several previous experiments (Brettell et al. 1981; Petolino and Jones 1986; Vergne et al. 1990). F1656 does not display any responsiveness to anther culture (Barloy et al. 1989; Vergne et al. 1990). In the same manner, Oh43 has never shown androgenic activity despite repeated trials over 1989 and 1990 (Vergne et al. 1990; P. Vergne, unpublished data).

Plants were either grown in growth chambers under strictly controlled conditions (Gaillard et al. 1991) or in a greenhouse at 18°/24° + / − 2 °C (day/night) with supplemental lighting provided by 1000 W high pressure sodium vapor bulbs carried on a mobile beam (16 h photoperiod). In this latter condition the photosynthetic photon flux density was 400 $\mu E\, m^{-2} s^{-1}$ when measured at the top of the plants. Under both conditions plants were irrigated daily with a nutritive solution (Coic and Lesaint 1971). In most of the experiments, only donor plants raised during the same period of time and in the same growing conditions were compared.

Cultural procedures

Anthers were essentially cultured using previously described procedures (Dieu and Beckert 1986). Briefly, tassels with anthers containing mid-uninucleate microspores were stored at 7 °C for 7 days. At least 150 anthers per plant were then excised and plated. After 1 week in the dark at 14 °C, the dishes were transferred to 28 °C under dim light. The number of embryogenic anthers and the total number of embryo-like structures (ELS) were recorded 6–8 weeks after plating. Androgenic embryos and plantlets were regenerated as described (Dieu and Beckert 1986). The regenerated plants were scored after adaptation to soil conditions. Alternatively, tassels were pretreated at 7 °C for 14 days and anthers were cultured directly at 28 °C. Pilot experiments have demonstrated that this procedure does not modify anther responsiveness (M. Beckert, unpublished data).

Anther sampling and microspore isolation

At different stages of the cold pretreatment, samples of 10–30 spikelets were taken from the tassels, and the anthers were then quickly dissected on ice and stored immediately in liquid nitrogen (LN_2) until protein extraction. Alternatively, the sampled spikelets were first stored directly in cryotubes in LN_2; prior to protein extraction, the anthers of these spikelets were dissected from the frozen material in a mortar regularly cooled with LN_2 so that no thawing could occur before extraction. For those experiments requiring the sampling of anthers/spikelets at intermediary durations of the cold treatment, tassel branches were divided into several homogeneous groups and wrapped separately in aluminium foil. For some experiments, including the correlation experiments (see Results), sets of 10–15 dissected spikelets to be used for anther protein analysis were pretreated with cold from the time of tassel sampling in closed 1.8-ml cryotubes and then stored in LN_2 at appropriate stages. These alternative protocols for cold pretreatment do not impair the androgenic response of the anthers (P. Vergne, unpublished data). Only anthers from spikelets pretreated in the same manner were compared in each analysis. Eighteen-day-long cold pretreatments were also evaluated as this is the most optimal time period for isolated microspore culture (Gaillard et al. 1991).

Microspores at the appropriate stage were isolated from pretreated tassels as described by Gaillard et al. (1991). The entire isolation procedure was performed at 0°–4 °C. The final suspension of viable isolated microspores was filtered on a nylon cloth (pore size: 50 μm) under light suction to collect the microspores. Excess liquid was quickly eliminated by blotting the filter onto a piece of tissue paper. The filter and the microspores were then placed into a cryotube and immediately frozen in LN_2. The microspore fractions were stored in LN_2 until protein extraction.

Labeling of anthers by [^{35}S]methionine

Anthers from 15 spikelets were dissected on ice and washed for 30 s with a sterile 0.05% Triton X-100 solution. They were then rinsed 3 times with sterile distilled water and transferred to 250 μl of sterile incubation medium (Murashige and Skoog 1962), with salts at 0.2 × strength and 0.5% sucrose. Then, 1.85×10^6 Bq[^{35}S]-methionine (NEN, sp. act. 4.17×10^{13}Bq/mmol) was added, and the anthers were incubated for 3 h at 28 °C in darkness. The anthers were then rinsed 3 times with sterile distilled water, quickly blotted dry on tissue paper and stored in LN_2.

Protein extraction and electrophoretic analysis

For the extraction of native soluble proteins, 30–90 anthers or up to 7×10^5 microspores were ground to powder in an Eppendorf 1.5-ml tube at the temperature of liquid nitrogen using a motor-driven pestle fitted to the tube. The powder was suspended in extraction buffer (25 m*M* Tris-HCl (pH 7.8), 5 m*M* DTT, 2% insoluble PVP), and the material was further homogenized on ice for a few seconds. The slurries were centrifuged for 15 min at 15,000 *g* at 2 °C; then the supernatants were pipetted off, recentrifuged for 5 min and stored at −80 °C until use. The protein content of the extracts was determined by the Biorad dye binding assay (Bradford 1976) with BSA as the standard. In the case of [^{35}S]-labeled extracts, total incorporated counts were determined on trichloracetic acid (TCA) precipitates.

Proteins were separated by polyacrylamide slab gel isoelectric focusing (IEF) in a LKB Multiphor II unit, according to the directions of the manufacturer. Gels (0.4 mm × 10 cm × 24 cm, 7.5% T, 3% C) contained 3.2% carrier ampholytes (Pharmacia LKB preblended Ampholines, pH 4.0–6.5). The electrode solutions were 0.5 *M* H_3PO_4, 0.1 *M* glutamic acid for the anode and 0.1 *M* alanine for the cathode, and the initial settings were

2000 V, 20 mA, 20 W. The gels were prefocused for 10 min before applying the samples and then run for 50 min.

Selective two-dimensional electrophoresis was performed essentially according to Gaude et al. (1991). Briefly, triplicate filter paper wicks on IEF gels were loaded with up to 60 µl crude extract per lane; the applied volume was adjusted so as to load the same amount of protein (ca. 120–150 µg) or TCA precipitable counts per sample. The gels were run as described above, and the protein bands of interest were transiently visualized with Coomassie blue staining. They were then cut off from the gels and subjected to SDS-PAGE on 12% acrylamide gels (Laemmli 1970).

For one- or two-dimensional gels, the total separated proteins were detected by silver staining (Morrissey 1981). To visualize the newly synthesized protein by fluorography, the gels were impregnated with Amplify (Amersham), dried and exposed to Kodak X-Omat X-Ray films at −80 °C for 10–30 days.

Densitometric and correlation analysis

Silver-stained IEF patterns were scanned in a Jr. Plus densitometer (Helena Laboratories), and peak areas for the bands of interest were calculated. To account for the minor differences in protein loading between samples, these area values were transformed by calculating for each track the ratio: (peak area)/(background grey level value for the track) (see Fig. 2). To measure the background grey level values, gel images were recorded using an Imager documentation system (Appligene) and analyzed with a Scan Analysis software. Correlation coefficients for $P = 0.01$ and $P = 0.05$ ($r_{0.01}$ and $r_{0.05}$) were taken from Snedecor and Cochran (1957, Table A11). Only densitometric data for plants analyzed on the same gels were pooled and compared in the correlation analysis.

Results

Selective two-dimensional electrophoresis analysis revealed that a protein with an apparent molecular mass of 32 kDa is induced and accumulates in the anthers of the hybrid DH5 × DH7 during cold treatment. This protein, referred to as the maize androgenesis-related 32-kDa protein (MAR32) is hardly detectable at the time of tassel sampling but increases in quantity during an 18-day cold pretreatment (Fig. 1a). MAR32 represents the most prominent polypeptide component of the characteristic protein band of the one-dimensional IEF pattern of cold pretreated anthers (Fig. 1b and Vergne et al. 1990). No protein of a similar apparent molecular mass was detectable after silver staining in extracts from anthers of non-pretreated tassels developed normally in vivo and sampled after the early binucleate pollen stage (Fig. 1a).

Accumulation of MAR32 during the cold treatment was found to occur for most of the DH5 × DH7 plants analyzed. However, despite the controlled growing conditions of the donor plants, important plant to plant variations in the level of MAR32 in anthers after 7 days of cold treatment were generally detected by IEF analysis (Fig. 2). Therefore, we addressed the question of a possible relationship between the amount of MAR32 accumulated in the anthers and the actual androgenic response. Plants from several homogeneous sets with regard to their growing conditions were individually evaluated for their androgenic performance and for the accumulation of MAR32 in their anthers during the cold pretreatment. These experiments demonstrated a positive correlation between the mean number of ELS produced per plated anther and the amount of MAR32 after 7 days of cold treatment, as estimated by densitometric analysis of silver-stained IEF patterns of anthers excised from spikelets pretreated for 7 days. Furthermore, a significant ($P = 0.01$ level) linear relationship was found between the two parameters, for both greenhouse-and growth chamber-originated plants (Fig. 3a). A similar correlation between the percentage of embryogenic anthers and the

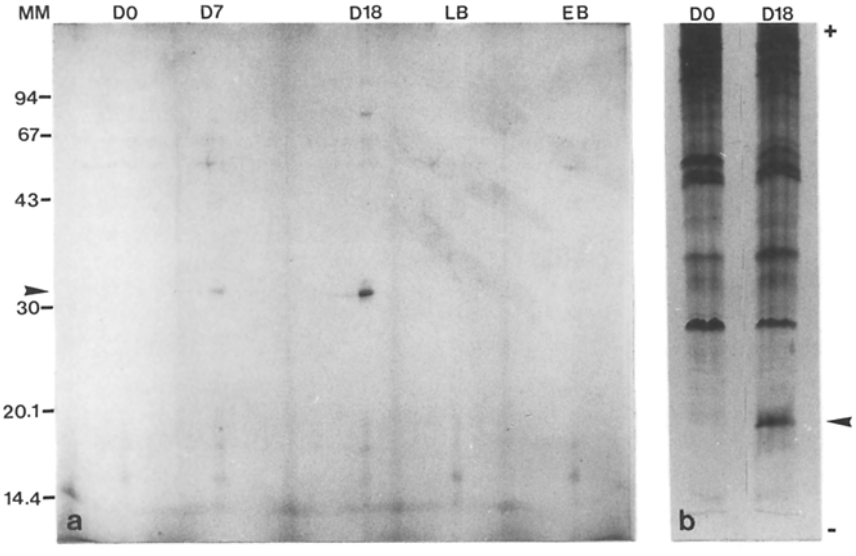

Fig. 1a, b. Analysis of DH5 × DH7 anther proteins. **a** SDS-PAGE separation and visualization by silver staining of polypeptides from selected IEF bands of anthers at different stages of the cold pretreatment (*D0*, *D7* and *D18*: days 0, 7 and 18, respectively) and from anthers developed normally in vivo (*LB* and *EB*: late and early bicellular pollen stage, respectively). *Arrowhead* points to the position of the MAR32 polypeptide. Molecular mass (*MM*) is in kDa. **b** Comparison of the IEF patterns of anthers at tassel sampling (*D0*) and after an 18-day cold treatment (*D18*). An aliquot of 35 µg protein was loaded per lane. Only the basic part of the gel is presented. *Arrowhead* indicates the MAR32 band. The positions of the anode (+) and the cathode (−) are indicated

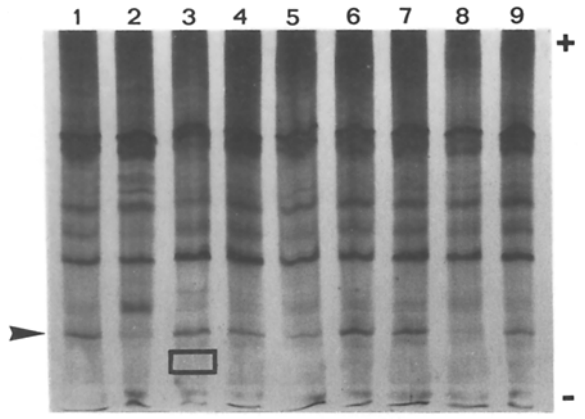

Fig. 2. Concentration of the MAR32 protein, as visualized by IEF analysis and silver staining, in the anthers of individual DH5 × DH7 plants after 7 days of cold treatment. The plants were grown in either a growth chamber (*lanes 1–7*) or a greenhouse (*lanes 8 and 9*). An aliquot of 35 µg protein was loaded per lane. *Arrowhead* indicates the MAR32 band. The *boxed area* exemplifies the portion of the track image used to measure the background grey level of each track, as described in Materials and methods

amount of MAR32 in the anthers after 7 days of cold treatment was revealed by this analysis (data not illustrated). In contrast to this, no clear correlation could be found between the number of regenerated plants per plated anther and the level of MAR32 accumulation (Fig. 3b).

As silver staining solely characterizes the amount of the protein of interest present at the time of sampling, we decided to determine if the accumulation of the MAR32 protein results only from post-translational modification (e.g. proteolytic degradation) of pre-existing anther proteins or/and from the translation of mRNAs. The analysis of [^{35}S]methionine-labeled proteins from anther taken at different stages of the cold pretreatment revealed that cold-pretreated anthers are capable of synthesizing the MAR32 polypeptide and that the highest rate of synthesis is displayed by anthers from tassels pretreated for 7 days (Fig. 4).

This demonstration of MAR32 accumulation in anthers during the cold pretreatment raises the question as to which cells of the anther accumulate the MAR32 protein. In order to clarify the tissular origin of MAR32 accumulation, proteins extracted from both isolated microspores and whole anthers of the same pretreated tassels were run on IEF gels. Figure 5 shows that microspores isolated from anthers which exhibit noticeable levels of MAR32 protein do not display any visualizable level of the protein, thus indicating that MAR32 accumulation takes place in the locular space or in sporophytic tissues of the anther, but not in the microspore cells.

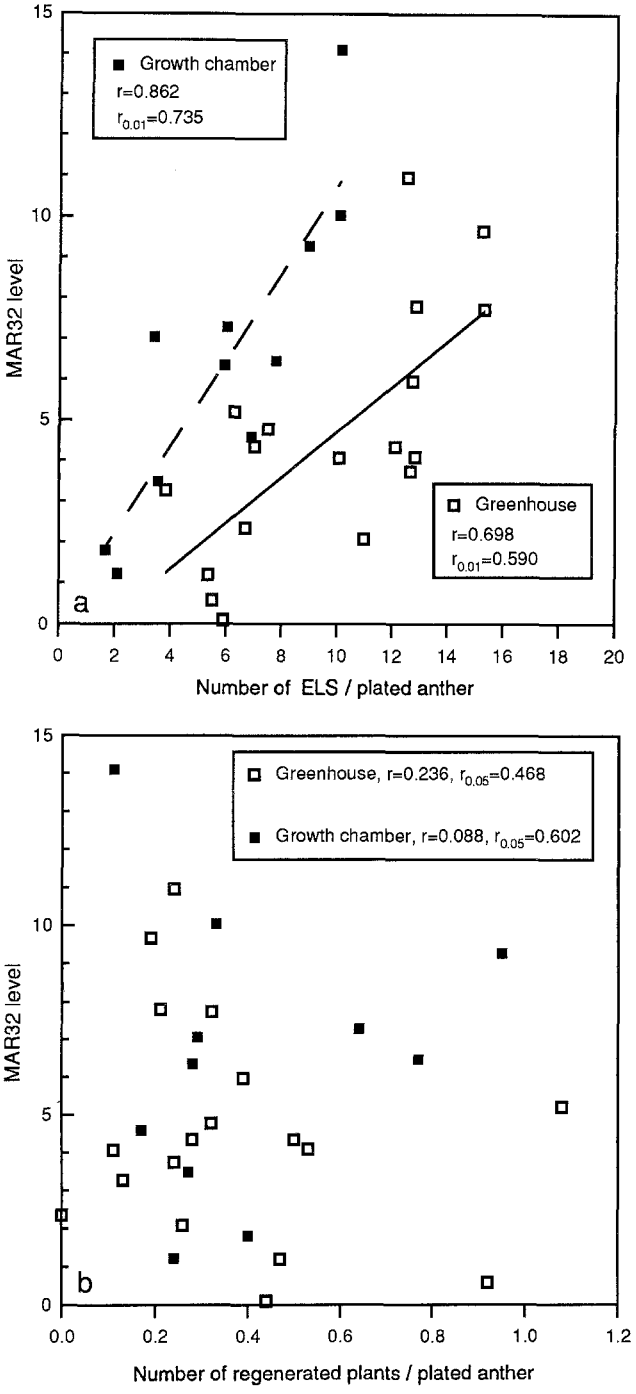

Fig. 3a, b. Relationship between DH5 × DH7 anther responsiveness and the level of MAR32 accumulation after 7 days of cold treatment. Each point represents an individual tassel (plant). MAR32 levels are expressed in arbitrary units and were calculated as described in Materials and methods

Since the aptitude for androgenesis is strongly genetically determined, we extended the study of variations in the anther protein pattern during the cold treatment to a set of responsive and non-responsive

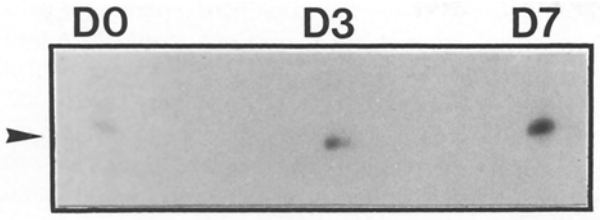

Fig. 4. Selective two-dimensional analysis and fluorography of in vivo-synthesized polypeptides from selected IEF bands of [^{35}S]methionine-labeled DH5 × DH7 anthers at different stages of the cold pretreatment (*D0*, *D3* and *D7*: days 0, 3 and 7, respectively). *Arrowhead* indicates the 32 kDa position

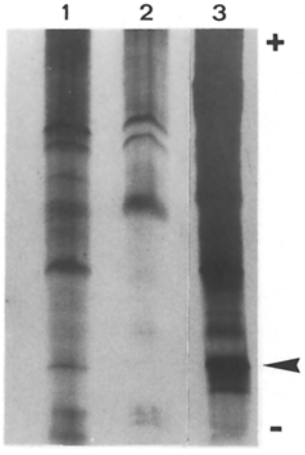

Fig. 5. Comparison of the silver-stained IEF patterns of whole anthers (*lanes 1 and 3*) and isolated microspores (*lane 2*), taken from cold pretreated DH5 × DH7 tassels. Spikelets were treated at 7 °C for 11 days (*lane 1*) and 18 days (*lanes 2 and 3*). The samples analyzed in *lanes 2 and 3* originated from the same tassel. An aliquot of 35 µg protein was loaded per lane. *Arrowhead* indicates the MAR32 band

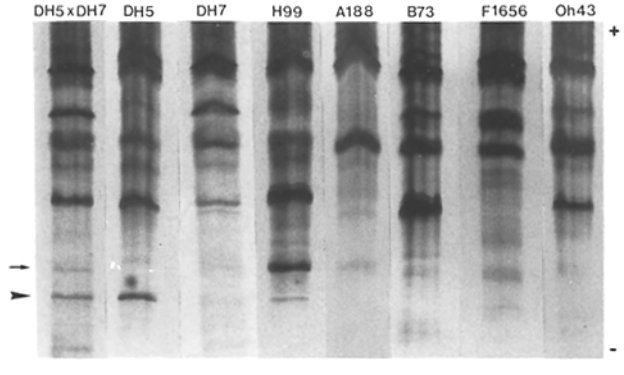

Fig. 6. Comparison of the silver-stained IEF patterns of anthers of different genotypes, taken from tassels treated at 7 °C for 7 days. An aliquot of 35 µg protein was loaded per lane. *Arrowhead* indicates the MAR32 band and the *arrow* points to the H99 prominent IEF band

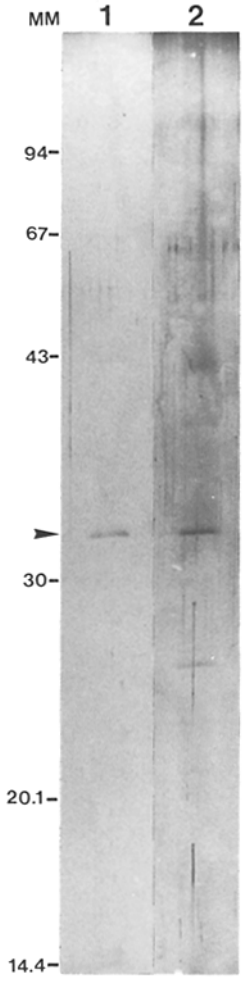

Fig. 7. SDS-PAGE separation and visualization by silver staining of polypeptides from selected IEF bands of DH5 × DH7 and H99 anthers taken from tassels pretreated for 7 days. *Lane 1* MAR32 band (DH5 × DH7), *lane 2* H99 prominent IEF band (See Fig. 6). *Arrowhead* indicates the MAR32 band. Molecular mass (*MM*) is in kDa

genotypes with various genetic backgrounds. These experiments demonstrated that none of the four non-androgenic lines tested (A188, B73, F1656 and Oh43) accumulate at detectable levels any protein similar to MAR32 in their anthers during the cold pretreatment (Fig. 6). The line DH5, used as the female in the cross DH5 × DH7, exhibits a similar pattern of accumulation of a MAR32 protein band as the hybrid itself. On the other hand, the male parent DH7 does not display such an accumulation, as indicated by the lack of a MAR32-like protein band in the IEF pattern of the treated anthers of this genotype (Fig. 6). Interestingly, anthers of the androgenic inbred line H99 were found to accumulate two proteins that focus in the basic part of the gels: one corresponding to MAR32, and a more

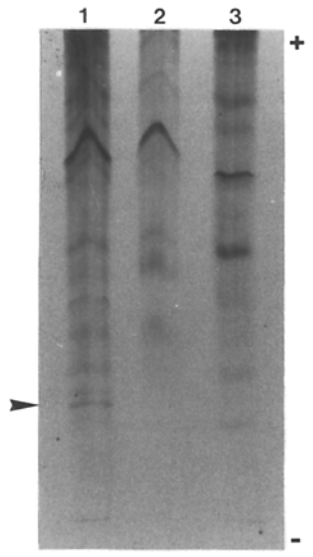

Fig. 8. IEF analysis and fluorography of in vivo-synthesized proteins extracted from anthers taken from tassels treated at 7 °C for 7 days. *Lane 1* DH5 × DH7, *lane 2* A188, *lane 3* DH7. Anthers have been labeled with [^{35}S]methionine as described in Materials and methods. *Arrowhead* indicates the MAR32 band

abundant species with a more acidic pI point (Fig. 6). Selective two-dimensional electrophoresis analysis revealed that the major polypeptide component of this H99 prominent IEF band displays the same apparent molecular mass of 32 kDa as the DH5 × DH7 protein MAR32 (Fig. 7). Some DH5 × DH7 plants also display the accumulation of low levels of a protein similar to the H99 specific protein (Fig. 6).

Finally, the possibility that genotypes which do not display the accumulation of MAR32-like protein could nonetheless synthesize similar proteins with a different turn-over rate was investigated. For the three genotypes DH5 × DH7, DH7 and A188, equivalent amounts of TCA-precipitable counts from cold-treated anthers labeled with [^{35}S]methionine were loaded onto IEF gels, focused and fluorographed. These experiments demonstrated that neither DH7 nor A188 anthers are able to synthesize any MAR32-like protein at detectable levels when the spikelets bearing them are subjected to cold treatment (Fig. 8).

Discussion

In the work reported here, we show that the cold pretreatment-induced accumulation of a 32-kDa anther protein, MAR32, is correlated in a maize competent genotype with a morphogenetic event, the development in vitro of embryo-like structures (ELS) from microspores. In order to quantify this correlation, we have used the effect that significant plant to plant variation in the androgenic response could be found during the course of our experiments with the hybrid DH5 × DH7, despite its overall high response to anther culture (Barloy et al. 1989). The occurrence of non-genotypic plant to plant variation is in accordance with results reported previously in maize (Petolino and Thompson 1987; Barloy 1990).

It is notable that the densitometric analysis of silver-stained IEF gels revealed significant linear relationships between the estimated amount of MAR32 protein and both the mean number of ELS per plated anther and the percentage of embryogenic anthers. Generally, silver staining methods for proteins in polyacrylamide gels are not considered to yield linear dose/response plots. However, such linear responses have been obtained and used for the quantitation of proteins by Oulad Abdelghani et al. (1991). In the same way, we have evaluated protein loads ranging from 5 to 45 µg using a MAR32-rich sample and have found a linear relationship between the relative amounts of MAR32 and corresponding densitometric values (data not shown). However, owing to the number of factors that influence the silver staining of proteins, we cannot rule out that the linear correlations reported here do not reflect exactly the association between the level of the MAR32 protein and the androgenic response. Nevertheless, it is clear that the trend documented (Fig. 3a) is consistent, i.e. that the level of the MAR32 protein after 7 days of cold pretreatment is positively correlated with the androgenic induction rate in the genotype DH5 × DH7. There is, however, no clear association between the detectable amount of MAR32 and the mean number of regenerated plants per plated anther. This lack of direct correlation may reflect the diversity and the complexity of interactions that determine this final response parameter. On the other hand, data from several cereal species, including maize, suggest that the intensity of androgenic induction (i.e. the formation of ELS) and the efficiency of ELS regeneration are governed by independent genetic factors and different mechanisms (Pace et al. 1987; Barloy 1990). Therefore, it would appear from this analysis that the accumulation of MAR32 in the anthers of DH5 × DH7 is linked to the expression of genes that govern ELS induction from microspores in this genotype.

While the female parent DH5 exhibits pretreatment-induced accumulation of MAR32, the male parent DH7 does not appear to accumulate MAR32. This difference is in accordance with the hypothesis that DH5 and DH7 carry separate genetic factors for competence, which show good complementation in the hybrid progeny (Barloy et al. 1989). Classical genetic studies have estimated that several genes (4–7) determine androgenic aptitude in the DH5 × DH7 genetic background (Barloy 1990). In addition to this, recent

RFLP analysis has enabled the detection of at least four major quantitative trait loci for ELS inductibility in maize (Cowen et al. 1992; A. Murigneux et al., submitted).

It is likely that MAR32 and the protein accumulated by the genetically distant androgenic line H99 (Figs. 6 and 7) are related in their molecular structure. Although differing in pI points, they are resolved into major polypeptide components of similar molecular mass on SDS gels. Thus, we assume that MAR32 is a putative genetic marker for a class of competence factors carried by both DH5 and H99. The fact that none of the non-responsive lines displays a MAR32-like protein supports this hypothesis. However, a broader screening of androgenic and non-androgenic genotypes with specific reagents such as anti-MAR32 antibodies is necessary to test this assumption.

In order to label in vivo-synthesized proteins to a sufficient specific activity, labeling experiments were performed at 28 °C, and not at the pretreatment temperature of 7 °C. Thus, it is likely that the observed protein patterns do not represent exactly in vivo translational activity in the anthers during the cold treatment. With regard to MAR32, we assume that the documented rates of synthesis at different stages of the pretreatment (Fig. 4) were influenced by the incubations conditions at 28 °C versus 7 °C. Nevertheless, our experiments clearly demonstrate that synthesis of the MAR32 protein can be induced, at least upon rewarming, in cold pretreated anthers in a stage-dependent fashion. However, it is not yet possible to ascertain the primary causes of accumulation of the protein. The fact that anthers pretreated for 7 days exhibit a higher rate of MAR32 synthesis (Fig. 4) indicates that an increase in the corresponding mRNA synthesis during the cold treatment is probable. Studies investigating differentially expressed mRNAs in cold treated versus non-treated anthers should provide a clearer answer to this issue.

IEF analysis of the proteins of the isolated microspores versus the whole anthers demonstrated that MAR32 accumulation does not take place within the microspore cells (Fig. 5). However, we cannot rule out the possibility that MAR32 is an extracellular protein secreted in the anther locule by the microspores. Interestingly, De Vries and co-workers reported that extracellular proteins are functionally implicated in the induction and the early stages of carrot somatic embryo development (De Jong et al. 1992; Van Engelen and De Vries 1992). MAR32 may also be produced in one of the anther wall cell layers and thus not be closely associated with the ELS induction mechanism. On the other hand, androgenic responsiveness in maize is probably partially sporophytically determined (Barloy et al. 1989). Furthermore, data from a recent RFLP study suggest that genes conditioning high anther culture response function at the sporophytic level (Cowen et al. 1992). Thus, MAR32 also represents a putative marker of a type of sporophytically determined competence for androgenesis in maize. Further studies are required, however, to clarify these points. The analysis of in vitro-translated proteins from microspore and anther mRNAs coupled with immunochemical and in situ experiments should help to ascertain precisely the tissular origin of MAR32 synthesis.

In conclusion, MAR32 represents a marker protein for a form of androgenic responsiveness in maize. However, it is not possible at present to specify the precise function, if any, of the MAR32 protein in ELS induction. Microsequence analysis and the development of antibodies directed against the protein will help us to understand the relationships between androgenic induction and MAR32 accumulation.

Acknowledgements. We wish to thank Richard Blanc and Maurice Giroux for taking care of the plants, Anne-Marie Thierry and Jeanine Tucci for excellent technical assistance and continuous support, and Pierre Audenis for photographic work. We acknowledge Steve Pescitelli for communicating results prior to publication, Pierre Jalinot and Marie-Claire Ronzière for their help with densitometric analysis, and Liz Bates for critical reading of the manuscript. F. Riccardi was supported by a fellowship from INRA ("bourse de DEA"). This work was supported by grants from INRA (AIP 88/4684 and 89/4684).

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